



THE UNIVERSITY
of **ADELAIDE**

**FUNCTION OF LATENT TRANSFORMING GROWTH
FACTOR- β BINDING PROTEIN-2 (LTBP-2) IN
ELASTINOGENESIS AND MODULATION OF GROWTH
FACTOR STORAGE, EXPRESSION AND ACTIVITY IN
NORMAL AND FIBROTIC TISSUES**

Mohamed Arshad Mohamed Sideek

Discipline of Anatomy and Pathology

School of Medicine

The University of Adelaide

April 2016

A thesis submitted in fulfilment of the requirement for the Degree of

Doctor of Philosophy in Medicine and Surgery

Table of Contents

Summary	8
Declaration	10
Acknowledgements	11
Publication Arising During PhD Candidature	12
Scientific Communications	13
Awards and Achievements During Candidature.....	15
Abbreviations	16
CHAPTER 1: The Role of LTBP-2 in Elastinogenesis and Fibrotic Diseases.....	19
1.1 The extracellular matrix (ECM) and its components.....	19
1.2 Collagens.....	21
1.3 Proteoglycans	23
1.3.1 Heparin/Heparan sulphate proteoglycans	24
1.4 Glycoprotein	26
1.5 Elastic fibres.....	28
1.5.1 Elastin	28
1.5.2 Fibrillin-microfibrils	30
1.6 Fibulins	32
1.6.1 Fibulin-5.....	34
1.7 Fibrotic diseases.....	37
1.8 Growth factors	39
1.8.1 Transforming growth factor – beta (TGF- β)	39
1.8.2 Fibroblast growth factor -2 (FGF-2).....	42
1.9 Latent TGF- β binding proteins (LTBPs)	44
1.9.1 LTBP-2	45
1.10 Aims of the present study	50
1.10.1 Specific aims.....	50
1.10.2 Hypotheses.....	50
1.11 References.....	51
CHAPTER 2: LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis	76
2.1 Abstract.....	80
2.2 Introduction.....	81

2.3 Experimental procedures	83
2.3.1 Materials	83
2.3.2 Molecular binding assays.....	83
2.3.3 Confocal microscopy	84
2.4 Results and discussion	86
2.4.1 LTBP-2 and tropoelastin have similar binding affinities for fibulin-5.	86
2.4.2 LTBP-2 can completely block the interaction of tropoelastin with fibulin-5.....	86
2.4.3 Heparin shows only minor inhibition of fibulin-5 interaction with tropoelastin and LTBP-2.	87
2.4.4 LTBP-2 substantially inhibits the interaction of tropoelastin with heparin.....	87
2.4.5 Co-localization of LTBP-2 with fibrillin-1, fibulin-5 and heparan sulfate proteoglycans in fibroblast matrix.	88
2.4.6 Exogenous LTBP-2 blocks elastinogenesis by cultured chondrocytes.....	88
2.5 Acknowledgments.....	91
2.6 References.....	91
2.7 Figures.....	96
Figure 2.1 LTBP-2 and tropoelastin bind fibulin-5 with similar affinities.....	96
Figure 2.2 Inhibition of the fibulin-5- tropoelastin interaction by LTBP-2 and its N- terminal fragment (LTBP-2 NT(H))	98
Figure 2.3 Heparin shows minor but specific inhibition of LTBP-2 interaction with fibulin-5.....	100
Figure 2.4 LTBP-2 inhibits the interaction of tropoelastin with heparin in a dose- dependent manner.	102
Figure 2.5 Confocal co-localisation of LTBP-2 with fibulin-5, fibrillin-1, fibronectin and heparan sulfate proteoglycans in fibroblast matrix.	104
Figure 2.6 Exogenous LTBP-2 inhibits elastinogenesis by ear cartilage chondrocytes in culture.	107
Figure 2.7 LTBP-2 and elastinogenesis	108
2.8 Mini-research paper: LTBP-2 inhibits elastin and fibrillin assembly in matrix of fetal bovine ear cartilage chondrocytes.....	110
2.8.1 Introduction.....	111
2.8.2 Method	111
2.8.3 Results and Discussion	112
2.8.4 References.....	116

CHAPTER 3: LTBP-2 has a single high-affinity binding site for FGF-2 and blocks FGF-2-induced cell proliferation.	119
3.1 Abstract.....	124
3.2 Introduction.....	125
3.3 Materials and Methods.....	127
3.3.1 Recombinant protein production and purification	127
3.3.2 Solid Phase Binding Assays.....	128
3.3.3 Cell proliferation assay	129
3.3.4 Detection and quantitation of FGFR1 activation	129
3.3.5 Immunohistochemistry	130
3.4 Results and Discussion	131
3.4.1 FGF-2 has a strong affinity for LTBP-2	131
3.4.2 FGF-2 binding is confined to a small central region of the LTBP-2 molecule	132
3.4.3 The FGF-2 binding site is close to a heparin-binding region of LTBP-2.....	132
3.4.4 LTBP-2 blocks FGF-2-induced cell proliferation.....	133
3.4.5 LTBP-2 and FGF-2 show similar distributions in fibrotic skin.....	133
3.5 References.....	137
3.6 Figures.....	142
Figure 3.1 Recombinant LTBP-2 fragments.....	142
Figure 3.2 LTBP-2 specifically binds FGF-2 but not VEGF, BMP-4, BMP-7 or TGF-beta.....	143
Figure 3.3 LTBP-2 interacts strongly with FGF-2.....	144
Figure 3.4 FGF-2 has a single binding domain in the central region of LTBP-2.....	145
Figure 3.5 The FGF-2 binding site is close to the central heparin binding site on LTBP-2	147
Figure 3.6 LTBP-2 blocks FGF-2-induced cell proliferation	148
Figure 3.7 LTBP-2 and fibrillin-1 colocalize in fibrotic skin.....	150
Figure 3.8 LTBP-2 and FGF-2 co-localize in keloid tissue.....	151
Figure 3.9 Quantitation of LTBP-2 and FGF-2 in normal skin and keloid.	152
CHAPTER 4: Co-localization of LTBP-2 with FGF-2 in fibrotic human keloid and hypertrophic scar.....	153
4.1 Abstract.....	158
4.2 Introduction.....	159
4.3 Materials and Methods.....	162

4.3.1 Human tissue samples.....	162
4.3.2 Antibodies.....	162
4.3.3 Immunohistochemistry.....	162
4.3.4 Quantitative Polymerase Chain Reaction.....	164
4.3.5 Statistical analysis.....	165
4.4 Results.....	166
4.4.1 LTBP-2 and FGF-2 do not co-localize in fibroblast cell culture.....	166
4.4.2 LTBP-2 and FGF-2 show extensive co-localizations in fibrotic skin lesions.....	166
4.4.3 LTBP-2 shows similar distribution with elastin and fibrillin-1 in fibrotic skin ...	167
4.4.4 Both LTBP-2 and FGF-2 mRNA expression levels are greatly elevated in hypertrophic scar.....	168
4.5 Discussion.....	169
4.6 References.....	173
4.7 Figures.....	179
Figure 4.1 Immunofluorescence staining for LTBP-2 and FGF-2 in human foreskin fibroblast (HFF) cultures.....	179
Figure 4.2 LTBP-2 and FGF-2 co-localize in fibrotic skin lesions.....	180
Figure 4.3 Quantitative immunofluorescence analysis of LTBP-2 and FGF-2 in normal skin, mature scar, hypertrophic scar and keloid tissue.....	182
Figure 4.4 LTBP-2 co-localizes with fibrillin-1 and elastin in fibrotic skin.....	183
Figure 4.5 The expression levels of mRNA for LTBP-2, FGF-2 and TGF- β in fibrotic skin tissues.....	184
4.8 Supplementary figures.....	185
Figure 4.6 Specificity of LTBP-2 and FGF-2 antibodies.....	185
Figure 4.7 Specificity of secondary antibodies and laser channels.....	186
CHAPTER 5: LTBP-2 stimulates the expression of TGF-β in human fibroblasts via Akt & p38 MAPK signalling pathways.....	187
5.1 Abstract.....	192
5.2 Introduction.....	193
5.3 Materials and Methods.....	196
5.3.1 Reagents.....	196
5.3.2 Expression and purification of recombinant LTBP-2 and fragments.....	196
5.3.3 Cell culture and treatment.....	196
5.3.4 Detection and quantitation of TGF- β	197

5.3.5 Real-time PCR	198
5.3.6 Measurement of signal phosphorylation	198
5.3.7 Statistical analysis	199
5.4 Results	200
5.4.1 Exogenous LTBP-2 stimulates expression and secretion of TGF- β 1 in MSU 1.1 fibroblasts.....	200
5.4.2 LTBP-2 stimulation of TGF- β synthesis and secretion is dose-dependent.	201
5.4.3 Time course for LTBP-2 stimulation of TGF- β upregulation	202
5.4.4 A short exposure to exogenous LTBP-2 is sufficient to stimulate TGF- β upregulation	202
5.4.5 The TGF- β stimulating activity maps to a central region of LTBP-2 consisting of an eight -cys motif flanked by pairs of EGF-like repeats.	203
5.4.6 Induction of Akt and p38 phosphorylation by LTBP-2	204
5.4.7 LTBP-2 stimulates the expression of TGF- β via Akt and p38 MAPK signalling pathways	205
5.4.8 Blocking of integrin α V β 3 receptors partially attenuates TGF- β production by LTBP-2	205
5.5 Discussion	207
5.6 References.....	213
Figure 5.1 Exogenous LTBP-2 increases TGF- β in conditioned medium which is independent of extracellular matrix.	220
Figure 5.2 LTBP-2 upregulates TGF- β expression in MSU 1.1 cells	221
Figure 5.3 LTBP-2 upregulates TGF- β in a dose-dependent manner.....	222
Figure 5.4 Time course for LTBP-2 stimulation of TGF- β production.	223
Figure 5.5 A short incubation of MSU 1.1 cells with LTBP-2 is sufficient to upregulate TGF- β expression and secretion.	224
Figure 5.6 A central region of LTBP-2 consisting of an 8-cys motif flanked by pairs of EGF-like repeats (fragment LTBP-2C F3) contains the stimulatory activity.....	225
Figure 5.7 Exogenous LTBP-2 stimulates phosphorylation of AKT and p38 MAPK in human fibroblasts.....	226
Figure 5.8 LTBP-2 stimulation of TGF- β upregulation involves Akt and p38 MAPK signalling pathways.....	227
Figure 5.9 Blocking of integrin α V β 3 receptors partially attenuates TGF- β production induced by LTBP-2.....	228
Figure 5.10. Schematic representation of possible signalling pathways involved in the production of TGF- β 1 by LTBP-2.....	229

CHAPTER 6: Discussion.....	230
6.1 LTBP-2 plays an important role in elastinogenesis and elastic fibre assembly.....	230
6.2 Role of LTBP-2 in the regulation of growth factor activities	233
6.3 Co-localization of LTBP-2 and FGF-2 in fibrotic human tissues.....	234
6.4 LTBP-2 stimulates the expression and secretion of TGF- β	236
6.5 The domain organization of the human LTBP-2 protein.....	243
6.6 References	245
CHAPTER 7: Conclusions and Future Directions.....	258
7.1 Conclusion	258
7.2 Future directions	258

Summary

LTBP-2 is tightly associated with fibrillin microfibrils and elastic fibres in a range of tissues mainly in the lung, heart, skeletal muscle, placenta, liver and the aorta. LTBP-2 belongs to the fibrillin-LTBP superfamily of extracellular matrix proteins. Unlike other LTBPs, LTBP-2 does not covalently bind TGF-beta and its molecular function remains unclear. LTBP-2 complexes with fibulin-5, an elastin-chaperone protein critical for normal elastic fibre assembly, and it has been suggested that LTBP-2 may preferentially direct fibulin-5-elastin globules onto fibrillin-1 (rather than fibrillin-2) microfibrils during elastinogenesis. However, we have now shown that LTBP-2 inhibits rather than enhances the interaction of tropoelastin with fibulin-5 in vitro. In addition LTBP-2 inhibited elastic fibre assembly in ear cartilage chondrocyte cultures largely at the stage of elastin deposition onto the fibrillin microfibril scaffold. In parallel experiments, LTBP-2 was shown to significantly inhibit the binding of heparin to tropoelastin suggesting LTBP-2 may compete with tropoelastin for binding to certain cell surface HSPGs and contribute to controlling the release of elastin microassemblies from the cell surface. Confocal microscopy showed strong co-distribution of LTBP-2 with fibulin-5 and fibrillin-1 and partial co-distribution with HSPGs, perlecan and syndecan-4 in fibroblast matrix. Thus it is evident that LTBP-2 is a negative modulator of elastinogenesis and that LTBP-2 levels may regulate the rate and extent of elastinogenesis in some tissues.

A recent study has linked LTBP-2 gene mutations to recessive form of Weill-Marchesani syndrome which is characterised by short stature, thick fibrotic skin and ectopia lentis. Since fibrillin-1 mutations can also cause this syndrome it is now clear that LTBP-2 is linked to fibrillin biology, growth factor regulation and fibrosis. To investigate growth factor binding to LTBP-2, our laboratory screened a number of cytokines involved in the pathogenesis of fibrotic disorders and identified a very strong specific interaction of FGF-2. The activity was confined to a central region of the LTBP-2 consisting of 6 EGF-like repeats, suggesting a single binding sequence. The finding presented in this thesis found that 5-fold molar excess LTBP-2 can completely block FGF-2 stimulation of fibroblast proliferation via its receptor. In addition increased levels and extensive co-localisation of LTBP-2 and FGF-2 were observed and quantitated in human hypertrophic scars and keloids. Furthermore, qPCR confirmed consistent elevation of LTBP-2 and FGF-2 expression in samples of these fibrotic tissues. The results

support the concept that increased LTBP-2 expression in fibrotic disorders may increase FGF-2 binding and reduce FGF-2 activity, inhibiting normal repair processes.

Previously we have shown that LTBP-2 competes with LTBP-1 for binding to fibrillin *in vitro*, suggesting that LTBP-2 may modulate TGF- β storage and activation. In experiments designed to measure displacement of TGF- β complexes from fibrillin microfibrils, our laboratory discovered addition of LTBP-2, or a small bioactive fragment LTBP-2C F3 to MSU 1.1 skin fibroblasts resulted in a large increase in TGF- β levels in culture medium. However the increase in TGF- β the medium was cycloheximide sensitive indicating elevated cellular expression and secretion of TGF- β rather than release of matrix-stored TGF- β . Exogenous LTBP-2 or fragment F3 significantly increased levels of latent TGF- β in the medium after 9h peaking at 15h. The signalling mechanism appears to involve the PI3K/Akt and p38 MAPK pathways, as incubation of cells with LTBP-2 (10 μ g/ml) elevated Akt 1/2/3 Ser473 and P38 D-8 phosphorylation and inhibition of each pathway completely blocked the synthesis of TGF- β . Investigation of the cell surface receptor for the bioactive fragment of LTBP-2 was less informative. Inhibitory antibody to β 1 integrins did not affect the TGF- β upregulation but it was partially inhibited by an antibody to the integrin α V β 3 receptor, suggesting it may be involved in LTBP-2-cell interaction(s) resulting in elevated TGF- β expression.

In conclusion, these findings are consistent with LTBP-2 having novel regulatory functions in elastinogenesis, growth factor modulation and fibrosis which may lead to novel therapy development for fibrotic diseases and tissue repair.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due references has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Beyond this, I do not wish to place any restriction on access to this thesis.

.....

(Mohamed Arshad Mohamed Sideek)

1154273 The University of Adelaide

Date: 15/04/2016

Acknowledgements

I am very grateful to a lot of people for their support throughout my Ph.D journey and the production of this thesis. The highest gratitude to my primary supervisor, **Dr. Mark Gibson** for his constant guidance and encouragement. Thank you for being patience and understanding, and for standing by my side when times get hard. Thank you for teaching me that every mistake is just a learning experience. I am forever indebted to the best supervision over the last four years. I really appreciate it from the bottom of my heart. I would like to thank my co-supervisor, **Prof. Allison Cowin**, and his research team especially **Dr. Zlatko Kopecki**, for the opportunity to work in their reputable laboratory. I thank the fantastic members of the Gibson's lab, **Mahroo Parsi**, **Clementine Menz**, and **Josh Smith** for their help and advice throughout my Ph.D work.

I wholeheartedly thank the greatest gift I have ever had, my mother, **Fazila Begum (Amma)** for her warm hugs, pleasing smiles and encouraging words, and for all the countless times she has been there for me. To my wonderful dad, **Mohamed Sideek (Atta)**, I could not begin to list all the ways his love has made all the difference in my life. Amma and Atta, I am blessed to have both of you in my life, I love you so much! To my one and only brother and sister, **Jamal Mohideen** and **Nur Saminah**, I cannot thank you enough for their amazing support, unconditional love and care. I could not imagine my life without you.

My deepest appreciation belongs to my wife and my soul mate, **Noor Shafiq**, who has always been my strength. I do not know how I can ever thank you for being such a loving and caring person. Thank you to other family members, **Abdul Hadi** (grandfather), **Mumtaj Begum** (grandmother), **Haji Mohamed** (father-in-law), **Fathimunnisa** (mother-in-law), **Mohamed Zahirudin** (brother-in-law), **Thilsath Yasmine** (sister-in-law), **Imthiyaz** (brother), **Rosmah akka** (sister), **Mohamed Hussain** (uncle), **Jalifah Nachiya** (aunty), **Ruknudin mama** (uncle), **Sajeetha mami** (aunty) and **Dato' Haji Mohamed Mustafa** (uncle). Without your persistent love and dedication over the past years, none of this would have been possible.

Special thanks to **Iqbal Jamaludin** and **Azuwan Musa**, who have been an awesome friends and colleagues. Thank you for always being a good listener and making me laugh when I had almost forgotten how to do so.

Publication Arising During PhD Candidature

CHAPTER 2

LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis

Mohamed A. Sideek, Clementine Menz, Mahroo K. Parsi, Mark A. Gibson

Matrix Biology 34 (2014) 114-123 (Impact factor: 5.074)

LTBP-2 inhibits elastin and fibrillin assembly in matrix of fetal bovine ear cartilage chondrocytes

Mohamed A. Sideek and Mark A. Gibson

(manuscript in preparation)

CHAPTER 3

LTBP-2 has a single high-affinity binding site for FGF-2 and blocks FGF-2-induced cell proliferation

Clementine Menz, Mahroo K. Parsi, Julian R.J. Adams, **Mohamed A. Sideek**, Zlatko Kopecki, Allison J. Cowin, Mark A. Gibson

PLOS ONE 10(8): e013557 (Impact factor: 3.234)

CHAPTER 4

Co-localization of LTBP-2 with FGF-2 in fibrotic human keloid and hypertrophic scar

Mohamed A. Sideek, Abdulrahman Teia, Zlatko Kopecki, Allison J. Cowin, Mark A. Gibson

Journal of Molecular Histology (Impact factor: 1.815)

CHAPTER 5

LTBP-2 stimulates the expression of TGF- β via Akt & p38 MAPK signalling pathway in human fibroblast

Mohamed A. Sideek, Josh Smith, Clementine Menz, Julian R.J. Adams, Allison J. Cowin, Mark A. Gibson

Matrix Biology (Impact factor: 5.074) (manuscript in preparation)

Scientific Communications

INTERNATIONAL:

2011

The Elastin and Elastic Fibers Gordon Research Conference, University of New England, Biddeford, ME, United States (oral and poster)

NATIONAL AND LOCAL:

2015

Australian Society of Medical Research (ASMR) South Australia Annual Scientific Meeting, National Wine Centre, Adelaide, Australia (poster)

2014

Matrix Biology Society of Australia and New Zealand (MBSANZ) 38th Annual Scientific Meeting, Queenscliff, Victoria, Australia (oral and poster)

Florey International Postgraduate Research Conference, Faculty of Health Sciences (FHS) Postgraduate Research Conference 2014, The University of Adelaide, Adelaide, Australia (poster)

Australian Society of Medical Research (ASMR) South Australia Annual Scientific Meeting, Adelaide Convention Center, Adelaide, Australia (poster)

2013

Matrix Biology Society of Australia and New Zealand (MBSANZ) 37th Annual Scientific Meeting, McCracken Country Club, South Australia, Australia (poster)

Faculty of Health Sciences (FHS) Postgraduate Research Conference 2013, Adelaide, Australia (poster)

Australian Society of Medical Research (ASMR) South Australia Annual Scientific Meeting, Adelaide Convention Center, Adelaide, Australia (poster)

1st Malaysian Postgraduate Student Symposium of South Australia 2013, Adelaide, Australia (oral and poster)

2012

Matrix Biology Society of Australia and New Zealand (MBSANZ) 36th Annual Scientific Meeting, Mantra Legends, Gold Coast, Queensland, Australia (poster)

Postgraduate Research Expo, Faculty of Health Science, University of Adelaide, The National Wine Centre, Adelaide, South Australia, Australia (poster)

Australian Society of Medical Research (ASMR) South Australia Annual Scientific Meeting, Adelaide Convention Center, Adelaide, Australia (poster)

School of Medical Sciences, The University of Adelaide. PhD Introductory Seminar (oral)

Awards and Achievements During Candidature

2014

Shortlisted for the Dennis Lowther Award at Matrix Biology Society of Australia and New Zealand 38th Annual Scientific Meeting, Queenscliff, Victoria, Australia.

School of Medical Sciences Postgraduate Travel Award (National Conference Attendance), Faculty of Health Sciences (FHS), The University of Adelaide, Australia.

2013

Shortlisted for the Dennis Lowther Award at Matrix Biology Society of Australia and New Zealand 37th Annual Scientific Meeting, Victor Harbour, South Australia, Australia.

Academic and Character Excellence Award 2013 (Anugerah Kecemerlangan Akademik dan Sahniah (AKASIA)). Australian-Malaysian Muslim Solidarity of South Australia (ISMA SA), South Australia, Australia.

2012

Shortlisted for the Dennis Lowther Award at Matrix Biology Society of Australia and New Zealand 36th Annual Scientific Meeting, Mantra Legends, Gold Coast, Queensland, Australia.

IIUM Scholarship (Full), Kulliyah of Allied Health Sciences, International Islamic University Malaysia (IIUM), Pahang, Malaysia.

SLAB/SLAI (PhD) Scholarship (Full), Ministry of Education (MoE), Malaysia.

Abbreviations

BCIP-	5-bromo-4-chloro-3-indolylphosphate toluidine salt
BMP-	bone morphogenetic protein
BSA-	bovine serum albumin
C-	carboxy-terminus
C-6-S-	chondroitin-6-sulphate
Ca ²⁺ -	calcium ions
CaCl ₂ -	calcium chloride
cbEGF-	calcium binding epidermal growth factor
cDNA-	complementary deoxyribonucleic acid
CHX-	cycloheximide
CS-	chondroitin sulphate
DAPI-	4',6-Diamidino-2-phenylindole dihydrochloride
ddH ₂ O-	double distilled water
DMEM-	Dulbecco's Modification of Eagles Medium
DMSO-	dimethyl sulphoxide
DNA-	deoxyribonucleic acid
DTT-	dithiothreitol
ECM-	extracellular matrix
EDTA-	ethylene diamine tetraacetic acid
EGF-	epidermal growth factor
ELISA-	enzyme-linked immuno-sorbent assay
EMILIN-	elastinmicrofibril interface located protein
FCS-	foetal calf serum
FGF-2	fibroblast growth factor -2
FGFRs	FGF receptors
FITC-	fluorescein isothiocyanate
GAG-	glycosaminoglycan
GuHCL-	guanidine hydrochloride
HAC-	heparin-albumin conjugates
HEK-	human embryo kidney
HFF-	human foreskin fibroblasts
his ₆ -	6-histidine
HS-	heparan sulphate
HSPGs-	heparan sulphate proteoglycans
HTS-	hypertrophic scar
IgG-	Immunoglobulin G
IL-	interleukin
K _d -	dissociation constant

kDa-	kiloDalton
LAP-	latency-associated protein
LLC-	large latent complex
LTBP-	latent transforming growth factor- β binding protein
LTBP-2C (H)-	LTBP-2 C-terminal
LTBP-2NT (H)-	LTBP-2 N-terminal
M-	molar
MAGP-	microfibrillar-associated glycoprotein
MAPK-	mitogen-activated protein kinases
MFS-	Marfan syndrome
min-	minutes
mM-	millimolar
MMP-	matrix metalloprotease
mRNA-	messenger RNA
N-	amino-terminus
NaCl-	sodium chloride
NBCS-	new born calf serum
NBT-	nitro-blue tetrazolium chloride
NEAA-	non-essential amino acids
ng-	nanogram
Ni-	nickel
NRS-	normal rabbit serum
PBS-	phosphate-buffered saline
PCR-	polymerase chain reactions
PG-	proteoglycan
PVDF-	polyvinylidene difluoride
r-	recombinant
RGD-	arginine-glycine-aspartic acid motif
RNA-	ribonucleic acid
RT-	room temperature
SD-	standard deviation
SDS-PAGE-	sodium sulphate polyacrylamide gel electrophoresis
SLC-	small latent complex
SMA-	smooth muscle actin
SMC-	smooth muscle cell
TBS-	tris buffered saline
TGF- β -	transforming growth factor- β
TIMP-	tissue inhibitor of metalloproteinases

TMB-	tetramethylbenzidine
v or vol-	volume
w-	weight
WMS-	Weill-Marchesani syndrome
α -	alpha
$\alpha V\beta 3$ -	alpha V beta 3
$\alpha V\beta 5$ -	alpha V beta 5
β -	beta
μ l-	microliter
Δ	delta
8-cys-	8-cysteine containing motif

CHAPTER 1: The Role of LTBP-2 in Elastinogenesis and Fibrotic Diseases

1.1 The extracellular matrix (ECM) and its components

The extracellular matrix (ECM) is a compilation of various molecules that surround cells, and provide structural and functional integrity to connective tissues and organs (Schultz *et al.*; Yanagishita, 1993; Gentili and Cancedda, 2009; Gillies and Lieber, 2011). The ECM is not simply a uniform scaffold; it is a complex mixture of structural molecules, including collagens, glycoproteins, proteoglycans and elastin (Hohenester and Engel, 2002; Halper and Kjaer, 2014). These proteins serve many functions including the provision of structural support; resilience and tensile strength, scaffold for migration, and attachment sites for cell surface receptors. Moreover, these proteins also act as reservoir for signaling factors that modulate such diverse processes as angiogenesis and vasculogenesis, cell migration, cell proliferation and orientation, inflammation, immune responsiveness and wound healing (Werb, 1997; Lukashev and Werb, 1998; Badylak, 2002).

The collagen protein superfamily provides the major structural components of most connective tissues and the most abundant insoluble proteins of the ECM (Kjaer *et al.*, 2009; Ricard-Blum, 2011b). Collagens are ubiquitous proteins responsible for maintaining the structural integrity of vertebrates and many other organisms (Ricard-Blum, 2011a; Hwang *et al.*, 2014). In tissues that have to resist shear, tensile, or pressure forces, such as tendons, bone cartilage, and skin, collagen is arranged in fibrils, responsible for providing the tensile strength (Diamant *et al.*, 1972; Weber *et al.*, 1994). Only collagen type I, II, III, V, and XI self-assemble into fibrils. Some collagens form networks such as type IV, VIII, and X (Bosman and Stamenkovic, 2003). An example of such a network is the basement membrane, made of collagen IV, (together with perlecan and laminin) (Kühn, 1995; Poschl *et al.*, 2004). Basement membranes are sheet-like extracellular matrices which underlay almost all epithelia and endothelia, support soft tissue structure and acting as selective barriers to permeability (Kielty *et al.*, 2002b). Other collagens associate with fibril surfaces such as types IX, XII, and XIV. There are also transmembrane proteins (type XIII and XVIII) and collagens that form periodic beaded microfibrils (type VI) (Gordon and Hahn, 2009).

Elastic fibres are essential for the structure and function of various types of organs that require elasticity, such as large arteries, lung and skin (Sherratt, 2009a). Lung tissue has a high content of elastic fibres that provide tissues with the resilience they require to distend and contract during tidal breathing (Starcher, 2000; Shifren and Mecham, 2006). Moreover, elastic fibres allow blood vessels to stretch and recoil, maintaining a constant blood pressure and preventing the vessels from rupture (Rosenbloom *et al.*, 1993; Cain *et al.*, 2008c). Elastic fibres consist of two main components, a hydrophobic amorphous elastin core and a fibrillin containing microfibril, which acts as template for elastin deposition (Kielty *et al.*, 2002a; Shifren and Mecham, 2006). There are also other macromolecules that play essential roles in the functional and biological characteristics of the matrix and connective tissues including; proteoglycans, hyaluronan, and glycoproteins such as fibronectin, thrombospondin, and microfibrillar-associated proteins (Culav *et al.*, 1999; Bruckner-Tuderman *et al.*, 2010).

The ECM also appears to serve as a reservoir for growth factor cytokines where components of the matrix have the potential to interact with growth factors to store these cytokines in tissues and subsequently regulate their activation (Rosso *et al.*, 2004). Although growth factors were originally defined as soluble molecules, increasing evidence indicates that the binding of growth factors to the ECM is a major mechanism regulating growth factor activity (Taipale and Keski-Oja, 1997). Many of the ECM growth factors develop complex interactions with surface molecules of neighbouring cells (Aumailley and Gayraud, 1998b; Badylak, 2002). Such interactions may provide a mechanism for the local control of cellular behaviour such as cellular differentiation, tissue morphogenesis, maintenance of homeostasis, growth factor expression and cellular response to injury, indicating that the role for each ECM molecule is sophisticated and highly specific (Yamaguchi *et al.*, 1990). Due to the direct connections between cells and their milieu, any quantitative and qualitative modification of the ECM microenvironment will influence cell functions. For example, disruption of transforming growth factor-beta (TGF- β) signalling has been implicated in many human diseases, including cancer, and fibrotic, autoimmune and cardiovascular diseases (Matrisian and Hogan, 1990; Border and Ruoslahti, 1992; Yamamoto *et al.*, 1994; Ruiz-Ortega *et al.*, 2007).

1.2 Collagens

Collagens are major components of the ECM and are the most abundant proteins found in the body. Collagens are secreted by multiple cell types and at least 26 different types of collagens have been described in vertebrates, and each has distinct properties in the matrix (Bunyaratavej and Wang, 2001; Canty and Kadler, 2002; Ricard-Blum, 2011a). The collagen family represents a group of diverse molecules linked by presence of the collagen triple-helix structure as a common structural element (Brodsky and Ramshaw, 1997; Brodsky and Persikov, 2005). Collagens are trimeric molecules composed of three polypeptide α chains, which contain the sequence repeat (G-X-Y)_n, where the first position of repeat is glycine, the second and third positions of the repeat being frequently proline and hydroxyproline, allowing the formation of a triple helix (Brodsky and Ramshaw, 1997; Brodsky and Persikov, 2005). Each member of the collagen family contains at least one triple-helical domain (COL) and most collagens are able to form supramolecular aggregates (Ricard-Blum, 2011a).

Various types of collagens are involved in the formation of fibrils and microfibril networks of both basement membranes and interstitial ECM (Trelstad and Hayashi, 1979; Kadler *et al.*, 2008). The structure and function of different collagens varies significantly and multiple types of collagens are often combined to form major structural elements of the ECM. The triple helix constitutes a rod-like structure important for fibril formation and structural integrity, but it is now clear that the triple helix also interacts with a wide range of molecules important in extracellular matrix organization and function (Brodsky and Ramshaw, 1997; Brodsky and Persikov, 2005). For example, collagen IV, a network forming collagen, plays important roles in the structure and assembly of basement membranes (Miner and Sanes, 1994; Pöschl *et al.*, 2004; Khoshnoodi *et al.*, 2008). Members of the collagen IV family are found in all basement membranes and are characterized by their ability to form complex and covalently-linked structural scaffolds, required for the basement membrane assembly process (Miner and Sanes, 1994; Pöschl *et al.*, 2004; Khoshnoodi *et al.*, 2008). Type I and type III collagens, both of which are fibrillar or rod-shaped collagens, provide tensile strength to the skin and other connective tissues (Lapiere *et al.*, 1977; Epstein and Munderloh, 1978). The tensile strength of skin is due predominantly to these fibrillar collagen molecules, which self-assemble into microfibrils in a head-to-tail and staggered side-to-side lateral arrangement (Brodsky and Ramshaw, 1997; Ottani *et al.*, 2001; Brodsky and Persikov, 2005). Type VII collagen forms anchoring fibrils, attachment structures in the basement membrane zone of skin that adhere the

epidermal layer of skin onto the dermis (Sakai *et al.*, 1986c; Keene *et al.*, 1987; Burgeson, 1993). Different types of collagen are found in the matrix and each of the collagen have specific localization and functions in the extracellular matrix (Table 1.1).

Missense mutations can cause alterations in the stability of the triple helical structure of collagens, contribute to a range of heritable connective tissue disorders. For example, mutations in the type I collagen gene causes osteogenesis imperfect, mutation in type III collagen causes Ehlers-Danlos syndrome type IV, and mutation in type IV collagen causes Alport syndrome (Superti-Furga *et al.*, 1989; Hudson *et al.*, 2003; Marini *et al.*, 2007).

Type	Chain Composition	Structural Details	Localization
I	$[\alpha 1(I)]_2[\alpha(I)]$	300nm, 67nm banded fibrils	skin, tendon, bone, etc.
II	$[\alpha 1(II)]_3$	300nm, small 67nm fibrils	cartilage, intervertebral disk
III	$[\alpha 1(III)]_3$	300nm, small 67nm fibrils	Blood vessels, skin, muscle, frequently with type I
IV	$[\alpha 1(IV)]_2[\alpha 2(IV)]$	390nm C-term globular domain, nonfibrillar	all basal lamina
V	$[\alpha 1(V)][\alpha 2(V)][\alpha 3(V)]$	390nm N-term globular domain, small fibers	most interstitial tissue, assoc. with type I
VI	$[\alpha 1(VI)][\alpha 2(VI)][\alpha 3(VI)]$	150nm, N+C term. globular domains, microfibrils, 100nm banded fibrils	most interstitial tissue, assoc. with type I
VII	$[\alpha 1(VII)]_3$	450nm, dimer	epithelia; oral mucosa, cervix
VIII	$[\alpha 1(VIII)]_3$		some endothelial cells
IX	$[\alpha 1(IX)][\alpha 2(IX)][\alpha 3(IX)]$	200nm, N-term. globular domain, bound proteoglycan	cartilage, assoc. with type II
X	$[\alpha 1(X)]_3$	150nm, C-term. globular domain	hypertrophic and mineralizing cartilage
XI	$[\alpha 1(XI)][\alpha 2(XI)][\alpha 3(XI)]$	300nm, small fibers	cartilage
XII	$\alpha 1(XII)$		interacts with types I and III

Table 1.1 Details of the different types of collagens found in the matrix. Table adapted and modified from Bateman JF (1996).

1.3 Proteoglycans

Proteoglycans (PGs) are major contributors to the structure and function of the ECM. They are primarily composed of a core protein that has covalently attached one or more long unbranched polyanionic sugar side-chains known as glycosaminoglycans (GAG) (Hardingham and Fosang, 1992) (Figure 1.1). The ECM proteoglycans can be divided into four groups, which are the small leucine repeat (LRR) family (e.g. biglycan, decorin), the aggrecan/versican family, the collagen family (e.g. type IX, XII and XIV collagen) and others that are specialised and expressed in the basement membrane and neuromuscular junctions (e.g. perlecan, agrin) (Junqueira and Montes, 1983; Hassell *et al.*, 1986; Hardingham and Fosang, 1992). The protein cores of proteoglycans are rich in serine and tyrosine residues to allow for multiple GAG attachment, resulting in very complex structures of high molecular weight. GAGs are extended structures and are highly negatively charged with many sulphate and carboxylate groups and they often dominate the physical properties of the protein to which they are attached (U Lindahl and Hook, 1978; Jackson *et al.*, 1991). GAG chains are repeating disaccharide units containing either of two modified sugars, N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GLcNAc) with an uronic acid. There are five sub-classes of GAGs, defined by their sugar compositions which are hyaluronic acid (HA), dermatan sulphate (DS), chondroitin sulphate (CS), heparin, heparan sulphate (HS) and keratan sulphate (KS). HS, CS, and DS are assembled via a serine residue to the protein cores, defining them as PGs (U Lindahl and Hook, 1978; Jackson *et al.*, 1991; Sasisekharan *et al.*, 2006; Taylor and Gallo, 2006) (Figure 1.1).

Recently, heparin and heparan sulphate proteoglycans (HSPGs) have been shown to bind fibrillin-1 and are essential for formation of fibrillin-microfibrils which in turn provide a scaffold for elastin deposition (Tiedemann *et al.*, 2001b). In addition, heparan sulphate chains have been shown to interact with tropoelastin and promote the coacervation of tropoelastin to form elastin-microassemblies (Gheduzzi *et al.*, 2005). However the precise HSPGs involved in these processes are not yet clear. This study will investigate the influence of heparin/heparan sulphate proteoglycan on the assembly of elastic fibre which will be discussed in more detail in section 1.5.

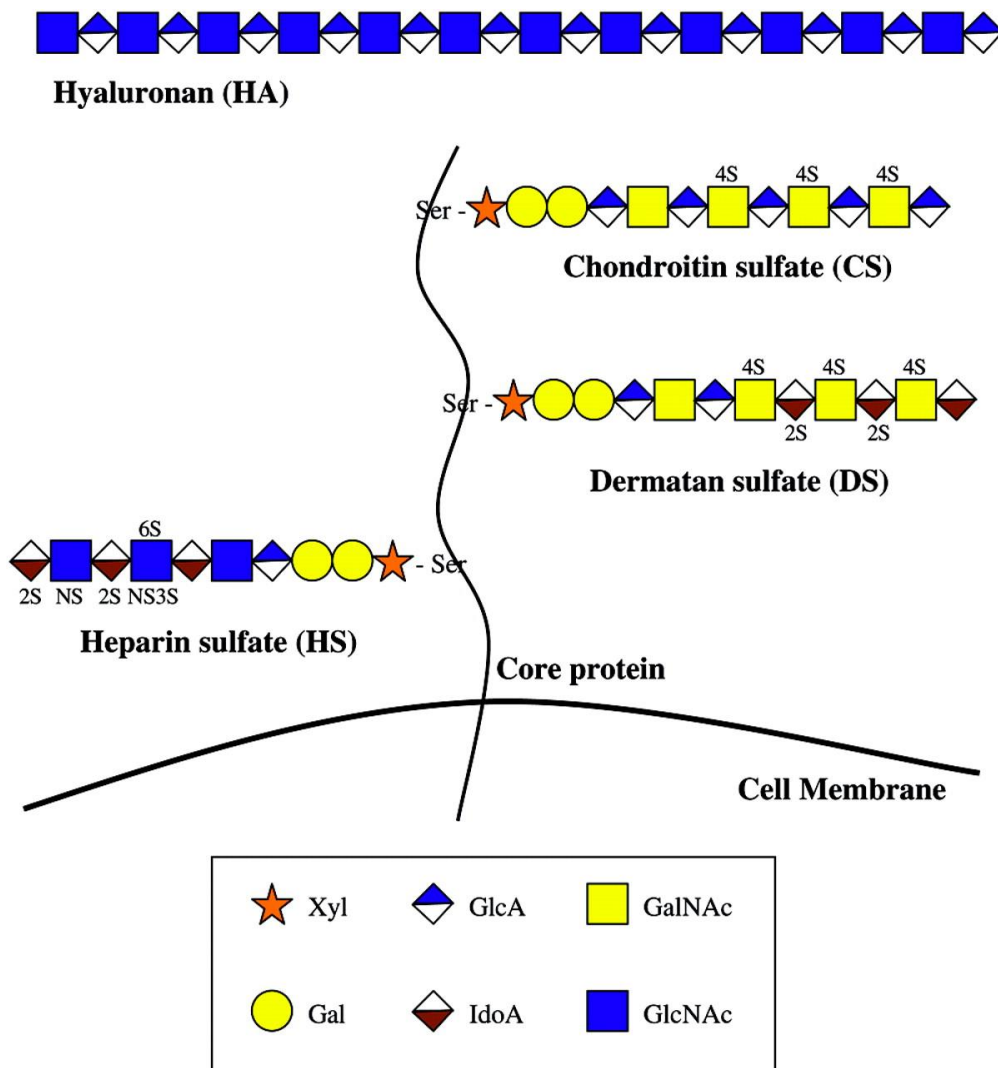


Figure 1.1 Structure of glycosaminoglycans (GAGs) and proteoglycans (PGs). Hyaluronic acid (HA) is not covalently linked to a PG, but synthesized directly into the extracellular space. Heparan sulphate (HS), chondroitin sulphate (CS) and dermatan sulphate (DC) GAG side chains are linked to a protein core via a serine residue. Members of the glycosaminoglycan family vary in the type of disaccharide unit they contain (e.g. N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), glucuronic acid (GlcA), iduronic acid (IdoA), galactose (Gal), and xylose (Xyl)). Diagram taken from Taylor and Gallo (2006).

1.3.1 Heparin/Heparan sulphate proteoglycans

There are several reports in the literature suggesting that HSPGs may participate in the formation of elastic fibres and in particular may play important role in the assembly of the microfibrillar component (Gheduzzi *et al.*, 2005). The involvement of PGs containing heparan

sulphate GAGs chains in elastic fibrogenesis has been observed, where heparin sulphates bind tropoelastin and mediate its coacervation (Wu *et al.*, 1999). Cell surface heparin sulphate proteoglycans appears to be an essential participant both for the assembly of the fibrillin microfibrils and for elastin deposition onto the microfibrillar scaffold during elastic fibre assembly (Tiedemann *et al.*, 2001a; Cain *et al.*, 2005a; Cain *et al.*, 2008a; Cain *et al.*, 2008c).

Heparan sulphate as an anionic polymer composed of a repeated disaccharide structure which is modified to varying degrees by N-deacetylation and N-sulfation of the glucosamine, and by additional O-sulfation on both sugars (Sasisekharan *et al.*, 2006). This anionic polymer is comprised of 50-200 disaccharide units covalently attached to specific protein core to form proteoglycans at cell surface (e.g. syndecans) or in the ECM (e.g. perlecan) (Kramer and Yost, 2003). Structurally, heparin is distinguished from heparan sulphate by being more heavily modified, consisting of an abundance of disaccharides modified with N- and O-sulfation along its entire length, instead of being clustered as is usually found in heparan sulphate (Taylor and Gallo, 2006). In addition, heparin is less abundant *in vivo*, compared to heparan sulphate which is more predominantly found on cell surfaces and in the ECM as part of a PG (Cohlberg *et al.*, 2002; Rose and Page, 2004).

So far, three broad classes of HSPGs have been identified which are those that are secreted by the cells and located in the ECM such as perlecan and agrin; those associated with the cell surface such as glypicans, and the transmembrane HSPGs such as syndecan family (syndecan-1, -2, -3 & -4) (Bernfield *et al.*, 1992; Kim *et al.*, 1994; Fuki *et al.*, 1997). Fibrillin-1 is a major extracellular heparin/heparan sulphate binding molecules and four high affinity heparin-binding regions on fibrillin-1 have been identified (Cain *et al.*, 2005b; Cain *et al.*, 2008b; Kirn-Safran *et al.*, 2009). It was shown by blocking heparan sulphate attachment to core proteins with β -D-xylosides (inhibiting the attachment the glycosaminoglycans to core protein) and blocking glycosaminoglycans sulfation, disruption of microfibril assembly has been observed indicating the importance of the heparan sulphate proteoglycans during microfibril assembly (Cain *et al.*, 2005a; Cain *et al.*, 2008b). Recent published data from our lab show that LTBP-2 binds strongly to syndecan-4 and perlecan, suggesting these molecules act as candidates for the cell surface interaction during elastic fibres assembly (Parsi *et al.*, 2010) and will discuss in detail later.

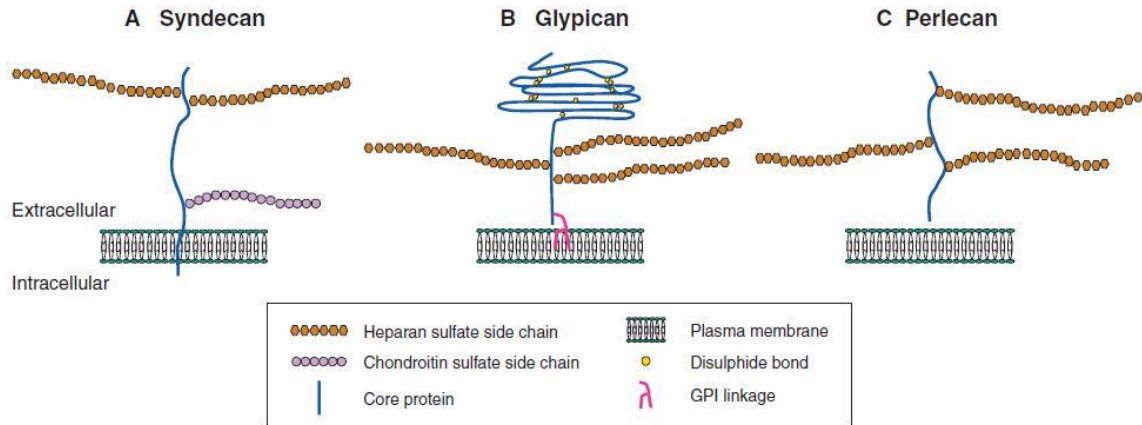


Figure 1.2 The three main classes of cell-surface heparan sulphate proteoglycans (HSPGs). (A) Syndecans are a family of transmembrane core proteins that contain a highly conserved C-terminal cytoplasmic domain and an extracellular domain capable of carrying HS and CS chain. (B) The glypican core protein is attached to the cell membrane covalently via a glycosylphosphatidylinositol (GPI) anchor through the C-terminus of the protein. (C) Perlecan, a basement membrane-type HSPG that carries HS chains (Lin, 2004).

1.4 Glycoprotein

Glycoproteins are proteins covalently bound to a carbohydrate consisting of amino-sugars, acidic sugars or neutral sugars all of which are very hydrophilic. In contrast with proteoglycans, glycoproteins have much shorter truncated carbohydrate chains with greater diversity of carbohydrate types (Pierce and Parsons, 1981; Dean *et al.*, 1991; Mark, 2002). Glycoproteins generally have structural and adhesive properties, which are able to mediate cellular activities both directly through interaction with cell surface and indirectly by organising a defined 3-dimensional extracellular matrix which is essential for most cells to express their tissue specific features (Pierce and Parsons, 1981; Dean *et al.*, 1991; Mark, 2002). Some of the glycoproteins which have adhesive properties are fibronectins, thrombospondin, and laminins (Pierce and Parsons, 1981; Dean *et al.*, 1991; Mark, 2002).

Fibronectin is an extracellular matrix protein that acts as a substrate for cell migration and adhesion during development (Dean *et al.*, 1991). Moreover, fibronectin is an important glycoprotein which is required for normal cell growth (Clark *et al.*, 1982; Pankov and Yamada, 2002; Mao and Schwarzbauer, 2005). Fibronectin consists of a soluble dimer with molecular weight of 540 kDa in plasma or as fibrillar form on the cell surface in the interstitial space.

Fibronectin is one of the first glycoproteins to be produced during the formation of the matrix, associated with cell surface integrin receptors (Mao and Schwarzbauer, 2005). Wide ranges of biological activities of fibronectin have been identified through *in vitro* and *in vivo* studies such as binding to integrin, and protecting cells from apoptosis due to stresses caused by the lack of growth factors (George *et al.*, 1993; Giancotti and Ruoslahti, 1999). Fibronectin has been shown to play a critical role in early development, since fibronectin knockout mice have an embryonic lethal phenotype (George *et al.*, 1993; Hines *et al.*, 1994). In addition, extracellular assembly of fibrillin microfibrils is markedly reduced in a fibronectin mutant mouse strain, indicating that interactions between fibronectin and fibrillins is important for elastic fibres formation (Kinsey *et al.*, 2008b; Kinsey *et al.*, 2008a; Sabatier *et al.*, 2009).

Like some proteoglycans, certain glycoproteins also have important roles in elastic fibres, such as fibulin-5, LTBP-2 and fibrillins (Sakai *et al.*, 1986a; Kielty *et al.*, 2002a; Nakamura *et al.*, 2002; Yanagisawa *et al.*, 2002; Rock *et al.*, 2004; Hirai *et al.*, 2007), which have specific implications for the present study. Fibrillins, play an essential role in the ECM, polymerising to form the major structural element structure of 10nm microfibrils. Fibrillins polymerize into a characteristic beads-on-a-string structure, which gives rise to the microfibril lattice by lateral association of the polymers (Kielty and Shuttleworth, 1995; Handford *et al.*, 2000; Ramirez *et al.*, 2004). They have adhesive properties as well as being a structural component of the matrix (Pfaff *et al.*, 1996; Bax *et al.*, 2003; Bax *et al.*, 2007). Moreover, fibrillin-microfibrils play a role in targeting growth factors by acting as storage depots in ECM (Charbonneau *et al.*, 2004; Sengle *et al.*, 2008; Sengle *et al.*, 2012).

1.5 Elastic fibres

1.5.1 Elastin

Elastin is a highly insoluble extracellular matrix protein and the core protein of the elastic fibres. Elastic fibres are major class of extracellular matrix fibres that are abundant in dynamic connective tissues. Their structural role is to provide resilience to elastic tissues such as skin, ligaments, lungs, and arterial walls (Montes, 1996; Kielty *et al.*, 2002a; Sherratt, 2009c). This allows the tissues to stretch and recoil without damage (Mecham, 1994; Kielty, 2006; Sherratt, 2009b). Moreover, they also act as an important adhesion template for cells and are essential in the regulation and activation of growth factors such as TGF- β (Dallas *et al.*, 1995; Kielty, 2006).

A study using an *in vivo* model has shown that mice lacking the elastin gene die within a few days of birth from vascular obstruction due to vascular smooth muscle cell over proliferation. The vessel wall is thickened and smooth muscle cells are misarranged at the time of death in these mice indicating elastin plays an important role in vascular (Li *et al.*, 1998a; Li *et al.*, 1998b). Loss of elasticity is observed in a range of serious diseases or age-related lesions, such as emphysema, arteriosclerosis, and skin aging (Rosenbloom *et al.*, 1993) (Table 1.2). A major health problem, emphysema, common form of obstructive pulmonary disease (COPD) is generally characterised slowly progressive airflow obstruction. In emphysema the elastic fibres in alveolar ducts and sacs are degraded causing loss of elasticity in small airways causes the development of airflow obstruction (Stone *et al.*, 1995; Santos *et al.*, 2002; Black *et al.*, 2008). Reduced amounts of elastin also contribute to the development of supra-aortic stenosis (SVAS), a congenital disorder caused by mutation in the elastin genes. SVAS is characterized by vascular abnormalities that caused systemic hypertension, myocardial infarction, cerebrovascular accident and obstructive cardiomyopathy with heart failure (Curran *et al.*, 1993; Ewart *et al.*, 1994; Li *et al.*, 1997a; Arteaga-Solis *et al.*, 2000). Vascular lesions showed disorganized, irregular and thickened elastic fibres, caused by quantitative deficiency of elastin. Reduced amounts of elastin may make the elastic fibres more susceptible to hemodynamic stress and damage (Curran *et al.*, 1993; Ewart *et al.*, 1994; Li *et al.*, 1997b; Arteaga-Solis *et al.*, 2000).

In addition to the mechanical properties of elasticity, elastic fibres are unique in several other aspects. Once synthesized in early development, elastic fibres undergo very little turnover

in normal adult tissues (Urbán and Boyd, 2000). For example, the elastic fibres deposited in one's aorta as a young child are the same elastic fibres that one usually dies with. However, new elastin-fibre synthesis in a variety of elastic-tissues diseases results in the aberrant accumulation of dysfunctional elastic fibres such as hypertension, emphysema, aortic aneurysms and actinic elastosis (Milewicz *et al.*, 2000; Urban *et al.*, 2000).

While importance of elastin and fibrillin are relatively well established, effective therapeutic approaches for elastic fibre replacement in these diseases are not been discovered, because the mechanism of elastic fibre formation and maintenance still remain unclear. Moreover, little is known about how elastic fibre associated molecules such as fibulin-5, heparan sulphate proteoglycan, and LTBP-2 contribute to their assembly and function. This study will investigate in more detail how these molecules involved during the formation of elastic fibres.

The elastic properties of many tissues such as the lung, dermis, and large blood vessels are dependent on the correct assembly of elastic fibres. These fibers have been shown by biochemical and ultrastructural analysis to be composed of two distinct components, a more abundant amorphous elastin and a 10-12 nm microfibrillar component made of fibrillins, which is located primarily around the periphery of the amorphous component (Cleary, 1996; Kielty *et al.*, 2002a; Cain *et al.*, 2008c).

The protein elastin makes up the highly insoluble amorphous component and approximately 90% of the mature fibre, which form a central core (Cleary, 1996; Mithieux and Weiss, 2005). Unlike all other families of the ECM macromolecules, there is only one elastin gene (Cleary, 1996; Debelle and Tamburro, 1999; Mithieux and Weiss, 2005). The 72-kDa biosynthetic precursor, tropoelastin, is secreted into the extracellular space where it becomes highly cross-linked into a rubber-like network through the activity of the copper-requiring enzyme lysyl oxidase (Narayanan *et al.*, 1978; Kozel *et al.*, 2004; Clarke *et al.*, 2006). In each tropoelastin monomer, approximately 35 of the 40 lysine residues are involved in cross-linking, which gives elastin a high degree of stability and insolubility (Wagenseil and Mecham, 2007). Moreover, the highly cross-linked structure of elastin contributes to its ability to stretch and recoil to its original shape repeatedly (Vrhovski *et al.*, 1997; Mithieux and Weiss, 2005). Aggregated tropoelastin molecules are deposited onto performed microfibrillar templates made of fibrillins (Kozel *et al.*, 2004; El-Hallous *et al.*, 2007). However, the exact mechanism of

elastin deposition onto microfibrils still unclear. Many other macromolecules are believed to be involved in the formation of elastic fibres such as LTBP-2, fibulin-5 and heparan sulphate proteoglycans and these will be discussed in more detail in sections 2.3, 2.4 and 2.5.

1.5.2 Fibrillin-microfibrils

As mentioned previously, TGF- β s are stored in latent form anchored to ECM particularly on fibrillin-rich microfibrils. Fibrillin molecules have been identified as the primary component of the 10-12nm elastin-associated microfibrils found within the connective tissues, and to date, three members of the fibrillin family, fibrillin-1, -2, -3, have been identified (Sakai *et al.*, 1986b; Corson *et al.*, 1993; Zhang *et al.*, 2005). Structurally, fibrillins are 350kDa glycoproteins that show a high degree of sequence homology (Ramirez and Sakai, 2010). All three fibrillins are expressed during human development, but fibrillin-1 is the most abundant isoform in adult tissues (Quondamatteo *et al.*, 2002; Corson *et al.*, 2004). Fibrillin-1 and fibrillin-2 are both extended linear molecules that have been identified within microfibrils as components of elastic fibres and in elastin-free bundles (Sakai *et al.*, 1991). Fibrillin-1 has widespread expression patterns within the extracellular matrix including skin, lung, tendon, kidney, cornea, and muscle (Sakai *et al.*, 1986b). In contrast to fibrillin-1, fibrillin-2 & fibrillin-3 are mainly expressed in foetal tissues associated with microfibrils close to amorphous elastin. Interestingly, fibrillin-3 is not present in rodents and this suggests that fibrillin-3 is not important for normal development (Corson *et al.*, 2004).

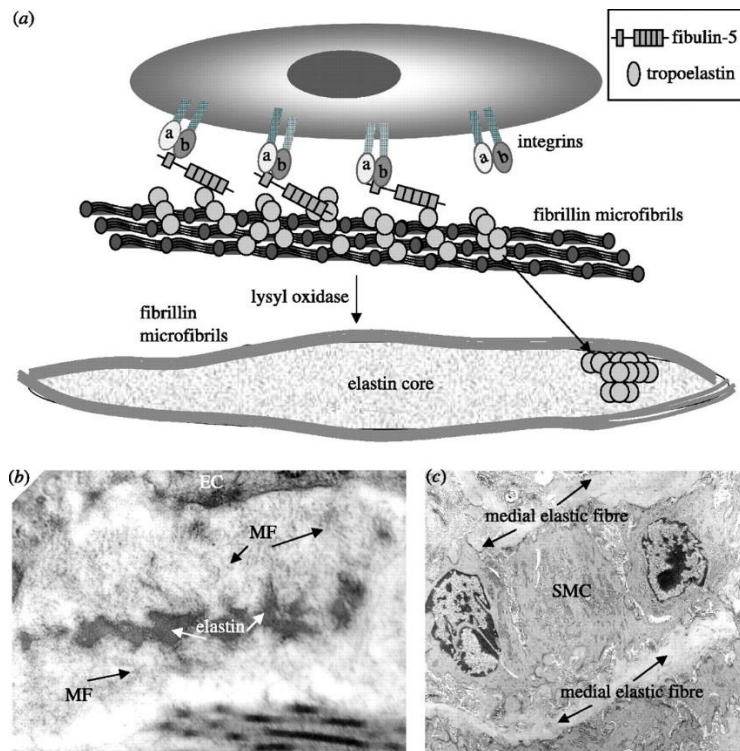


Figure 1.3 Elastic fibre formation (a) Diagram illustrating the deposition of tropoelastin onto fibrillin microfibrils in potential association with fibulin-5. (b) Transmission electron micrograph of elastic fibre structure in the subendothelium. The elastin core is surrounded by microfibrils (MF). (c) Electron micrograph displaying mature elastic fibre lamellae surrounding a medial smooth muscle cell (SMC) (Kielty *et al.*, 2007).

Generally, fibrillins do not function in tissue as monomers; instead they form into microfibrillar polymers, acting as a scaffold or template for elastin deposition (Wagenseil and Mecham, 2007). Microfibrils usually form non-striated fibrils with a uniform diameter and are found in both elastic and non-elastic tissues (Sakai *et al.*, 1991). Microfibrils mainly consist of fibrillin-1 and fibrillin-2, together with several kinds of proteins that are associated with them, such as latent transforming growth factor β -binding proteins (LTBPs) (Gibson *et al.*, 1995; Taipale *et al.*, 1995) and microfibrils-associated glycoproteins (MAGPs) (Gibson *et al.*, 1986; Gibson *et al.*, 1996). Recently, a mouse model has been used to demonstrate that the loss of fibrillin-1 containing microfibrils leads to several phenotypic features that may result from TGF- β activation (Holm *et al.*, 2011). A deficiency of fibrillin-1 gene expression in mice leads to TGF- β -mediated failure resulting in apoptosis in developing lung (Neptune *et al.*, 2003). Overall, the loss of fibrillins indicates the latent form of TGF- β is activated inappropriately and highlights fibrillins as crucial components of matrix that regulate TGF- β bioavailability. The

dysregulation of TGF- β activation and signaling could occur from loss of interaction between the fibrillins and LTBP_s since LTBP_s are covalently bound to the latent inactive form of TGF- β and have been shown to interact with fibrillins (Isogai *et al.*, 2003; Holm *et al.*, 2011).

1.6 Fibulins

Fibulins are a family of glycoproteins with distinctive structures containing a series of endothelial growth factor (EGF)-like repeat motifs and a globular C-terminal domain (Argraves *et al.*, 1990; Timpl *et al.*, 2003). The fibulin family consists of five 50-200kDa proteins which are widely distributed in tissues and are often associated with vasculature and elastic tissues (Kobayashi *et al.*, 2007). The five-member family can be further classified into two subgroups (figure 2.4). The first subgroup, fibulin-1 and fibulin-2 are considerably larger (90-200 kDa) than other three members of family because of the higher numbers of calcium binding EGF modules and the presence of an extra N-terminal domain. The members of the second subgroup, fibulin-3, -4, and -5, are similarly small in size (50-60 kDa) and highly homologous to one another in modular structure (figure 2.4) (Kobayashi *et al.*, 2007; de Vega *et al.*, 2009). All members of fibulin family consist of modules grouped into domains I, II, and III where domain I represents the N terminus which is variable between the family members, domain II represents the central portion consist of variable number of EGF-like modules in a tandem array, and domain III represents the C-terminal portion called the fibulin type module (Aumailley and Gayraud, 1998a; Chu and Tsuda, 2004; de Vega *et al.*, 2009)

Of the five fibulins, only two have been found to be essential for elastic fibre assembly. Fibulin-4 has been shown to colocalise with microfibrils (Choudhury *et al.*, 2009; Horiguchi *et al.*, 2009) and fibulin-5 has been demonstrated to colocalise with elastic fibers (Nakamura *et al.*, 2002; Freeman *et al.*, 2005; Nonaka *et al.*, 2009). Unlike fibulin-4 and fibulin-5 deficient mice (see below), the absence of fibulin-1, fibulin-2 or fibulin-3 in mice does not have apparent effects on elastic fibre homeostasis (Kostka *et al.*, 2001; Timpl *et al.*, 2003; de Vega *et al.*, 2009). However recently, both fibulin-4 and fibulin-5 have been shown to interact with fibrillin-1, the major component of the microfibril associated with elastic fibers (Freeman *et al.*, 2005; El-Hallous *et al.*, 2007) suggesting these two fibulins likely serve as connecting proteins between elastin and fibrillin microfibrils, by aiding in the transfer of tropoelastin aggregates to microfibrils.

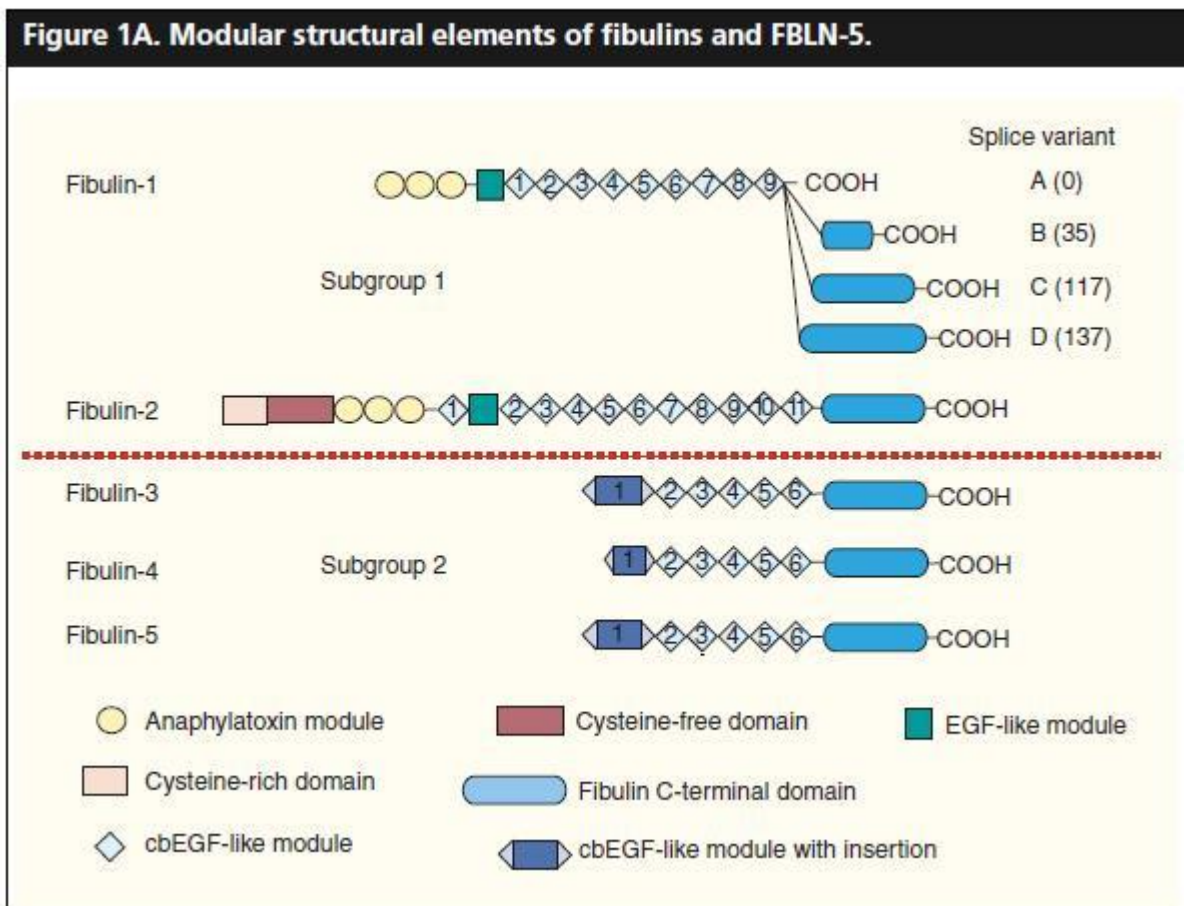


Figure 1.4 Domain structure of fibulin family proteins. The five members of the family display similar modular arrangement, consisting of domains I, II, and III (Albig and Schiemann, 2005).

Fibulin-4 is expressed by adult human fibroblasts and is located in vessel walls and basement membranes, strongly expressed in the heart, moderately in skeletal muscle, and weakly in the placenta, lungs, kidneys and brain (Giltay *et al.*, 1999; de Vega *et al.*, 2009). Fibulin-4 knockout mice do not develop intact elastic fibres, and exhibit lung and vascular defects such as emphysema, aneurysm, and haemorrhages, indicating a key role of fibulin-4 in vascular homeostasis (McLaughlin *et al.*, 2006a; Hanada *et al.*, 2007). In addition, defects in fibulin-4, such as the missense mutation G169A causes autosomal recessive cutis laxa characterised by loose skin and organs enriched in elastic fibers, such as the lung and the arteries, resulting from paucity and fragmentation of elastic fibers (Huchtagowder *et al.*, 2006; McLaughlin *et al.*, 2006b). Recent studies have shown that strong binding between fibulin-4 and lysyl oxidase enhanced the interaction of fibulin-4 with tropoelastin, forming ternary complexes that may direct and contribute to the elastin cross-linking (Choudhury *et al.*, 2009).

In contrast, fibulin-5 did not bind lysyl oxidase strongly but bound tropoelastin in terminal and central region (Wachi *et al.*, 2008; Nonaka *et al.*, 2009). The structural and function of fibulin-5 will discuss in more detail in section 2.3.1.

1.6.1 Fibulin-5

Fibulin-5 (molecular mass 56 kDa) is a calcium-dependent, elastin-binding protein that localises to the surface of elastic fibres in vivo (Nakamura *et al.*, 2002; Nonaka *et al.*, 2009). It also known as DANCE (developmental arteries and neural crest epidermal growth factor-like) which consists of six tandem repeats of cbEGF like domains and a C-terminal fibulin module (Nakamura *et al.*, 2002; Nonaka *et al.*, 2009). The first cbEGF like repeat of fibulin-5 also contains a RGD sequence, which mediates interaction with cell surface integrins (Preis *et al.*, 2006; Yanagisawa *et al.*, 2009). Fibulin-5 is predominantly expressed in developing arteries and has been found to be prominent in elastic-fibre-rich tissues such as lung and blood vessels (Tsuruga *et al.*, 2004; Hisanaga *et al.*, 2009). These data suggested that fibulin-5 is an important regulator of vascular development and elastic fibre assembly.

Studies of fibulin-5-deficient mice have demonstrated that fibulin-5 knockout mouse showed several vascular defects such as disorganisation of elastic fibres in the lung and aorta (Zheng *et al.*, 2006; Zheng *et al.*, 2007a; Wachi *et al.*, 2008; Choi *et al.*, 2009a). The fibulin-5 knockout mice also have severe loose skin with a remarkable near absence of dermal elastic fibres indicating an essential role of fibulin-5 in elastic fibres (Choi *et al.*, 2009b). Moreover, the importance of fibulin-5 for elastic fibre assembly has also been supported by genetic mutations found in three cutis laxa patients (Loeys *et al.*, 2002; Markova *et al.*, 2003; Hu *et al.*, 2006) fibulin-5 mutations also caused age-related macular degeneration in humans (Stone *et al.*, 2004; Lotery *et al.*, 2006; Mullins *et al.*, 2007).

Previous studies have shown that fibulin-5 interacted with fibrillin-1 microfibrils indicating it plays a role in the pericellular space during elastic fibre assembly (Freeman *et al.*, 2005). In addition, binding of fibulin-5 to the integrin receptors in adhesion assays suggests that fibulin-5 may serve to anchor tropoelastin to surrounding cells during assembly and/or final organization of functional elastic fibres (Zheng *et al.*, 2007b). Recently, studies have shown that the molecular interaction of fibulin-5 with tropoelastin was enhanced by increase in temperature and the whole tropoelastin molecules was required for the interaction with

fibulin-5 indicating that the conformational structure of tropoelastin (Wachi *et al.*, 2008). In addition, the molecules interaction of fibulin-5 with tropoelastin is also important for coacervation of the tropoelastin molecules (Wachi *et al.*, 2008).

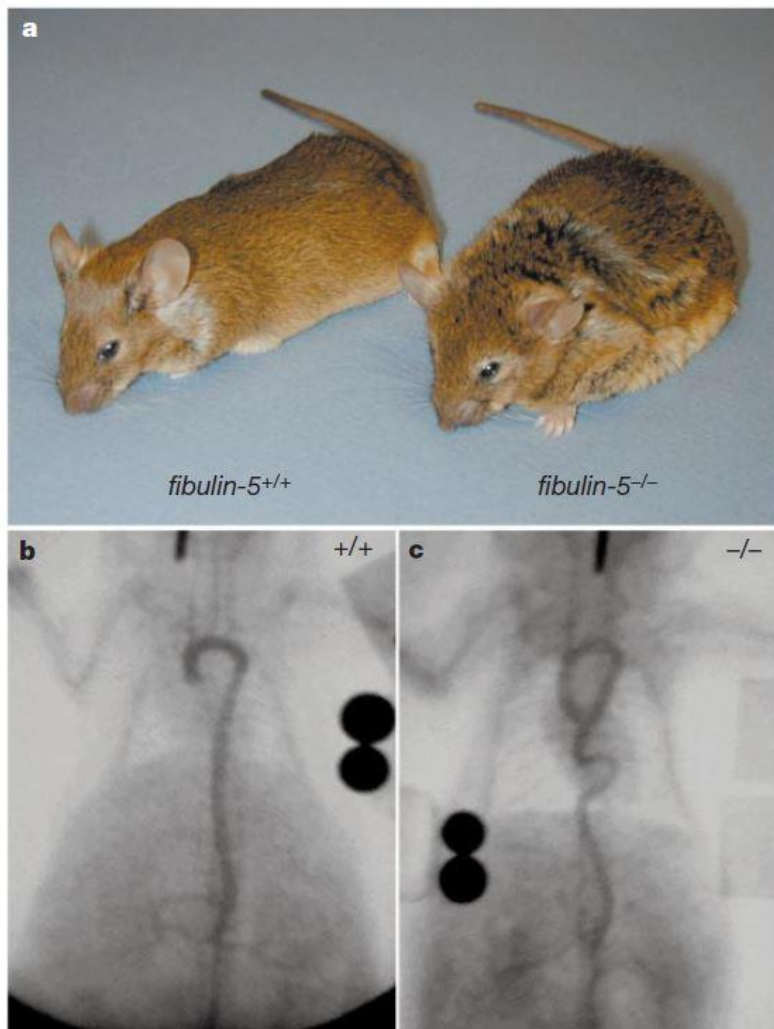


Figure 1.5 Fibulin-5 is essential for elastinogenesis. (A) Appearance of wild-type mice and fibulin-5 knockout mice. Mice deficient in in the fibulin-5 gene exhibit loose and wrinkled skin, short nose and large cheeks. (B) and (C), aortography of wild-type mice and fibulin-5 knockout mice. Fibulin-5 knockout mice have a tortuous aorta with loss of compliance (Nakamura *et al.*, 2002).

While fibulin-5 is clearly essential for normal elastic fibre formation, its precise contribution and exact mechanism of interaction during elastic fibres assembly remains unclear. Several groups have previously identified some fibulin-5 binding proteins, such as

elastin (Wachi *et al.*, 2008), fibrillin-1 (Freeman *et al.*, 2005), lysyl oxidase-like 1 (Liu *et al.*, 2004), EMILIN (Zanetti *et al.*, 2004), apolipoprotein (Kapetanopoulos *et al.*, 2002), and extracellular superoxide dismutase (Nguyen *et al.*, 2004). Recently, fibulin-5 has been shown to interact with LTBP-2 (Hirai *et al.*, 2007), indicating LTBP-2 may influence the interaction of fibulin-5 with other molecules and/or contribute to the elastic fibres. It has been shown that the sixth cbEGF-like domain of fibulin-5 specifically interacts with the amino- (N-) terminal of LTBP-2 (Hirai *et al.*, 2007). It has been proposed by Hirai *et al.*, 2007 that when present, LTBP-2 binds to fibulin-5/tropoelastin complex and promotes fibulin-5/tropoelastin complex deposition on fibrillin-1 and inhibits deposition onto fibrillin-2 microfibrils, indicating LTBP-2 may regulate the differential usage of microfibrils in elastic fibre assembly (Hirai *et al.*, 2007). However, on fibulin-5, the tropoelastin and LTBP-2 binding sites appear to overlap, suggesting they compete for binding. If proven then, LTBP-2 may be involved in displacing fibulin-5 from the elastin (rather than guiding the complex to the fibrillin-1 microfibrils), to enable the deposition of elastin into the microfibrils. In this study, we want to investigate the role of LTBP-2 on interaction with fibulin-5/tropoelastin complex, particularly whether LTBP-2 enhances or blocks the binding of fibulin-5 to tropoelastin.

1.7 Fibrotic diseases

Fibrosis is the formation or development of excess fibrous connective tissues in an organ or tissues, following injury or repeated insult to a tissue or organ. Fibrosis is an important cause of morbidity and mortality involving tissues such as lung, kidney, skin, liver and cardiovascular (Bottinger and Bitzer, 2002; Kissin and Korn, 2003; Afdhal and Nunes, 2004; Lakos *et al.*, 2004; Kang *et al.*, 2007). Currently there is no effective treatment for these diseases. In human patients, idiopathic pulmonary fibrosis is characterized by inflammatory cell infiltrates and increased collagen deposition, resulting in loss of alveolar architecture and impaired gas exchange with most patient dying within 3-8 years (Broekelmann *et al.*, 1991; Kang *et al.*, 2007). The prognosis of primary renal fibrotic disease is often dependent on the degree of tubulointerstitial scarring caused by proliferation and excessive matrix production of renal fibroblasts and possibility other cellular elements (Bottinger and Bitzer, 2002; Strutz and Neilson, 2003). Scleroderma (or systemic sclerosis) is characterised by skin fibrosis but severe forms of the disease can involve the lungs, kidneys, gastrointestinal tract, and heart (Lakos *et al.*, 2004). In 2001, cirrhosis (liver fibrosis) disease was identified as the 10th leading cause of death for men and the 12th for women in the United States, killing about 27,000 people each year, while new cases of idiopathic pulmonary fibrosis have been conservatively estimated at 270/million/year (Anderson and Smith, 2003; Raghu *et al.*, 2006).

The mechanism of tissue fibrosis is complex and poorly understood. Generally, during the inflammatory stage of the healing process, activated fibroblasts are stimulated to produce collagenous matrix mainly by cytokines, such as TGF- β (Strutz and Neilson, 2003). Occasionally however, when inflammation subsides, the matrix synthesis in the post-inflammatory stages of fibrogenesis continues, stimulated by aberrant TGF- β activity which results in massive progressive deposition of fibrous matrix (Strutz *et al.*, 2001; Strutz and Neilson, 2003). In idiopathic pulmonary fibrosis, activated TGF- β stimulates the expression of many ECM proteins and down regulates their degradation by matrix metalloproteinase (MMP) through upregulation of tissue inhibitor of metalloproteinases (TIMP) (Gharaee-Kermani *et al.*, 2009).

In this study, we will focus on fibrotic skin lesions, namely HTS and keloid samples to address the regulation of growth factors involved during the fibrosis. Both keloid and HTS tissues represent fibrotic conditions caused by abnormal wound healing causing excessive

collagen production by fibroblasts (Aarabi *et al.*, 2007; Verhaegen *et al.*, 2009). HTS usually occurs within 4-8 weeks after injury and remains confined within the boundaries of the wound while in keloid, the scars develop several months after an initial trauma with overgrowth of fibrotic tissue beyond the original wound boundaries (Tuan and Nichter, 1998; Verhaegen *et al.*, 2009). Both lesions also can be distinguished by histo-pathological characteristic where HTS is generally identified by the presence of α -SMA-expressing myofibroblasts and fine collagen fibres running parallel with the epithelial surface while in keloids, myofibroblast are absent and collagen bundles are randomly orientated (Tuan and Nichter, 1998; Verhaegen *et al.*, 2009).

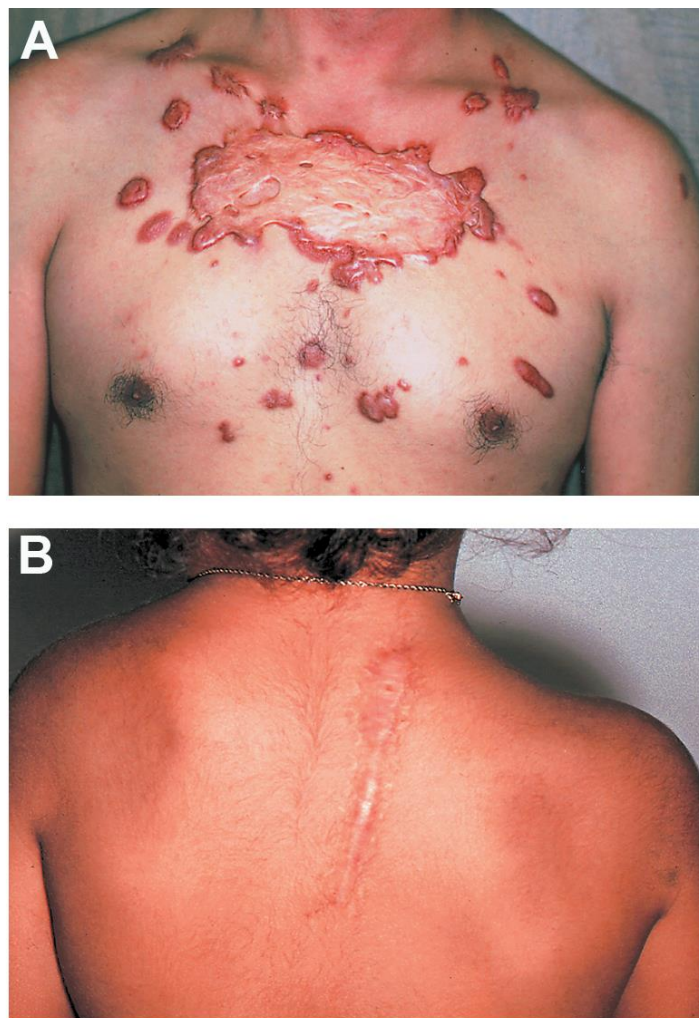


Figure 1.6 (A) Keloids on patient's chest. (B) Hypertrophic scar on a patient's back. Hypertrophic scars typically remain confined within the boundaries of the original wound whereas keloid scars develop beyond the margins of the original injury. Images adapted from Kelly (2008).

1.8 Growth factors

1.8.1 Transforming growth factor – beta (TGF- β)

TGF- β superfamily consists of more than 40 members including TGF- β s, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins and inhibins (Clark and Coker, 1998; Roberts, 1998). They are multifunctional regulators and exert a variety of effects on mammalian cells such as, differentiation, growth, migration, adhesion, wound healing, tissues homeostasis, embryogenesis and promoting ECM production (Roberts, 1998; Massague and Gomis, 2006; Gordon and Blobel, 2008).

Three different TGF- β isoforms have been described, TGF- β 1, TGF- β 2, and TGF- β 3, which have a wide range of biological responses and play important roles in many normal cellular functions (Oklu and Hesketh, 2000). The functions of TGF- β varies between tissues since TGF- β isoforms can act as growth-promoting factors in some cell types, such as mesodermal cells and smooth muscle cells, but also act as growth inhibitors of many normal and transformed cells, with keratinocytes and epithelial cells being particularly susceptible (Massague *et al.*, 1990; Elliott and Blobel, 2005). Moreover, TGF- β can also stimulate ECM biosynthesis including suppression of lymphocyte function and regulation both bone formation and angiogenesis (Massague *et al.*, 1990; Oklu and Hesketh, 2000).

TGF- β isoforms are synthesized within cells as an inactive protein, named latent TGF- β , which consists of an active region and a latency associated peptide (LAP). Each of TGF- β is usually secreted in large latent complexes (LLCs), and comprises a disulphide-bonded homodimer of TGF- β associated non-covalently with LAP and covalently attached molecules of LTBP (LTBP-1,-3,-4) (Kanzaki *et al.*, 1990; Miyazono and Heldin, 1991; Oklu and Hesketh, 2000) (Figure 1). The LLC then subsequently targets TGF- β to fibrillin-rich microfibrils in the extracellular matrix where it awaits activation by agents such as thrombospondin, integrin α V β 6, reactive oxygen species or low pH during tissue remodelling, inflammation and wound repair (Annes *et al.*, 2003). Therefore, fibrillin-1 has a biologically important role in controlling the storage and activation of TGF- β growth factor (Kanzaki *et al.*, 1990; Gleizes *et al.*, 1996; Nunes *et al.*, 1997). However, LTBP-2 is the only LTBP that does not bind to TGF- β , and its function is uncertain. LTBP-2 is the focus of this study and will be discussed in more detail in section 1.9.1.

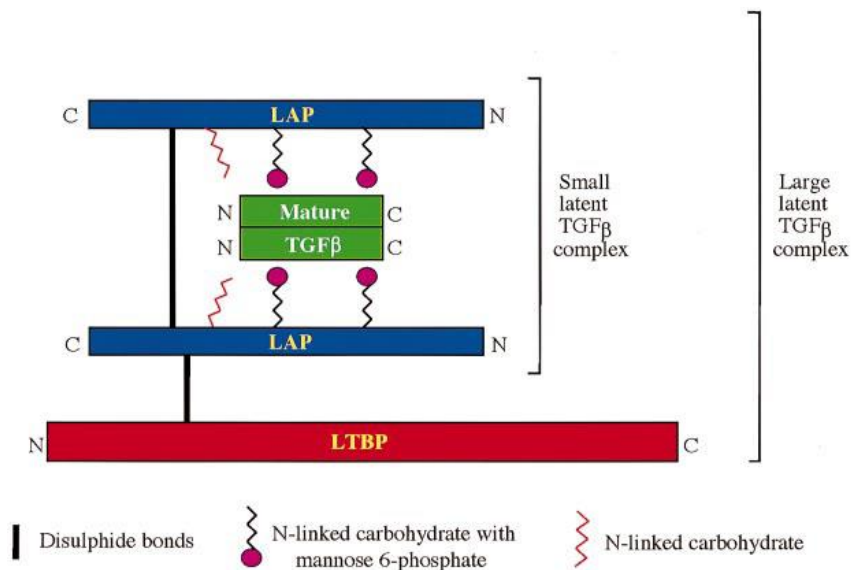


Figure 1.7 Structure of TGF- β latent complexes. The small latent TGF- β complex (SLC) comprises an active TGF- β homodimer associated non-covalently with a LAP homodimer. The large latent TGF- β complex (LLC) contains an additional protein, LTBP, which is disulphide-bonded to LAP. Diagram adapted from Oklu and Hesketh (2000).

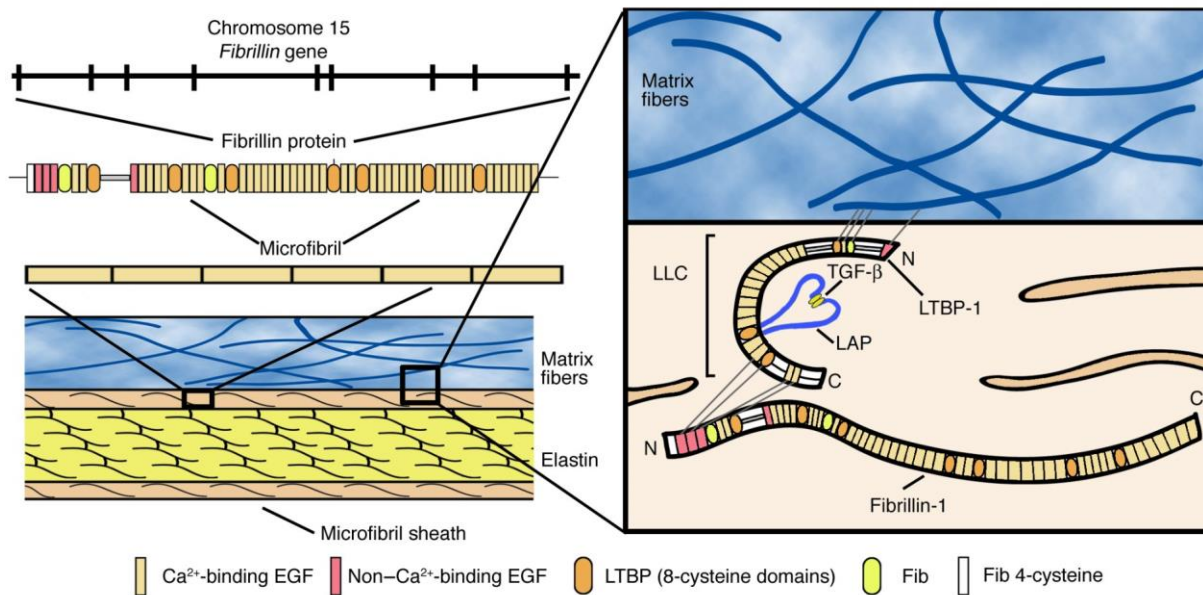


Figure 1.8 Schematic representation of the model for TGF- β storage. LTBP-1, a binding protein for latent form of TGF- β is associated with fibrillin-1 and this interaction appears to influence the activation of TGF- β (Byers, 2004).

It has been well documented TGF- β has a major role in tissue homeostasis and the disturbance of the TGF- β signalling pathways may contribute to many human diseases, including fibrotic, cancer, autoimmune, and cardiovascular diseases (Bottinger *et al.*, 1997; Branton and Kopp, 1999; Gold, 1999; Bottinger and Bitzer, 2002; Ruiz-Ortega *et al.*, 2007; Akhurst and Hata, 2012; Doyle *et al.*, 2012). TGF- β is a key mediator of fibrosis by inducing recruitment of inflammatory cells and stimulating the proliferation and myofibroblastic differentiation of fibroblasts (Branton and Kopp, 1999). Moreover, TGF- β stimulates ECM synthesis and deposition during the fibrotic process by inducing the production of protease inhibitors which block matrix degradation (Wynn, 2008). The overexpression of TGF- β alone is sufficient to induce renal fibrosis and idiopathic pulmonary fibrosis, and the inhibition of TGF- β or TGF- β R activation can prevent fibrotic progression (Li *et al.*, 2002; Li *et al.*, 2011; Lepparanta *et al.*, 2012).

TGF- β exerts its biologic effect by activating signalling pathways through a heteromeric receptor complex consisting of one type I and one type II transmembrane receptor serine-threonine kinase; TGF- β receptor I (TGF β R1) and TGF- β receptor II (TGF β R2), respectively (Roberts, 1999). Once activated, TGF- β receptors directly activate signals within the cell through the Smad family of transcriptional activators, Smad2 and Smad3, the receptor-activated Smads (R-Smads) (Roberts, 1999; Shi and Massague, 2003). Activated R-Smads resulting in heterodimerization with the common mediator Smad4, and these complexes regulate translocation and activation of TGF- β target genes. Inhibitory Smads (I-Smads), including Smad6 and Smad7 have been shown to block the phosphorylation of Smad2 and Smad3, thus inhibiting TGF- β signalling (Roberts, 1999; Shi and Massague, 2003).

The exact mechanism leading to TGF- β synthesis still remains unclear at this point. A study by Otsuka *et al.* (2007) demonstrated that the PI3K/Akt pathway is involved in the elevation of TGF- β by macrophages treated with liposomes composed of phosphatidylserine. PI3K/Akt and ERK inhibitors suppressed the production of TGF- β by inhibiting the activation of Akt and ERK signalling pathways suggesting these signalling pathways are intimately involved in the production of TGF- β . Moreover, statins reduce the mRNA expression of TGF- β as angiogenic factor in a mouse osteosarcoma cell line by blocking the Ras/MEK/Erk and Ras/PI3K/Akt pathways (Tsubaki *et al.*, 2011). Similar studies were reported by Zhou *et al.* (2008) where the elevated TGF- β production induced by neuropeptide Y could be blocked by

PI3K/Akt inhibitor. Recent studies have shown that MAPKs (p38 MAPK, ERK and JNK) signalling pathway involved in up-regulation of TGF- β mRNA expression in apoptotic cells (Xiao *et al.*, 2008). Taken together, the above evidence strongly suggests the involvement of PI3K/Akt, ERK, p38 MAPK and JNK in TGF- β gene stimulation and in this study, we will identify major intracellular signalling molecules and pathways triggered by LTBP-2 leading to stimulation of TGF- β expression.

1.8.2 Fibroblast growth factor -2 (FGF-2)

FGF-2 belongs to the large family of heparin-binding molecules, originally purified from bovine pituitary extracts (Beenken and Mohammadi, 2009). FGF-2 was initially identified as an 18 kDa protein. Larger forms of FGF-2 (21.5, 22 and 24kDa) have also been identified resulting from alternate CUG-translation start sites (Eriksson *et al.*, 1991; Bikfalvi *et al.*, 1997; Nugent and Iozzo, 2000; Okada-Ban *et al.*, 2000). FGF-2 activity is mediated by binding to heparan sulphate proteoglycans and to high affinity membrane FGF receptors (FGFR1 to FGFR4) (Yayon *et al.*, 1991; Faham *et al.*, 1996; Chuang *et al.*, 2010).

FGF-2 is secreted from cells by an energy-dependent process that is independent of the ER-Golgi, and it becomes strongly bound to glycosaminoglycan (GAG) chains of heparan sulphate proteoglycans (HSPGs) in the matrix and basement membranes. Following tissue injury, the FGF-2 molecules are released by proteases and heparinases (Kardami *et al.*, 2007; Schultz and Wysocki, 2009). Multiple FGF-2 molecules remain attached to released HS chains and their interaction with cell-surface FGF-receptors causes clustering of two such receptors necessary to activate intracellular signalling pathways (Kardami *et al.*, 2007; Schultz and Wysocki, 2009).

FGF-2 was first described as a mitogenic factor stimulating proliferation of fibroblasts (Beenken and Mohammadi, 2009). FGF-2 is also involved in tissue regeneration and remodelling (Basilico and Moscatelli, 1992; Murakami *et al.*, 1999; Kawai *et al.*, 2000). FGF-2 has extensive roles involving many cell types and tissues including smooth muscle cells, endothelial cells and neurons. FGF-2 affects cell proliferation, differentiation, migration as well as survival. Evidence from various experimental systems has demonstrated that FGF-2 stimulates TGF- β gene expression and secretion (Phillips *et al.*, 1997; Dhandapani *et al.*, 2007).

For some years, FGF-2 were considered to have pro-fibrotic role during fibrosis by upregulating the expression of TGF- β (Phillips *et al.*, 1997; Dhandapani *et al.*, 2007). This results in increased synthesis of ECM proteins, including collagens, fibronectin, tenascin, tissue inhibitor of metalloproteinase-1 (TIMP-1) and secretion of fibrogenic connective tissue growth factor (CTGF) (Plisov *et al.*, 2001; Strutz *et al.*, 2001). The expression of basic FGF-2 and TGF- β is robustly up-regulated in human kidneys with tubulointerstitial fibrosis indicating that FGF-2 plays an essential role as a potent inducer of fibroblast proliferation (Strutz *et al.*, 2001). However in contrast, multiple recent studies have revealed that FGF-2 may also have anti-fibrotic functions in wound healing (Xie *et al.*, 2008; Eto *et al.*, 2012; Shi *et al.*, 2013). Post-operative administration of FGF-2 during wound healing inhibits hypertrophy and widening of the lesion without any serious side effects (Ono *et al.*, 2007). Similarly, FGF-2 enhanced acute and chronic wound healing in both animal models and clinical use (Bikfalvi *et al.*, 1997; Fu *et al.*, 2000; Akita *et al.*, 2008). Moreover, FGF-2 inhibits TGF- β activity by blocking the Smad2/Smad3 signalling in the TGF- β 1/SMAD dependent pathway, attenuating excessive ECM production during fibrosis (Shi *et al.*, 2013). However, the mechanism of storage and activation of FGF-2 in fibrotic diseases remains unclear and will be a focus of interest in this study.

1.9 Latent TGF- β binding proteins (LTBPs)

Fibrillins share a similar domain structure to LTBPs (Hyytiainen *et al.*, 1998). There are four human LTPB genes that have been identified and characterised (Oklu and Hesketh, 2000). LTBPs 1-4 are large extracellular glycoproteins that range in molecular mass from 125 to 240-kDa (Kanzaki *et al.*, 1990), with all members sharing some structural homology with fibrillins (Figure 2). They consist mainly of two types of repeating cysteine-rich-domains, epidermal growth factor (EGF)-like repeats and eight-cysteine motifs (8-Cys). The 8-cysteine motif are found only in LTBPs and fibrillins, and the presence of this domain defines the family (reviewed by (Hyytiainen *et al.*, 2004). The LTBPs are smaller in size compared to fibrillins and they are most structurally similar in the central regions and vary considerably around the terminal regions (Isogai *et al.*, 2003; Charbonneau *et al.*, 2004).

The domain structure of the LTPB family members is similar and it can be divided into four specific regions; the N-terminal region, the adjacent hinge region, a central cluster of EGF-like repeats and the C-terminal region (Saharinen *et al.*, 1996). In LTPB-1, the N-terminal domain is able to attach to unidentified ECM fibres, whereas the C-terminal domain binds to fibrillin microfibrils (Isogai *et al.*, 2003).

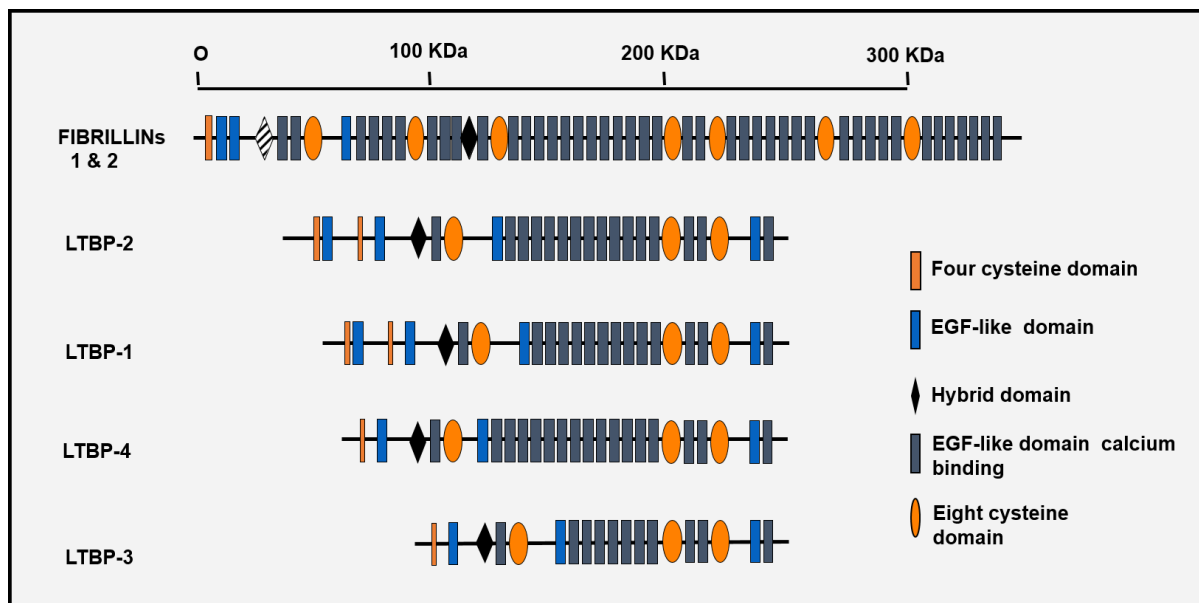


Figure 1.9 Fibrillins and LTBPs share a high degree of structural similarity since they both contain unique 8-cysteine motifs.

The expression patterns of four LTBP and their isoforms in different tissues are only partially overlapping (Saharinen *et al.*, 1998) and tissue specific expressions of various types of LTBP have been observed (Charbonneau *et al.*, 2004). LTBP-1 seems to be expressed mostly in the dorsal tissues of the developing embryo (Altmann *et al.*, 2002). LTBP-2 is expressed in the lung, arterial vessels, dermis, pericardium, epicardium and heart valves (Shipley *et al.*, 2000). LTBP-3 expressed widely in mesenchymal cells, developing bone, and in the central nervous system (Yin *et al.*, 1995) and LTBP-4 is predominantly expressed in aorta, heart, small intestine, and ovaries (Giltay *et al.*, 1997). LTBP were first identified as carriers for growth factor TGF- β and play an important role in the sequestration and activation of the growth factor (Charbonneau *et al.*, 2004). The roles of these proteins in matrix have been demonstrated by mouse models where mutation in LTBP-1, LTBP-2 and LTBP-4 genes cause abnormalities in the development of the heart, bones and lungs respectively (Dabovic *et al.*, 2002a; Koli *et al.*, 2004; Oklu *et al.*, 2011). For example, severely reduced expression of LTBP-1 in mice leads to congenital heart defects caused by decreased in TGF- β activity (Todorovic *et al.*, 2007). Studies from Dabovic *et al.*, (2002) demonstrated that mice lacking LTBP-3 genes suffer from osteoarthritis and osteosclerosis caused from impaired TGF- β signaling on bone physiology (Dabovic *et al.*, 2002a; Dabovic *et al.*, 2002b). In contrast, decreased in TGF-activity in mice deficient in LTBP-4 caused severe pulmonary emphysema and colorectal cancer (Sterner-Kock *et al.*, 2002; Dabovic *et al.*, 2009; Urban *et al.*, 2009).

1.9.1 LTBP-2

LTBP-2 is a 195-240kDa protein consisting of 20 cbEGF repeats, 16 of which have been shown to be calcium binding domains and 4 8-cysteine motifs. LTBP-2 is known to have 80% structural similarity to LTBP-1 (Gibson *et al.*, 1995) and 25% similarity to fibrillin-1 (Moren *et al.*, 1994) which is the greatest sequence homology between a LTBP and fibrillin-1. In situ hybridisation and immunohistochemical studies show that LTBP-2 is found in a variety of adult tissues; such as lung, heart, testes, maternal deciduas, liver, spleen & skeletal muscles (Moren *et al.*, 1994). In addition, LTBP-2 has been shown to associate in abundance with fibrillin microfibrils in developing tissue such as the developing human foetal aorta (Gibson *et al.*, 1995; Hirani *et al.*, 2007). Furthermore, the expression of LTBP-2 was revealed to be largely parallel to tropoelastin expression by in situ hybridisation (Giltay *et al.*, 1997). LTBP-2 and tropoelastin are co-expressed in rat lung, epicardium, pericardium and heart valves, while in the young adult mouse, both proteins are expressed in the capsule of the spleen and the

mesenchyme of the lung (Shipley *et al.*, 2000). Interestingly, LTBP-2 is present in the tissues within the body that are subjected to tightly regulated amounts of growth factor and cytokines such as testes and maternal deciduas, suggesting LTBP-2 may play roles other than as a structural molecule.

As mentioned previously, LTBP-2 function is largely unknown and different from other members of the LTBP family since it does not bind latent forms of TGF- β (Saharinen and Keski-Oja, 2000). A study by Lack *et al.* (2003) demonstrated that the third 8-Cys domains of LTBP-2 and fibrillins lack the required latent TGF- β binding consensus sequence. Previously in our laboratory, LTBP-2 was investigated for interaction with other elastic fibre associated proteins and we found no direct *in vitro* interaction with tropoelastin, MAGPs 1 and 2, or dermatan sulphate proteoglycans, biglycan and decorin (Hirani *et al.*, 2007). Since LTBP-2 have most structurally similar to the fibrillins compare to other members in the family, it has been suggested that LTBP-2 may functionally associate with fibrillin protein and form a structural component of elastic fibres. In recent years, LTBP-2 has been reported to bind specifically with C-terminal domain of fibulin-5, and the interactions have an essential influence on the assembly of microfibril and elastic fibres (Hirai *et al.*, 2007). Studied by Hirai *et al.* (2007) demonstrated that LTBP-2 might interact with fibulin-5/tropoelastin complexes and direct them to fibrillin-1 rather than fibrillin-2 microfibrils during elastinogenesis. Interestingly, Zheng *et al.* (2007b) reported that elastin binding sites on fibulin-5 located in both N-terminal region and C-terminal region of fibulin-5. This observation have led to our suggestion that one major binding site for tropoelastin on fibulin-5 is overlapping with that of LTBP-2 since both LTBP-2 and tropoelastin attachment sites are contained in the C-terminal EGF repeat of fibulin-5. This suggesting LTBP-2 may be involved in release of elastin globules from fibulin-5 complexes during the deposition process and it seems unlikely LTBP-2 directly bind to the complexes as suggested by Hirai *et al.* (2007) since its binding site on fibulin-5 will be blocked by tropoelastin. This hypothesis will be tested in the present study. LTBP-2 also has been shown to modulate the coalescence of oxytalan fibres, which consist of elastin-free fibrillin microfibril bundles, by negatively controlling the function of fibulin-5 (Tsuruga *et al.*, 2012). Recently published data from our laboratory (Parsi *et al.*, 2010) have shown that LTBP-2 binds strongly to heparin and HSPGs, syndecan-4 and perlecan. The study identified at least four distinct heparin binding regions on LTBP-2 and defined three major interaction sites in the N-terminal region. These heparan sulphate binding activities of LTBP-2 may influence microfibril interaction with proteoglycans during elastic fibre assembly and/or at microfibril-

basement membrane interfaces, since LTBP-2 has extensive localization to microfibrils in many developing tissues.

To investigate the importance of LTBP-2 during development and function in the ECM, a gene knockout mice study was conducted (Shipley *et al.*, 2000). LTBP-2 knockout mice were reported to die around the implantation period indicating some essential role of LTBP-2 in early embryogenesis (Shipley *et al.*, 2000). This early lethality prevented this knockout mice model from providing any information about the function of LTBP-2 in later stages of tissues development. However recent contradictory evidence demonstrated a very viable LTBP-2 null mouse strain with a mild ocular phenotype including lens luxation caused by compromised ciliary zonule formation (Inoue *et al.*, 2014). The authors also demonstrated that the addition of recombinant LTBP-2 to cultured cells and organ cultured eyeballs from the mice promoted the assembly of mature microfibrils in ciliary zonules suggesting the important role of LTBP-2 in eye development.

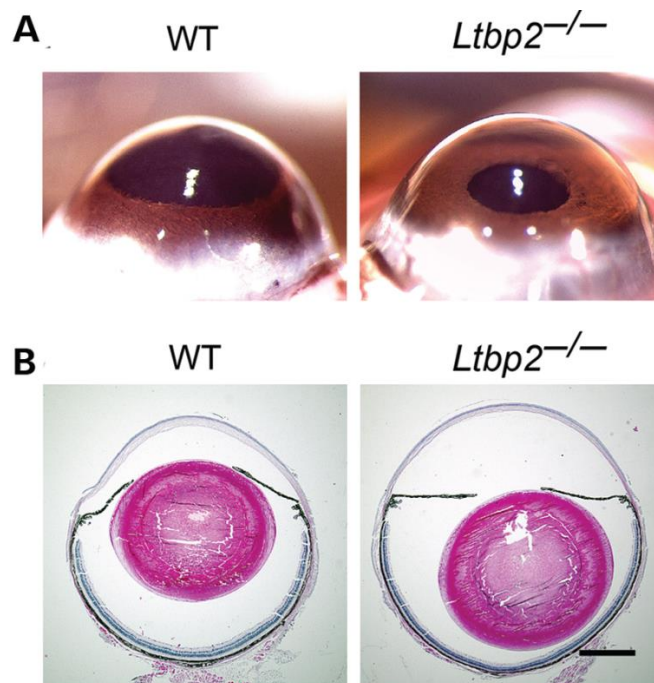


Figure 1.10 LTBP-2 knockout mice develop ciliary zonule fragmentation. (A) Eyes of wild-type and LTBP-2 null mice at 12 months. The irises of LTBP-2 null mice appeared flat compared to wild-type where eyes were convex as they were pushed by lenses. (B) HE-stained histological sections of mouse eyes. The LTBP-2 null mice developed lens dislocation into the posterior chamber of the eyes. Images adapted from Inoue *et al.* (2014).

Although LTBP-2 does not directly bind latent TGF- β , multiple studies have shown that LTBP-2 may be involved in the regulation of TGF- β storage and activation. LTBP-2 expression was found to significantly increased in tumour tissues when compared to normal tissues (Yoshihara *et al.*, 2009; Turtoi *et al.*, 2011; Ren *et al.*, 2015). Moreover, LTBP-2 expression positively correlated significantly with the survival of esophageal carcinoma (ESCC) patients. Increased levels of TGF- β correlated with tumor progression and prognosis in a wide variety of cancers including esophageal squamous cell carcinoma (ESCC) (Chan *et al.*, 2011). These findings indicated that LTBP-2 could have a TGF- β regulatory role independent of TGF- β storage and activation. Studies within our laboratory have shown that LTBP-2 competes with LTBP-1 for attachment to fibrillin-1 *in vitro* suggesting LTBP-2 may have the ability to displace the LTBP-1/latent TGF- β complex from binding to fibrillin-1 microfibrils and thus LTBP-2 may have an indirect role in the modulation of TGF- β storage and activation in tissues (Hirani *et al.*, 2007). We have not subsequently found evidence for this. However recently we showed LTBP-2 can directly elevate TGF- β expression in a variety of cell types. Thus, one of the aims of this present study is to determine how LTBP-2 elevates TGF- β expression. Interaction between the cell surface and ECM components is important for events during development of many tissues and cells types (Gumbiner, 1996). Such interactions may provide cells with biological information and act as a scaffold for adhesion and migration. Integrins are the most common cell surface receptors for extracellular matrix proteins. Generally LTBP-2 acts as an antiadhesive matrix component that decreases fibroblast adhesion and linked to the modulation of fibroblast migration in the lung (Hyytiainen and Keski-Oja, 2003). However recent studies showed that LTBP-2 can mediate melanoma cell adhesion through the $\alpha 3\beta 1$ integrin receptor, implicating a role for LTBP-2 in the modulation of cell adhesion (Hyytiainen and Keski-Oja, 2003; Vehvilainen *et al.*, 2003). However, the full spectrum of the LTBP-2 cell interactions and how LTBP-2 modulates cell behaviour is still unclear.

Recent evidence indicates that LTBP-2 null humans have relatively mild phenotypes (Ali *et al.*, 2009). The principal phenotype is primary congenital glaucoma apparently resulting from changes in the structure of the elastic fibre-rich ocular trabecular meshwork and ciliary processes (Ali *et al.*, 2009). However, some patients had varied Marfan-like characteristics including Marfanoid habitus, overlong limbs, joint hypermobility, osteopenia and aortic valve problems (Ali *et al.*, 2009). These findings point to altered TGF- β signalling in these patients.

Interestingly, mutation of the LTBP-2 gene is also linked to Weill-Marchesani syndrome (WMS) (Haji-Seyed-Javadi *et al.*, 2012). WMS is characterised by fibrotic skin, ocular and skeletal deformities which appears to be caused by altered TGF- β levels and signalling suggesting connections between LTBP-2 and fibrosis and TGF- β dysregulation. Since fibrillin-1 mutations also cause Marfan syndrome and WMS, this finding clearly confirmed the functional link between LTBP-2 and fibrillin-1 biology, probably involving abnormal TGF- β signalling. Moreover, preliminary data from our lab showed that LTBP-2 has also a strong interaction with FGF-2, suggesting LTBP-2 may play a role as a matrix store for latent FGF-2 and thus may be involved in fibrotic diseases.

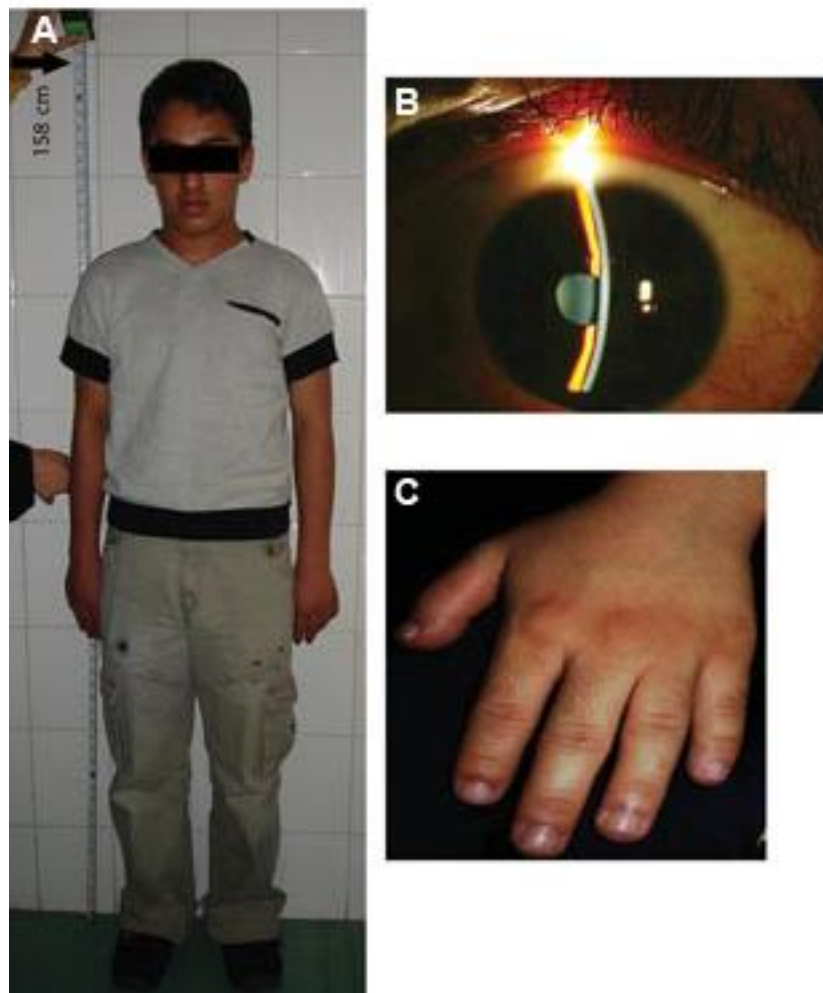


Figure 1.11 (A) Proband of WMS family showing short stature. (B) Eye of proband showing extreme shallow anterior chamber with forward subluxation of the lens. (C) Hand of patients showing short fingers (brachydactyly) (Haji-Seyed-Javadi *et al.*, 2012)

1.10 Aims of the present study

This project will define the function of the poorly understood matrix protein LTBP-2. In particular, the aim is to define the roles of LTBP-2 in a) elastic fibre assembly, b) modulating FGF-2 activity and storage, and c) stimulating TGF- β expression. A particular aim is to determine the mechanism of TGF- β elevation by investigating precise intracellular signalling systems. Furthermore, this study aims to characterize the surface receptors mediating the LTBP-2 cell interaction resulting in TGF- β elevation. Preliminary data from our lab showed that a LTBP-2 fragment, LTBP-2 C(H) F2 binds strongly to FGF-2. Therefore, the influences of LTBP-2 on FGF-2 bioactivity will be determined by using cell culture models. This project also aims to determine if LTBP-2 and growth factor levels, especially FGF-2 and TGF- β are modulated in fibrotic tissues by analysing a range of fibrosis tissues samples. Overall, the project will increase the knowledge about fibrotic disorders with a goal of identification of novel therapeutic targets for attenuation of fibrotic diseases.

1.10.1 Specific aims

1. To determine the influence of LTBP-2 on the interaction of fibulin-5 with tropoelastin during elastic fibre assembly
2. To further define and characterize molecular interaction of LTBP-2 with FGF-2 and determine LTBP-2 effects on FGF-2 activity.
3. To determine the function of LTBP-2 in TGF- β activation and signaling.
4. To investigate the role of LTBP-2 in fibrotic diseases.

1.10.2 Hypotheses

1. LTBP-2 acts as negative modulator during elastic fibre assembly.
2. LTBP-2 acts as matrix store for latent FGF-2 and modulates bioactivity of FGF-2.
3. LTBP-2 upregulates TGF- β expression via direct interaction with unidentified cell receptors activating specific intracellular signaling systems.
4. Elevated LTBP-2 correlates with increased expression of TGF- β and FGF-2 in fibrotic tissues.

1.11 References

- Aarabi, S., Longaker, M. T. and Gurtner, G. C. (2007) 'Hypertrophic scar formation following burns and trauma: new approaches to treatment', *PLoS Med*, 4(9), p. e234.
- Afdhal, N. H. and Nunes, D. (2004) 'Evaluation of liver fibrosis: a concise review', *Am J Gastroenterol*, 99(6), pp. 1160-74.
- Akhurst, R. J. and Hata, A. (2012) 'Targeting the TGFbeta signalling pathway in disease', *Nat Rev Drug Discov*, 11(10), pp. 790-811.
- Akita, S., Akino, K., Imaizumi, T. and Hirano, A. (2008) 'Basic fibroblast growth factor accelerates and improves second-degree burn wound healing', *Wound Repair Regen*, 16(5), pp. 635-41.
- Albig, A. R. and Schiemann, W. P. (2005) 'Fibulin-5 function during tumorigenesis', *Future Oncol*, 1(1), pp. 23-35.
- Ali, M., McKibbin, M., Booth, A., Parry, D. A., Jain, P., Riazuddin, S. A., Hejtmancik, J. F., Khan, S. N., Firasat, S., Shires, M., Gilmour, D. F., Towns, K., Murphy, A. L., Azmanov, D., Tournev, I., Cherninkova, S., Jafri, H., Raashid, Y., Toomes, C., Craig, J., Mackey, D. A., Kalaydjieva, L., Riazuddin, S. and Inglehearn, C. F. (2009) 'Null mutations in LTBP2 cause primary congenital glaucoma', *Am J Hum Genet*, 84(5), pp. 664-71.
- Altmann, C. R., Chang, C., Munoz-Sanjuan, I., Bell, E., Heke, M., Rifkin, D. B. and Brivanlou, A. H. (2002) 'The latent-TGFbeta-binding-protein-1 (LTBP-1) is expressed in the organizer and regulates nodal and activin signaling', *Dev Biol*, 248(1), pp. 118-27.
- Anderson, R. N. and Smith, B. L. (2003) 'Deaths: leading causes for 2001', *Natl Vital Stat Rep*, 52(9), pp. 1-85.
- Annes, J. P., Munger, J. S. and Rifkin, D. B. (2003) 'Making sense of latent TGF- β activation', *J Cell Sci*, 116(Pt 2), pp. 217-24.
- Argaves, W. S., Tran, H., Burgess, W. H. and Dickerson, K. (1990) 'Fibulin is an extracellular matrix and plasma glycoprotein with repeated domain structure', *The Journal of Cell Biology*, 111(6), pp. 3155-3164.
- Arteaga-Solis, E., Gayraud, B. and Ramirez, F. (2000) 'Elastic and collagenous networks in vascular diseases', *Cell Struct Funct*, 25(2), pp. 69-72.
- Aumailley, M. and Gayraud, B. (1998a) 'Structure and biological activity of the extracellular matrix', *Journal of Molecular Medicine*, 76(3-4), pp. 253-265.

- Aumailley, M. and Gayraud, B. (1998b) 'Structure and biological activity of the extracellular matrix', *J Mol Med (Berl)*, 76(3-4), pp. 253-65.
- Badylak, S. F. (2002) 'The extracellular matrix as a scaffold for tissue reconstruction', *Semin Cell Dev Biol*, 13(5), pp. 377-83.
- Basilico, C. and Moscatelli, D. (1992) 'The FGF family of growth factors and oncogenes', *Adv Cancer Res*, 59, pp. 115-65.
- Bateman JF, L. S., Ramshaw JA (1996) *Collagen Superfamily*.
- Bax, D. V., Bernard, S. E., Lomas, A., Morgan, A., Humphries, J., Shuttleworth, C. A., Humphries, M. J. and Kielty, C. M. (2003) 'Cell adhesion to fibrillin-1 molecules and microfibrils is mediated by alpha 5 beta 1 and alpha v beta 3 integrins', *J Biol Chem*, 278(36), pp. 34605-16.
- Bax, D. V., Mahalingam, Y., Cain, S., Melody, K., Freeman, L., Younger, K., Shuttleworth, C. A., Humphries, M. J., Couchman, J. R. and Kielty, C. M. (2007) 'Cell adhesion to fibrillin-1: identification of an Arg-Gly-Asp-dependent synergy region and a heparin-binding site that regulates focal adhesion formation', *J Cell Sci*, 120(Pt 8), pp. 1383-92.
- Beenken, A. and Mohammadi, M. (2009) 'The FGF family: biology, pathophysiology and therapy', *Nat Rev Drug Discov*, 8(3), pp. 235-53.
- Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L. and Lose, E. J. (1992) 'Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans', *Annu Rev Cell Biol*, 8(1), pp. 365-93.
- Bikfalvi, A., Klein, S., Pintucci, G. and Rifkin, D. B. (1997) 'Biological roles of fibroblast growth factor-2', *Endocr Rev*, 18(1), pp. 26-45.
- Black, P. N., Ching, P. S., Beaumont, B., Ransinghe, S., Taylor, G. and Merrilees, M. J. (2008) 'Changes in elastic fibres in the small airways and alveoli in COPD', *Eur Respir J*, 31(5), pp. 998-1004.
- Border, W. A. and Ruoslahti, E. (1992) 'Transforming growth factor-beta in disease: the dark side of tissue repair', *Journal of Clinical Investigation*, 90(1), pp. 1-7.
- Bosman, F. T. and Stamenkovic, I. (2003) 'Functional structure and composition of the extracellular matrix', *J Pathol*, 200(4), pp. 423-8.
- Bottinger, E. P. and Bitzer, M. (2002) 'TGF-beta signaling in renal disease', *J Am Soc Nephrol*, 13(10), pp. 2600-10.
- Bottinger, E. P., Letterio, J. J. and Roberts, A. B. (1997) 'Biology of TGF- β in knockout and transgenic mouse models', *Kidney Int*, 51(5), pp. 1355-60.

- Branton, M. H. and Kopp, J. B. (1999) 'TGF- β and fibrosis', *Microbes Infect*, 1(15), pp. 1349-65.
- Brodsky, B. and Persikov, A. V. (2005) 'Molecular Structure of the Collagen Triple Helix', in *Advances in Protein Chemistry*. Academic Press, pp. 301-339.
- Brodsky, B. and Ramshaw, J. A. M. (1997) 'The collagen triple-helix structure', *Matrix Biology*, 15(8-9), pp. 545-554.
- Broekelmann, T. J., Limper, A. H., Colby, T. V. and McDonald, J. A. (1991) 'Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis', *Proc Natl Acad Sci U S A*, 88(15), pp. 6642-6.
- Bruckner-Tuderman, L., von der Mark, K., Pihlajaniemi, T. and Unsicker, K. (2010) 'Cell interactions with the extracellular matrix', *Cell Tissue Res*, 339(1), pp. 1-5.
- Bunyaratavej, P. and Wang, H. L. (2001) 'Collagen membranes: a review', *J Periodontol*, 72(2), pp. 215-29.
- Burgeson, R. E. (1993) 'Type VII Collagen, Anchoring Fibrils, and Epidermolysis Bullosa', *J Invest Dermatol*, 101(3), pp. 252-255.
- Byers, P. H. (2004) 'Determination of the molecular basis of Marfan syndrome: a growth industry', *Journal of Clinical Investigation*, 114(2), pp. 161-163.
- Cain, S. A., Baldock, C., Gallagher, J., Morgan, A., Bax, D. V., Weiss, A. S., Shuttleworth, C. A. and Kielty, C. M. (2005a) 'Fibrillin-1 interactions with heparin. Implications for microfibril and elastic fiber assembly', *J Biol Chem*, 280(34), pp. 30526-37.
- Cain, S. A., Baldock, C., Gallagher, J., Morgan, A., Bax, D. V., Weiss, A. S., Shuttleworth, C. A. and Kielty, C. M. (2005b) 'Fibrillin-1 Interactions with Heparin: IMPLICATIONS FOR MICROFIBRIL AND ELASTIC FIBER ASSEMBLY', *Journal of Biological Chemistry*, 280(34), pp. 30526-30537.
- Cain, S. A., Baldwin, A. K., Mahalingam, Y., Raynal, B., Jowitt, T. A., Shuttleworth, C. A., Couchman, J. R. and Kielty, C. M. (2008a) 'Heparan sulfate regulates fibrillin-1 N- and C-terminal interactions', *J Biol Chem*, 283(40), pp. 27017-27.
- Cain, S. A., Baldwin, A. K., Mahalingam, Y., Raynal, B., Jowitt, T. A., Shuttleworth, C. A., Couchman, J. R. and Kielty, C. M. (2008b) 'Heparan Sulfate Regulates Fibrillin-1 N- and C-terminal Interactions', *Journal of Biological Chemistry*, 283(40), pp. 27017-27027.
- Cain, S. A., Raynal, B., Hodson, N., Shuttleworth, A. and Kielty, C. M. (2008c) 'Biomolecular analysis of elastic fibre molecules', *Methods*, 45(1), pp. 42-52.

- Canty, E. G. and Kadler, K. E. (2002) 'Collagen fibril biosynthesis in tendon: a review and recent insights', *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 133(4), pp. 979-985.
- Chan, S. H., Yee Ko, J. M., Chan, K. W., Chan, Y. P., Tao, Q., Hyytiainen, M., Keski-Oja, J., Law, S., Srivastava, G., Tang, J., Tsao, S. W., Chen, H., Stanbridge, E. J. and Lung, M. L. (2011) 'The ECM protein LTBP-2 is a suppressor of esophageal squamous cell carcinoma tumor formation but higher tumor expression associates with poor patient outcome', *Int J Cancer*, 129(3), pp. 565-73.
- Charbonneau, N. L., Ono, R. N., Corson, G. M., Keene, D. R. and Sakai, L. Y. (2004) 'Fine tuning of growth factor signals depends on fibrillin microfibril networks', *Birth Defects Res C Embryo Today*, 72(1), pp. 37-50.
- Choi, J., Bergdahl, A., Zheng, Q., Starcher, B., Yanagisawa, H. and Davis, E. C. (2009a) 'Analysis of dermal elastic fibers in the absence of fibulin-5 reveals potential roles for fibulin-5 in elastic fiber assembly', *Matrix Biology*, 28(4), pp. 211-220.
- Choi, J., Bergdahl, A., Zheng, Q., Starcher, B., Yanagisawa, H. and Davis, E. C. (2009b) 'Analysis of dermal elastic fibers in the absence of fibulin-5 reveals potential roles for fibulin-5 in elastic fiber assembly', *Matrix Biol*, 28(4), pp. 211-20.
- Choudhury, R., McGovern, A., Ridley, C., Cain, S. A., Baldwin, A., Wang, M. C., Guo, C., Mironov, A., Jr., Drymoussi, Z., Trump, D., Shuttleworth, A., Baldock, C. and Kielty, C. M. (2009) 'Differential regulation of elastic fiber formation by fibulin-4 and -5', *J Biol Chem*, 284(36), pp. 24553-67.
- Chu, M.-L. and Tsuda, T. (2004) 'Fibulins in development and heritable disease', *Birth Defects Research Part C: Embryo Today: Reviews*, 72(1), pp. 25-36.
- Chuang, C. Y., Lord, M. S., Melrose, J., Rees, M. D., Knox, S. M., Freeman, C., Iozzo, R. V. and Whitelock, J. M. (2010) 'Heparan sulfate-dependent signaling of fibroblast growth factor 18 by chondrocyte-derived perlecan', *Biochemistry*, 49(26), pp. 5524-32.
- Clark, D. A. and Coker, R. (1998) 'Transforming growth factor- β (TGF- β)', *Int J Biochem Cell Biol*, 30(3), pp. 293-8.
- Clark, R. A. F., Lanigan, J. M., DellaPelle, P., Manseau, E., Dvorak, H. F. and Colvin, R. B. (1982) 'Fibronectin and Fibrin Provide a Provisional Matrix for Epidermal Cell Migration During Wound Reepithelialization', *J Invest Dermatol*, 79(5), pp. 264-269.

- Clarke, A. W., Arnspang, E. C., Mithieux, S. M., Korkmaz, E., Braet, F. and Weiss, A. S. (2006) 'Tropoelastin massively associates during coacervation to form quantized protein spheres', *Biochemistry*, 45(33), pp. 9989-96.
- Cleary, E. G. a. G., M. A (1996) *Elastic tissue, elastin and elastin-associated microfibrils*. Edited by Comper, W. D. Amsterdam: Harwood Academic Publishers.
- Cohlberg, J. A., Li, J., Uversky, V. N. and Fink, A. L. (2002) 'Heparin and Other Glycosaminoglycans Stimulate the Formation of Amyloid Fibrils from α -Synuclein in Vitro†', *Biochemistry*, 41(5), pp. 1502-1511.
- Corson, G. M., Chalberg, S. C., Dietz, H. C., Charbonneau, N. L. and Sakai, L. Y. (1993) 'Fibrillin binds calcium and is coded by cDNAs that reveal a multidomain structure and alternatively spliced exons at the 5' end', *Genomics*, 17(2), pp. 476-84.
- Corson, G. M., Charbonneau, N. L., Keene, D. R. and Sakai, L. Y. (2004) 'Differential expression of fibrillin-3 adds to microfibril variety in human and avian, but not rodent, connective tissues', *Genomics*, 83(3), pp. 461-72.
- Culav, E. M., Clark, C. H. and Merrilees, M. J. (1999) 'Connective tissues: matrix composition and its relevance to physical therapy', *Phys Ther*, 79(3), pp. 308-19.
- Curran, M. E., Atkinson, D. L., Ewart, A. K., Morris, C. A., Leppert, M. F. and Keating, M. T. (1993) 'The elastin gene is disrupted by a translocation associated with supravalvular aortic stenosis', *Cell*, 73(1), pp. 159-168.
- Dabovic, B., Chen, Y., Choi, J., Vassallo, M., Dietz, H. C., Ramirez, F., von Melchner, H., Davis, E. C. and Rifkin, D. B. (2009) 'Dual functions for LTBP in lung development: LTBP-4 independently modulates elastogenesis and TGF-beta activity', *J Cell Physiol*, 219(1), pp. 14-22.
- Dabovic, B., Chen, Y., Colarossi, C., Obata, H., Zambuto, L., Perle, M. A. and Rifkin, D. B. (2002a) 'Bone abnormalities in latent TGF-[beta] binding protein (Ltbp)-3-null mice indicate a role for Ltbp-3 in modulating TGF-[beta] bioavailability', *J Cell Biol*, 156(2), pp. 227-32.
- Dabovic, B., Chen, Y., Colarossi, C., Zambuto, L., Obata, H. and Rifkin, D. B. (2002b) 'Bone defects in latent TGF-beta binding protein (Ltbp)-3 null mice; a role for Ltbp in TGF-beta presentation', *J Endocrinol*, 175(1), pp. 129-41.
- Dallas, S. L., Miyazono, K., Skerry, T. M., Mundy, G. R. and Bonewald, L. F. (1995) 'Dual role for the latent transforming growth factor-beta binding protein in storage of latent TGF-beta in the extracellular matrix and as a structural matrix protein', *The Journal of Cell Biology*, 131(2), pp. 539-549.

- de Vega, S., Iwamoto, T. and Yamada, Y. (2009) 'Fibulins: multiple roles in matrix structures and tissue functions', *Cell Mol Life Sci*, 66(11-12), pp. 1890-902.
- Dean, D. C., Birkenmeier, T. M., Rosen, G. D. and Weintraub, S. J. (1991) 'Glycoprotein synthesis and secretion. Expression of fibronectin and its cell surface receptors', *Am Rev Respir Dis*, 144(3 Pt 2), pp. S25-8.
- Debelle, L. and Tamburro, A. M. (1999) 'Elastin: molecular description and function', *The International Journal of Biochemistry & Cell Biology*, 31(2), pp. 261-272.
- Dhandapani, K. M., Khan, M. M., Wade, F. M., Wakade, C., Mahesh, V. B. and Brann, D. W. (2007) 'Induction of transforming growth factor-beta1 by basic fibroblast growth factor in rat C6 glioma cells and astrocytes is mediated by MEK/ERK signaling and AP-1 activation', *J Neurosci Res*, 85(5), pp. 1033-45.
- Diamant, J., Keller, A., Baer, E., Litt, M. and Arridge, R. G. C. (1972) 'Collagen; Ultrastructure and Its Relation to Mechanical Properties as a Function of Ageing', *Proceedings of the Royal Society of London B: Biological Sciences*, 180(1060), pp. 293-315.
- Doyle, J. J., Gerber, E. E. and Dietz, H. C. (2012) 'Matrix-dependent perturbation of TGFbeta signaling and disease', *FEBS Lett*, 586(14), pp. 2003-15.
- El-Hallous, E., Sasaki, T., Hubmacher, D., Getie, M., Tiedemann, K., Brinckmann, J., Batge, B., Davis, E. C. and Reinhardt, D. P. (2007) 'Fibrillin-1 interactions with fibulins depend on the first hybrid domain and provide an adaptor function to tropoelastin', *J Biol Chem*, 282(12), pp. 8935-46.
- Elliott, R. L. and Blobel, G. C. (2005) 'Role of transforming growth factor Beta in human cancer', *J Clin Oncol*, 23(9), pp. 2078-93.
- Epstein, E. H. and Munderloh, N. H. (1978) 'Human skin collagen. Presence of type I and type III at all levels of the dermis', *Journal of Biological Chemistry*, 253(5), pp. 1336-1337.
- Eriksson, A. E., Cousens, L. S., Weaver, L. H. and Matthews, B. W. (1991) 'Three-dimensional structure of human basic fibroblast growth factor', *Proceedings of the National Academy of Sciences*, 88(8), pp. 3441-3445.
- Eto, H., Suga, H., Aoi, N., Kato, H., Doi, K., Kuno, S., Tabata, Y. and Yoshimura, K. (2012) 'Therapeutic potential of fibroblast growth factor-2 for hypertrophic scars: upregulation of MMP-1 and HGF expression', *Lab Invest*, 92(2), pp. 214-23.
- Ewart, A. K., Jin, W., Atkinson, D., Morris, C. A. and Keating, M. T. (1994) 'Supravalvular aortic stenosis associated with a deletion disrupting the elastin gene', *Journal of Clinical Investigation*, 93(3), pp. 1071-1077.

- Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J. and Rees, D. C. (1996) 'Heparin Structure and Interactions with Basic Fibroblast Growth Factor', *Science*, 271(5252), pp. 1116-1120.
- Freeman, L. J., Lomas, A., Hodson, N., Sherratt, M. J., Mellody, K. T., Weiss, A. S., Shuttleworth, A. and Kielty, C. M. (2005) 'Fibulin-5 interacts with fibrillin-1 molecules and microfibrils', *Biochem J*, 388(Pt 1), pp. 1-5.
- Fu, X., Shen, Z., Chen, Y., Xie, J., Guo, Z., Zhang, M. and Sheng, Z. (2000) 'Recombinant bovine basic fibroblast growth factor accelerates wound healing in patients with burns, donor sites and chronic dermal ulcers', *Chin Med J (Engl)*, 113(4), pp. 367-71.
- Fuki, I. V., Kuhn, K. M., Lomazov, I. R., Rothman, V. L., Tuszynski, G. P., Iozzo, R. V., Swenson, T. L., Fisher, E. A. and Williams, K. J. (1997) 'The syndecan family of proteoglycans. Novel receptors mediating internalization of atherogenic lipoproteins in vitro', *Journal of Clinical Investigation*, 100(6), pp. 1611-1622.
- Gentili, C. and Cancedda, R. (2009) 'Cartilage and bone extracellular matrix', *Curr Pharm Des*, 15(12), pp. 1334-48.
- George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H. and Hynes, R. O. (1993) 'Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin', *Development*, 119(4), pp. 1079-91.
- Gharaee-Kermani, M., Hu, B., Phan, S. H. and Gyetko, M. R. (2009) 'Recent advances in molecular targets and treatment of idiopathic pulmonary fibrosis: focus on TGFbeta signaling and the myofibroblast', *Curr Med Chem*, 16(11), pp. 1400-17.
- Gheduzzi, D., Guerra, D., Bochicchio, B., Pepe, A., Tamburro, A. M., Quaglino, D., Mithieux, S., Weiss, A. S. and Pasquali Ronchetti, I. (2005) 'Heparan sulphate interacts with tropoelastin, with some tropoelastin peptides and is present in human dermis elastic fibers', *Matrix Biol*, 24(1), pp. 15-25.
- Giancotti, F. G. and Ruoslahti, E. (1999) 'Integrin signaling', *Science*, 285(5430), pp. 1028-32.
- Gibson, M. A., Hatzinikolas, G., Davis, E. C., Baker, E., Sutherland, G. R. and Mecham, R. P. (1995) 'Bovine latent transforming growth factor β 1-binding protein 2: molecular cloning, identification of tissue isoforms, and immunolocalization to elastin-associated microfibrils', *Mol Cell Biol*, 15(12), pp. 6932-42.
- Gibson, M. A., Hatzinikolas, G., Kumaratilake, J. S., Sandberg, L. B., Nicholl, J. K., Sutherland, G. R. and Cleary, E. G. (1996) 'Further characterization of proteins

- associated with elastic fiber microfibrils including the molecular cloning of MAGP-2 (MP25)', *J Biol Chem*, 271(2), pp. 1096-103.
- Gibson, M. A., Hughes, J. L., Fanning, J. C. and Cleary, E. G. (1986) 'The major antigen of elastin-associated microfibrils is a 31-kDa glycoprotein', *J Biol Chem*, 261(24), pp. 11429-36.
- Gillies, A. R. and Lieber, R. L. (2011) 'Structure and function of the skeletal muscle extracellular matrix', *Muscle Nerve*, 44(3), pp. 318-31.
- Giltay, R., Kostka, G. and Timpl, R. (1997) 'Sequence and expression of a novel member (LTBP-4) of the family of latent transforming growth factor-beta binding proteins', *FEBS Lett*, 411(2-3), pp. 164-8.
- Giltay, R., Timpl, R. and Kostka, G. (1999) 'Sequence, recombinant expression and tissue localization of two novel extracellular matrix proteins, fibulin-3 and fibulin-4', *Matrix Biology*, 18(5), pp. 469-480.
- Gleizes, P. E., Beavis, R. C., Mazzieri, R., Shen, B. and Rifkin, D. B. (1996) 'Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-beta binding protein-1 that mediates bonding to the latent transforming growth factor-beta1', *J Biol Chem*, 271(47), pp. 29891-6.
- Gold, L. I. (1999) 'The role for transforming growth factor- β (TGF- β) in human cancer', *Crit Rev Oncog*, 10(4), pp. 303-60.
- Gordon and Hahn, R. A. (2009) 'Collagens', *Cell and Tissue Research*, 339(1), pp. 247-257.
- Gordon, K. J. and Blobel, G. C. (2008) 'Role of transforming growth factor-beta superfamily signaling pathways in human disease', *Biochim Biophys Acta*, 1782(4), pp. 197-228.
- Gumbiner, B. M. (1996) 'Cell adhesion: the molecular basis of tissue architecture and morphogenesis', *Cell*, 84(3), pp. 345-57.
- Haji-Seyed-Javadi, R., Jelodari-Mamaghani, S., Paylakhi, S. H., Yazdani, S., Nilforushan, N., Fan, J. B., Klotzle, B., Mahmoudi, M. J., Ebrahimian, M. J., Chelich, N., Taghiabadi, E., Kamyab, K., Boileau, C., Paisan-Ruiz, C., Ronaghi, M. and Elahi, E. (2012) 'LTBP2 mutations cause Weill-Marchesani and Weill-Marchesani-like syndrome and affect disruptions in the extracellular matrix', *Hum Mutat*, 33(8), pp. 1182-7.
- Halper, J. and Kjaer, M. (2014) 'Basic components of connective tissues and extracellular matrix: elastin, fibrillin, fibulins, fibrinogen, fibronectin, laminin, tenascins and thrombospondins', *Adv Exp Med Biol*, 802, pp. 31-47.

- Hanada, K., Vermeij, M., Garinis, G. A., de Waard, M. C., Kunen, M. G. S., Myers, L., Maas, A., Duncker, D. J., Meijers, C., Dietz, H. C., Kanaar, R. and Essers, J. (2007) 'Perturbations of Vascular Homeostasis and Aortic Valve Abnormalities in Fibulin-4 Deficient Mice', *Circulation Research*, 100(5), pp. 738-746.
- Handford, P. A., Downing, A. K., Reinhardt, D. P. and Sakai, L. Y. (2000) 'Fibrillin: from domain structure to supramolecular assembly', *Matrix Biology*, 19(6), pp. 457-470.
- Hardingham, T. E. and Fosang, A. J. (1992) 'Proteoglycans: many forms and many functions', *FASEB J*, 6(3), pp. 861-70.
- Hassell, J. R., Kimura, J. H. and Hascall, V. C. (1986) 'Proteoglycan core protein families', *Annu Rev Biochem*, 55, pp. 539-67.
- Hines, K. L., Kulkarni, A. B., McCarthy, J. B., Tian, H., Ward, J. M., Christ, M., McCartney-Francis, N. L., Furcht, L. T., Karlsson, S. and Wahl, S. M. (1994) 'Synthetic fibronectin peptides interrupt inflammatory cell infiltration in transforming growth factor beta 1 knockout mice', *Proc Natl Acad Sci U S A*, 91(11), pp. 5187-91.
- Hirai, M., Horiguchi, M., Ohbayashi, T., Kita, T., Chien, K. R. and Nakamura, T. (2007) 'Latent TGF-beta-binding protein 2 binds to DANCE/fibulin-5 and regulates elastic fiber assembly', *EMBO J*, 26(14), pp. 3283-95.
- Hirani, R., Hanssen, E. and Gibson, M. A. (2007) 'LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein', *Matrix Biol*, 26(4), pp. 213-23.
- Hisanaga, Y., Nakashima, K., Tsuruga, E., Nakatomi, Y., Hatakeyama, Y., Ishikawa, H. and Sawa, Y. (2009) 'Fibulin-5 contributes to microfibril assembly in human periodontal ligament cells', *Acta Histochem Cytochem*, 42(5), pp. 151-7.
- Hohenester, E. and Engel, J. (2002) 'Domain structure and organisation in extracellular matrix proteins', *Matrix Biol*, 21(2), pp. 115-28.
- Holm, T. M., Habashi, J. P., Doyle, J. J., Bedja, D., Chen, Y., van Erp, C., Lindsay, M. E., Kim, D., Schoenhoff, F., Cohn, R. D., Loeys, B. L., Thomas, C. J., Patnaik, S., Marugan, J. J., Judge, D. P. and Dietz, H. C. (2011) 'Noncanonical TGFbeta signaling contributes to aortic aneurysm progression in Marfan syndrome mice', *Science*, 332(6027), pp. 358-61.
- Horiguchi, M., Inoue, T., Ohbayashi, T., Hirai, M., Noda, K., Marmorstein, L. Y., Yabe, D., Takagi, K., Akama, T. O., Kita, T., Kimura, T. and Nakamura, T. (2009) 'Fibulin-4 conducts proper elastogenesis via interaction with cross-linking enzyme lysyl oxidase', *Proc Natl Acad Sci U S A*, 106(45), pp. 19029-34.

- Hu, Q., Reymond, J. L., Pinel, N., Zabet, M. T. and Urban, Z. (2006) 'Inflammatory destruction of elastic fibers in acquired cutis laxa is associated with missense alleles in the elastin and fibulin-5 genes', *J Invest Dermatol*, 126(2), pp. 283-90.
- Huchtagowder, V., Sausgruber, N., Kim, K. H., Angle, B., Marmorstein, L. Y. and Urban, Z. (2006) 'Fibulin-4: a novel gene for an autosomal recessive cutis laxa syndrome', *Am J Hum Genet*, 78(6), pp. 1075-80.
- Hudson, B. G., Tryggvason, K., Sundaramoorthy, M. and Neilson, E. G. (2003) 'Alport's Syndrome, Goodpasture's Syndrome, and Type IV Collagen', *New England Journal of Medicine*, 348(25), pp. 2543-2556.
- Hwang, E. Y., Jeong, M. S., Park, E. K., Kim, J. H. and Jang, S. B. (2014) 'Structural characterization and interaction of periostin and bone morphogenetic protein for regulation of collagen cross-linking', *Biochem Biophys Res Commun*, 449(4), pp. 425-31.
- Hyytiainen, M. and Keski-Oja, J. (2003) 'Latent TGF-beta binding protein LTBP-2 decreases fibroblast adhesion to fibronectin', *J Cell Biol*, 163(6), pp. 1363-74.
- Hyytiainen, M., Penttinen, C. and Keski-Oja, J. (2004) 'Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation', *Crit Rev Clin Lab Sci*, 41(3), pp. 233-64.
- Hyytiainen, M., Taipale, J., Heldin, C. H. and Keski-Oja, J. (1998) 'Recombinant latent transforming growth factor beta-binding protein 2 assembles to fibroblast extracellular matrix and is susceptible to proteolytic processing and release', *J Biol Chem*, 273(32), pp. 20669-76.
- Inoue, T., Ohbayashi, T., Fujikawa, Y., Yoshida, H., Akama, T. O., Noda, K., Horiguchi, M., Kameyama, K., Hata, Y., Takahashi, K., Kusumoto, K. and Nakamura, T. (2014) 'Latent TGF- β binding protein-2 is essential for the development of ciliary zonule microfibrils', *Hum Mol Genet*, 23(21), pp. 5672-82.
- Isogai, Z., Ono, R. N., Ushiro, S., Keene, D. R., Chen, Y., Mazzieri, R., Charbonneau, N. L., Reinhardt, D. P., Rifkin, D. B. and Sakai, L. Y. (2003) 'Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein', *J Biol Chem*, 278(4), pp. 2750-7.
- Jackson, R. L., Busch, S. J. and Cardin, A. D. (1991) 'Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes', *Physiological Reviews*, 71(2), pp. 481-539.

- Junqueira, L. C. and Montes, G. S. (1983) 'Biology of collagen-proteoglycan interaction', *Archivum histologicum Japonicum = Nihon soshikigaku kiroku*, 46(5), pp. 589-629.
- Kadler, K. E., Hill, A. and Canty-Laird, E. G. (2008) 'Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators', *Current Opinion in Cell Biology*, 20(5), pp. 495-501.
- Kang, H. R., Lee, C. G., Homer, R. J. and Elias, J. A. (2007) 'Semaphorin 7A plays a critical role in TGF-beta1-induced pulmonary fibrosis', *J Exp Med*, 204(5), pp. 1083-93.
- Kanzaki, T., Olofsson, A., Moren, A., Wernstedt, C., Hellman, U., Miyazono, K., Claesson-Welsh, L. and Heldin, C. H. (1990) 'TGF-beta 1 binding protein: a component of the large latent complex of TGF-beta 1 with multiple repeat sequences', *Cell*, 61(6), pp. 1051-61.
- Kapetanopoulos, A., Fresser, F., Millionig, G., Shaul, Y., Baier, G. and Utermann, G. (2002) 'Direct interaction of the extracellular matrix protein DANCE with apolipoprotein(a) mediated by the kringle IV-type 2 domain', *Molecular Genetics and Genomics*, 267(4), pp. 440-446.
- Kardami, E., Detillieux, K., Ma, X., Jiang, Z., Santiago, J. J., Jimenez, S. K. and Cattini, P. A. (2007) 'Fibroblast growth factor-2 and cardioprotection', *Heart Fail Rev*, 12(3-4), pp. 267-77.
- Kawai, K., Suzuki, S., Tabata, Y., Ikada, Y. and Nishimura, Y. (2000) 'Accelerated tissue regeneration through incorporation of basic fibroblast growth factor-impregnated gelatin microspheres into artificial dermis', *Biomaterials*, 21(5), pp. 489-499.
- Keene, D. R., Sakai, L. Y., Lunstrum, G. P., Morris, N. P. and Burgeson, R. E. (1987) 'Type VII collagen forms an extended network of anchoring fibrils', *The Journal of Cell Biology*, 104(3), pp. 611-621.
- Kelly, A. P. (2008) 'Hypertrophic Scars and Keloids', in Gloster, H. M. (ed.) *Complications in Cutaneous Surgery*. New York, NY: Springer New York, pp. 87-94.
- Khoshnoodi, J., Pedchenko, V. and Hudson, B. G. (2008) 'Mammalian collagen IV', *Microscopy Research and Technique*, 71(5), pp. 357-370.
- Kielty, C. M. (2006) 'Elastic fibres in health and disease', *Expert Rev Mol Med*, 8(19), pp. 1-23.
- Kielty, C. M., Sherratt, M. J. and Shuttleworth, C. A. (2002a) 'Elastic fibres', *Journal of Cell Science*, 115(14), pp. 2817-2828.
- Kielty, C. M., Sherratt, M. J. and Shuttleworth, C. A. (2002b) 'Elastic fibres', *J Cell Sci*, 115(Pt 14), pp. 2817-28.

- Kielty, C. M. and Shuttleworth, C. A. (1995) 'Fibrillin-containing microfibrils: Structure and function in health and disease', *The International Journal of Biochemistry & Cell Biology*, 27(8), pp. 747-760.
- Kielty, C. M., Stephan, S., Sherratt, M. J., Williamson, M. and Shuttleworth, C. A. (2007) 'Applying elastic fibre biology in vascular tissue engineering', *Philos Trans R Soc Lond B Biol Sci*, 362(1484), pp. 1293-312.
- Kim, C. W., Goldberger, O. A., Gallo, R. L. and Bernfield, M. (1994) 'Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue-, and development-specific patterns', *Molecular Biology of the Cell*, 5(7), pp. 797-805.
- Kinsey, R., Williamson, M. R., Chaudhry, S., Mellody, K. T., McGovern, A., Takahashi, S., Shuttleworth, C. A. and Kielty, C. M. (2008a) 'Fibrillin-1 microfibril deposition is dependent on fibronectin assembly', *Journal of Cell Science*, 121(16), pp. 2696-2704.
- Kinsey, R., Williamson, M. R., Chaudhry, S., Mellody, K. T., McGovern, A., Takahashi, S., Shuttleworth, C. A. and Kielty, C. M. (2008b) 'Fibrillin-1 microfibril deposition is dependent on fibronectin assembly', *J Cell Sci*, 121(Pt 16), pp. 2696-704.
- Kirn-Safran, C., Farach-Carson, M. and Carson, D. (2009) 'Multifunctionality of extracellular and cell surface heparan sulfate proteoglycans', *Cellular and Molecular Life Sciences*, 66(21), pp. 3421-3434.
- Kissin, E. Y. and Korn, J. H. (2003) 'Fibrosis in scleroderma', *Rheum Dis Clin North Am*, 29(2), pp. 351-69.
- Kjaer, M., Langberg, H., Heinemeier, K., Bayer, M. L., Hansen, M., Holm, L., Doessing, S., Kongsgaard, M., Krogsgaard, M. R. and Magnusson, S. P. (2009) 'From mechanical loading to collagen synthesis, structural changes and function in human tendon', *Scand J Med Sci Sports*, 19(4), pp. 500-10.
- Kobayashi, N., Kostka, G., Garbe, J. H., Keene, D. R., Bachinger, H. P., Hanisch, F. G., Markova, D., Tsuda, T., Timpl, R., Chu, M. L. and Sasaki, T. (2007) 'A comparative analysis of the fibulin protein family. Biochemical characterization, binding interactions, and tissue localization', *J Biol Chem*, 282(16), pp. 11805-16.
- Koli, K., Wempe, F., Sterner-Kock, A., Kantola, A., Komor, M., Hofmann, W. K., von Melchner, H. and Keski-Oja, J. (2004) 'Disruption of LTBP-4 function reduces TGF-beta activation and enhances BMP-4 signaling in the lung', *J Cell Biol*, 167(1), pp. 123-33.

- Kostka, G., Giltay, R., Bloch, W., Addicks, K., Timpl, R., Fassler, R. and Chu, M. L. (2001) 'Perinatal lethality and endothelial cell abnormalities in several vessel compartments of fibulin-1-deficient mice', *Mol Cell Biol*, 21(20), pp. 7025-34.
- Kozel, B. A., Ciliberto, C. H. and Mecham, R. P. (2004) 'Deposition of tropoelastin into the extracellular matrix requires a competent elastic fiber scaffold but not live cells', *Matrix Biol*, 23(1), pp. 23-34.
- Kramer, K. L. and Yost, H. J. (2003) 'Heparan sulfate core proteins in cell-cell signaling', *Annu Rev Genet*, 37, pp. 461-84.
- Kühn, K. (1995) 'Basement membrane (type IV) collagen', *Matrix Biology*, 14(6), pp. 439-445.
- Lakos, G., Takagawa, S., Chen, S. J., Ferreira, A. M., Han, G., Masuda, K., Wang, X. J., DiPietro, L. A. and Varga, J. (2004) 'Targeted disruption of TGF-beta/Smad3 signaling modulates skin fibrosis in a mouse model of scleroderma', *Am J Pathol*, 165(1), pp. 203-17.
- Lapiere, C. M., Nusgens, B. and Pierard, G. E. (1977) 'Interaction Between Collagen Type I and Type III in Conditioning Bundles Organization', *Connective Tissue Research*, 5(1), pp. 21-29.
- Lepparanta, O., Sens, C., Salmenkivi, K., Kinnula, V. L., Keski-Oja, J., Myllarniemi, M. and Koli, K. (2012) 'Regulation of TGF-beta storage and activation in the human idiopathic pulmonary fibrosis lung', *Cell Tissue Res*, 348(3), pp. 491-503.
- Li, D. Y., Brooke, B., Davis, E. C., Mecham, R. P., Sorensen, L. K., Boak, B. B., Eichwald, E. and Keating, M. T. (1998a) 'Elastin is an essential determinant of arterial morphogenesis', *Nature*, 393(6682), pp. 276-80.
- Li, D. Y., Faury, G., Taylor, D. G., Davis, E. C., Boyle, W. A., Mecham, R. P., Stenzel, P., Boak, B. and Keating, M. T. (1998b) 'Novel arterial pathology in mice and humans hemizygous for elastin', *J Clin Invest*, 102(10), pp. 1783-7.
- Li, D. Y., Toland, A. E., Boak, B. B., Atkinson, D. L., Ensing, G. J., Morris, C. A. and Keating, M. T. (1997a) 'Elastin Point Mutations Cause an Obstructive Vascular Disease, Supravalvular Aortic Stenosis', *Human Molecular Genetics*, 6(7), pp. 1021-1028.
- Li, D. Y., Toland, A. E., Boak, B. B., Atkinson, D. L., Ensing, G. J., Morris, C. A. and Keating, M. T. (1997b) 'Elastin point mutations cause an obstructive vascular disease, supravalvular aortic stenosis', *Hum Mol Genet*, 6(7), pp. 1021-8.
- Li, J. H., Zhu, H. J., Huang, X. R., Lai, K. N., Johnson, R. J. and Lan, H. Y. (2002) 'Smad7 inhibits fibrotic effect of TGF-Beta on renal tubular epithelial cells by blocking Smad2 activation', *J Am Soc Nephrol*, 13(6), pp. 1464-72.

- Li, M., Krishnaveni, M. S., Li, C., Zhou, B., Xing, Y., Banfalvi, A., Li, A., Lombardi, V., Akbari, O., Borok, Z. and Minoos, P. (2011) 'Epithelium-specific deletion of TGF-beta receptor type II protects mice from bleomycin-induced pulmonary fibrosis', *J Clin Invest*, 121(1), pp. 277-87.
- Lin, X. (2004) 'Functions of heparan sulfate proteoglycans in cell signaling during development', *Development*, 131(24), pp. 6009-6021.
- Liu, X., Zhao, Y., Gao, J., Pawlyk, B., Starcher, B., Spencer, J. A., Yanagisawa, H., Zuo, J. and Li, T. (2004) 'Elastic fiber homeostasis requires lysyl oxidase-like 1 protein', *Nat Genet*, 36(2), pp. 178-82.
- Loeys, B., Van Maldergem, L., Mortier, G., Coucke, P., Gerniers, S., Naeyaert, J. M. and De Paepe, A. (2002) 'Homozygosity for a missense mutation in fibulin-5 (FBLN5) results in a severe form of cutis laxa', *Hum Mol Genet*, 11(18), pp. 2113-8.
- Lotery, A. J., Baas, D., Ridley, C., Jones, R. P. O., Klaver, C. C. W., Stone, E., Nakamura, T., Luff, A., Griffiths, H., Wang, T., Bergen, A. A. B. and Trump, D. (2006) 'Reduced secretion of fibulin 5 in age-related macular degeneration and cutis laxa', *Human Mutation*, 27(6), pp. 568-574.
- Lukashev, M. E. and Werb, Z. (1998) 'ECM signalling: orchestrating cell behaviour and misbehaviour', *Trends Cell Biol*, 8(11), pp. 437-41.
- Mao, Y. and Schwarzbauer, J. E. (2005) 'Fibronectin fibrillogenesis, a cell-mediated matrix assembly process', *Matrix Biol*, 24(6), pp. 389-99.
- Marini, J. C., Forlino, A., Cabral, W. A., Barnes, A. M., San Antonio, J. D., Milgrom, S., Hyland, J. C., Körkkö, J., Prockop, D. J., De Paepe, A., Coucke, P., Symoens, S., Glorieux, F. H., Roughley, P. J., Lund, A. M., Kuurila-Svahn, K., Hartikka, H., Cohn, D. H., Krakow, D., Mottes, M., Schwarze, U., Chen, D., Yang, K., Kuslich, C., Troendle, J., Dalgleish, R. and Byers, P. H. (2007) 'Consortium for osteogenesis imperfecta mutations in the helical domain of type I collagen: regions rich in lethal mutations align with collagen binding sites for integrins and proteoglycans', *Human Mutation*, 28(3), pp. 209-221.
- Mark, K., Soroken, L., (2002) 'Adhesive Glycoproteins', in Royce, P. M., and Steinmann, B (ed.) *Connective Tissues and its Heritable Disorders-Molecular, Genetics and medical aspects*. New York: Wiley-Liss, pp. 159-221.
- Markova, D., Zou, Y., Ringpfeil, F., Sasaki, T., Kostka, G., Timpl, R., Uitto, J. and Chu, M. L. (2003) 'Genetic heterogeneity of cutis laxa: a heterozygous tandem duplication within the fibulin-5 (FBLN5) gene', *Am J Hum Genet*, 72(4), pp. 998-1004.

- Massague, J., Cheifetz, S., Boyd, F. T. and Andres, J. L. (1990) 'TGF-beta receptors and TGF-beta binding proteoglycans: recent progress in identifying their functional properties', *Ann N Y Acad Sci*, 593, pp. 59-72.
- Massague, J. and Gomis, R. R. (2006) 'The logic of TGFbeta signaling', *FEBS Lett*, 580(12), pp. 2811-20.
- Matrisian, L. M. and Hogan, B. L. (1990) 'Growth factor-regulated proteases and extracellular matrix remodeling during mammalian development', *Current topics in developmental biology*, 24, pp. 219-259.
- McLaughlin, P. J., Chen, Q., Horiguchi, M., Starcher, B. C., Stanton, J. B., Broekelmann, T. J., Marmorstein, A. D., McKay, B., Mecham, R., Nakamura, T. and Marmorstein, L. Y. (2006a) 'Targeted disruption of fibulin-4 abolishes elastogenesis and causes perinatal lethality in mice', *Mol Cell Biol*, 26(5), pp. 1700-9.
- McLaughlin, P. J., Chen, Q., Horiguchi, M., Starcher, B. C., Stanton, J. B., Broekelmann, T. J., Marmorstein, A. D., McKay, B., Mecham, R., Nakamura, T. and Marmorstein, L. Y. (2006b) 'Targeted Disruption of Fibulin-4 Abolishes Elastogenesis and Causes Perinatal Lethality in Mice', *Molecular and Cellular Biology*, 26(5), pp. 1700-1709.
- Mecham, R. P., and Davis, E (1994) in P.D Yurchenco, D. E. B., and R.P Mecham (ed.) *Elastic fiber structure and assembly: Extracellular Matrix Assembly and Structure*. New York: Academic Press, pp. 281-314.
- Milewicz, D. M., Urban, Z. and Boyd, C. (2000) 'Genetic disorders of the elastic fiber system', *Matrix Biol*, 19(6), pp. 471-80.
- Miner, J. H. and Sanes, J. R. (1994) 'Collagen IV alpha 3, alpha 4, and alpha 5 chains in rodent basal laminae: sequence, distribution, association with laminins, and developmental switches', *The Journal of Cell Biology*, 127(3), pp. 879-891.
- Mithieux, S. M. and Weiss, A. S. (2005) 'Elastin', *Adv Protein Chem*, 70, pp. 437-61.
- Miyazono, K. and Heldin, C. H. (1991) 'Latent forms of TGF- β : molecular structure and mechanisms of activation', *Ciba Found Symp*, 157, pp. 81-9; discussion 89-92.
- Montes, G. S. (1996) 'Structural biology of the fibres of the collagenous and elastic systems', *Cell Biol Int*, 20(1), pp. 15-27.
- Moren, A., Olofsson, A., Stenman, G., Sahlin, P., Kanzaki, T., Claesson-Welsh, L., ten Dijke, P., Miyazono, K. and Heldin, C. H. (1994) 'Identification and characterization of LTBP-2, a novel latent transforming growth factor- β -binding protein', *J Biol Chem*, 269(51), pp. 32469-78.

- Mullins, R. F., Olvera, M. A., Clark, A. F. and Stone, E. M. (2007) 'Fibulin-5 distribution in human eyes: Relevance to age-related macular degeneration', *Experimental Eye Research*, 84(2), pp. 378-380.
- Murakami, S., Takayama, S., Ikezawa, K., Sltimabukuro, Y., Kitamura, M., Nozaki, T., Terashima, A., Asano, T. and Okada, H. (1999) 'Regeneration of periodontal tissues by basic fibroblast growth factor', *Journal of Periodontal Research*, 34(7), pp. 425-430.
- Nakamura, T., Lozano, P. R., Ikeda, Y., Iwanaga, Y., Hinek, A., Minamisawa, S., Cheng, C. F., Kobuke, K., Dalton, N., Takada, Y., Tashiro, K., Ross Jr, J., Honjo, T. and Chien, K. R. (2002) 'Fibulin-5/DANCE is essential for elastogenesis in vivo', *Nature*, 415(6868), pp. 171-5.
- Narayanan, A. S., Page, R. C., Kuzan, F. and Cooper, C. G. (1978) 'Elastin cross-linking in vitro. Studies on factors influencing the formation of desmosines by lysyl oxidase action on tropoelastin', *Biochem J*, 173(3), pp. 857-62.
- Neptune, E. R., Frischmeyer, P. A., Arking, D. E., Myers, L., Bunton, T. E., Gayraud, B., Ramirez, F., Sakai, L. Y. and Dietz, H. C. (2003) 'Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome', *Nat Genet*, 33(3), pp. 407-11.
- Nguyen, A. D., Itoh, S., Jeney, V., Yanagisawa, H., Fujimoto, M., Ushio-Fukai, M. and Fukai, T. (2004) 'Fibulin-5 is a novel binding protein for extracellular superoxide dismutase', *Circ Res*, 95(11), pp. 1067-74.
- Nonaka, R., Onoue, S., Wachi, H., Sato, F., Urban, Z., Starcher, B. C. and Seyama, Y. (2009) 'DANCE/fibulin-5 promotes elastic fiber formation in a tropoelastin isoform-dependent manner', *Clin Biochem*, 42(7-8), pp. 713-21.
- Nugent, M. A. and Iozzo, R. V. (2000) 'Fibroblast growth factor-2', *The International Journal of Biochemistry & Cell Biology*, 32(2), pp. 115-120.
- Nunes, I., Gleizes, P. E., Metz, C. N. and Rifkin, D. B. (1997) 'Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta', *J Cell Biol*, 136(5), pp. 1151-63.
- Okada-Ban, M., Thiery, J. P. and Jouanneau, J. (2000) 'Fibroblast growth factor-2', *The International Journal of Biochemistry & Cell Biology*, 32(3), pp. 263-267.
- Oklu, R. and Hesketh, R. (2000) 'The latent transforming growth factor beta binding protein (LTBP) family', *Biochem J*, 352 Pt 3, pp. 601-10.

- Oklu, R., Hesketh, R., Wicky, S. and Metcalfe, J. C. (2011) 'Expression of mRNA isoforms of latent transforming growth factor-beta binding protein-1 in coronary atherosclerosis and human tissues', *Biochem Genet*, 49(3-4), pp. 213-25.
- Ono, I., Akasaka, Y., Kikuchi, R., Sakemoto, A., Kamiya, T., Yamashita, T. and Jimbow, K. (2007) 'Basic fibroblast growth factor reduces scar formation in acute incisional wounds', *Wound Repair Regen*, 15(5), pp. 617-23.
- Otsuka, M., Negishi, Y. and Aramaki, Y. (2007) 'Involvement of phosphatidylinositol-3-kinase and ERK pathways in the production of TGF- β 1 by macrophages treated with liposomes composed of phosphatidylserine', *FEBS Lett*, 581(2), pp. 325-30.
- Ottani, V., Raspanti, M. and Ruggeri, A. (2001) 'Collagen structure and functional implications', *Micron*, 32(3), pp. 251-260.
- Pankov, R. and Yamada, K. M. (2002) 'Fibronectin at a glance', *Journal of Cell Science*, 115(20), pp. 3861-3863.
- Parsi, M. K., Adams, J. R., Whitelock, J. and Gibson, M. A. (2010) 'LTBP-2 has multiple heparin/heparan sulfate binding sites', *Matrix Biol*, 29(5), pp. 393-401.
- Pfaff, M., Reinhardt, D. P., Sakai, L. Y. and Timpl, R. (1996) 'Cell adhesion and integrin binding to recombinant human fibrillin-1', *FEBS Lett*, 384(3), pp. 247-50.
- Phillips, A. O., Topley, N., Morrissey, K., Williams, J. D. and Steadman, R. (1997) 'Basic fibroblast growth factor stimulates the release of preformed transforming growth factor beta 1 from human proximal tubular cells in the absence of de novo gene transcription or mRNA translation', *Lab Invest*, 76(4), pp. 591-600.
- Pierce, J. G. and Parsons, T. F. (1981) 'Glycoprotein hormones: structure and function', *Annu Rev Biochem*, 50(1), pp. 465-95.
- Plisov, S. Y., Yoshino, K., Dove, L. F., Higinbotham, K. G., Rubin, J. S. and Perantoni, A. O. (2001) 'TGF beta 2, LIF and FGF2 cooperate to induce nephrogenesis', *Development*, 128(7), pp. 1045-57.
- Poschl, E., Schlotzer-Schrehardt, U., Brachvogel, B., Saito, K., Ninomiya, Y. and Mayer, U. (2004) 'Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development', *Development*, 131(7), pp. 1619-28.
- Pöschl, E., Schlötzer-Schrehardt, U., Brachvogel, B., Saito, K., Ninomiya, Y. and Mayer, U. (2004) 'Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development', *Development*, 131(7), pp. 1619-1628.

- Preis, M., Cohen, T., Sarnatzki, Y., Ben Yosef, Y., Schneiderman, J., Gluzman, Z., Koren, B., Lewis, B. S., Shaul, Y. and Flugelman, M. Y. (2006) 'Effects of fibulin-5 on attachment, adhesion, and proliferation of primary human endothelial cells', *Biochem Biophys Res Commun*, 348(3), pp. 1024-33.
- Quondamatteo, F., Reinhardt, D. P., Charbonneau, N. L., Pophal, G., Sakai, L. Y. and Herken, R. (2002) 'Fibrillin-1 and fibrillin-2 in human embryonic and early fetal development', *Matrix Biol*, 21(8), pp. 637-46.
- Raghu, G., Weycker, D., Edelsberg, J., Bradford, W. Z. and Oster, G. (2006) 'Incidence and prevalence of idiopathic pulmonary fibrosis', *Am J Respir Crit Care Med*, 174(7), pp. 810-6.
- Ramirez, F. and Sakai, L. Y. (2010) 'Biogenesis and function of fibrillin assemblies', *Cell Tissue Res*, 339(1), pp. 71-82.
- Ramirez, F., Sakai, L. Y., Dietz, H. C. and Rifkin, D. B. (2004) 'Fibrillin microfibrils: multipurpose extracellular networks in organismal physiology', *Physiol Genomics*, 19(2), pp. 151-4.
- Ren, Y., Lu, H., Zhao, D., Ou, Y., Yu, K., Gu, J., Wang, L., Jiang, S., Chen, M., Wang, J., Zhang, R. and Xu, C. (2015) 'LTPB2 acts as a prognostic factor and promotes progression of cervical adenocarcinoma', *Am J Transl Res*, 7(6), pp. 1095-105.
- Ricard-Blum, S. (2011a) 'The Collagen Family', *Cold Spring Harbor Perspectives in Biology*, 3(1).
- Ricard-Blum, S. (2011b) 'The collagen family', *Cold Spring Harbor perspectives in biology*, 3(1), p. a004978.
- Roberts, A. B. (1998) 'Molecular and cell biology of TGF- β ', *Miner Electrolyte Metab*, 24(2-3), pp. 111-9.
- Roberts, A. B. (1999) 'TGF-beta signaling from receptors to the nucleus', *Microbes Infect*, 1(15), pp. 1265-73.
- Rock, M. J., Cain, S. A., Freeman, L. J., Morgan, A., Mellody, K., Marson, A., Shuttleworth, C. A., Weiss, A. S. and Kielty, C. M. (2004) 'Molecular basis of elastic fiber formation. Critical interactions and a tropoelastin-fibrillin-1 cross-link', *J Biol Chem*, 279(22), pp. 23748-58.
- Rose, M. J. and Page, C. (2004) 'Glycosaminoglycans and the regulation of allergic inflammation', *Curr Drug Targets Inflamm Allergy*, 3(3), pp. 221-5.
- Rosenbloom, J., Abrams, W. R. and Mecham, R. (1993) 'Extracellular matrix 4: the elastic fiber', *FASEB J*, 7(13), pp. 1208-18.

- Rosso, F., Giordano, A., Barbarisi, M. and Barbarisi, A. (2004) 'From cell-ECM interactions to tissue engineering', *J Cell Physiol*, 199(2), pp. 174-80.
- Ruiz-Ortega, M., Rodriguez-Vita, J., Sanchez-Lopez, E., Carvajal, G. and Egido, J. (2007) 'TGF- β signaling in vascular fibrosis', *Cardiovasc Res*, 74(2), pp. 196-206.
- Sabatier, L., Chen, D., Fagotto-Kaufmann, C., Hubmacher, D., McKee, M. D., Annis, D. S., Mosher, D. F. and Reinhardt, D. P. (2009) 'Fibrillin Assembly Requires Fibronectin', *Molecular Biology of the Cell*, 20(3), pp. 846-858.
- Saharinen, J. and Keski-Oja, J. (2000) 'Specific sequence motif of 8-Cys repeats of TGF- β binding proteins, LTBPs, creates a hydrophobic interaction surface for binding of small latent TGF- β ', *Mol Biol Cell*, 11(8), pp. 2691-704.
- Saharinen, J., Taipale, J. and Keski-Oja, J. (1996) 'Association of the small latent transforming growth factor-beta with an eight cysteine repeat of its binding protein LTBP-1', *EMBO J*, 15(2), pp. 245-53.
- Saharinen, J., Taipale, J., Monni, O. and Keski-Oja, J. (1998) 'Identification and characterization of a new latent transforming growth factor-beta-binding protein, LTBP-4', *J Biol Chem*, 273(29), pp. 18459-69.
- Sakai, L. Y., Keene, D. R. and Engvall, E. (1986a) 'Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils', *The Journal of Cell Biology*, 103(6), pp. 2499-2509.
- Sakai, L. Y., Keene, D. R. and Engvall, E. (1986b) 'Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils', *J Cell Biol*, 103(6 Pt 1), pp. 2499-509.
- Sakai, L. Y., Keene, D. R., Glanville, R. W. and Bachinger, H. P. (1991) 'Purification and partial characterization of fibrillin, a cysteine-rich structural component of connective tissue microfibrils', *J Biol Chem*, 266(22), pp. 14763-70.
- Sakai, L. Y., Keene, D. R., Morris, N. P. and Burgeson, R. E. (1986c) 'Type VII collagen is a major structural component of anchoring fibrils', *The Journal of Cell Biology*, 103(4), pp. 1577-1586.
- Santos, S., Peinado, V. I., Ramírez, J., Melgosa, T., Roca, J., Rodriguez-Roisin, R. and Barberà, J. A. (2002) 'Characterization of pulmonary vascular remodelling in smokers and patients with mild COPD', *European Respiratory Journal*, 19(4), pp. 632-638.
- Sasisekharan, R., Raman, R. and Prabhakar, V. (2006) 'Glycomics approach to structure-function relationships of glycosaminoglycans', *Annu Rev Biomed Eng*, 8, pp. 181-231.

- Schultz, G. S., Ladwig, G. and Wysocki, A. 'Extracellular matrix: review of its roles in acute and chronic wounds'.
- Schultz, G. S. and Wysocki, A. (2009) 'Interactions between extracellular matrix and growth factors in wound healing', *Wound Repair Regen*, 17(2), pp. 153-62.
- Sengle, G., Charbonneau, N. L., Ono, R. N., Sasaki, T., Alvarez, J., Keene, D. R., Bächinger, H. P. and Sakai, L. Y. (2008) 'Targeting of Bone Morphogenetic Protein Growth Factor Complexes to Fibrillin', *Journal of Biological Chemistry*, 283(20), pp. 13874-13888.
- Sengle, G., Tsutsui, K., Keene, D. R., Tufa, S. F., Carlson, E. J., Charbonneau, N. L., Ono, R. N., Sasaki, T., Wirtz, M. K., Samples, J. R., Fessler, L. I., Fessler, J. H., Sekiguchi, K., Hayflick, S. J. and Sakai, L. Y. (2012) 'Microenvironmental regulation by fibrillin-1', *PLoS Genet*, 8(1), p. e1002425.
- Sherratt (2009a) 'Tissue elasticity and the ageing elastic fibre', *Age (Dordr)*, 31(4), pp. 305-25.
- Sherratt, M. (2009b) 'Tissue elasticity and the ageing elastic fibre', *AGE*, 31(4), pp. 305-325.
- Sherratt, M. J. (2009c) 'Tissue elasticity and the ageing elastic fibre', *Age (Dordr)*, 31(4), pp. 305-25.
- Shi, H. X., Lin, C., Lin, B. B., Wang, Z. G., Zhang, H. Y., Wu, F. Z., Cheng, Y., Xiang, L. J., Guo, D. J., Luo, X., Zhang, G. Y., Fu, X. B., Bellusci, S., Li, X. K. and Xiao, J. (2013) 'The anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo', *PLoS One*, 8(4), p. e59966.
- Shi, Y. and Massague, J. (2003) 'Mechanisms of TGF-beta signaling from cell membrane to the nucleus', *Cell*, 113(6), pp. 685-700.
- Shifren, A. and Mecham, R. P. (2006) 'The stumbling block in lung repair of emphysema: elastic fiber assembly', *Proc Am Thorac Soc*, 3(5), pp. 428-33.
- Shipley, J. M., Mecham, R. P., Maus, E., Bonadio, J., Rosenbloom, J., McCarthy, R. T., Baumann, M. L., Frankfater, C., Segade, F. and Shapiro, S. D. (2000) 'Developmental expression of latent transforming growth factor β binding protein 2 and its requirement early in mouse development', *Mol Cell Biol*, 20(13), pp. 4879-87.
- Starcher, B. C. (2000) 'Lung elastin and matrix', *Chest*, 117(5 Suppl 1), pp. 229S-34S.
- Sternier-Kock, A., Thorey, I. S., Koli, K., Wempe, F., Otte, J., Bangsow, T., Kuhlmeier, K., Kirchner, T., Jin, S., Keski-Oja, J. and von Melchner, H. (2002) 'Disruption of the gene encoding the latent transforming growth factor-beta binding protein 4 (LTBP-

- 4) causes abnormal lung development, cardiomyopathy, and colorectal cancer', *Genes Dev*, 16(17), pp. 2264-73.
- Stone, E. M., Braun, T. A., Russell, S. R., Kuehn, M. H., Lotery, A. J., Moore, P. A., Eastman, C. G., Casavant, T. L. and Sheffield, V. C. (2004) 'Missense Variations in the Fibulin 5 Gene and Age-Related Macular Degeneration', *New England Journal of Medicine*, 351(4), pp. 346-353.
- Stone, P. J., Gottlieb, D. J., O'Connor, G. T., Ciccolella, D. E., Breuer, R., Bryan-Rhadfi, J., Shaw, H. A., Franzblau, C. and Snider, G. L. (1995) 'Elastin and Collagen Degradation Products in Urine of Smokers With and Without Chronic Obstructive Pulmonary Disease', *American Journal of Respiratory and Critical Care Medicine*, 151(4), pp. 952-959.
- Strutz, F. and Neilson, E. G. (2003) 'New insights into mechanisms of fibrosis in immune renal injury', *Springer Semin Immunopathol*, 24(4), pp. 459-76.
- Strutz, F., Zeisberg, M., Renziehausen, A., Raschke, B., Becker, V., van Kooten, C. and Muller, G. (2001) 'TGF-beta 1 induces proliferation in human renal fibroblasts via induction of basic fibroblast growth factor (FGF-2)', *Kidney Int*, 59(2), pp. 579-92.
- Superti-Furga, A., Steinmann, B., Ramirez, F. and Byers, P. (1989) 'Molecular defects of type III procollagen in Ehlers-Danlos syndrome type IV', *Human Genetics*, 82(2), pp. 104-108.
- Taipale, J. and Keski-Oja, J. (1997) 'Growth factors in the extracellular matrix', *FASEB J*, 11(1), pp. 51-9.
- Taipale, J., Lohi, J., Saarinen, J., Kovanen, P. T. and Keski-Oja, J. (1995) 'Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells', *J Biol Chem*, 270(9), pp. 4689-96.
- Taylor, K. R. and Gallo, R. L. (2006) 'Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation', *FASEB J*, 20(1), pp. 9-22.
- Tiedemann, K., Batge, B., Muller, P. K. and Reinhardt, D. P. (2001a) 'Interactions of fibrillin-1 with heparin/heparan sulfate, implications for microfibrillar assembly', *J Biol Chem*, 276(38), pp. 36035-42.
- Tiedemann, K., Bätge, B., Müller, P. K. and Reinhardt, D. P. (2001b) 'Interactions of Fibrillin-1 with Heparin/Heparan Sulfate, Implications for Microfibrillar Assembly', *Journal of Biological Chemistry*, 276(38), pp. 36035-36042.

- Timpl, R., Sasaki, T., Kostka, G. and Chu, M. L. (2003) 'Fibulins: a versatile family of extracellular matrix proteins', *Nat Rev Mol Cell Biol*, 4(6), pp. 479-89.
- Todorovic, V., Friendewey, D., Gutstein, D. E., Chen, Y., Freyer, L., Finnegan, E., Liu, F., Murphy, A., Valenzuela, D., Yancopoulos, G. and Rifkin, D. B. (2007) 'Long form of latent TGF-beta binding protein 1 (Ltbp1L) is essential for cardiac outflow tract septation and remodeling', *Development*, 134(20), pp. 3723-32.
- Trelstad, R. L. and Hayashi, K. (1979) 'Tendon collagen fibrillogenesis: Intracellular subassemblies and cell surface changes associated with fibril growth', *Developmental Biology*, 71(2), pp. 228-242.
- Tsubaki, M., Yamazoe, Y., Yanae, M., Satou, T., Itoh, T., Kaneko, J., Kidera, Y., Moriyama, K. and Nishida, S. (2011) 'Blockade of the Ras/MEK/ERK and Ras/PI3K/Akt pathways by statins reduces the expression of bFGF, HGF, and TGF- β as angiogenic factors in mouse osteosarcoma', *Cytokine*, 54(1), pp. 100-7.
- Tsuruga, E., Oka, K., Hatakeyama, Y., Isokawa, K. and Sawa, Y. (2012) 'Latent transforming growth factor-beta binding protein 2 negatively regulates coalescence of oxytalan fibers induced by stretching stress', *Connect Tissue Res*, 53(6), pp. 521-7.
- Tsuruga, E., Yajima, T. and Irie, K. (2004) 'Induction of fibulin-5 gene is regulated by tropoelastin gene, and correlated with tropoelastin accumulation in vitro', *Int J Biochem Cell Biol*, 36(3), pp. 395-400.
- Tuan, T. L. and Nichter (1998) 'The molecular basis of keloid and hypertrophic scar formation', *Mol Med Today*, 4, pp. 19-24.
- Turtoi, A., Musmeci, D., Wang, Y., Dumont, B., Somja, J., Bevilacqua, G., De Pauw, E., Delvenne, P. and Castronovo, V. (2011) 'Identification of novel accessible proteins bearing diagnostic and therapeutic potential in human pancreatic ductal adenocarcinoma', *J Proteome Res*, 10(9), pp. 4302-13.
- U Lindahl, a. and Hook, M. (1978) 'Glycosaminoglycans and Their Binding to Biological Macromolecules', *Annual Review of Biochemistry*, 47(1), pp. 385-417.
- Urbán, Z. and Boyd, C. D. (2000) 'Elastic-Fiber Pathologies: Primary Defects in Assembly—and Secondary Disorders in Transport and Delivery', *The American Journal of Human Genetics*, 67(1), pp. 4-7.
- Urban, Z., Huchtagowder, V., Schurmann, N., Todorovic, V., Zilberberg, L., Choi, J., Sens, C., Brown, C. W., Clark, R. D., Holland, K. E., Marble, M., Sakai, L. Y., Dabovic, B., Rifkin, D. B. and Davis, E. C. (2009) 'Mutations in LTBP4 cause a syndrome of

- impaired pulmonary, gastrointestinal, genitourinary, musculoskeletal, and dermal development', *Am J Hum Genet*, 85(5), pp. 593-605.
- Urban, Z., Michels, V. V., Thibodeau, S. N., Davis, E. C., Bonnefont, J. P., Munnich, A., Eyskens, B., Gewillig, M., Devriendt, K. and Boyd, C. D. (2000) 'Isolated supravalvular aortic stenosis: functional haploinsufficiency of the elastin gene as a result of nonsense-mediated decay', *Hum Genet*, 106(6), pp. 577-88.
- Vehvilainen, P., Hyytiainen, M. and Keski-Oja, J. (2003) 'Latent transforming growth factor- β -binding protein 2 is an adhesion protein for melanoma cells', *J Biol Chem*, 278(27), pp. 24705-13.
- Verhaegen, P. D., van Zuijlen, P. P., Pennings, N. M., van Marle, J., Niessen, F. B., van der Horst, C. M. and Middelkoop, E. (2009) 'Differences in collagen architecture between keloid, hypertrophic scar, normotrophic scar, and normal skin: An objective histopathological analysis', *Wound Repair Regen*, 17(5), pp. 649-56.
- Vrhovski, B., Jensen, S. and Weiss, A. S. (1997) 'Coacervation characteristics of recombinant human tropoelastin', *Eur J Biochem*, 250(1), pp. 92-8.
- Wachi, H., Nonaka, R., Sato, F., Shibata-Sato, K., Ishida, M., Iketani, S., Maeda, I., Okamoto, K., Urban, Z., Onoue, S. and Seyama, Y. (2008) 'Characterization of the molecular interaction between tropoelastin and DANCE/fibulin-5', *J Biochem*, 143(5), pp. 633-9.
- Wagenseil, J. E. and Mecham, R. P. (2007) 'New insights into elastic fiber assembly', *Birth Defects Res C Embryo Today*, 81(4), pp. 229-40.
- Weber, K. T., Sun, Y., Tyagi, S. C. and Cleutjens, J. P. M. (1994) 'Collagen Network of the Myocardium: Function, Structural Remodeling and Regulatory Mechanisms', *Journal of Molecular and Cellular Cardiology*, 26(3), pp. 279-292.
- Werb, Z. (1997) 'ECM and cell surface proteolysis: regulating cellular ecology', *Cell*, 91(4), pp. 439-42.
- Wu, W. J., Vrhovski, B. and Weiss, A. S. (1999) 'Glycosaminoglycans mediate the coacervation of human tropoelastin through dominant charge interactions involving lysine side chains', *J Biol Chem*, 274(31), pp. 21719-24.
- Wynn, T. A. (2008) 'Cellular and molecular mechanisms of fibrosis', *J Pathol*, 214(2), pp. 199-210.
- Xiao, Y. Q., Freire-de-Lima, C. G., Schiemann, W. P., Bratton, D. L., Vandivier, R. W. and Henson, P. M. (2008) 'Transcriptional and Translational Regulation of

- Transforming Growth Factor- β Production in Response to Apoptotic Cells', *J. Immunol*, 181(5), pp. 3575-3585.
- Xie, J. L., Bian, H. N., Qi, S. H., Chen, H. D., Li, H. D., Xu, Y. B., Li, T. Z., Liu, X. S., Liang, H. Z., Xin, B. R. and Huan, Y. (2008) 'Basic fibroblast growth factor (bFGF) alleviates the scar of the rabbit ear model in wound healing', *Wound Repair Regen*, 16(4), pp. 576-81.
- Yamaguchi, Y., Mann, D. M. and Ruoslahti, E. (1990) 'Negative regulation of transforming growth factor-beta by the proteoglycan decorin', *Nature*, 346(6281), pp. 281-4.
- Yamamoto, T., Noble, N. A., Miller, D. E. and Border, W. A. (1994) 'Sustained expression of TGF-[beta]1 underlies development of progressive kidney fibrosis', *Kidney Int*, 45(3), pp. 916-927.
- Yanagisawa, H., Davis, E. C., Starcher, B. C., Ouchi, T., Yanagisawa, M., Richardson, J. A. and Olson, E. N. (2002) 'Fibulin-5 is an elastin-binding protein essential for elastic fibre development in vivo', *Nature*, 415(6868), pp. 168-71.
- Yanagisawa, H., Schluterman, M. and Brekken, R. (2009) 'Fibulin-5, an integrin-binding matricellular protein: its function in development and disease', *Journal of Cell Communication and Signaling*, 3(3-4), pp. 337-347.
- Yanagishita, M. (1993) 'Function of proteoglycans in the extracellular matrix', *Acta Pathol Jpn*, 43(6), pp. 283-93.
- Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991) 'Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor', *Cell*, 64(4), pp. 841-848.
- Yin, W., Smiley, E., Germiller, J., Mecham, R. P., Florer, J. B., Wenstrup, R. J. and Bonadio, J. (1995) 'Isolation of a novel latent transforming growth factor-beta binding protein gene (LTBP-3)', *J Biol Chem*, 270(17), pp. 10147-60.
- Yoshihara, K., Tajima, A., Komata, D., Yamamoto, T., Kodama, S., Fujiwara, H., Suzuki, M., Onishi, Y., Hatae, M., Sueyoshi, K., Fujiwara, H., Kudo, Y., Inoue, I. and Tanaka, K. (2009) 'Gene expression profiling of advanced-stage serous ovarian cancers distinguishes novel subclasses and implicates ZEB2 in tumor progression and prognosis', *Cancer Sci*, 100(8), pp. 1421-8.
- Zanetti, M., Braghetta, P., Sabatelli, P., Mura, I., Doliana, R., Colombatti, A., Volpin, D., Bonaldo, P. and Bressan, G. M. (2004) 'EMILIN-1 deficiency induces elastogenesis and vascular cell defects', *Mol Cell Biol*, 24(2), pp. 638-50.

- Zhang, Y., Conrad, A. H., Tasheva, E. S., An, K., Corpuz, L. M., Kariya, Y., Suzuki, K. and Conrad, G. W. (2005) 'Detection and quantification of sulfated disaccharides from keratan sulfate and chondroitin/dermatan sulfate during chick corneal development by ESI-MS/MS', *Invest Ophthalmol Vis Sci*, 46(5), pp. 1604-14.
- Zheng, Q., Choi, J., Rouleau, L., Leask, R. L., Richardson, J. A., Davis, E. C. and Yanagisawa, H. (2006) 'Normal Wound Healing in Mice Deficient for Fibulin-5, an Elastin Binding Protein Essential for Dermal Elastic Fiber Assembly', *J Invest Dermatol*, 126(12), pp. 2707-2714.
- Zheng, Q., Davis, E. C., Richardson, J. A., Starcher, B. C., Li, T., Gerard, R. D. and Yanagisawa, H. (2007a) 'Molecular Analysis of Fibulin-5 Function during De Novo Synthesis of Elastic Fibers', *Molecular and Cellular Biology*, 27(3), pp. 1083-1095.
- Zheng, Q., Davis, E. C., Richardson, J. A., Starcher, B. C., Li, T., Gerard, R. D. and Yanagisawa, H. (2007b) 'Molecular analysis of fibulin-5 function during de novo synthesis of elastic fibers', *Mol Cell Biol*, 27(3), pp. 1083-95.
- Zhou, J. R., Xu, Z. and Jiang, C. L. (2008) 'Neuropeptide Y promotes TGF-beta1 production in RAW264.7 cells by activating PI3K pathway via Y1 receptor', *Neurosci Bull*, 24(3), pp. 155-9.

CHAPTER 2: LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis

Mohamed A. Sideek^{a,b}, Clementine Menz^a, Mahroo K. Parsi^a, Mark A. Gibson^{a*}

^a Discipline of Anatomy and Pathology, School of Medicine, University of Adelaide, Adelaide, South Australia 5005, Australia

^b Department of Biomedical Sciences, Kulliyah of Allied Health Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia

* corresponding author. Tel: +61 88313 5337 Email address: mark.gibson@adelaide.edu.au

Received: 4 December 2012, Accepted: 15 October 2013, Available online: 19 October 2013

Matrix Biology 34 (2014) 114-123

Statement of Authorship

Title of Paper	LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis
Publication Status	<input checked="" type="checkbox"/> Published <input type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
Publication Details	Sideek, M. A., Menz, C., Parsi, M. K. and Gibson, M. A. (2014) 'LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis', <i>Matrix Biology</i> , 34, pp. 114-23.

Principal Author

Name of Principal Author (Candidate)	Mohamed Arshad Sideek			
Contribution to the Paper	<p>Performed all experiments.</p> <p>Performed analysis on all samples presented.</p> <p>Interpreted the data.</p> <p>Wrote the manuscript.</p>			
Overall percentage (%)	85%			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.			
Signature	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 60%;"></td> <td style="width: 10%; text-align: center;">Date</td> <td style="width: 30%;">30/11/2015</td> </tr> </table>		Date	30/11/2015
	Date	30/11/2015		

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Clementine Menz		
Contribution to the Paper	Helped in data interpretation. Manuscript evaluation and feedback.		
Signature		Date	29/11/2015

Name of Co-Author	Dr. Mahroo K. Parsi		
Contribution to the Paper	Supervised the development of work and data interpretation. Manuscript evaluation and feedback.		
Signature		Date	29/11/2015

Name of Co-Author	Dr. Mark A. Gibson		
Contribution to the Paper	Principal supervisor of candidate's PhD Supervise development and planning of the research Involved in data interpretation and manuscript evaluation and editing		
Signature		Date	26/11/2015

LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis

Mohamed A. Sideek^{a,b}, Clementine Menz^a, Mahroo K. Parsi^a, Mark A. Gibson^{a*}

^a Discipline of Anatomy and Pathology, School of Medicine, University of Adelaide, Adelaide, South Australia 5005, Australia

^b Department of Biomedical Sciences, Kulliyah of Allied Health Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia

* corresponding author. Tel: +61 88313 5337 Email address: mark.gibson@adelaide.edu.au

Keyword

LTBP-2, tropoelastin, fibulin-5, heparin, heparan sulfate

2.1 Abstract

Latent transforming growth factor-beta-1 binding protein-2 (LTBP-2) is a protein of ill-defined function associated with elastic fibers during elastinogenesis. Although LTBP-2 binds fibrillin-1, fibulin-5, and heparin/heparan sulfate, molecules critical for normal elastic fiber assembly, it does not interact directly with elastin or its precursor, tropoelastin. We investigated the modulating effect of LTBP-2 on two key interactions of tropoelastin during elastinogenesis a) with fibulin-5 and b) with heparan sulfate (using heparin). Firstly, using solid phase assays we showed that LTBP-2 bound fibulin-5 ($K_d = 26.47 \pm 5.68$ nM) with an affinity similar to that of the tropoelastin-fibulin-5 interaction ($K_d = 24.66 \pm 5.64$ nM). Then using a competitive binding assay we showed that LTBP-2 inhibited the tropoelastin-fibulin-5 interaction in a dose dependent manner with almost complete inhibition obtained with 5-fold molar excess of LTBP-2. Interestingly, a fragment of LTBP-2 containing the fibulin-5 binding sequence only partially inhibited the tropoelastin-fibulin-5 interaction suggesting that LTBP-2 was directly blocking only the C-terminal tropoelastin binding site on fibulin-5 and indirectly blocking tropoelastin binding to the N-terminal region. In parallel experiments heparin was shown to have minor inhibitory effects on fibulin-5 interactions with tropoelastin and LTBP-2. However, LTBP-2 was shown to significantly inhibit the binding of heparin to tropoelastin with 50% inhibition achieved with 10 fold molar excess of LTBP-2. Confocal microscopy of fibroblast matrix showed strong co-distribution of LTBP-2 with fibulin-5 and fibrillin-1 and partial co-distribution with heparan sulfate proteoglycans, perlecan and syndecan-4. Also addition of exogenous LTBP-2 to ear cartilage chondrocyte cultures blocked elastinogenesis in a concentration-dependent manner. Overall the results indicate that LTBP-2 may have a negative regulatory role during elastic fiber assembly, perhaps in displacing elastin microassemblies from complexes with fibulin-5 and/or cell surface heparan sulfate proteoglycans.

2.2 Introduction

LTBP-2 is a member of the fibrillin / LTBP super family of extracellular matrix proteins. Fibrillins 1-3 form microfibrils which, together with a core of elastin, are the main structural components of elastic fibers (Kielty et al., 2005; Ramirez and Sakai, 2010). LTBPs -1, 3, and 4, covalently bind latent growth factor TGF-beta and direct the growth factor to storage depots within the extracellular matrix (Hyytiainen et al., 2004; Rifkin, 2005). Fibrillin microfibrils are considered to be a principal storage location for these latent complexes (Ramirez and Rifkin, 2009). LTBP-2 is structurally similar to the other LTBPs, but like fibrillins, it does not directly bind TGF-beta and LTBP-2 function remains largely unknown (Gibson et al., 1995; Saharinen and Keski-Oja, 2000). Progress has been hampered by the report that LTBP-2 null mice have early lethality (Shipley et al., 2000), although more recently LTBP-2 null humans are recorded to present with mild ocular phenotypes including glaucoma, megalocornea, ectopia lentis and microspherophakia (Ali et al., 2009; Desir et al., 2010; Kumar et al., 2010; Narooie-Nejad et al., 2009). It has long been documented that LTBP-2 is associated with elastic fibers in developing elastic tissues (Gibson et al., 1995) and it appears that fibrillin-1 microfibrils are required for its incorporation into the extracellular matrix (Vehvilainen et al., 2009). In vitro studies have shown that LTBP-2 specifically binds to fibrillin-1 rather than fibrillin-2 and that LTBP-2 can compete with LTBP-1 for binding to the fibrillin-1 molecule, suggesting that LTBP-2 may indirectly affect TGF- beta bioavailability (Hirani et al., 2007). This idea is supported by a recent study linking LTBP-2 gene mutations to a recessive form of Weill-Marchesani syndrome (Haji-Seyed-Javadi et al., 2012) which is characterized by short stature, brachydactyly, thick skin and ectopia lentis (WMS, OMIM # 608328). Previously, mutations in the fibrillin-1 gene have been shown to cause some presentations of WMS (Faivre et al., 2003) as well as the more common, related disorder, Marfan Syndrome (MFS, OMIM 3154700) (Ramirez and Dietz, 2007). Many of the characteristics of these diseases have been attributed to aberrant TGF-beta signaling (Doyle et al., 2012). Interestingly, Cain et al (Cain et al., 2012) have recently shown that fibrillin-1 interactions with heparan sulfate may be disrupted in WMS. LTBP-2 also has multiple high affinity binding sites for heparin/heparan sulfate (Parsi et al., 2010) and it is possible that LTBP-2 / heparan sulfate interactions are affected in WMS cases linked to LTBP-2 gene mutations. The LTBP-2 gene has been linked to other conditions including tumor suppression in squamous cell carcinoma and meningioma (Chan et al., 2011; Perez-Magan et al., 2012) and a marker for pulmonary deaths following acute dyspnoea (Breidhardt et al., 2012).

In this paper we focus on the potential role of LTBP-2 in elastic fiber assembly. Elastic fiber formation is a complex process which involves deposition of the elastin precursor tropoelastin onto a framework of microfibrils principally composed of fibrillin polymers, usually fibrillin-1 and / or fibrillin-2 (Kielty, 2006; Wagenseil and Mecham, 2007). Tropoelastin initially forms spherical microassemblies in association with cell surface heparan sulfate proteoglycans (Cain et al., 2005; Tiedemann et al., 2001; Wagenseil and Mecham, 2007). The tropoelastin molecules are crosslinked to form elastin in association with lysyl oxidase and fibulin-4 (Horiguchi et al., 2009). The elastin microassemblies are transferred from the cell surface onto the fibrillin scaffold by an unclear mechanism involving fibulin-5 (Hirai et al., 2007; Nakamura et al., 2002; Schiemann et al., 2002). Recently LTBP-2 has been found to bind to fibulin-5 and evidence suggests that LTBP-2 may selectively direct elastin-fibulin-5 complexes onto microfibrils containing fibrillin-1, rather than fibrillin-2, during elastic fiber formation (Hirai et al., 2007). However, examination of the published binding sequences indicates that there is a major overlap between the binding sites for LTBP-2 and tropoelastin within the C-terminal region of fibulin-5 (Hirai et al., 2007; Zheng et al., 2007). Since LTBP-2 does not bind tropoelastin (Hirani et al., 2007), we investigated whether LTBP-2 enhanced or inhibited the interaction of tropoelastin with fibulin-5. LTBP-2 also has three high affinity heparin/ heparan sulfate binding sites located in the N-terminal region of the molecule (Parsi et al., 2010). Thus we also investigated whether LTBP-2 could compete with tropoelastin for binding to heparin/heparan sulfate which is a key early stage of elastinogenesis. The results indicate that LTBP-2 inhibits rather than enhances the critical interactions of tropoelastin with fibulin-5 and heparin/heparan sulfate and that excess LTBP-2 can block elastinogenesis in chondrocyte cultures.

2.3 Experimental procedures

2.3.1 Materials

Recombinant human LTBP-2 and fragments were produced using the pCEP4 expression vector in 293EBNA cells as described previously (Hirani et al., 2007; Parsi et al., 2010). Recombinant human tropoelastin was a kind gift from Prof. A Weiss, University of Sydney. Recombinant human fibulin-5 and anti-[DDK tag] antibody for recombinant fibulin-5 detection were purchased from Origene, Rockville, MD, USA. Heparin-albumin conjugate (HAC) was prepared and characterized as described previously (Parsi et al., 2010) following the method of Hennink et al. (Hennink et al., 1983). Sodium heparin from porcine intestinal mucosa and BSA were purchased from Sigma-Aldrich, St. Louis, MO. Chondroitin-6-sulfate from shark cartilage was obtained from Seikagaku Corp. Tokyo, Japan. Rabbit anti-[human LTBP-2 peptide] antibody 3504 has been described previously (Hirani et al., 2007). Anti-His4 antibodies were obtained from Qiagen (Valencia, CA). Anti-fibulin-5 (DANCE) antibody MAB3095 was purchased from R and D systems Inc., Minneapolis, MN, USA. Anti-fibrillin-1 antibody MAB1919 was purchased from Millipore Australia Ltd., Klisyth, Victoria. Anti-[human perlecan domain 1] monoclonal antibody CSI-071 and anti-[cellular fibronectin] antibody ab6328 were purchased from Abcam Plc, Cambridge, UK. Mouse anti [human syndecan-4] monoclonal antibody 5G9 was obtained from Santa Cruz inc., Santa Cruz, Ca, USA. Rabbit anti- BSA antibody A11133 was supplied by Invitrogen, Carlsbad, CA, USA. Mouse anti-human elastin monoclonal antibody BA4 was purchased from Sigma, St. Louis, MO.

2.3.2 Molecular binding assays

Solid phase binding was detected using a peroxide-ELISA system as described previously (Finnis and Gibson, 1997). Briefly, plastic flat-bottomed multiwell microtiter plates (Immuno-Maxisorp, Nalge-Nunc International, Denmark) were coated with triplicate samples of up to 400 ng of the test molecule, (tropoelastin or LTBP-2) in 100 μ l of TBS, 2 mM CaCl₂ at 4 °C overnight. Control wells were coated with BSA instead of the test protein. The wells were rinsed with TBS and blocked with 5% low-fat dried milk in TBS. A second test molecule was then added (full-length rLTBP-2 or fragment, rfibulin-5 or heparin-BSA conjugate (up to 1 μ g / 100 μ l of TBS, 2 mM CaCl₂) and incubated for 3 h at 37 °C. The wells were rinsed

three times with TBS, CaCl₂ buffer and an appropriate dilution of appropriate primary antibody (anti-His4 or anti-LTBP-2 antibody for LTBP-2, anti-DDK for fibulin-5 or anti-BSA for heparin-albumin conjugate) in TBS/ 2mM CaCl₂ / 0.05% low fat milk was added and incubated for 2.5 hours at 37 °C. Wells were then washed three times with TBS / 0.05% (v/v) tween-20 before incubation with the appropriate anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) in TBS/ 2mM CaCl₂ for 1.5 hours at 37 °C in humidity chamber. After washing four times with TBS/tween-20, binding was detected using 100µl of 3',3',5',5' -tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO) following 20 min incubation. Absorbance was measured at 450 nm, using a Titertek Multiskan plate reader (Flow Laboratories, North Ryde, NSW, Australia).

To determine the strength of interactions, increasing concentrations of rFibulin-5 (0-40 nM) was incubated with a constant amount of tropoelastin (35 ng) or LTBP-2 (200 ng) coated on the wells and binding was detected with anti-DDK antibody as described above. Background binding, calculated from BSA-coated control wells, was then subtracted. The amount of bound protein was measured from a standard ELISA curve, produced concurrently with each experiment, for fibulin-5 coated directly onto microtiter wells. Dissociation constants were calculated from the resulting quantitative binding curves, using non-linear regression analysis (Prism 4.0 program, Graphpad software, San Diego, CA). For inhibition of fibulin-5/tropoelastin interactions, rFibulin-5 (100 ng [2 pmol]/well) was pre-treated with up to 10-fold molar excess of rLTBP-2 or fragment LTBP-2 NT(H) for 15 min with gentle shaking, prior to incubation with tropoelastin (400 ng) coated on the wells. Similarly, for inhibition of heparin/tropoelastin interactions, HAC [50 ng (1.4 pmol heparin equivalent)/well] was pre-treated with up to 10 fold molar excess of rLTBP-2 prior to incubation with tropoelastin-coated wells. HAC binding was detected using anti-BSA antibody.

2.3.3 Confocal microscopy

Human skin fibroblasts (from biopsy of deltoid region of healthy adult, subject 239/05) were plated onto microscope chamber slides (Lab-Tek [Nunc]) at an initial density of 4 x 10⁵ /ml and grown for 3 weeks post-confluency in DMEM plus 10% fetal calf serum. The cell layer was fixed by incubation with 3% para-formaldehyde in PBS for 10 min followed by cold acetone/methanol mixture for 1 min, then rehydrated in PBS for 5 min. To expose reactive epitopes, the slides were incubated with 2m GuHCl/50 mM Tris pH 8.0 containing 50 mM

dithiothreitol for 5 min then with iodoacetamide (110 mM) for 5 min in the dark. The slides were blocked with ovalbumin (1% in TBS) for 15 min then incubated overnight at 4 °C with appropriate pairs of primary antibodies. Control sections were incubated with matching concentrations of rabbit and mouse IgG. After washing with PBS, the slides were incubated with appropriate secondary antibodies conjugated to fluorophore Alexa 488 (Invitrogen) or Cy3 (Jackson Immunoresearch, West Grove, PA, USA) for 90 min in the dark. After washing the slides were mounted in 90% glycerol/10% TBS containing anti-fade reagent p-phenylenediamine prior to scanning on a Leica SP5 spectral confocal microscope. Images were obtained sequentially, firstly for alexa488 with excitation at 488 nm and emission filtering between 498 and 551 nm then for cy3 with excitation at 561 nm and emission filtering between 571 and 650 nm. Confocal images were processed using Confocal Assistant software version 4.02 and all images were assimilated into figures using Adobe Photoshop version 6.0 (Adobe Ltd., San Jose, CA, USA).

Chondrocytes were grown from explants of dissected ear cartilage from a 220-day-old fetal bovine. After passage, the cells were cultured on microscope chamber slides to 7 days post-confluence in DMEM plus 10% fetal calf serum (0.4 ml/well). The cells were then cultured in DMEM plus 3% fetal calf serum for a further 7 days with the daily addition of recombinant human LTBP-2 (0-10 µg/ ml). The cell layer was lightly fixed in acetone / methanol, rinsed 3x with tris-buffered saline and incubated for 18h at 4 °C with 1 µg/ml of anti-elastin monoclonal antibody BA4 (Sigma). Following washing in TBS, antibody binding was detected using FITC conjugated to goat anti-mouse IgG antibody and confocal microscopy.

2.4 Results and discussion

2.4.1 LTBP-2 and tropoelastin have similar binding affinities for fibulin-5.

Both LTBP-2 and tropoelastin have been reported to bind to fibulin-5 (Hirai et al., 2007; Zheng et al., 2007) but their relative binding affinities have not previously been measured. We therefore use solid phase binding assays to determine the Kds for fibulin-5 interactions with LTBP-2 and tropoelastin. Firstly we confirmed that rFibulin-5 in the liquid phase strongly bound to both rLTBP-2 (Fig 2.1A) and rtropoelastin (Fig 2.1B) coated on microtiter wells, and that rLTBP-2 did not bind to rtropoelastin (Fig 2.1C). We then produced binding curves for rFibulin-5 interactions with LTBP-2 and tropoelastin by adding increasing amounts of fibulin-5 in the liquid phase to a constant amount of the other protein coated on the wells. The amount of fibulin-5 bound was quantitated from standard ELISA curves, constructed concurrently, of fibulin-5 coated directly on the wells (data not shown). Finally, graphs were plotted of fibulin-5 bound versus fibulin-5 added for LTBP-2 (Fig 2.1D) and tropoelastin (Fig 2.1E). Using the Prism program, the Kds for fibulin-5 binding to LTBP-2 (26.47 ± 5.68 nM) and tropoelastin (24.66 ± 5.64 nM) were calculated from non-linear regression analysis of the curves. The results show that fibulin-5 binds LTBP-2 and tropoelastin with similar affinities.

2.4.2 LTBP-2 can completely block the interaction of tropoelastin with fibulin-5.

Since LTBP-2 and tropoelastin bind to the same C-terminal domain of fibulin-5 (Hirai et al., 2007; Zheng et al., 2007) we determined if LTBP-2 could modulate the fibulin-5/tropoelastin interaction. Initially LTBP-2 was found to substantially inhibit, rather than enhance, this interaction when added in 5-fold molar excess, presumably by competing with tropoelastin for binding to fibulin-5 (Fig 2.2A). The experiment was repeated using the N-terminal LTBP-2 fragment, LTBP-2 NT(H) which contained the fibulin-5 binding activity (Fig 2.2B). Fragment LTBP-2 NT(H) also inhibited the tropoelastin-fibulin-5 interaction but apparently to a lesser extent than the full-length molecule (Fig 2.2C). This finding was confirmed following the production of inhibition curves for LTBP-2 and fragment LTBP-2 NT(H) (Fig 2.2D). LTBP-2 showed concentration dependent inhibition of the tropoelastin-fibulin-5 interaction with complete inhibition being achieved at 10 fold molar excess of LTBP-2 (Fig 2.2D). In contrast, fragment LTBP-2 NT(H) was only able to reduce the interaction by approx. 50% at the same molar concentration even though this fragment contains all of the

fibulin-5 binding activity. The reason for this difference is unclear but it may be due to indirect blockade by bound full-length LTBP-2 of the second tropoelastin binding site at the N-terminal end of the fibulin-5 molecule (Zheng et al., 2007). The shorter fragment LTBP-2 NT(H) may not obstruct this site when bound to fibulin-5.

2.4.3 Heparin shows only minor inhibition of fibulin-5 interaction with tropoelastin and LTBP-2.

HSPGs appear to play a critical if ill-defined role in elastic fiber assembly (Cain et al., 2005; Tiedemann et al., 2001; Wagenseil and Mecham, 2007). LTBP-2 has a strong affinity for heparin/heparan sulfate and has several binding sites in its N-terminal region (Parsi et al., 2010), one of which is adjacent to the documented fibulin-5 binding site (Hirai et al., 2007). We therefore determined if heparin could modulate the binding of fibulin-5 to LTBP-2. Ten-fold molar excess of heparin was found to have a minor but significantly negative effect on LTBP-2 binding to fibulin-5 (Fig 2.3B). The result was similar to the effect of heparin on the interaction between tropoelastin and fibulin-5 (Fig 2.3A). Incubation with chondroitin sulfate instead of heparin had no effect on the LTBP-2-fibulin-5 interaction, indicating that the inhibitory effect of heparin was specific to that class of glycosaminoglycan (Fig 2.3C).

2.4.4 LTBP-2 substantially inhibits the interaction of tropoelastin with heparin.

The interaction of tropoelastin with cell surface heparan sulfate proteoglycans appears to be essential for the early stages of elastin assembly (Wagenseil and Mecham, 2007). It is unclear how the nascent elastin microassemblies are detached from the cell surface to be deposited on to the fibrillin microfibril scaffold. Since LTBP-2 is associated with elastic fibers during elastinogenesis and has a very high affinity for heparin/heparan sulfate it is likely LTBP-2 interacts with cell surface HSPGs during elastic fiber assembly. We therefore determined if LTBP-2 enhanced or inhibited the interaction of tropoelastin with heparin. Fivefold molar excess of LTBP-2 over heparin showed substantial but incomplete inhibition of the tropoelastin-heparin interaction (Fig 2.4A). In a subsequent experiment the inhibition by LTBP-2 was found to be concentration dependent with maximum inhibition of around 65% attained with 10 fold molar excess of LTBP-2 (Fig 2.4B). The finding indicates that LTBP-2 can compete with some but not all tropoelastin binding motifs on the heparin molecule. Thus,

LTBP-2 may be able to compete with tropoelastin for binding to certain cell surface heparan sulfate proteoglycans, depending on the microstructure of their heparan sulfate chains.

2.4.5 Co-localization of LTBP-2 with fibrillin-1, fibulin-5 and heparan sulfate proteoglycans in fibroblast matrix.

Using confocal microscopy, the distribution of LTBP-2 in the matrix elaborated by fibroblasts in culture was compared to those of fibrillin-1, fibulin-5, fibronectin and heparan sulfate proteoglycans perlecan and syndecan-4. (Fig 2.5). Extensive co-distribution of LTBP-2 was observed with fibrillin-1 (Fig 2.5A-C), fibulin-5 (Fig 2.5D-E), and fibronectin (Fig 2.5G-I) indicating that both LTBP-2 and fibulin-5 have the opportunity to interact with fibrillin and fibronectin microfibrils. This is consistent with reports that fibrillin-1 requires fibronectin to form microfibrils (Sabatier et al., 2009) and that LTBP-2 requires fibrillin-1 for its incorporation into the matrix (Vehvilainen et al., 2009). Clearly fibulin-5 is also in close association with fibrillin-1 indicating that molecular interaction between LTBP-2 and fibulin-5 on the surface of the microfibrils is a strong possibility. Although the fibroblasts make tropoelastin it showed poor incorporation into the matrix (data not shown). LTBP-2 localization showed partial overlap with those of perlecan (Fig 2.5J-L) and syndecan-4 (Fig 2.5M-O). Perlecan appeared to be mainly matrix associated whereas syndecan-4 showed mainly a cell surface distribution. Thus it is apparent that LTBP-2 also has opportunity for molecular interactions with HSPGs at the cell surface and within the extracellular matrix.

2.4.6 Exogenous LTBP-2 blocks elastinogenesis by cultured chondrocytes.

Initial experiments using skin fibroblasts were hampered by poor and inconsistent elastinogenesis evident in culture even after 3 weeks post-confluency. We therefore used chondrocytes cultured from fetal bovine elastic ear cartilage which consistently produced extensive elastin-rich networks two weeks after confluency in the presence of reduced levels of FCS (3%) (Fig 2.6A). In the experiment cultures were grown to 7 days post confluency to establish an extensive network of fibrillin microfibrils within the matrix. No elastin was evident at this stage (data not shown). Some cultures were then supplemented with exogenous LTBP-2 daily for a further 7 days. LTBP-2 was found to inhibit elastinogenesis in a dose dependent manner. At 1 μ g/ml LTBP-2 caused evident reduction in elastin staining (Fig 2.6B), at 2.5 μ g/ml LTBP-2 almost completely blocked elastinogenesis (Fig 2.6C) and no elastin deposition was

evident with LTBP-2 at 10 µg/ ml (Fig 2.6D). The results indicated that exogenous LTBP-2 caused critical disruption of elastin deposition into the chondrocyte matrix and thus that LTBP-2 can act as a negative modulator of elastinogenesis in a cell culture model. It is also apparent that spatiotemporal production and organization of LTBP-2 must be tightly controlled by cells during normal elastinogenesis.

The mechanism(s) whereby exogenous LTBP-2 inhibits elastinogenesis are unclear but the experiments above point to two key interactions inhibited by LTBP-2 in vitro which are important in the elastinogenic process, the binding of tropoelastin to heparin, mimicking the interaction of the elastin precursor with cell surface HSPGs, and the binding of tropoelastin to fibulin-5, evidently important for deposition of elastin microassemblies onto the fibrillin microfibril scaffold (Wagenseil and Mecham, 2007). Thus at face value LTBP-2 appears to be a negative modulator of elastic fiber assembly by interacting with fibulin-5 and inhibiting the binding of elastin to fibulin-5. LTBP-2 may be involved in specifying spatiotemporal release of elastin globules from fibulin-5 complexes for deposition onto the fibrillin microfibrils. It has been suggested by Hirai et al (Hirai et al., 2007) that LTBP-2 may attach to nascent elastin-fibulin-5 complexes and direct these microassemblies specifically to fibrillin-1 microfibrils. However since we have shown that LTBP-2 is inhibitory to TE-fibulin-5 interactions it seems unlikely that LTBP-2 can directly participate in these aggregates since its binding site on fibulin-5 will be blocked by (tropo)elastin. An alternative possibility is that LTBP-2 is attached to the surface of fibrillin-1 microfibrils, rather than the elastin complex, where it facilitates the release of the nascent elastin globules from the complex with fibulin-5 for deposition onto the microfibril by binding to and displacing the fibulin-5. Similarly, LTBP-2 may also contribute to controlling the rate of release of elastin-microassemblies from the cell surface HSPGs (candidates include syndecan-4 and perlecan) by a similar competitive displacement mechanism. These concepts are represented schematically in Fig 2.8.

Conversely, the complete lack of LTBP-2 does not grossly affect elastic fiber assembly in all tissues, as evidenced by LTBP-2 null humans which have elastic fiber and microfibril defects largely confined to ocular tissues (Ali et al., 2009; Desir et al., 2010; Kumar et al., 2010; Narooie-Nejad et al., 2009). Moreover the documented LTBP-2 gene mutations linked to WMS result in tissue specific disruption of the elastic fiber and microfibril organization within the skin and the eyes of the affected patients (Haji-Seyed-Javadi et al., 2012). Thus it seems likely that LTBP-2 plays a subtle role during elastinogenesis perhaps influencing the fine organization of the nascent elastic fibers in particular tissues. Without LTBP-2, the fine

control may be lost but the assembled fiber may be functionally viable except where fine, specialized elastic fiber architecture is required, such as in the ocular trabecular network and the skin. In these tissues, elastic fiber distribution is complex with elastin-rich and elastin-free microfibrillar bundles often in close proximity to each other (Cotta-Pereira et al., 1976; Kielty, 2006; Schlotzer-Schrehardt et al., 1997). Recent studies using stretched periodontal ligament fibroblasts in culture have indicated that LTBP-2, in coordination with fibulin-5, controls the diameter and organization of fibrillin microfibrils during aggregation into thick bundles (termed oxytalan fibers) (Hisanaga et al., 2009; Nakashima et al., 2009; Tsuruga et al., 2009). Interestingly stretching the cells induced aggregation and this was accompanied by up-regulation of fibulin-5 expression by the cells and a loss of LTBP-2 from the microfibrils which suggested an exchange of the two proteins on the surface of these structures (Hisanaga et al., 2009; Tsuruga et al., 2012). These observations are consistent with LTBP-2 fulfilling its functions as part of an interactive complex of molecules on the surface of microfibrils, involving fibulin-5, fibrillin-1, heparan sulfate and perhaps members of the ADAMTS protease family, genetic mutations of which have also been linked to WMS (Hubmacher and Apte, 2011; Sengle et al., 2012).

2.5 Acknowledgments

This work was supported by the National Health and Medical Research Council of Australia project grant number 519211. Part of the work was supported by a scholarship to M.A.S. from the International Islamic University of Malaysia and the Malaysian Government.

2.6 References

- Ali, M., McKibbin, M., Booth, A., Parry, D.A., Jain, P., Riazuddin, S.A., Hejtmancik, J.F., Khan, S.N., Firasat, S., Shires, M., Gilmour, D.F., Towns, K., Murphy, A.L., Azmanov, D., Tournev, I., Cherninkova, S., Jafri, H., Raashid, Y., Toomes, C., Craig, J., Mackey, D.A., Kalaydjieva, L., Riazuddin, S., Inglehearn, C.F., 2009. Null mutations in LTBP2 cause primary congenital glaucoma. *Am J Hum Genet* 84, 664-671.
- Breidhardt, T., Vanpoucke, G., Potocki, M., Mosimann, T., Ziller, R., Thomas, G., Laroy, W., Moerman, P., Socrates, T., Drexler, B., Mebazaa, A., Kas, K., Mueller, C., 2012. The novel marker LTBP2 predicts all-cause and pulmonary death in patients with acute dyspnoea. *Clin Sci (Lond)* 123, 557-566.
- Cain, S.A., Baldock, C., Gallagher, J., Morgan, A., Bax, D.V., Weiss, A.S., Shuttleworth, C.A., Kielty, C.M., 2005. Fibrillin-1 interactions with heparin. Implications for microfibril and elastic fiber assembly. *J Biol Chem* 280, 30526-30537.
- Cain, S.A., McGovern, A., Baldwin, A.K., Baldock, C., Kielty, C.M., 2012. Fibrillin-1 mutations causing Weill-Marchesani syndrome and acromicric and geleophysic dysplasias disrupt heparan sulfate interactions. *PloS one* 7, e48634.
- Chan, S.H., Yee Ko, J.M., Chan, K.W., Chan, Y.P., Tao, Q., Hyytiainen, M., Keski-Oja, J., Law, S., Srivastava, G., Tang, J., Tsao, S.W., Chen, H., Stanbridge, E.J., Lung, M.L., 2011. The ECM protein LTBP-2 is a suppressor of esophageal squamous cell carcinoma tumor formation but higher tumor expression associates with poor patient outcome. *Int J Cancer* 129, 565-573.
- Cotta-Pereira, G., Guerra Rodrigo, F., Bittencourt-Sampaio, S., 1976. Oxytalan, elaunin, and elastic fibers in the human skin. *The Journal of investigative dermatology* 66, 143-148.
- Desir, J., Sznajder, Y., Depasse, F., Roulez, F., Schrooyen, M., Meire, F., Abramowicz, M., 2010. LTBP2 null mutations in an autosomal recessive ocular syndrome with

- megalocornea, spherophakia, and secondary glaucoma. *Eur J Hum Genet* 18, 761-767.
- Doyle, J.J., Gerber, E.E., Dietz, H.C., 2012. Matrix-dependent perturbation of TGFbeta signaling and disease. *FEBS Lett* 586, 2003-2015.
- Faivre, L., Gorlin, R.J., Wirtz, M.K., Godfrey, M., Dagoneau, N., Samples, J.R., Le Merrer, M., Collod-Beroud, G., Boileau, C., Munnich, A., Cormier-Daire, V., 2003. In frame fibrillin-1 gene deletion in autosomal dominant Weill-Marchesani syndrome. *Journal of medical genetics* 40, 34-36.
- Finnis, M.L., Gibson, M.A., 1997. Microfibril-associated glycoprotein-1 (MAGP-1) binds to the pepsin-resistant domain of the alpha3(VI) chain of type VI collagen. *J Biol Chem* 272, 22817-22823.
- Gibson, M.A., Hatzinikolas, G., Davis, E.C., Baker, E., Sutherland, G.R., Mecham, R.P., 1995. Bovine latent transforming growth factor beta 1-binding protein 2: molecular cloning, identification of tissue isoforms, and immunolocalization to elastin-associated microfibrils. *Mol Cell Biol* 15, 6932-6942.
- Haji-Seyed-Javadi, R., Jelodari-Mamaghani, S., Paylakhi, S.H., Yazdani, S., Nilforushan, N., Fan, J.B., Klotzle, B., Mahmoudi, M.J., Ebrahimian, M.J., Chelich, N., Taghiabadi, E., Kamyab, K., Boileau, C., Paisan-Ruiz, C., Ronaghi, M., Elahi, E., 2012. LTBP2 mutations cause Weill-Marchesani and Weill-Marchesani-like syndrome and affect disruptions in the extracellular matrix. *Human mutation* 33, 1182-1187.
- Hennink, W.E., Feijen, J., Ebert, C.D., Kim, S.W., 1983. Covalently bound conjugates of albumin and heparin: synthesis, fractionation and characterization. *Thromb Res* 29, 1-13.
- Hirai, M., Horiguchi, M., Ohbayashi, T., Kita, T., Chien, K.R., Nakamura, T., 2007. Latent TGF-beta-binding protein 2 binds to DANCE/fibulin-5 and regulates elastic fiber assembly. *EMBO J* 26, 3283-3295.
- Hirani, R., Hanssen, E., Gibson, M.A., 2007. LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein. *Matrix biology : journal of the International Society for Matrix Biology* 26, 213-223.
- Hisanaga, Y., Nakashima, K., Tsuruga, E., Nakatomi, Y., Hatakeyama, Y., Ishikawa, H., Sawa, Y., 2009. Fibulin-5 contributes to microfibril assembly in human periodontal ligament cells. *Acta Histochem Cytochem* 42, 151-157.

- Horiguchi, M., Inoue, T., Ohbayashi, T., Hirai, M., Noda, K., Marmorstein, L.Y., Yabe, D., Takagi, K., Akama, T.O., Kita, T., Kimura, T., Nakamura, T., 2009. Fibulin-4 conducts proper elastogenesis via interaction with cross-linking enzyme lysyl oxidase. *Proceedings of the National Academy of Sciences of the United States of America* 106, 19029-19034.
- Hubmacher, D., Apte, S.S., 2011. Genetic and functional linkage between ADAMTS superfamily proteins and fibrillin-1: a novel mechanism influencing microfibril assembly and function. *Cell Mol Life Sci* 68, 3137-3148.
- Hyytiainen, M., Penttinen, C., Keski-Oja, J., 2004. Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci* 41, 233-264.
- Kielty, C.M., 2006. Elastic fibres in health and disease. *Expert Rev Mol Med* 8, 1-23.
- Kielty, C.M., Sherratt, M.J., Marson, A., Baldock, C., 2005. Fibrillin microfibrils. *Adv Protein Chem* 70, 405-436.
- Kinsey, R., Williamson, M.R., Chaudhry, S., Mellody, K.T., McGovern, A., Takahashi, S., Shuttleworth, C.A., Kielty, C.M., 2008. Fibrillin-1 microfibril deposition is dependent on fibronectin assembly. *J Cell Sci* 121, 2696-2704.
- Kumar, A., Duvvari, M.R., Prabhakaran, V.C., Shetty, J.S., Murthy, G.J., Blanton, S.H., 2010. A homozygous mutation in LTBP2 causes isolated microspherophakia. *Hum Genet* 128, 365-371.
- Nakamura, T., Lozano, P.R., Ikeda, Y., Iwanaga, Y., Hinek, A., Minamisawa, S., Cheng, C.F., Kobuke, K., Dalton, N., Takada, Y., Tashiro, K., Ross Jr, J., Honjo, T., Chien, K.R., 2002. Fibulin-5/DANCE is essential for elastogenesis in vivo. *Nature* 415, 171-175.
- Nakashima, K., Tsuruga, E., Hisanaga, Y., Ishikawa, H., Sawa, Y., 2009. Stretching stimulates fibulin-5 expression and controls microfibril bundles in human periodontal ligament cells. *J Periodontal Res* 44, 622-627.
- Narooie-Nejad, M., Paylakhi, S.H., Shojaei, S., Fazlali, Z., Rezaei Kanavi, M., Nilforushan, N., Yazdani, S., Babrzadeh, F., Suri, F., Ronaghi, M., Elahi, E., Paisan-Ruiz, C., 2009. Loss of function mutations in the gene encoding latent transforming growth factor beta binding protein 2, LTBP2, cause primary congenital glaucoma. *Human molecular genetics* 18, 3969-3977.
- Ono, R.N., Sengle, G., Charbonneau, N.L., Carlberg, V., Bachinger, H.P., Sasaki, T., Lee-Arteaga, S., Zilberberg, L., Rifkin, D.B., Ramirez, F., Chu, M.L., Sakai, L.Y., 2009. Latent transforming growth factor beta-binding proteins and fibulins compete for

- fibrillin-1 and exhibit exquisite specificities in binding sites. *J Biol Chem* 284, 16872-16881.
- Parsi, M.K., Adams, J.R., Whitelock, J., Gibson, M.A., 2010. LTBP-2 has multiple heparin/heparan sulfate binding sites. *Matrix biology : journal of the International Society for Matrix Biology* 29, 393-401.
- Perez-Magan, E., Campos-Martin, Y., Mur, P., Fiano, C., Ribalta, T., Garcia, J.F., Rey, J.A., Rodriguez de Lope, A., Mollejo, M., Melendez, B., 2012. Genetic alterations associated with progression and recurrence in meningiomas. *J Neuropathol Exp Neurol* 71, 882-893.
- Ramirez, F., Dietz, H.C., 2007. Fibrillin-rich microfibrils: Structural determinants of morphogenetic and homeostatic events. *Journal of cellular physiology* 213, 326-330.
- Ramirez, F., Rifkin, D.B., 2009. Extracellular microfibrils: contextual platforms for TGFbeta and BMP signaling. *Curr Opin Cell Biol* 21, 616-622.
- Ramirez, F., Sakai, L.Y., 2010. Biogenesis and function of fibrillin assemblies. *Cell Tissue Res* 339, 71-82.
- Rifkin, D.B., 2005. Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *J Biol Chem* 280, 7409-7412.
- Sabatier, L., Chen, D., Fagotto-Kaufmann, C., Hubmacher, D., McKee, M.D., Annis, D.S., Mosher, D.F., Reinhardt, D.P., 2009. Fibrillin assembly requires fibronectin. *Mol Biol Cell* 20, 846-858.
- Saharinen, J., Keski-Oja, J., 2000. Specific sequence motif of 8-Cys repeats of TGF-beta binding proteins, LTBPs, creates a hydrophobic interaction surface for binding of small latent TGF-beta. *Mol Biol Cell* 11, 2691-2704.
- Schiemann, W.P., Blobel, G.C., Kalume, D.E., Pandey, A., Lodish, H.F., 2002. Context-specific effects of fibulin-5 (DANCE/EVEC) on cell proliferation, motility, and invasion. Fibulin-5 is induced by transforming growth factor-beta and affects protein kinase cascades. *J Biol Chem* 277, 27367-27377.
- Schlotzer-Schrehardt, U., von der Mark, K., Sakai, L.Y., Naumann, G.O., 1997. Increased extracellular deposition of fibrillin-containing fibrils in pseudoexfoliation syndrome. *Invest Ophthalmol Vis Sci* 38, 970-984.
- Sengle, G., Tsutsui, K., Keene, D.R., Tufa, S.F., Carlson, E.J., Charbonneau, N.L., Ono, R.N., Sasaki, T., Wirtz, M.K., Samples, J.R., Fessler, L.I., Fessler, J.H., Sekiguchi, K., Hayflick, S.J., Sakai, L.Y., 2012. Microenvironmental regulation by fibrillin-1. *PLoS Genet* 8, e1002425.

- Shiple, J.M., Mecham, R.P., Maus, E., Bonadio, J., Rosenbloom, J., McCarthy, R.T., Baumann, M.L., Frankfater, C., Segade, F., Shapiro, S.D., 2000. Developmental expression of latent transforming growth factor beta binding protein 2 and its requirement early in mouse development. *Mol Cell Biol* 20, 4879-4887.
- Tiedemann, K., Batge, B., Muller, P.K., Reinhardt, D.P., 2001. Interactions of fibrillin-1 with heparin/heparan sulfate, implications for microfibrillar assembly. *J Biol Chem* 276, 36035-36042.
- Tsuruga, E., Nakashima, K., Ishikawa, H., Yajima, T., Sawa, Y., 2009. Stretching modulates oxytalan fibers in human periodontal ligament cells. *Journal of Periodontal Research* 44, 170-174.
- Tsuruga, E., Oka, K., Hatakeyama, Y., Isokawa, K., Sawa, Y., 2012. Latent transforming growth factor-beta binding protein 2 negatively regulates coalescence of oxytalan fibers induced by stretching stress. *Connect Tissue Res* 53, 521-527.
- Vehvilainen, P., Hyytiainen, M., Keski-Oja, J., 2009. Matrix association of latent TGF-beta binding protein-2 (LTBP-2) is dependent on fibrillin-1. *Journal of cellular physiology* 221, 586-593.
- Wagenseil, J.E., Mecham, R.P., 2007. New insights into elastic fiber assembly. *Birth Defects Res C Embryo Today* 81, 229-240.
- Zheng, Q., Davis, E.C., Richardson, J.A., Starcher, B.C., Li, T., Gerard, R.D., Yanagisawa, H., 2007. Molecular analysis of fibulin-5 function during de novo synthesis of elastic fibers. *Mol Cell Biol* 27, 1083-1095.

2.7 Figures

Figure 2.1 LTBP-2 and tropoelastin bind fibulin-5 with similar affinities.

A-C. Fibulin-5 binds tropoelastin and LTBP-2 but LTBP-2 does not bind tropoelastin. Microtiter wells were coated with rLTBP-2 [100 ng (0.5 pmol) /well] (A), or tropoelastin [200 ng (3 pmol)/well] (B and C). Control wells were coated with a molar equivalent of BSA. After blocking, each well was incubated at 37 °C for 3h with rFibulin-5 [200 ng (4 pmol)/ well, concentration = 40 nM] (A and B) or rLTBP-2 [100 ng (0.5 pmol) /well, concentration = 5 nM] (C). In A and B, specific binding of rFibulin-5 was detected using anti-[DDK tag] antibody and in C specific binding of rLTBP-2 was detected using affinity-purified anti-[LTBP-2 peptide] antibody 3504 (1:2000 dilution), followed by a peroxidase detection method (see Materials section). Means \pm S.D. of triplicate determinations are shown. D-E. Kinetic Analysis of the interactions of fibulin-5 with LTBP-2 and tropoelastin. Saturation curves were established for rFibulin-5 [0-40 nM] binding to LTBP-2 and tropoelastin and the A450 values (minus average BSA background, A450 = 0.26) were converted to fmol of fibulin-5 using a standard ELISA curve (not shown). Additional graphs of bound versus added rFibulin-5 were plotted for D) LTBP-2 and E) tropoelastin. The K_d for each interaction was calculated by non-linear regression analysis of the curve using the Prism 4.0 program.

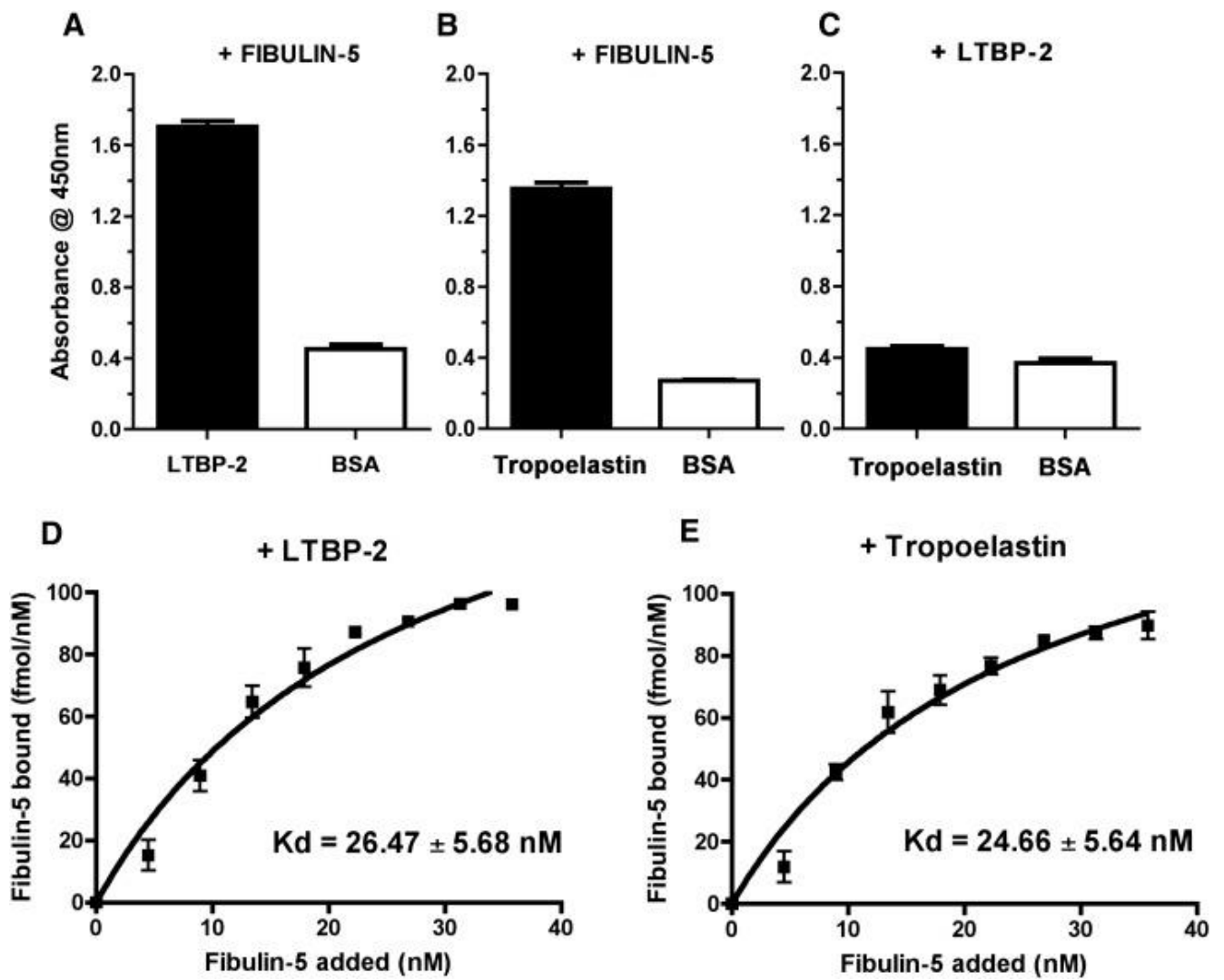


Figure 2.1. LTBP-2 and tropoelastin bind fibulin-5 with similar affinities.

Figure 2.2 Inhibition of the fibulin-5- tropoelastin interaction by LTBP-2 and its N-terminal fragment (LTBP-2 NT(H))

A. LTBP-2 substantially inhibits, rather than enhances, binding of tropoelastin to fibulin-5. Tropoelastin or BSA control [400 ng (6 pmol)/well] was coated onto microtiter plates. After blocking, the wells were incubated at 37 °C for 3h with fibulin-5 [100 ng (2 pmol) /well, concentration =20 nM] which had been pre-incubated for 30 min at 37 °C with control TBS (black columns) or with the same buffer containing 5-fold molar equivalent of rLTBP-2 (100 nM). B. Fragment LTBP-2 NT(H) contains the major fibulin-5 binding activity. Full-length LTBP-2 [200 ng (1 pmol) / well] and non-overlapping fragments spanning the LTBP-2 molecule, LTBP-2 NT(H) [85 ng (1 pmol) /well], LTBP-2 C(H) [85 ng (1 pmol/well, and LTBP-2 CT(H) [32 ng (1 pmol / well,] were coated onto microtiter wells. Control wells were coated with a molar equivalent of BSA. After blocking, each well was incubated at 37 °C for 3 h with fibulin-5 [200 ng (4 pmol)/ well, concentration = 40 nM]. Specific binding of fibulin-5 was detected as described in Fig.1. C. Fragment LTBP-2 NT(H) only partially inhibits tropoelastin binding to fibulin-5. Microtiter wells were coated with tropoelastin or BSA as described in A. After blocking, the wells were incubated at 37 °C for 3h with fibulin-5 [100 ng (2 pmol) /well, concentration = 20 nM] which had been pre-incubated for 30 min at 37 °C with control TBS (black columns) or with 10-fold molar excess of fragment LTBP-2 NT(H) (200 nM). D. Full-length LTBP-2 blocks the fibulin-5-tropoelastin interaction more effectively than fragment LTBP-2 NT(H), in a dose-dependent manner. Microtiter wells were coated with tropoelastin or BSA as described in A. After blocking, each well was incubated with fibulin-5 [50 ng (1 pmol/well, concentration = 10 nM] which had been pre-incubated for 30 min with 0.25 to 10 fold-molar equivalent of LTBP-2 (squares) or fragment LTBP-2NT(H) (triangles). After removal of the average background BSA signal [A450 = 0.21], binding was expressed as percentage fibulin-5 binding in the absence of LTBP-2. In all experiments the means \pm S.D. of triplicate determinations are shown.

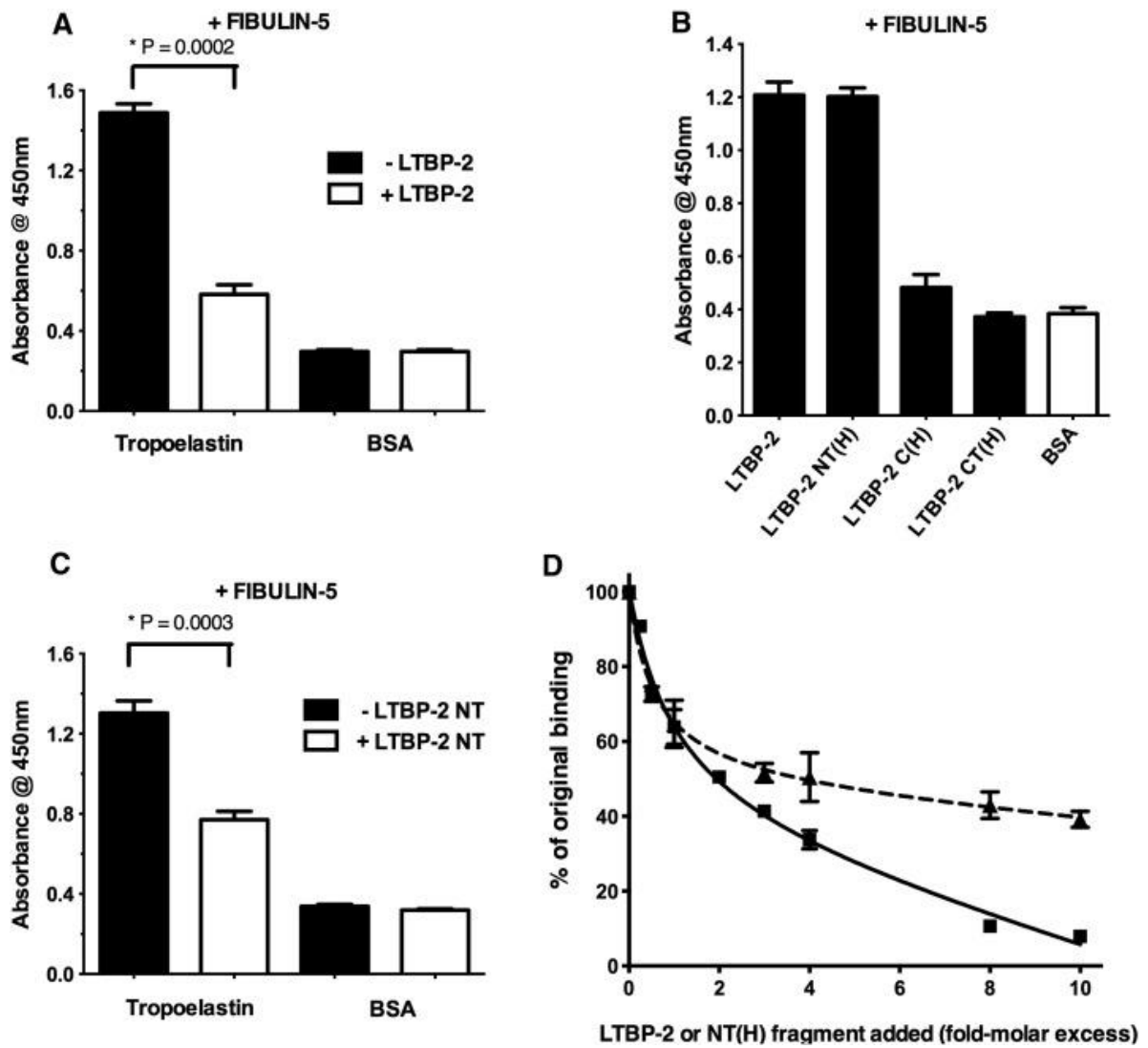


Figure 2.2 Inhibition of the fibulin-5- tropoelastin interaction by LTBP-2 and its N-terminal fragment (LTBP-2 NT(H))

Figure 2.3 Heparin shows minor but specific inhibition of LTBP-2 interaction with fibulin-5

A. R-LTBP-2 or BSA control [200 ng/well] was coated onto microtiter plates. After blocking, the wells were incubated at 37 °C for 3h with fibulin-5 [100 ng (2 pmol) /well, concentration = 20 nM] which had been pre-incubated for 30 min at 37 °C with control TBS (black columns) or with the same buffer containing 10-fold molar equivalent of heparin (white columns) (200 nM). Specific binding of fibulin-5 was detected as described in the experimental section. B. Heparin also partially inhibits the interaction of tropoelastin with fibulin-5. Experimental as in A except wells were coated with tropoelastin (in place of LTBP-2) or BSA control [400 ng/well]. C. Chondroitin-6- sulfate does not inhibit the interaction of fibulin-5 with LTBP-2. Experimental as in A except heparin was replaced with a molar equivalent of chondroitin sulfate. Means \pm S.D. of triplicate determinations are shown. * indicates statistical significance of $P \leq 0.05$.

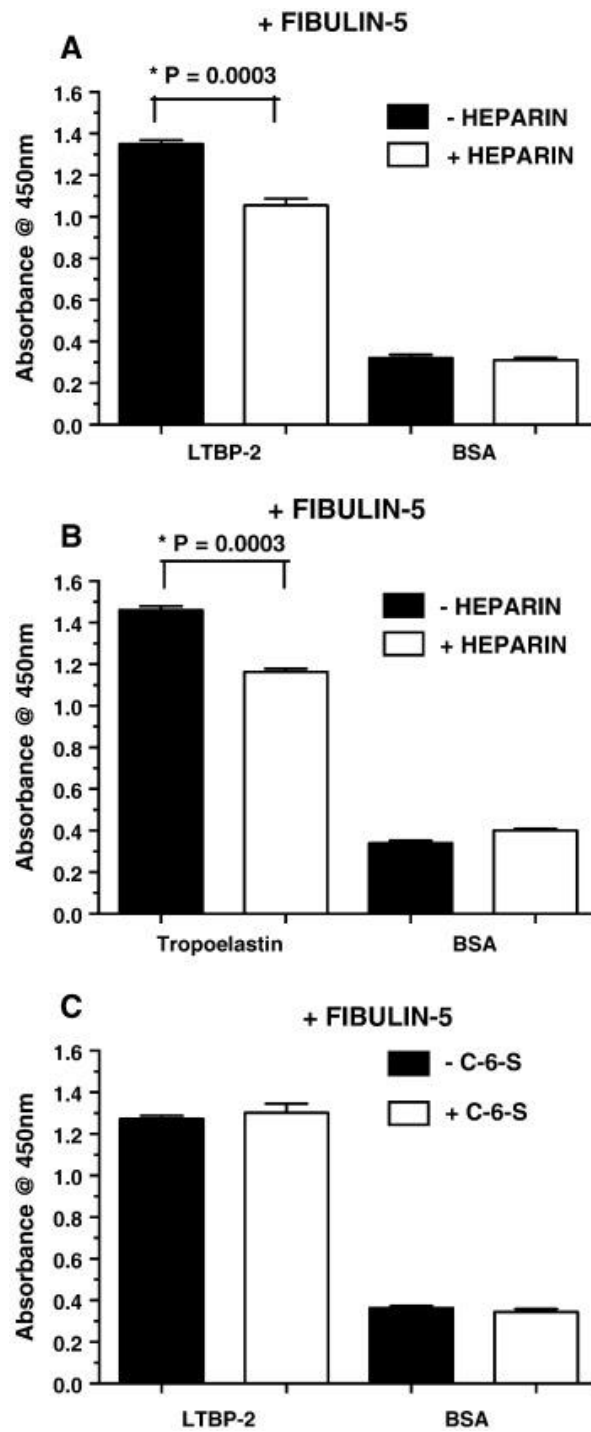


Figure 2.3 Heparin shows minor but specific inhibition of LTBP-2 interaction with fibulin-5

Figure 2.4 LTBP-2 inhibits the interaction of tropoelastin with heparin in a dose-dependent manner.

A. LTBP-2 partially inhibits interaction of tropoelastin with heparin. Tropoelastin [400 ng (6 pmol) /well] was coated onto microtiter plates. After blocking, the wells were incubated at 37 °C for 3h with heparin-conjugated to albumin (HAC) [50 ng/well (1.4 pmol [14 nM] heparin)] which had been pre-incubated for 30 min at 37 °C with 5-fold molar equivalent of rLTBP-2 [70 nM] (white columns) or with control buffer, TBS (black columns). Further control wells were incubated without HAC as indicated. Specific binding of HAC was detected using anti-BSA antibody followed by a peroxidase detection method.

Means \pm S.D. of triplicate determinations are shown. * indicates statistical significance of $P < 0.05$. B. The inhibition is dose -dependent. Microtiter wells were coated with tropoelastin as described in A. Control wells were coated with ovalbumin. After blocking, the wells were incubated as above with HAC [25 ng / well (0.7 pmol [7 nM] heparin)], which had been pre-incubated for 30 min at 37 °C with 0.25 to 10 fold-molar equivalents of LTBP-2 (0.175 pmol to 7 pmol) Following subtraction of the average ovalbumin background signal [A450= 0.40], an inhibition curve was plotted.

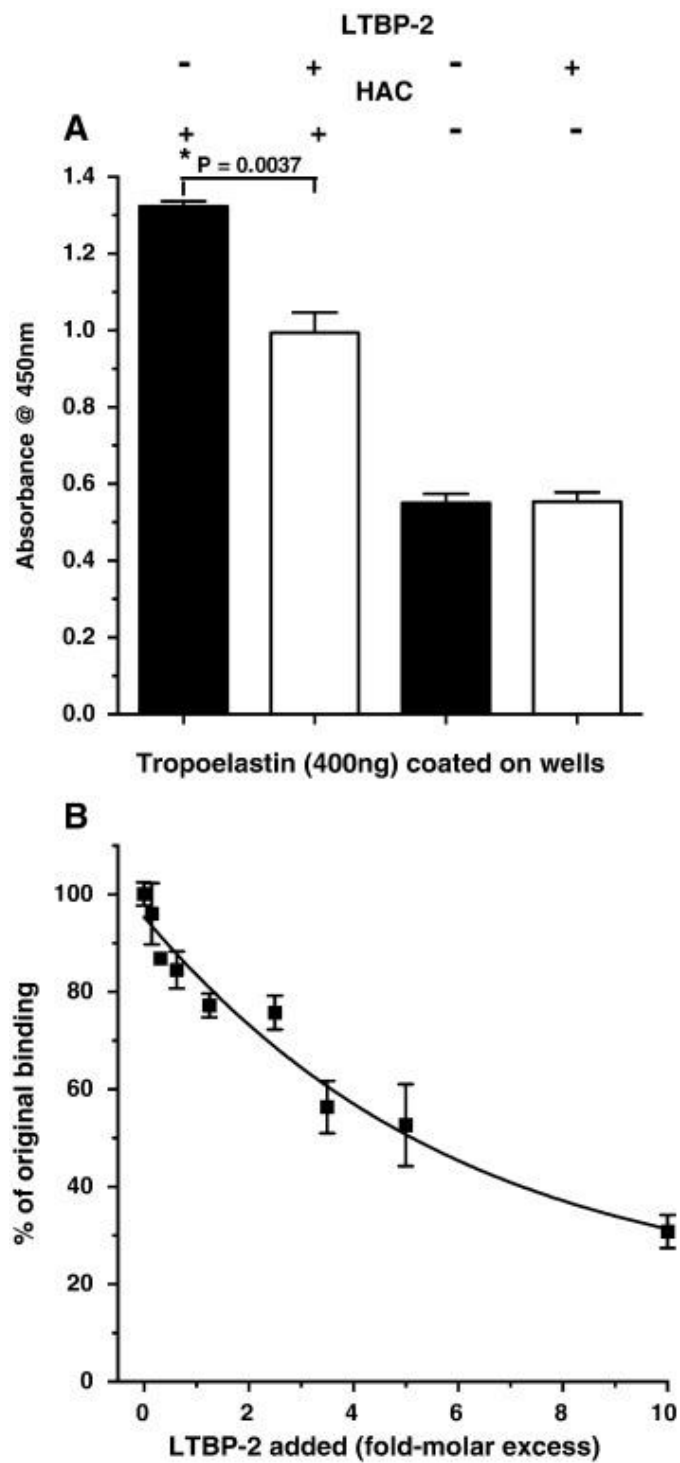


Figure 2.4 LTBP-2 inhibits the interaction of tropoelastin with heparin in a dose-dependent manner.

Figure 2.5 Confocal co-localisation of LTBP-2 with fibulin-5, fibrillin-1, fibronectin and heparan sulfate proteoglycans in fibroblast matrix.

Human skin fibroblast cultures were cultured for 21 days and then stained for confocal microscopy with antibodies to LTBP-2 in combination with antibodies to fibulin-5, fibrillin-1, fibronectin, perlecan or syndecan-4. Primary antibody was detected using an appropriate secondary antibody conjugated to fluorophore Alexa488 (red) or Cy3 (green). (see experimental section). A,D,G,J, and M, rabbit anti-[LTBP-2 peptide] antibody 3504 [16 $\mu\text{g/ml}$]; B, mouse anti-fibrillin-1 antibody MAB1919 [5 $\mu\text{g/ml}$]; C = A and B merged; E, mouse anti-fibulin-5 antibody MAB3095 [25 $\mu\text{g/ml}$]; F = D and E merged; H, mouse anti-[cellular fibronectin] antibody ab6328 [10 $\mu\text{g/ml}$]; I = G and H merged; K, mouse anti-perlecan antibody [2.5 mg / ml]; L = J and K merged; N, mouse syndecan-4 antibody [4 $\mu\text{g/ml}$]; O = M and N merged; P, rabbit IgG [15.6 $\mu\text{g/ml}$] control for A,D,G,J and M; Q, mouse IgG (25 $\mu\text{g/ml}$) control for B,E,H,K and N; R = P and Q merged. Bar = 200 μM .

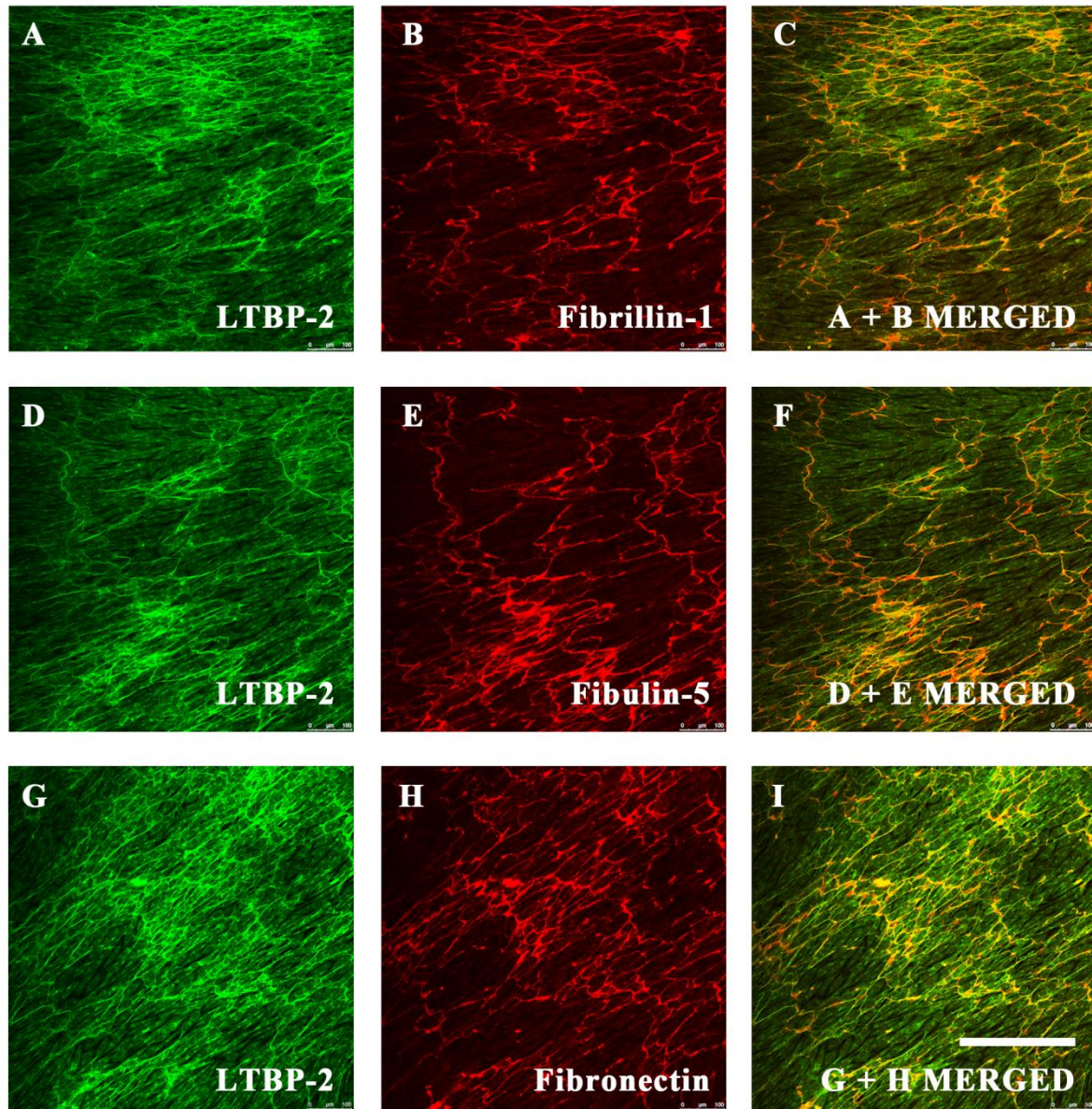


Figure 2.5 Confocal co-localisation of LTBP-2 with fibulin-5, fibrillin-1, fibronectin and heparan sulfate proteoglycans in fibroblast matrix.

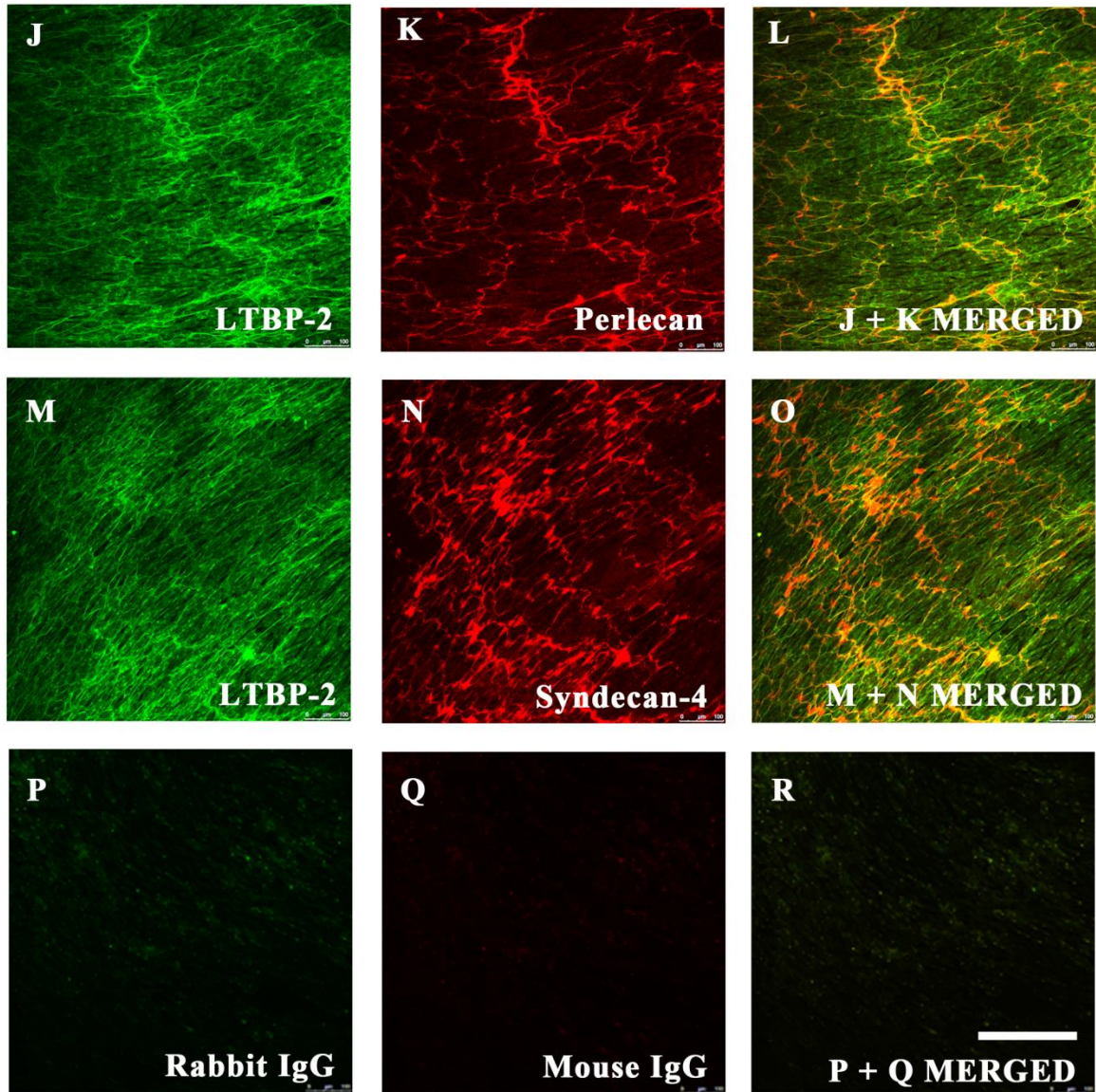


Figure 2.5 (*continued*).

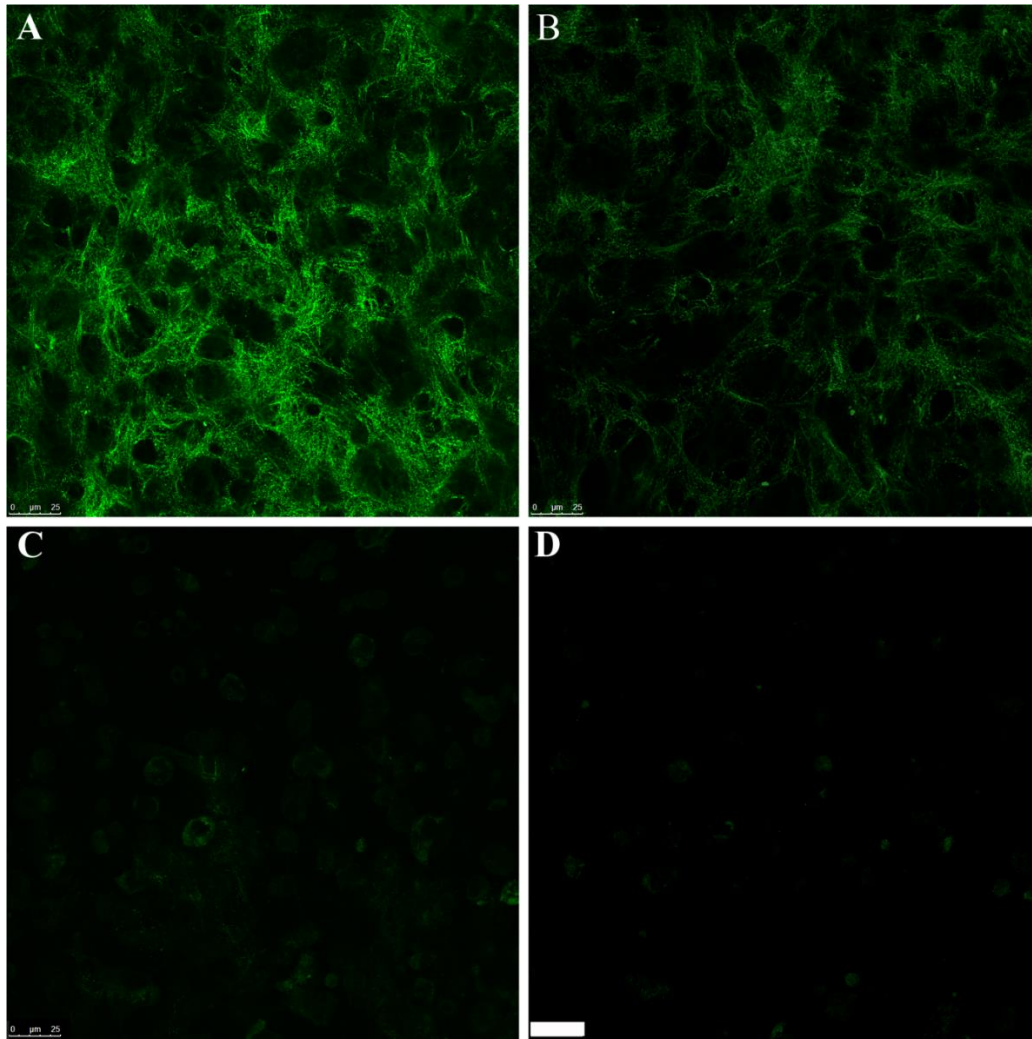


Figure 2.6 Exogenous LTBP-2 inhibits elastinogenesis by ear cartilage chondrocytes in culture.

Ear cartilage chondrocytes were cultured to 7 days post-confluency to allow a fibrillin-microfibril rich matrix to be established. The cells were then cultured for a further 7 days with daily supplementation of exogenous recombinant LTBP-2, (final concentrations 0 to 10 $\mu\text{g/ml}$). The cell layer was then stained for elastin with anti-tropoelastin monoclonal antibody BA4 and immunofluorescence detection as described in section 3.3. Fig 6A, no LTBP-2 added; Fig 6B, 1 $\mu\text{g/ml}$ LTBP-2; Fig 6C, 2.5 $\mu\text{g/ml}$ LTBP-2; Fig 6D 10 $\mu\text{g/ml}$ LTBP-2. Note the dose-dependent reduction of elastin staining by addition of exogenous LTBP-2, with little elastin evident at 2.5 $\mu\text{g/ml}$ LTBP-2. Bar = 25 μm

Figure 2.7 LTBP-2 and elastinogenesis

The complex process of elastinogenesis involves several steps and a number of proteins (Wagenseil and Mecham, 2007). For clarity we will focus on those proteins relevant to this study. An early stage involves the formation of small elastin globules from precursor tropoelastin close to the cell surface possibly in association with heparan sulfate proteoglycans. These elastin globules become complexed with fibulin-5 and are transferred onto a pre-established template of fibrillin-microfibrils where they coalesce to form the highly cross-linked elastin core of the elastic fibre. We have shown in this study that LTBP-2 can compete with tropoelastin for binding to fibulin-5 and heparin (similar to heparan sulfate). Thus the blocking by exogenous LTBP-2 of elastinogenesis observed in cell culture may involve displacement of tropoelastin from cell surface heparan sulfate preventing microglobule formation (A) and/or blocking elastin-fibulin-5 interactions preventing transfer of the microglobules onto the fibrillin template (B). For elastinogenesis to occur LTBP-2 production and location must be highly regulated by the fibre producing cell. LTBP-2 is abundant during the elastinogenic process in many tissues but is located specifically on the surface of the fibrillin-microfibrils (Gibson et al., 1995). In this highly controlled situation LTBP-2 may be involved in regulating the rate of docking of the elastin-fibulin-5 complexes onto the microfibrils and the subsequent release of fibulin-5 from the elastin. The binding sites for LTBP-2 and fibulin-5 on fibrillin-1 are in close proximity (Hirani et al., 2007; Ono et al., 2009) and thus competition for binding to the microfibrils may also occur (C).

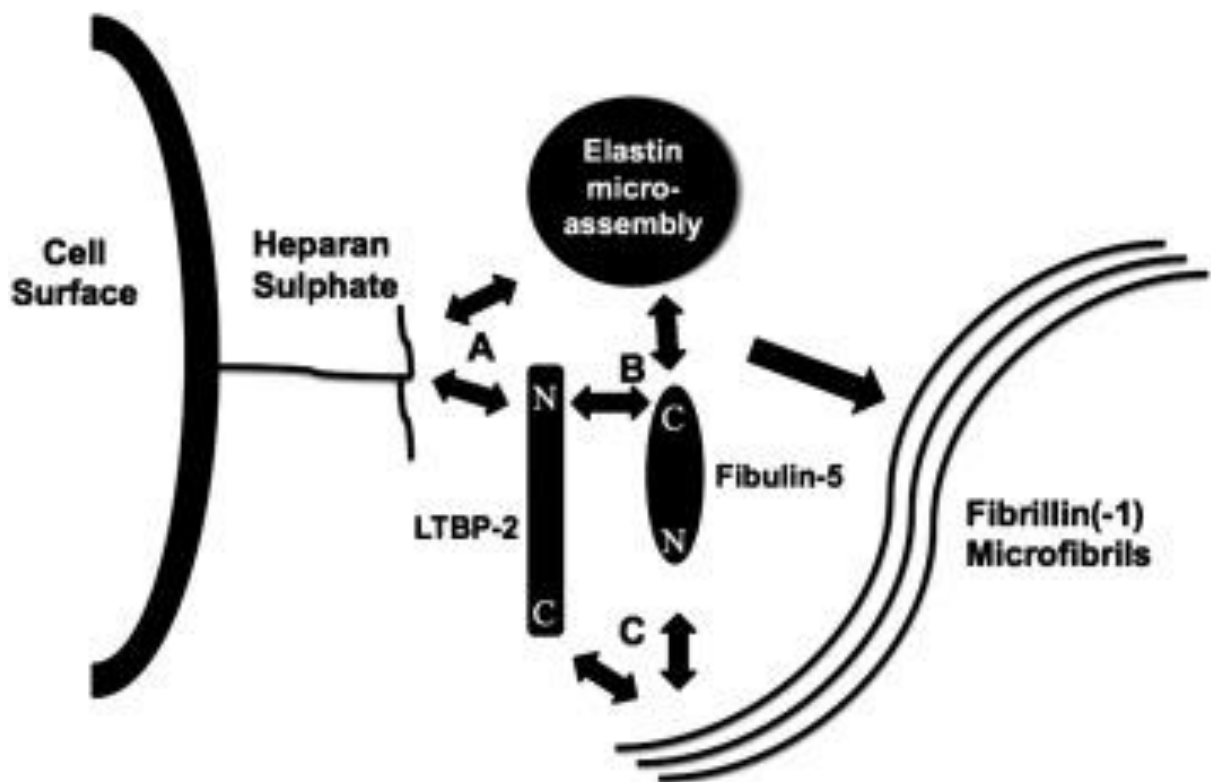


Figure 7. LTBP-2 and elastinogenesis

2.8 Mini-research paper: LTBP-2 inhibits elastin and fibrillin assembly in matrix of fetal bovine ear cartilage chondrocytes

Mohamed A. Sideek^{a,b} and Mark A. Gibson^{a*}

^a Discipline of Anatomy and Pathology, School of Medicine, University of Adelaide, Adelaide, South Australia 5005, Australia

^b Department of Biomedical Sciences, Kulliyah of Allied Health Sciences, International University Malaysia, 25200 Kuantan, Pahang, Malaysia

* corresponding author. Tel: +61 88313 5337 Email address: mark.gibson@adelaide.edu.au

Keyword

LTBP-2, elastin, fibrillin, fibronectin, elastinogenesis, fibrillogenesis

2.8.1 Introduction

Latent transforming growth factor-beta-1 binding protein-2 (LTBP-2) is a protein of ill-defined function which is abundant in developing elastic tissues such as aorta and nuchal ligament where it is specifically localised on fibrillin-containing microfibrils (Moren *et al.*, 1994). Recent discoveries have shown that LTBP-2 binds strongly *in vitro* to heparin/heparan sulphate proteoglycans (Parsi *et al.*, 2010) and to fibulin-5 (Hirai *et al.*, 2007), an elastin-binding protein essential for elastin deposition onto microfibrils. It has been proposed that during elastinogenesis LTBP-2 may complex with fibulin-5/elastin-microassemblies and direct these specifically to fibrillin-1 microfibrils for elastin deposition (Hirai *et al.*, 2007). However, our recent study has shown that LTBP-2 inhibits rather than enhances tropoelastin-fibulin-5 complexing. LTBP-2 was also found to compete with tropoelastin for binding the heparan/heparan sulphate. Additional experiments using ear cartilage chondrocyte cultures showed that addition of exogenous LTBP-2 to the culture blocked elastinogenesis in a concentration-dependent manner suggesting LTBP-2 acts as negative modulator during elastic fibre assembly (Sideek *et al.*, 2014). This has led to our suggestion that LTBP-2 may modulate two key aspects of elastic fibre assembly which are; a) displacement of elastin-microassemblies from cell surface heparan sulphate proteoglycans and b) inhibition of tropoelastin/fibulin-5 complexing during elastin deposition into fibrillin-containing microfibrils. Although the above studies have proved informative, the influences of LTBP-2 on the different stages of elastic fibre assembly remained unclear. In this study we have extended the above study to investigate the effect of LTBP-2 on elastin, fibrillin and fibronectin assembly in the chondrocyte cell culture model. The results indicate that LTBP-2 inhibits the elastinogenesis stage at lower concentrations than its inhibition of fibrillin microfibril assembly. LTBP-2 had no effect on fibronectin network formation.

2.8.2 Method

Cells were cultured and stained as described previously (Sideek *et al.*, 2014). Briefly, ear cartilage chondrocytes from a 220-day-old fetal bovine (P4) were plated onto microscope chamber slides at an initial density of 4×10^5 /ml and grown for 7 days post-confluence in DMEM plus 10% fetal calf serum. The cells were then cultured in DMEM plus 3% fetal calf serum for a further 7 days with daily addition of recombinant human LTBP-2 (2.5 or 10 μ g/ml). The cell layers were fixed with cold acetone and methanol and incubated overnight at 4 °C with

monoclonal antibody specific for elastin (BA4), fibrillin (MAB1919) or fibronectin (IST-9). Control sections were incubated with a matched concentration of mouse IgG. Sections were washed with TBS/methiolate and incubated with the goat anti-mouse IgG antibody conjugated to FITC. Sections were washed and mounted in 90% glycerol/ 10% TBS/methiolate containing anti-fade reagent p-phenylenediamine prior to viewing on a Leica TCS SP5 confocal microscope using a 488nm laser with emission settings at 496-533nm.

2.8.3 Results and Discussion

The elastin deposition stage of elastic fibre assembly is most sensitive to exogenous LTBP-2.

Assembly of elastic fibres appears to involve 3 distinct stages, fibronectin fibril assembly, fibrillin microfibril assembly and elastin deposition onto the fibrillin scaffold. (R. Kinsey *et al.*, 2008a. Previously we have shown exogenous LTBP-2 blocks elastinogenesis by cultured chondrocytes (Sideek *et al.*, 2014). However, it was unclear which stage(s) in the assembly of the elastic fibre was affected by LTBP-2. In the present study we tested the effect of exogenous LTBP-2 at 2.5 µg/ml and 10 µg/ml on the assembly of these three proteins into the chondrocyte extracellular matrix. In the absence of LTBP-2 the chondrocytes established a matrix rich in elastin, fibrillin-1 and fibronectin after 2 weeks of culture (Fig 1 E-G). Addition of exogenous LTBP-2 has a different affect on the assembly of each protein into the matrix. Firstly LTBP-2 did not inhibit fibronectin assembly at either concentration used (Fig 1 K and O). In contrast at both concentrations tested, LTBP-2 completely blocked elastin assembly (Fig 1 I and M). Fibrillin assembly appeared to be unaffected by LTBP-2 at 2.5 µg/ml (Fig 1 J) but was partially inhibited at 10 µg/ml LTBP-2 (Fig 1 N).

Thus the elastin deposition stage was most sensitive to inhibition by LTBP-2 as predicted from the *in vitro* binding studies. It is well documented that fibulin-5 interaction with tropoelastin is essential for the formation of elastic fibre assembly (Nakamura *et al.*, 2002; Nonaka *et al.*, 2009; Yanagisawa *et al.*, 2009a). In addition, fibulin-5 gene knockout mice exhibit severe disorganized elastic fibre system throughout the body (Nakamura *et al.*, 2002). Although the present study did not directly examine the interaction of LTBP-2 and fibulin-5 during elastic fibre assembly, the results together with *in vitro* studies provide evidence that LTBP-2 may act as a negative modulator of elastic fibre assembly by binding with fibulin-5 and inhibiting the

interaction of elastin to fibulin-5. Interestingly, elastinogenesis occurs through a series of highly regulated steps where fibulin-5 is important for the formation and maturation of tropoelastin microassemblies by controlling the efficiency of coacervation (Hirai *et al.*, 2007; Cirulis *et al.*, 2008; Wachi *et al.*, 2008). Fibulin-5 is also essential for the tropoelastin cross-linking stage of elastic fiber assembly by regulating the size of self-aggregates to achieve optimal cross-linking (Choi *et al.*, 2009). Since LTBP-2 binds fibulin-5 and negatively regulates the function of fibulin-5, it is possible that LTBP-2 could involve in the coacervation and cross-linking of tropoelastin. Moreover, LTBP-2 may also regulate the function of fibulin-5 in other biological process and diseases including angiogenesis, tumorigenesis and fibrosis (Xie *et al.*, 2008; Yanagisawa *et al.*, 2009b; Nakasaki *et al.*, 2015). LTBP-2 also inhibits the interaction of tropoelastin with heparan *in vitro* suggesting that it may influence another interaction important in elastinogenesis, the interaction of tropoelastin with cell surface heparan sulphate proteoglycans (HSPGs) (Tiedemann *et al.*, 2001; Cain *et al.*, 2005; Wagenseil and Mecham, 2007; Sideek *et al.*, 2014). Multiple studies have shown that the addition of heparan sulphate to cultured cells alter the coacervation properties of tropoelastin and deposition of tropoelastin into the extracellular matrix (McGowan *et al.*, 1993; Wu *et al.*, 1999; Kozel *et al.*, 2004). More recently, a study by Baughman *et al.* (2011) demonstrated that heparin treatment increases elastin mRNA levels and stimulates elastinogenesis in adult human smooth muscle cells. Since LTBP-2 binds strongly to heparin, it is likely LTBP-2 could inhibit elastin assembly by modulating HSPGs function at multiple levels during the elastinogenic process.

The inhibitory effect of LTBP-2 on fibrillin microfibril assembly required much greater concentrations of LTBP-2 suggesting that LTBP-2 has less influence in controlling microfibrillogenesis compared to elastinogenesis. Fibrillin-1 is a major heparin/HS binding protein containing at least 7 heparin binding sites (Cain *et al.*, 2005; Sabatier *et al.*, 2014a). It is possible that LTBP-2 may disrupt the fibrillin deposition by interfering with fibrillin-heparan sulphate interactions essential for early stages of microfibril assembly. Heparin has been suggested to influence microfibril and elastic fibre assembly by regulating the interaction between MAGP-1 and tropoelastin with fibrillin-1. Moreover, cell surface HS may be involved in multimerisation and alignment of fibrillin-1 during microfibril deposition (Cain *et al.*, 2005). Addition of heparin/HS to skin fibroblast cultures inhibited fibrillin-1 microfibril network formation (Tiedemann *et al.*, 2001; Ritty *et al.*, 2003). It has been demonstrated that the N terminus of fibrillin-1 strongly binds to itself and to the fibrillin-1 C terminus, and these interactions can effectively be inhibited by heparin indicating the importance of HSPGs to

regulate linear fibrillin-1 assembly into supramolecular microfibril assemblies (Sabatier *et al.*, 2014b). The potential binding of LTBP-2, at high concentration, to matrix and cell surface HSPGs may inhibit their crucial interactions with fibrillin-1 during microfibril assembly.

In addition a study by Hisanaga *et al.* (2009) found that fibulin-5 colocalized with fibrillin microfibrils and controls the biogenesis of microfibril bundles suggesting that fibulin-5 plays important roles in two steps of elastic fibres assembly, fibrillogenesis in addition to elastinogenesis. Very high concentrations of LTBP-2 may also interfere with this process via negatively modulation of fibulin-5 function of during fibrillin assembly. It is noteworthy that a recent study has shown LTBP-2, in coordination with fibulin-5, may control the diameter and organization of fibrillin microfibrils (Tsuruga *et al.*, 2012) suggesting exogenous LTBP-2 may negatively controlled the function of fibulin-5 during coalescence of microfibrils and oxytalan fibres which is consistent with our findings. A study by Kissin *et al.* (2002) showed that TGF- β stimulates fibrillin microfibrils assembly without affecting fibrillin gene expression or protein synthesis suggesting high level of LTBP-2 could directly modulate TGF- β bioavailability which may lead to inhibition and disruption of fibrillin-1 formation as found in our studies.

Exogenous LTBP-2 had no effect on fibronectin assembly showing the effects were specific to elastic fibre assembly and not to matrix production in general. Assembly of fibronectin is important for deposition of fibrillin microfibrillar arrays since knockdown of fibronectin in human dermal fibroblast cultures resulted in severe reduction in fibrillin-microfibril deposition (Kinsey *et al.*, 2008; Sabatier *et al.*, 2009). Since the fibronectin assembly is unaffected by the LTBP-2, we can confirm that the disruption of fibrillin assembly in ear chondrocytes cultures was directly caused by LTBP-2 and not by disruption of fibronectin assembly. We propose next to investigate effects of individual domains of LTBP-2 on the elastinogenesis to delineate the importance of LTBP-2–heparin and LTBP-2-fibulin-5 interactions in the inhibition of this process. The discovery of the negative impact of LTBP-2 on two stages of elastic fibre assembly may eventually lead to development of novel therapeutic agents, for the prevention of elastic fibre-degenerative conditions and regeneration of damaged elastic fibres.

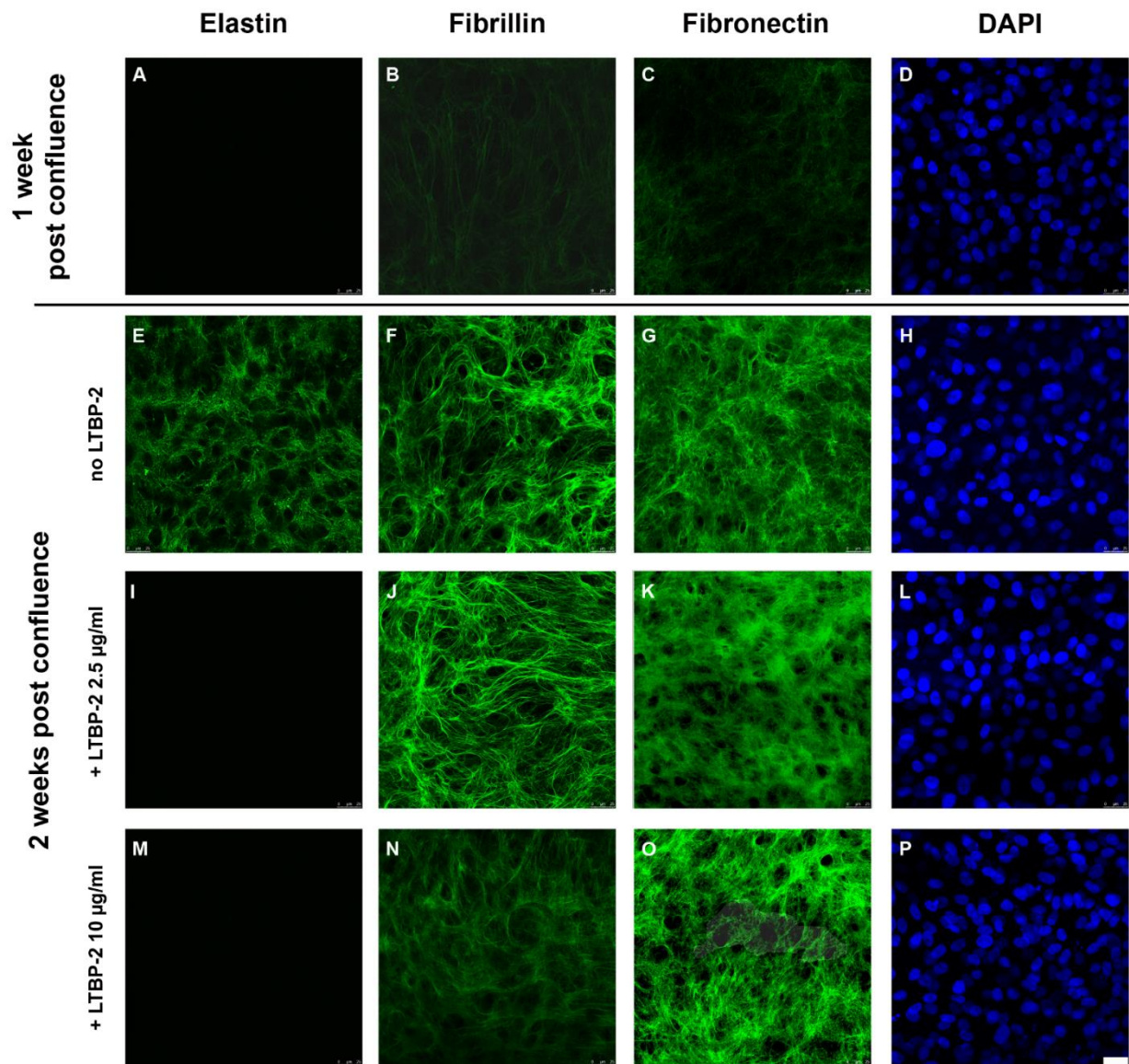


Fig. 1. LTBP-2 blocks elastinogenesis and inhibits fibrillogenesis by cultured chondrocytes.

Ear cartilage chondrocytes were cultured to 7 or 14 days post-confluency and stained for elastin (Fig 7A, 7E, 7I, 7M), fibrillin (Fig 7B, 7F, 7J, 7N), or fibronectin (Fig 7C, 7G, 7K, 7O) and DAPI nuclear stain (Fig 7D, 7H, 7L, 7P). Fig 7A-7H, no LTBP-2 added; Fig 7I-7L, 2.5µg/ml LTBP-2; Fig 7M-7P, 10 µg/ml LTBP-2. Bar = 25 µm.

2.8.4 References

- Baughman, C., Kaplan, D. L. and Castellot, J. J. (2011) 'Heparin Stimulates Elastogenesis: Application to Silk-Based Vascular Grafts', *Matrix biology : journal of the International Society for Matrix Biology*, 30(0), pp. 346-355.
- Cain, S. A., Baldock, C., Gallagher, J., Morgan, A., Bax, D. V., Weiss, A. S., Shuttleworth, C. A. and Kielty, C. M. (2005) 'Fibrillin-1 interactions with heparin. Implications for microfibril and elastic fiber assembly', *J Biol Chem*, 280(34), pp. 30526-37.
- Choi, J., Bergdahl, A., Zheng, Q., Starcher, B., Yanagisawa, H. and Davis, E. C. (2009) 'Analysis of dermal elastic fibers in the absence of fibulin-5 reveals potential roles for fibulin-5 in elastic fiber assembly', *Matrix Biology*, 28(4), pp. 211-220.
- Cirulis, J. T., Bellingham, C. M., Davis, E. C., Hubmacher, D., Reinhardt, D. P., Mecham, R. P. and Keeley, F. W. (2008) 'Fibrillins, Fibulins, and Matrix-Associated Glycoprotein Modulate the Kinetics and Morphology of in Vitro Self-Assembly of a Recombinant Elastin-like Polypeptide', *Biochemistry*, 47(47), pp. 12601-12613.
- Hirai, M., Horiguchi, M., Ohbayashi, T., Kita, T., Chien, K. R. and Nakamura, T. (2007) 'Latent TGF-beta-binding protein 2 binds to DANCE/fibulin-5 and regulates elastic fiber assembly', *EMBO J*, 26(14), pp. 3283-95.
- Hisanaga, Y., Nakashima, K., Tsuruga, E., Nakatomi, Y., Hatakeyama, Y., Ishikawa, H. and Sawa, Y. (2009) 'Fibulin-5 Contributes to Microfibril Assembly in Human Periodontal Ligament Cells', *Acta Histochemica et Cytochemica*, 42(5), pp. 151-157.
- Kinsey, R., Williamson, M. R., Chaudhry, S., Mellody, K. T., McGovern, A., Takahashi, S., Shuttleworth, C. A. and Kielty, C. M. (2008) 'Fibrillin-1 microfibril deposition is dependent on fibronectin assembly', *Journal of Cell Science*, 121(16), pp. 2696-2704.
- Kissin, E. Y., Lemaire, R., Korn, J. H. and Lafyatis, R. (2002) 'Transforming growth factor beta induces fibroblast fibrillin-1 matrix formation', *Arthritis Rheum*, 46(11), pp. 3000-9.
- Kozel, B. A., Ciliberto, C. H. and Mecham, R. P. (2004) 'Deposition of tropoelastin into the extracellular matrix requires a competent elastic fiber scaffold but not live cells', *Matrix Biology*, 23(1), pp. 23-34.

- McGowan, S. E., Liu, R. G. and Harvey, C. S. (1993) 'Effects of Heparin and Other Glycosaminoglycans on Elastin Production by Cultured Neonatal Rat Lung Fibroblasts', *Archives of Biochemistry and Biophysics*, 302(2), pp. 322-331.
- Moren, A., Olofsson, A., Stenman, G., Sahlin, P., Kanzaki, T., Claesson-Welsh, L., ten Dijke, P., Miyazono, K. and Heldin, C. H. (1994) 'Identification and characterization of LTBP-2, a novel latent transforming growth factor- β -binding protein', *J Biol Chem*, 269(51), pp. 32469-78.
- Nakamura, T., Lozano, P. R., Ikeda, Y., Iwanaga, Y., Hinek, A., Minamisawa, S., Cheng, C. F., Kobuke, K., Dalton, N., Takada, Y., Tashiro, K., Ross Jr, J., Honjo, T. and Chien, K. R. (2002) 'Fibulin-5/DANCE is essential for elastogenesis in vivo', *Nature*, 415(6868), pp. 171-5.
- Nakasaki, M., Hwang, Y., Xie, Y., Kataria, S., Gund, R., Hajam, E. Y., Samuel, R., George, R., Danda, D., M, J. P., Nakamura, T., Shen, Z., Briggs, S., Varghese, S. and Jamora, C. (2015) 'The matrix protein Fibulin-5 is at the interface of tissue stiffness and inflammation in fibrosis', *Nat Commun*, 6, p. 8574.
- Nonaka, R., Onoue, S., Wachi, H., Sato, F., Urban, Z., Starcher, B. C. and Seyama, Y. (2009) 'DANCE/fibulin-5 promotes elastic fiber formation in a tropoelastin isoform-dependent manner', *Clin Biochem*, 42(7-8), pp. 713-21.
- Parsi, M. K., Adams, J. R., Whitelock, J. and Gibson, M. A. (2010) 'LTBP-2 has multiple heparin/heparan sulfate binding sites', *Matrix Biol*, 29(5), pp. 393-401.
- Ritty, T. M., Broekelmann, T. J., Werneck, C. C. and Mecham, R. P. (2003) 'Fibrillin-1 and -2 contain heparin-binding sites important for matrix deposition and that support cell attachment', *Biochem J*, 375(Pt 2), pp. 425-32.
- Roberts, A. B., Heine, U. I., Flanders, K. C. and Sporn, M. B. (1990) 'Transforming growth factor-beta. Major role in regulation of extracellular matrix', *Ann N Y Acad Sci*, 580, pp. 225-32.
- Sabatier, L., Chen, D., Fagotto-Kaufmann, C., Hubmacher, D., McKee, M. D., Annis, D. S., Mosher, D. F. and Reinhardt, D. P. (2009) 'Fibrillin assembly requires fibronectin', *Mol Biol Cell*, 20(3), pp. 846-58.
- Sabatier, L., Djokic, J., Hubmacher, D., Dzafik, D., Nelea, V. and Reinhardt, D. P. (2014a) 'Heparin/heparan sulfate controls fibrillin-1, -2 and -3 self-interactions in microfibril assembly', *FEBS Lett*, 588(17), pp. 2890-7.

- Sabatier, L., Djokic, J., Hubmacher, D., Dzafik, D., Nelea, V. and Reinhardt, D. P. (2014b) 'Heparin/heparan sulfate controls fibrillin-1, -2 and -3 self-interactions in microfibril assembly', *FEBS Letters*, 588(17), pp. 2890-2897.
- Sideek, M. A., Menz, C., Parsi, M. K. and Gibson, M. A. (2014) 'LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis', *Matrix Biol*, 34, pp. 114-23.
- Tiedemann, K., Batge, B., Muller, P. K. and Reinhardt, D. P. (2001) 'Interactions of fibrillin-1 with heparin/heparan sulfate, implications for microfibrillar assembly', *J Biol Chem*, 276(38), pp. 36035-42.
- Tsuruga, E., Oka, K., Hatakeyama, Y., Isokawa, K. and Sawa, Y. (2012) 'Latent transforming growth factor-beta binding protein 2 negatively regulates coalescence of oxytalan fibers induced by stretching stress', *Connect Tissue Res*, 53(6), pp. 521-7.
- Wachi, H., Nonaka, R., Sato, F., Shibata-Sato, K., Ishida, M., Iketani, S., Maeda, I., Okamoto, K., Urban, Z., Onoue, S. and Seyama, Y. (2008) 'Characterization of the molecular interaction between tropoelastin and DANCE/fibulin-5', *J Biochem*, 143(5), pp. 633-9.
- Wagenseil, J. E. and Mecham, R. P. (2007) 'New insights into elastic fiber assembly', *Birth Defects Res C Embryo Today*, 81(4), pp. 229-40.
- Wu, W. J., Vrhovski, B. and Weiss, A. S. (1999) 'Glycosaminoglycans Mediate the Coacervation of Human Tropoelastin through Dominant Charge Interactions Involving Lysine Side Chains', *Journal of Biological Chemistry*, 274(31), pp. 21719-21724.
- Xie, L., Palmsten, K., MacDonald, B., Kieran, M. W., Potenta, S., Vong, S. and Kalluri, R. (2008) 'Basement membrane derived fibulin-1 and fibulin-5 function as angiogenesis inhibitors and suppress tumor growth', *Exp Biol Med (Maywood)*, 233(2), pp. 155-62.
- Yanagisawa, H., Schluterman, M. and Brekken, R. (2009a) 'Fibulin-5, an integrin-binding matricellular protein: its function in development and disease', *Journal of Cell Communication and Signaling*, 3(3-4), pp. 337-347.
- Yanagisawa, H., Schluterman, M. K. and Brekken, R. A. (2009b) 'Fibulin-5, an integrin-binding matricellular protein: its function in development and disease', *J Cell Commun Signal*, 3(3-4), pp. 337-47.

CHAPTER 3: LTBP-2 has a single high-affinity binding site for FGF-2 and blocks FGF-2-induced cell proliferation.

Clementine Menz^a, Mahroo K. Parsi^a, Julian R.J. Adams^a, **Mohamed A Sideek^{a,b}**, Zlatko Kopecki^c, Allison Cowin^c and Mark A. Gibson^{a*}

^a Discipline of Anatomy and Pathology, School of Medicine, University of Adelaide, Adelaide, Adelaide, South Australia 5005, Australia

^b Department of Biomedical Sciences, Kulliyah of Allied Health Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia

^c Regenerative Medicine, Mawson Institute, University of South Australia, Adelaide, South Australia 5095, Australia

* corresponding author. TEL: +61 88313 5337 Email address: mark.gibson@adelaide.edu.au

Received: September 14, 2014, Accepted: July 24, 2015, Published: August 11, 2015

PLOS ONE DOI:10.1371/journal.pone.0135577

Statement of Authorship

Title of Paper	LTBP-2 has a single high-affinity binding site for FGF-2 and blocks FGF-2 induced cell proliferation
Publication Status	<input checked="" type="checkbox"/> Published <input type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
Publication Details	Menz, C., Parsi, M. K., Adams, J. R., Sideek, M. A., Kopecki, Z., Cowin, A. J. and Gibson, M. A. (2015) 'LTBP-2 Has a Single High-Affinity Binding Site for FGF-2 and Blocks FGF-2-Induced Cell Proliferation', <i>PLoS One</i> , 10(8), p. e0135577.

Principal Author

Name of Principal Author (Candidate)	Mohamed Arshad Sideek			
Contribution to the Paper	<p>For cell proliferation assay, detection and quantitation of FGFR1 activation and immunohistochemistry of fibrotic tissues</p> <ul style="list-style-type: none"> • Performed all experiments • Performed all data analyses • Interpreted the data • Wrote the manuscript 			
Overall percentage (%)	70%			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.			
Signature	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 60%;"></td> <td style="width: 10%; text-align: center;">Date</td> <td style="width: 30%;">30/11/2015</td> </tr> </table>		Date	30/11/2015
	Date	30/11/2015		

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- iv. the candidate's stated contribution to the publication is accurate (as detailed above);
- v. permission is granted for the candidate to include the publication in the thesis; and
- vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Clementine Menz		
Contribution to the Paper	Generated recombinant of LTBP-2 fragment and performed binding activity experiments. Manuscript evaluation and feedback.		
Signature		Date	29/11/2015

Name of Co-Author	Dr. Mahroo K. Parsi		
Contribution to the Paper	Performed binding experiment. Manuscript evaluation and feedback.		
Signature		Date	29/11/2015

Name of Co-Author	Julian R.J Adams		
Contribution to the Paper	Helped with the experiments. Manuscript evaluation and feedback.		
Signature		Date	30/11/2015

Name of Co-Author	Dr. Zlatko Kopecki		
Contribution to the Paper	Collection of tissue and experimental training in IHC. Supervised and provided guidelines. Manuscript evaluation and feedback.		
Signature		Date	26/11/2015

Name of Co-Author	Prof. Allison J. Cowin		
Contribution to the Paper	Supervised the development of work and data interpretation. Manuscript evaluation and feedback.		
Signature		Date	01/04/2016

Name of Co-Author	Dr. Mark A. Gibson		
Contribution to the Paper	Principal supervisor of candidate's PhD Supervise development and planning of the research Involved in data interpretation and manuscript evaluation Wrote the manuscript		
Signature		Date	26/11/2015

LTBP-2 has a single high-affinity binding site for FGF-2 and blocks FGF-2-induced cell proliferation.

Clementine Menz^a, Mahroo K. Parsi^a, Julian R.J. Adams^a, **Mohamed A Sideek^a**, Zlatko Kopecki^b, Allison Cowin^b and Mark A. Gibson^{a*}

^a Discipline of Anatomy and Pathology, School of Medicine, University of Adelaide, Adelaide, Adelaide, South Australia 5005, Australia

^b Regenerative Medicine, Mawson Institute, University of South Australia, Adelaide, South Australia 5095, Australia

* corresponding author. TEL: +61 88313 5337 Email address: mark.gibson@adelaide.edu.au

3.1 Abstract

Latent transforming growth factor-beta-1 binding protein-2 (LTBP-2) belongs to the fibrillin-LTBP superfamily of extracellular matrix proteins. LTBPs and fibrillins are involved in the sequestration and storage of latent growth factors, particularly transforming growth factor β (TGF- β), in tissues. Unlike other LTBPs, LTBP-2 does not covalently bind TGF- β and its molecular functions remain unclear. We are screening LTBP-2 for binding to other growth factors and have found very strong saturable binding to fibroblast growth factor-2 (FGF-2) ($K_d = 1.1$ nM). Using a series of recombinant LTBP-2 fragments a single binding site for FGF-2 was identified in a central region of LTBP-2 consisting of six tandem epidermal growth factor-like (EGF-like) motifs (EGFs 9-14). This region was also shown to contain a heparin/heparan sulphate-binding site. FGF-2 stimulation of fibroblast proliferation was completely negated by the addition of 5-fold molar excess of LTBP-2 to the assay. Confocal microscopy showed strong co-localisation of LTBP-2 and FGF-2 in fibrotic keloid tissue suggesting that the two proteins may interact *in vivo*. Overall the study indicates that LTBP-2 is a potent inhibitor of FGF-2 that may influence FGF-2 bioactivity during wound repair particularly in fibrotic tissues.

3.2 Introduction

Latent transforming growth factor-beta-1 binding protein-2 (LTBP-2) is a member of the fibrillin-LTBP superfamily of extracellular matrix proteins. These proteins are all structurally similar, consisting of a rod-like molecule of tandem EGF-like 6-cys repeats interspersed with characteristic 8-cys motifs [1-5]. Fibrillins 1–3 form microfibrils which, together with a core of elastin, are the main structural components of elastic fibers [2, 5]. LTBPs -1, 3, and 4, covalently bind latent growth factor TGF- β and direct the growth factor to storage depots within the extracellular matrix [1, 6]. Fibrillin microfibrils are considered to be a principal storage location for these latent complexes and they act as critical regulators of TGF- β activation [7].

Structurally, LTBP-2 is more similar to the other LTBPs than fibrillins, but like fibrillins, it does not directly bind TGF- β [8, 9] and LTBP-2 function remains largely unclear. An early study reporting that LTBP-2 null mice have embryonic lethality [10], has recently been contradicted by Inoue et al. who presented a LTBP-2 null mouse with only a mild ocular phenotype [11]. This result agrees more closely with LTBP-2 null humans who also have mild ocular phenotypes including glaucoma, megalocornea, ectopia lentis and microspherophakia [12-15]. It has long been documented that LTBP-2 is associated with elastic fibers in developing elastic tissues [8] and there is evidence that LTBP-2 may play a negative regulatory role in elastinogenesis, inhibiting tropoelastin interactions with fibulin-5 and heparan sulphate proteoglycans [16]. In vitro studies have shown that LTBP-2 specifically binds to fibrillin-1 rather than fibrillin-2 and that LTBP-2 can compete with LTBP-1 for binding to the fibrillin-1 molecule, suggesting that LTBP-2 may indirectly affect TGF- β bioavailability [17]. This idea is supported by a recent study linking LTBP-2 gene mutations to a recessive form of Weill–Marchesani syndrome (WMS) [18] which is characterized by short stature, brachydactyly, thick fibrotic skin and ectopia lentis (WMS, Online Mendelian Inheritance in Man # 608328). This finding clearly links LTBP-2 to fibrillin biology as mutations in the fibrillin-1 gene also cause some presentations of WMS [19]. Fibrillin-1 gene mutations also cause Marfan Syndrome (MFS) (OMIM number 154700) and many of the characteristics of WMS and MFS have been attributed to aberrant TGF- β signaling [20]. However fibrillins and associated MAGP proteins have been documented to bind many other growth factors in latent and/or active forms, including bone morphogenic proteins (BMPs) 2, 4, 5, 7 and 10, and connective tissue growth factor [21-24]. Thus sequestration or release of these molecules may also

influence microfibril modulation of growth factor signaling and contribute to aberrant microfibril function in these genetic disorders and other diseases.

Given the above evidence it seems clear that LTBP-2 also has some as yet unidentified role in modulation of growth factor storage and activity. To investigate we have commenced screening LTBP-2 with candidate growth factor binding partners. In this paper we report a very strong interaction of LTBP-2 with fibroblast growth factor-2 (FGF-2). FGF-2 or basic FGF is an important member of a family of cytokines now numbering over 20, that modulate cellular behaviour through activation of FGF receptors (FGFRs)[25]. FGF-2 promotes proliferation, differentiation and migration in fibroblasts and a variety of other cell types [26] and has influence on a range of processes including angiogenesis, tissue remodeling, wound healing and tumour growth [27-29]. FGF-2 has prominent roles in the repair and regeneration stages of wound repair. In acute wound healing, FGF-2 promotes tissue repair by stimulating fibroblast motility and collagenase production for extracellular matrix remodeling, promoting granulation tissue formation, and increasing keratinocyte motility during re-epithelialization [30]. In chronic wounds such as hypertrophic scars and keloids, the growth factor can attenuate fibrosis and promote healing by down-regulating TGF- β induced collagen production, increasing matrix degrading enzymes such as matrix metalloprotein-1 and inducing myofibroblast apoptosis [31]. A role for FGF-2 in microfibril biology has yet to be documented.

We have found that FGF-2 has a single high-affinity binding site in a central region of LTBP-2. In addition LTBP-2 inhibited FGF-2 induced fibroblast proliferation in a bioassay and confocal microscopy showed strong co-localisation of LTBP-2 and FGF-2 in fibrotic keloid skin.

3.3 Materials and Methods

Rabbit anti-[human LTBP-2 peptide] antibody 3504 has been described previously [17]. Anti-His₄ antibodies were purchased from Qiagen (Valencia, CA). FGF-2 antibody (#610871) for immunohistochemistry was supplied by BD labs. Recombinant human FGF-2, VEGF, BMP-4, and BMP-7 and corresponding antibody detection systems (duo-set kits) were obtained from R and D systems. Mouse anti-fibrillin-1 monoclonal antibody MAB1919 and rabbit anti-phospho-FGFR1(Tyr653/Tyr654) antibody were obtained from Merck Millipore, Germany. Rabbit monoclonal anti-total FGFR1 antibody (D8E4) was purchased from Cell Signalling Technology (Danvers, MA).

3.3.1 Recombinant protein production and purification

CDNAs encoding recombinant human LTBP-2 and contiguous fragments NT(H), C(H) and CT(H) were cloned into episomal expression vector pCEP4 as described previously [32]. In addition, three contiguous sub-fragments of LTBP-2C(H), entitled F1, F2 and F3, were generated for the current study (Fig 3.1). Briefly, cDNAs encoding these sub-fragments were amplified by PCR from the LTBP-2C (H) cDNA [32] and ligated into a modified episomal expression vector BM40:his₆:pCEP-4, such that each recombinant fragment was flanked by a BM-40 secretion-signal peptide and a C-terminal His₆ tag. PCR amplification with Pfu turbo Cx DNA polymerase (Stratagene) used 25 cycles of 95 °C for 30 sec, annealing for 30 sec and extension for 1 min at 72 °C. For LTBP-2 C(H) F1, cDNA bases 775 to 1581 were amplified using sense primer 5'-CTGAAAGCTTGGACTCTCAGGCTGGCCAGG-3' and antisense primer 5'CACAAAGCTTCACATCTTGGCAGCCCTTCTCAT- 3', with an annealing temperature of 54°C, giving a product of 807 bp. For LTBP-2 C(H) F2, cDNA bases 1553 to 2343 were amplified using sense primer 5'-GAAGGGCTGCCAAAGCTTGGATGAGTGTGCCAG- 3' and antisense primer 5'-CGTTAAGCTTCTCGTCTATGTCAATGCAG- 3', with an annealing temperature of 62°C, giving a product of 791bp. For LTBP-2 C(H) F3, cDNA bases 2315 to 3180 were amplified using sense primer 5'-CTCCATTGAAAGCTTCGAGTGCGCCAACGACAC- 3' and antisense primer 5'-TTTTAAGCTTGATGTCCATGTGGATGTCGT-3', with an annealing temperature of 54°C, giving a product of 866 bp. The PCR products were purified by excision from agarose gels, A-tailed and ligated into the pGEM-T easy plasmid, as described previously [17]. The ligated constructs were transformed into JM109 competent cells, and individual

clones were propagated and sequenced. Error-free cDNAs were then excised by digestion with HindIII, and ligated into the HindIII site of the modified pCEP4 vector [17]. These expression constructs were transfected into 293-EBNA cells, and cells were grown with selection antibiotic hygromycin B. The cell culture medium was harvested and recombinant protein was purified using chelating Ni-Sepharose as previously described [33]. Samples of the recombinant proteins were dialyzed into TBS-0.5M NaCl, and analyzed by SDS-PAGE on a 12% gel under both reducing and non-reducing conditions to confirm size and purity.

3.3.2 Solid Phase Binding Assays

Solid phase binding assays were conducted as described previously [34] using an adaptation of the method provided with the growth factor DuoSet ELISA Development kits (R&D Systems). Briefly recombinant LTBP-2 or fragments was coated overnight onto microtitre wells, with BSA-coated wells as negative controls. After washing, growth factor, usually FGF-2, was added to the wells, at either a constant concentration, or in increasing amounts for the saturation curve, and incubation was continued for 2 h. After thorough washing, bound growth factor was detected by incubation for 2 h with biotinylated detection antibody (2.5 µg/ml) followed by 20 min with streptavidin conjugated with horseradish peroxidase. Colour development (absorbance at 450 nm) was detected using TMB substrate and quenched using 1M H₂SO₄. For the FGF-2 saturation binding curves, the amount of FGF-2 bound was calculated from a concurrent standard ELISA for FGF-2, following the protocol provided in the DuoSet ELISA Development kit. The K_d for each interaction was calculated by non-linear regression analysis of this saturation curve using GraphPad Prism (Version 4).

For heparin binding studies, binding assays were conducted with heparin-albumin conjugate [HAC] as described previously [32]. Briefly wells were coated overnight with HAC (or BSA control) in TBS, followed by blocking with 5% milk-TBS for 1 hour. LTBP-2C (H) or fragments F1, F2 and F3 were added to wells in TBS+2mM CaCl₂, and incubated at 37 °C for 3 hours. Following washing, primary antibody (usually anti-pentaHis) in TBS+2mM CaCl₂ was added to wells, and incubated at 37 °C for 2.5 h. Binding was detected using goat anti[mouse IgG] antibody-HRP conjugate followed by substrate development as described above.

3.3.3 Cell proliferation assay

Human foreskin fibroblasts (passage 4) were suspended in DMEM plus 10% fetal calf serum, non-essential amino acids, and penicillin/streptomycin. The cells were plated at a density of 4×10^4 cells per well into a 96-well culture plate (Costar #3596, Corning) and incubated overnight at 37 °C in a cell culture incubator (5% CO₂). The wells were rinsed with serum-free DMEM and further incubated at 37 °C for 48 h in 100 µl of serum-free DMEM containing FGF-2 (10 ng/ml, 0.625 nM). For some wells, the FGF-2 was pre-incubated for 15 min with five or ten fold molar excess of LTBP-2 or fragment LTBP-2C F2 prior to addition of the mixture to the wells. Follistatin (30 ng/well) was included in some incubations to ensure that observed effects were not due to undetectable quantities of TGF-β in the recombinant LTBP-2 preparations [35]. Cell proliferation was measured using metabolic substrate WST-1 (Roche) following the manufacturer's instructions. Briefly 10 µl of WST-1 was added to each well. The plate was rocked gently for 1 min to mix then returned to the cell culture incubator for 30 mins. A microplate spectrophotometer (Shimadzu UV-1601) was then used to read absorbances at 450 nm, and 595 nm and the reading at 595 nm was subtracted from the 450 nm reading to give final colour values.

3.3.4 Detection and quantitation of FGFR1 activation

Human foreskin fibroblasts were plated at a density of 4×10^5 cells per well into a 6-well plate (Nunc Surface, Nalge Nunc International, Denmark) and incubated overnight at 37°C in a cell culture incubator (5% CO₂) in DMEM plus 10% fetal calf serum. The wells were rinsed with PBS and further incubated at 37°C for 2 hours with 1 ml of serum-free DMEM containing FGF-2 (10ng/ml, 0.625 nM). For some wells, the FGF-2 was preincubated for 15 min with 10-fold molar excess of LTBP-2 or fragment LTBP-2C F2 prior to addition to the wells. The cells were then lysed using extraction buffer (containing 50 mM Tris (pH 6.8), 0.5% SDS, 2 mM EDTA and cocktails of phosphatase and protease inhibitors [#04906837001 and #11836153001, Roche, Germany]) and analysed by SDS-PAGE on 12 % gels (100 ug cell protein per well). The proteins were immunoblotted onto nitrocellulose membranes (0.2 µm, Pall Corporation, Pensacola, FL) as described previously [34]. The membranes were blocked in a 5% skim milk in TBS-Tween20 for 1 hr at RT with gentle shaking. The blots were horizontally cut into 2 halves and incubated at 4 °C overnight with 0.2ug/ml of anti-phospho-FGFR-1(Tyr653/Tyr654) antibody (#06-1433, Millipore, CA) or anti-total FGFR1 antibody

(#9740, CST, MA) (upper half) or 1 µg/ml of anti-β actin antibody (#SANTSC-47778, Santa Cruz Biotechnology, Inc, USA) (lower half). After washing the blot halves with TBS-Tween, bands were visualised with the appropriate anti-rabbit IgG or anti-mouse IgG antibodies conjugated with IR800 fluorescence dye (#SA5-35571 or #SA5-35521, Thermo Scientific, U.S.A). Membranes were imaged with the LI-COR Odyssey Infrared Imaging System. Bands were quantitated using ImageJ 1.48 software [National Institutes of Health (NIH), Bethesda, MD] and normalised to the internal β-actin signal. For comparison of the phospho-FGFR1 signal between samples, the ratio of the normalised phospho-FGFR1 signal to the total FGR1 signal was expressed as a percentage relative to the average value from cells treated with FGF-2 only (equaling 100%).

3.3.5 Immunohistochemistry

Paraffin-embedded tissue blocks of tissue from normal skin and keloid were prepared from biopsy or discarded surgery material from adult human subjects with informed written consent which is archived and human ethics clearances from the University of Adelaide (#H-16-2001) and the Calvary Hospital Research Ethics Committee (11-CHREC-F007). Sections (4 µm thickness) were cut via a microtome and dewaxed in xylene for 30 min and rehydrated gradually for 2 min each through a series of ethanol solutions (100 % to 30%) followed by water and finally PBS. The slides were then placed in 15% target retrieval solution [36] for 60 mins, starting at 90 °C and dropping to 65 °C. The sections were washed in PBS, incubated with trypsin (0.025% w/v) for 3 min at 37 °C then blocked with 3% normal goat serum for 30 min. After washing with PBS, the sections were incubated overnight at 4°C with primary antibodies (2 or 2.5 µg/ml) or matched concentrations of appropriate rabbit or mouse IgG as negative controls. After thorough washing in PBS, the sections were incubated for 1 h with a 1:200 dilution of appropriate secondary antibody (anti-rabbit IgG antibody conjugated to fluor Alexa 488 or anti-mouse IgG antibody conjugated to Alexa 594, Life Technologies). After further washing with PBS the sections were treated with 0.1 µg / ml of 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) [Sigma; D9542] and sealed under a coverslip in Dako fluorescence mounting medium. The slides were examined using a Leica TCS SP5 confocal microscope, sequentially excited at 488 nm for Alexa 488 (emission window 496-533 nm), 561 nm for Alexa 594 (emission window 569-753 nm) and 405 nm for DAPI (emission window

413 - 460 nm). For quantitation, 3 random areas (each 0.038 mm²) per section were analysed using the AnalySIS software package (Soft-Imaging System, Munster, Germany).

3.4 Results and Discussion

3.4.1 FGF-2 has a strong affinity for LTBP-2

Expression constructs in a modified pCEP4 vector for full-length human LTBP-2 and three contiguous fragments spanning the molecule have been described previously [32]. In addition similar constructs encoding three smaller recombinant fragments spanning the central region of the LTBP-2 molecule were made, each encoding an N-terminal BM40 signal peptide and a C-terminal His6 tag (Fig 3.1A). Each encoded fragment was produced in 293-EBNA cells and purified from the culture medium as previously described [32]. Each protein fragment gave a single band on SDS-PAGE (Fig 3.1B) indicating a high degree of purity. Fragments LTBP-2C F1, F2 and F3 (predicted molecule weights of 29 kDa, 28 kDa and 31 kDa respectively) migrated under non-reducing conditions with apparent molecular weights of 40 kDa, 30 kDa and 37 kDa respectively.

Full-length recombinant LTBP-2 was tested for binding to a range of growth factors including vascular endothelial growth factor, BMP-4, BMP-7 and FGF-2 in an established solid phase binding assay (Fig 3.2A) [34]. Initial screening identified FGF-2 and BMP-4 as candidate binding partners for LTBP-2. However a further experiment identified BMP-4 as a false positive, as the BMP-4 antibody showed binding to the LTBP-2 coated wells in the absence of BMP-4 protein (Fig 3.2B). Of the growth factors tested only FGF-2 showed strong saturable binding to LTBP-2 (Fig 3.3A). The binding curve was quantitated from a standard ELISA curve for FGF-2 coated onto microtitre wells. This enabled the K_d for the LTBP-2 / FGF-2 interaction to be calculated by non-linear regression analysis of the curve produced by plotting amount FGF-2 bound versus concentration of FGF-2 added (Fig 3.3B). The prism program calculated the K_d as 1.11 ± 0.17 nM for a single binding site. This finding indicated that the LTBP-2 / FGF-2 interaction is of high affinity.

3.4.2 FGF-2 binding is confined to a small central region of the LTBP-2 molecule

To identify the FGF-2 binding region(s) on LTBP-2, a range of recombinant LTBP-2 fragments were tested in the FGF-2 binding assay (Fig 3.4). Initially the three large fragments spanning the LTBP-2 molecule were tested with central fragment LTBP-2C(H) alone showing strong FGF-2 binding (Fig 3.4A). Subsequently three sub-fragments F1, F2, and F3 spanning LTBP-2C(H) were produced and tested with only the F2 showing strong FGF-2 binding (Fig 3.4C). This indicated that FGF-2 binding activity was confined to a small central region of LTBP-2 consisting of 6 calcium binding EGF-like repeat motifs (motifs 9-14) (see Fig 3.1A). Binding curves for fragment LTBP-2C(H) (not shown) and sub-fragment F2 (Fig 3.4D) were produced and the Kds for FGF-2 binding were calculated as previously for full length LTBP-2. The Kds for FGF-2 interaction with LTBP-2C(H) and F2 were calculated as 1.02 ± 0.19 nM (Fig 3.4B) and 1.03 ± 0.10 nM (Fig 3.4E) respectively indicating similar affinities for FGF-2 as the full-length LTBP-2 molecule. The binding affinity of LTBP-2 for FGF-2 was similar to that we reported for heparin [32] but was significantly higher than LTBP-2 interactions with fibrillin-1 (9 nM) and fibulin-5 (26 nM) using the same methodology [16, 17]. In an attempt to identify the precise binding site for FGF-2 on LTBP-2 we produced 6 peptides corresponding to each EGF-like motif of the FGF-2 binding region. However no direct FGF-2 binding or inhibition of the LTBP-2 -FGF-2 interaction was identified for any of the peptides (data not shown). This indicates that the binding site may span two or more EGF-like repeats.

3.4.3 The FGF-2 binding site is close to a heparin-binding region of LTBP-2

We have previously identified several heparin-binding regions on LTBP-2 including a central site of moderate affinity contained in fragment LTBP-2C(H) [32]. Since FGF-2 also has affinity for heparin/heparan sulphate we determined if the FGF-2 and heparin binding sites were contained in the same or distinct sub-fragments of LTBP-2C(H). Using the solid phase binding assay, fragments LTBP-2C(H) and sub-fragment F2 showed strong binding to heparin-albumin conjugate coated wells, whereas sub-fragments showed no binding above the control wells coated with BSA (Fig 3.5). Thus both the central heparin binding region and the FGF-2 binding site on LTBP-2 are present within six EGF-like repeats of each other. This site was

reported to have moderate affinity for heparin with a K_d estimated at 80 nM compared to a cluster of higher affinity sites identified in the N-terminal region of LTBP-2 [32].

3.4.4 LTBP-2 blocks FGF-2-induced cell proliferation

To determine if LTBP-2 enhanced or inhibited FGF-2 bioactivity a cell proliferation assay was conducted (Fig 3.6A). Addition of exogenous FGF-2 was found to significantly increase the rate of proliferation of fibroblasts in serum-free culture over 48 h, to a similar extent in the presence or absence of activin/TGF- β inhibitor follistatin. However pre-incubation of the FGF-2 with full-length LTBP-2 in 5-fold or 10-fold molar excess prevented any FGF-2-induced cell proliferation. Pre-incubation with fragment LTBP-2C-F2, which contains the FGF-2 binding site, also significantly inhibited, but did not completely block, FGF-2 induced cell proliferation. Controls conducted in the absence of FGF-2 showed that follistatin, LTBP-2 or fragment LTBP-2C F2 had no significant effect on cell proliferation. To determine if LTBP-2 blocked the activation of the FGF receptor, the experiment was repeated and cellular proteins were extracted after 2 hours and analysed by SDS-PAGE and immunoblotting (Fig 3.6B and 3.6C). The results clearly showed that the control cells had no detectable activated FGFR1 but the addition of FGF-2 resulted in a strong FGFR1 signal. Additional of excess full length LTBP-2 completely blocked the activation of the receptor but the same molarity of fragment LTBP-2CF2 greatly reduced but did not completely prevent FGFR1 activation. Overall the experiment indicated that LTBP-2 inhibits rather than enhances FGF-2 activity. It is noteworthy that the 6-EGF-like repeat fragment containing the FGF-2 binding sequence (LTBP-2C F2) only partially inhibited the mitogenic effect of FGF-2. Thus additional sequences adjacent to fragment F2 may be important for the full influence of LTBP-2 on FGF-2 bioactivity.

3.4.5 LTBP-2 and FGF-2 show similar distributions in fibrotic skin

To determine if the interaction of LTBP-2 and FGF-2 could have biological relevance we searched for overlapping of immunofluorescence localization patterns in normal and fibrotic skin. Neither protein showed discernible localization within the extracellular matrix of

normal adult skin (data not shown). LTBP-2 gene mutations have been linked to WMS which demonstrates thickened fibrotic skin suggesting a connection between LTBP-2 and fibrosis [18]. We therefore examined LTBP-2 expression in fibrotic keloid tissue that has elevated production of new elastic fibres [37, 38]. Keloids are fibrotic scars that are raised above skin level and project beyond the original wound margins [39]. The keloid tissue stained very strongly for LTBP-2 with a widespread, fibrous distribution (Fig 3.7A) which closely matched the distribution of fibrillin-1 (Fig 3.7B) as confirmed by the merged images (Fig 3.7C). Control sections incubated with rabbit or mouse IgG in place of antibody showed no discernible staining (Fig 3.7D and 3.7E). At high power, fine irregular fibres staining for both LTBP-2 (Fig 3.7F) and fibrillin-1 (Fig 3.7G) were evident in the intercellular matrix, visualized as yellow staining in the merged image (Fig 3.7H). The results indicate that LTBP-2 is predominantly associated with fibrillin-containing microfibrils, which are components of elastic fibres. These findings are consistent with previous studies showing strong co-localization of LTBP-2 and developing elastin fibres in fetal tissues and in tissue remodelling [8, 10, 40]. The elastic fibres generally ran parallel to the epithelium although some areas showed a more random distribution consistent with previous reports [37, 38]. Interestingly a similar intense immuno-staining pattern was found for FGF-2 in sections of fibrotic keloid skin from several patients. An example from one patient is shown in Fig 3.7. Low power images show intense discrete staining for LTBP-2 (Fig 3.8A-green) and FGF-2 (Fig 3.8B-red) to the same structures throughout the keloid as confirmed from the merged image (Fig 3.8C) where co-localization is visualized as yellow-orange. At higher power, LTBP-2 (Fig 3.8F- green) and FGF-2 (Fig 3.8G-red) antibodies stained the same fibres within the extracellular matrix as well as cellular elements (identified using the blue nuclear DAPI stain). The extensive overlap of staining for the two proteins is confirmed by the merged image (Fig 3.8H) where the co-localization is visualized as yellow staining. The appropriate immunoglobulin controls showed little background staining (Fig 3.8D and 3.8E). As an additional control a section was stained for LTBP-2 and VEGF which has no known affinity for fibrillin microfibrils (Fig 3.8I). No overlap in the distributions were observed, with VEGF detected only in association with some but not all of the stromal cells and showing no localization within the extracellular matrix. The close proximity of FGF-2 to LTBP-2 within the keloid indicates that the two proteins may directly interact in the matrix of fibrotic skin on the surface of newly generated elastic fibres where they may influence, *in vivo*, the biological activity of each other. The significance of the strong intracellular staining for both proteins is less clear. It seems likely that this simply reflects high synthesis rates for both proteins in fibrotic tissues although a direct intracellular

interaction cannot be ruled out. Quantitation of the relative immunofluorescence signals between normal skin and keloid showed around 9-fold increases in signals for both LTBP-2 and FGF-2 in the keloid tissue suggesting that production of both proteins was greatly increased in the fibrotic condition (Fig 3.9).

Our results have shown that LTBP-2 strongly binds and inactivates FGF-2 in vitro and that both proteins appear to co-localize with fibrillin-microfibrils in fibrotic tissues. However the importance of these observations in microfibril and elastic fibre biology, and pathophysiology of relevant congenital and fibrotic diseases, remains to be established. The paradigm of the congenital disease MFS and related disorders has demonstrated that fibrillin microfibrils are important for growth factor regulation. Mutations in fibrillin genes cause a reduction in the number of normal microfibrils in tissues, resulting in inappropriate or excessive activation of latent TGF- β during tissue development and growth [7, 20]. This aberrant TGF- β signaling is considered to be a major contributor to the malformation and dysfunction of the cardiovascular, skeletal, pulmonary and ocular systems characteristic of MFS. The mechanism of this TGF- β activation appears to be more complex than originally envisaged. Isogai et al showed that LTBP-1, 3 and 4 share a single binding site on fibrillin-1 and suggested that disruption of this binding activity would reduce matrix storage of the LTBP-TGF- β latent complexes resulting in excessive growth factor activation [41]. However subsequent research with mutant mice showed that total deletion of this binding site on fibrillin-1 caused no obvious disease phenotype [42]. More recently Zilberberg et al demonstrated that LTBP-1, the major contributor to latent TGF- β sequestration, required only fibronectin and not fibrillin 1 or 2 for matrix attachment [43]. The findings suggest that other mechanisms in addition to direct liberation of latent TGF- β from the fibrillin microfibrils may contribute to elevation of the TGF- β signalling. Since fibrillin and associated proteins also bind a range of other potent cytokines, it seems likely that disruption of normal microfibrils will activate other signalling pathways perhaps leading to indirect TGF- β elevation. It appears that LTBP-2 requires fibrillin-1 microfibrils for incorporation into the extracellular matrix [44] and thus loss of these structures is likely to disrupt matrix sequestration of LTBP-2 and any attached proteins such as FGF-2. Depending on context FGF-2 can stimulate TGF- β gene expression [45] and secretion [46] or can inhibit TGF- β induced fibrosis [31]. Thus it is difficult to predict possible effects of disrupting LTBP-2/FGF-2 interactions in WMS and other relevant diseases. The LTBP-2 gene has also been linked to tumor suppression in squamous cell carcinoma and meningioma, [47, 48] and as a marker for pulmonary deaths following acute dyspnea [49].

It also remains to be established how LTBP-2 relates to FGF-2 functional biology. FGF-2 lacks a secretion signal [50] and is secreted from cells by an unknown mechanism and becomes strongly bound to the GAG side-chains of HSPGs in the matrix and basement membranes [51, 52]. Following tissue injury, the FGF-2 molecules are released by protease and heparinase activity. Multiple FGF-2 molecules remain attached to released HS chains and subsequent interaction with cell surface FGF-receptors causes clustering of the FGFR molecules necessary to activate intracellular signaling pathways [51, 52]. FGF-2 achieves its diverse effects by stimulating several major cell signaling pathways including RAS/MAPK, PI3K/AKT and PLC- γ [53] and in complex with cell surface heparan sulphate proteoglycans, the ERK1/2 pathway [54].

We have shown here that the FGF-2 binding site of LTBP-2 is adjacent to a heparin binding site of moderate affinity. LTBP-2 also has multiple high affinity binding sites for heparin/heparan sulfate in its N-terminal region, binds HSPGs perlecan in vitro [32] and partially co-localizes with the proteoglycan in some tissues [55, 56]. The findings suggest that LTBP-2, in addition to free FGF-2, may also target and inhibit heparan sulphate-bound growth factor. Interestingly, Cain et al. have recently shown that fibrillin-1 interactions with heparan sulfate may be disrupted in WMS [57] and it is possible that LTBP-2 interactions with FGF-2 and heparan sulfate are affected in WMS cases linked to LTBP-2 gene mutations.

The association of LTBP-2 with elastic fibres is well documented during periods of active elastinogenesis [8, 40] but the protein is not ubiquitously associated with all elastic fibres [17]. This restriction may explain why FGF-2 localization to elastic fibres has not previously been reported since its association may be dependent on the presence of LTBP-2. The high levels of LTBP-2 in keloid tissue suggests a potential role for the protein in fibrosis. FGF-2 has an anti-fibrotic role in the later stages of wound healing and exogenous FGF-2 has been used to effect in treatment of hypertrophic scar and keloid tissues [31, 58]. An intriguing possibility is that in keloid and perhaps other fibrotic disorders elevated LTBP-2 may bind and inactivate FGF-2, inhibiting its contribution to resolution and healing of the condition and perpetuating the fibrotic process. This suggestion warrants further investigation.

3.5 References

1. Hyytiainen M, Penttinen C and Keski-Oja J (2004) Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci* 41: 233-264.
2. Kielty CM, Sherratt MJ, Marson A and Baldock C (2005) Fibrillin microfibrils. *Adv Protein Chem* 70: 405-436.
3. Rifkin DB (2005) Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *J Biol Chem* 280: 7409-7412.
4. Baldwin AK, Simpson A, Steer R, Cain SA and Kielty CM (2013) Elastic fibres in health and disease. *Expert Rev Mol Med* 15: e8.
5. Ramirez F and Sakai LY (2010) Biogenesis and function of fibrillin assemblies. *Cell Tissue Res* 339: 71-82.
6. Annes JP, Chen Y, Munger JS and Rifkin DB (2004) Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1. *J Cell Biol* 165: 723-734.
7. Ramirez F and Rifkin DB (2009) Extracellular microfibrils: contextual platforms for TGFbeta and BMP signaling. *Curr Opin Cell Biol* 21: 616-622.
8. Gibson MA, Hatzinikolas G, Davis EC, Baker E, Sutherland GR, et al. (1995) Bovine latent transforming growth factor beta 1-binding protein 2: molecular cloning, identification of tissue isoforms, and immunolocalization to elastin-associated microfibrils. *Mol Cell Biol* 15: 6932-6942.
9. Saharinen J and Keski-Oja J (2000) Specific sequence motif of 8-Cys repeats of TGF-beta binding proteins, LTBP2, creates a hydrophobic interaction surface for binding of small latent TGF-beta. *Mol Biol Cell* 11: 2691-2704.
10. Shipley JM, Mecham RP, Maus E, Bonadio J, Rosenbloom J, et al. (2000) Developmental expression of latent transforming growth factor beta binding protein 2 and its requirement early in mouse development. *Mol Cell Biol* 20: 4879-4887.
11. Inoue T, Ohbayashi T, Fujikawa Y, Yoshida H, Akama TO, et al. (2014) Latent TGF-beta binding protein-2 is essential for the development of ciliary zonule microfibrils. *Hum Mol Genet*.
12. Ali M, McKibbin M, Booth A, Parry DA, Jain P, et al. (2009) Null mutations in LTBP2 cause primary congenital glaucoma. *Am J Hum Genet* 84: 664-671.

13. Narooie-Nejad M, Paylakhi SH, Shojaee S, Fazlali Z, Rezaei Kanavi M, et al. (2009) Loss of function mutations in the gene encoding latent transforming growth factor beta binding protein 2, LTBP2, cause primary congenital glaucoma. *Hum Mol Genet* 18: 3969-3977.
14. Desir J, Sznajder Y, Depasse F, Roulez F, Schrooyen M, et al. (2010) LTBP2 null mutations in an autosomal recessive ocular syndrome with megalocornea, spherophakia, and secondary glaucoma. *Eur J Hum Genet* 18: 761-767.
15. Kumar A, Duvvari MR, Prabhakaran VC, Shetty JS, Murthy GJ, et al. (2010) A homozygous mutation in LTBP2 causes isolated microspherophakia. *Hum Genet* 128: 365-371.
16. Sideek MA, Menz C, Parsi MK and Gibson MA (2014) LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis. *Matrix Biol* 34: 114-123.
17. Hirani R, Hanssen E and Gibson MA (2007) LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein. *Matrix Biol* 26: 213-223.
18. Haji-Seyed-Javadi R, Jelodari-Mamaghani S, Paylakhi SH, Yazdani S, Nilforushan N, et al. (2012) LTBP2 mutations cause Weill-Marchesani and Weill-Marchesani-like syndrome and affect disruptions in the extracellular matrix. *Hum Mutat* 33: 1182-1187.
19. Faivre L, Gorlin RJ, Wirtz MK, Godfrey M, Dagoneau N, et al. (2003) In frame fibrillin-1 gene deletion in autosomal dominant Weill-Marchesani syndrome. *J Med Genet* 40: 34-36.
20. Doyle JJ, Gerber EE and Dietz HC (2012) Matrix-dependent perturbation of TGFbeta signaling and disease. *FEBS Lett* 586: 2003-2015.
21. Combs MD, Knutsen RH, Broekelmann TJ, Toennies HM, Brett TJ, et al. (2013) Microfibril-associated glycoprotein 2 (MAGP2) loss of function has pleiotropic effects in vivo. *J Biol Chem* 288: 28869-28880.
22. Weinbaum JS, Tranquillo RT and Mecham RP (2010) The matrix-binding domain of microfibril-associated glycoprotein-1 targets active connective tissue growth factor to a fibroblast-produced extracellular matrix. *Macromolecular bioscience* 10: 1338-1344.
23. Weinbaum JS, Broekelmann TJ, Pierce RA, Werneck CC, Segade F, et al. (2008) Deficiency in microfibril-associated glycoprotein-1 leads to complex phenotypes in multiple organ systems. *J Biol Chem* 283: 25533-25543.
24. Sengle G, Ono RN, Sasaki T and Sakai LY (2011) Prodomains of transforming growth factor beta (TGFbeta) superfamily members specify different functions: extracellular matrix interactions and growth factor bioavailability. *J Biol Chem* 286: 5087-5099.

25. Turner N and Grose R (2010) Fibroblast growth factor signalling: from development to cancer. *Nature reviews Cancer* 10: 116-129.
26. Yun YR, Won JE, Jeon E, Lee S, Kang W, et al. (2010) Fibroblast growth factors: biology, function, and application for tissue regeneration. *Journal of tissue engineering* 2010: 218142.
27. Mikroulis D, Papanas N, Maltezos E and Bougioukas G (2007) Angiogenic growth factors in the treatment of peripheral arterial disease. *Current vascular pharmacology* 5: 195-209.
28. Agasse F, Nicoleau C, Petit J, Jaber M, Roger M, et al. (2007) Evidence for a major role of endogenous fibroblast growth factor-2 in apoptotic cortex-induced subventricular zone cell proliferation. *The European journal of neuroscience* 26: 3036-3042.
29. Ribatti D, Vacca A, Rusnati M and Presta M (2007) The discovery of basic fibroblast growth factor/fibroblast growth factor-2 and its role in haematological malignancies. *Cytokine Growth Factor Rev* 18: 327-334.
30. Barrientos S, Stojadinovic O, Golinko MS, Brem H and Tomic-Canic M (2008) Growth factors and cytokines in wound healing. *Wound Repair Regen* 16: 585-601.
31. Shi HX, Lin C, Lin BB, Wang ZG, Zhang HY, et al. (2013) The anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo. *PLoS One* 8: e59966.
32. Parsi MK, Adams JR, Whitelock J and Gibson MA (2010) LTBP-2 has multiple heparin/heparan sulfate binding sites. *Matrix Biol* 29: 393-401.
33. Hanssen E, Hew FH, Moore E and Gibson MA (2004) MAGP-2 has multiple binding regions on fibrillins and has covalent periodic association with fibrillin-containing microfibrils. *J Biol Chem* 279: 29185-29194.
34. Finnis ML and Gibson MA (1997) Microfibril-associated glycoprotein-1 (MAGP-1) binds to the pepsin-resistant domain of the alpha3(VI) chain of type VI collagen. *J Biol Chem* 272: 22817-22823.
35. Kaur J and Reinhardt DP (2012) Immobilized metal affinity chromatography co-purifies TGF-beta1 with histidine-tagged recombinant extracellular proteins. *PLoS One* 7: e48629.
36. Kopecki Z, Ruzehaji N, Turner C, Iwata H, Ludwig RJ, et al. (2013) Topically applied flightless I neutralizing antibodies improve healing of blistered skin in a murine model of epidermolysis bullosa acquisita. *J Invest Dermatol* 133: 1008-1016.
37. Amadeu TP, Braune AS, Porto LC, Desmouliere A and Costa AM (2004) Fibrillin-1 and elastin are differentially expressed in hypertrophic scars and keloids. *Wound Repair Regen* 12: 169-174.

38. Sidgwick GP and Bayat A (2012) Extracellular matrix molecules implicated in hypertrophic and keloid scarring. *Journal of the European Academy of Dermatology and Venereology : JEADV* 26: 141-152.
39. Gauglitz GG, Korting HC, Pavicic T, Ruzicka T and Jeschke MG (2011) Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. *Molecular medicine* 17: 113-125.
40. Sinha S, Heagerty AM, Shuttleworth CA and Kielty CM (2002) Expression of latent TGF-beta binding proteins and association with TGF-beta 1 and fibrillin-1 following arterial injury. *Cardiovasc Res* 53: 971-983.
41. Isogai Z, Ono RN, Ushiro S, Keene DR, Chen Y, et al. (2003) Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein. *J Biol Chem* 278: 2750-2757.
42. Charbonneau NL, Carlson EJ, Tufa S, Sengle G, Manalo EC, et al. (2010) In vivo studies of mutant fibrillin-1 microfibrils. *J Biol Chem* 285: 24943-24955.
43. Zilberberg L, Todorovic V, Dabovic B, Horiguchi M, Courousse T, et al. (2012) Specificity of latent TGF-beta binding protein (LTBP) incorporation into matrix: role of fibrillins and fibronectin. *J Cell Physiol* 227: 3828-3836.
44. Vehvilainen P, Hyytiainen M and Keski-Oja J (2009) Matrix association of latent TGF-beta binding protein-2 (LTBP-2) is dependent on fibrillin-1. *J Cell Physiol* 221: 586-593.
45. Dhandapani KM, Khan MM, Wade FM, Wakade C, Mahesh VB, et al. (2007) Induction of transforming growth factor-beta1 by basic fibroblast growth factor in rat C6 glioma cells and astrocytes is mediated by MEK/ERK signaling and AP-1 activation. *J Neurosci Res* 85: 1033-1045.
46. Phillips AO, Topley N, Morrisey K, Williams JD and Steadman R (1997) Basic fibroblast growth factor stimulates the release of preformed transforming growth factor beta 1 from human proximal tubular cells in the absence of de novo gene transcription or mRNA translation. *Lab Invest* 76: 591-600.
47. Chan SH, Yee Ko JM, Chan KW, Chan YP, Tao Q, et al. (2011) The ECM protein LTBP-2 is a suppressor of esophageal squamous cell carcinoma tumor formation but higher tumor expression associates with poor patient outcome. *Int J Cancer* 129: 565-573.
48. Perez-Magan E, Campos-Martin Y, Mur P, Fiano C, Ribalta T, et al. (2012) Genetic alterations associated with progression and recurrence in meningiomas. *J Neuropathol Exp Neurol* 71: 882-893.

50. Breidhardt T, Vanpoucke G, Potocki M, Mosimann T, Ziller R, et al. (2012) The novel marker LTBP2 predicts all-cause and pulmonary death in patients with acute dyspnoea. *Clin Sci (Lond)* 123: 557-566.
51. Yu PJ, Ferrari G, Galloway AC, Mignatti P and Pintucci G (2007) Basic fibroblast growth factor (FGF-2): the high molecular weight forms come of age. *J Cell Biochem* 100: 1100-1108.
52. Schultz GS and Wysocki A (2009) Interactions between extracellular matrix and growth factors in wound healing. *Wound Repair Regen* 17: 153-162.
53. Kardami E, Detillieux K, Ma X, Jiang Z, Santiago JJ, et al. (2007) Fibroblast growth factor-2 and cardioprotection. *Heart failure reviews* 12: 267-277.
54. Goetz R and Mohammadi M (2013) Exploring mechanisms of FGF signalling through the lens of structural biology. *Nature reviews Molecular cell biology* 14: 166-180.
55. Chua CC, Rahimi N, Forsten-Williams K and Nugent MA (2004) Heparan sulfate proteoglycans function as receptors for fibroblast growth factor-2 activation of extracellular signal-regulated kinases 1 and 2. *Circulation research* 94: 316-323.
56. Hayes AJ, Gibson MA, Shu C and Melrose J (2014) Confocal microscopy demonstrates association of LTBP-2 in fibrillin-1 microfibrils and colocalisation with perlecan in the disc cell pericellular matrix. *Tissue & cell* 46: 185-197.
57. Hayes AJ, Smith SM, Gibson MA and Melrose J (2011) Comparative immunolocalization of the elastin fiber-associated proteins fibrillin-1, LTBP-2, and MAGP-1 with components of the collagenous and proteoglycan matrix of the fetal human intervertebral disc. *Spine (Phila Pa 1976)* 36: E1365-1372.
58. Cain SA, McGovern A, Baldwin AK, Baldock C and Kielty CM (2012) Fibrillin-1 mutations causing weill-marchesani syndrome and acromicric and geleophysic dysplasias disrupt heparan sulfate interactions. *PLoS One* 7: e48634.
59. Eto H, Suga H, Aoi N, Kato H, Doi K, et al. (2012) Therapeutic potential of fibroblast growth factor-2 for hypertrophic scars: upregulation of MMP-1 and HGF expression. *Lab Invest* 92: 214-223.

3.6 Figures

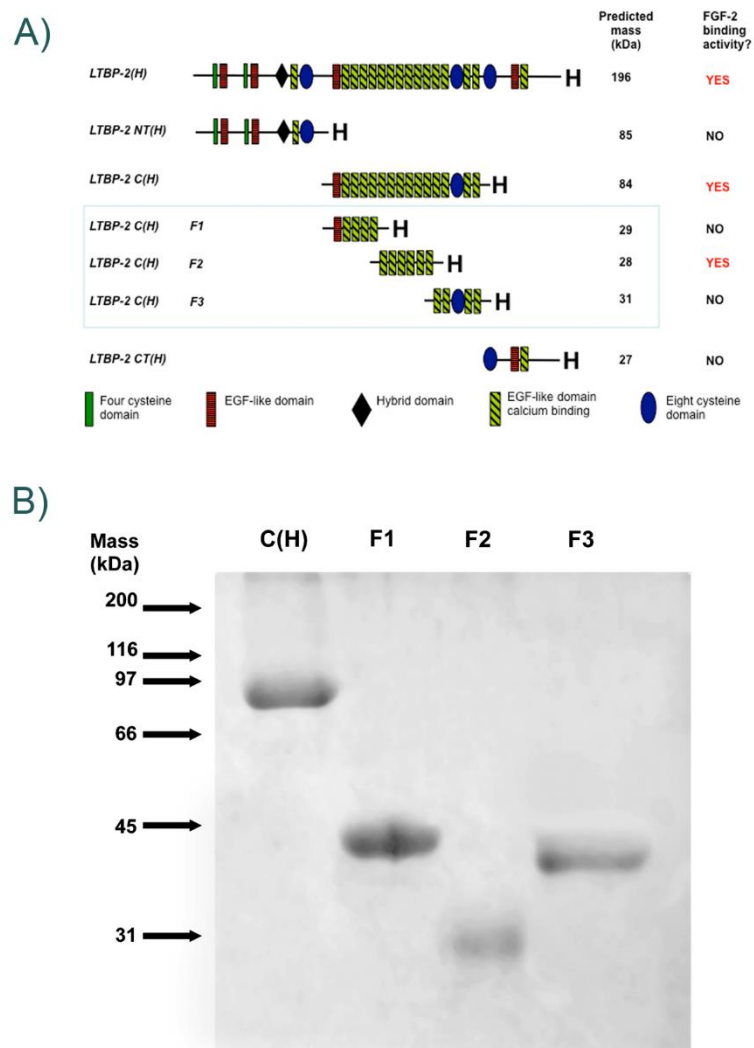


Figure 3.1 Recombinant LTBP-2 fragments

A. Schematic diagram of recombinant LTBP-2 fragments. Protein fragments generated specifically for this study (LTBP-2C(H) F1, F2 and F3) are highlighted within the blue box. FGF-2 binding was confined to a single central region of the LTBP-2 molecule consisting of 6 EGF-like repeats (fragment LTBP-2C(H) F2). **B.** SDS-PAGE of purified recombinant LTBP-2 fragments. Samples of purified fragments LTBP-2 C(H), LTBP-2 C(H) F1, LTBP-2 C(H) F2 and LTBP-2 C(H) F3 were analyzed on a 12% gel under non-reducing conditions and stained with Coomassie blue. The relative mobilities of protein standards are indicated by arrows.

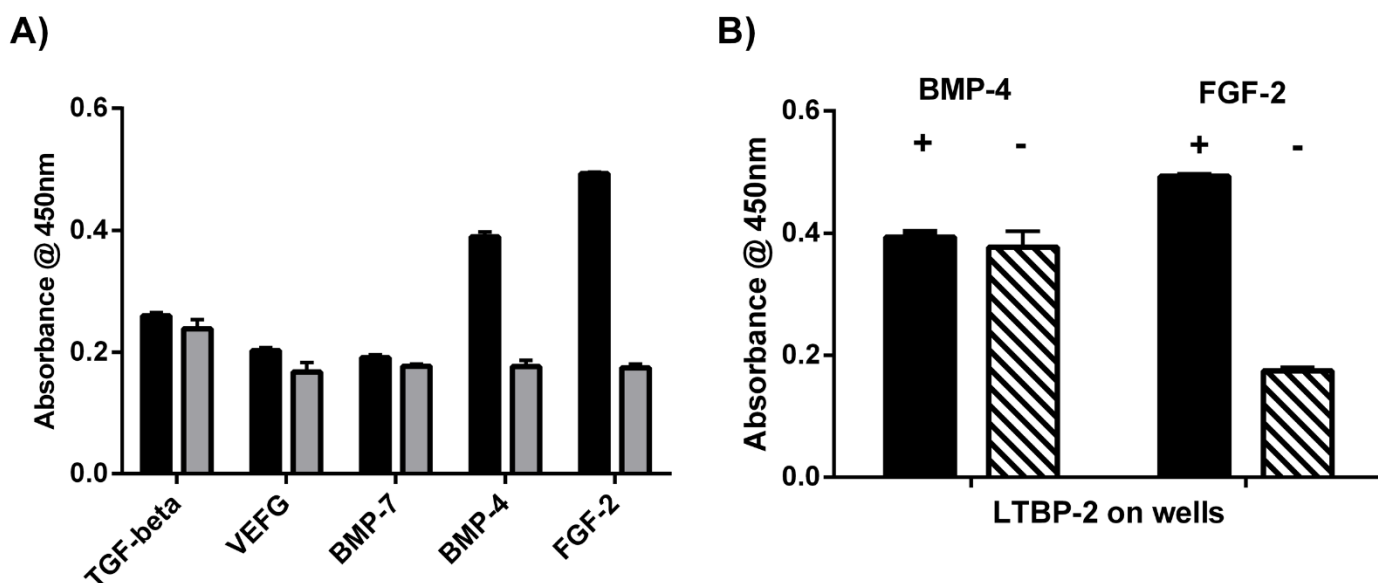


Figure 3.2 LTBP-2 specifically binds FGF-2 but not VEGF, BMP-4, BMP-7 or TGF-beta

A. Microtitre wells were coated with rLTBP-2 (black columns) or BSA (shaded columns) (100 ng/ well). After blocking, triplicate wells were incubated at 37°C for 2h with TGF-beta (13 ng / well), VEGF (21 ng / well), BMP-7 (4 ng/well), BMP-4 (4 ng / well) or FGF-2 (10 ng / well). Growth factor binding was detected using specific biotinylated antibodies from DuoSet kits as described in material and methods. Mean values \pm S.D. from triplicate wells are shown.

B. Microtitre wells coated with rLTBP-2 (100ng/well) were coated onto microtitre plates. After blocking, triplicate wells were incubated at 37°C for 2h with (black columns) or without (cross-hatched) growth factor, (BMP-4 (4ng/well) or FGF-2 (10ng/well). Binding of growth factor to LTBP-2 was detected using biotinylated anti-BMP-4 detection antibody (0.5ug/ml) or anti-FGF-2 detection antibody (0.25ug/ml), followed by a peroxidase detection method (see material and methods). Mean values \pm S.D. from triplicate wells are shown. Note the anti-BMP-4 antibody bound to the wells equally strongly in the presence or absence of added BMP-4, indicating the interaction was non-specific.

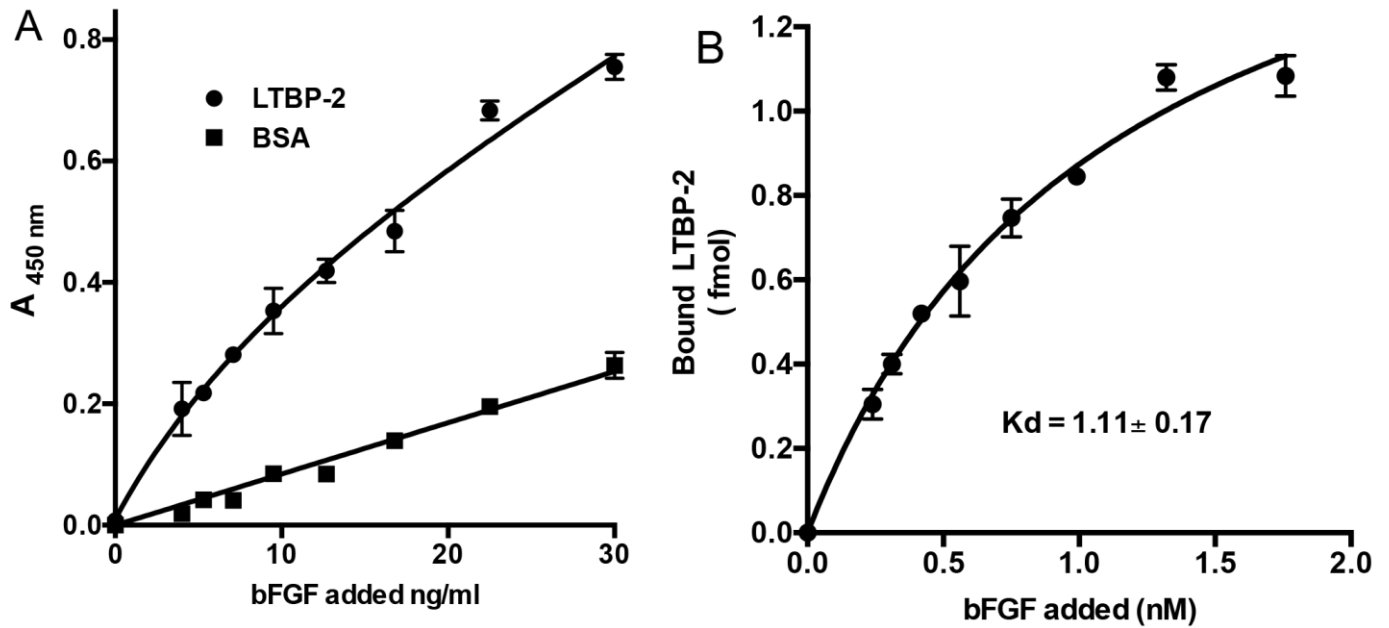


Figure 3.3 LTBP-2 interacts strongly with FGF-2.

A). Microtitre wells were coated with 200 ng rLTBP-2 or BSA control. After blocking, triplicate wells were incubated with 0- 1.8 nM concentrations of FGF-2 (0- 30 ng/ml) for 3 h at 37°C. FGF-2 binding was detected following sequential incubation of the wells with biotinylated mouse anti-[human FGF-2] antibody and streptavidin-HRP conjugate following the duoset protocol. Circles, LTBP-2; squares, BSA. Mean values \pm S.D. of triplicate determinations are shown

B). K_d calculation. Following subtraction of the average BSA signal, the A_{450nm} values were converted to fmol of FGF-2 using a standard ELISA curve (not shown). An additional graph was plotted of bound versus added FGF-2 and the K_d for interaction with LTBP-2 was calculated by non-linear regression analysis of the curve using the prism 4.0 program. Mean values \pm S.D. from triplicate determinations are shown.

Figure 3.4 FGF-2 has a single binding domain in the central region of LTBP-2.

A). Three recombinant fragments spanning the LTBP-2 molecule were tested for binding to FGF-2 in a solid phase assay. Full length LTBP-2(H), fragments LTBP-2 NT (H), LTBP-2C (H), LTBP-2 CT (H) or BSA control were coated onto wells at 100 ng/ml, followed by incubation with FGF-2 (100ng/ml) for 3h at 37 °C. Strong specific binding to central fragment LTBP-2C(H) was detected as described in Fig 3.2A. Mean values \pm S.D. from triplicate wells are shown.

B). A binding curve was produced for the FGF-2 interaction with fragment LTBP-2C(H) following the protocol described under figure 3.2, with 400 ng/well (4.8 pmol) of LTBP-2C (H) or BSA control coated on the wells incubated with increasing concentrations FGF-2 (0- 1.5 nM). The Kd for binding of FGF-2 to fragment LTBP-2C (H) was calculated as 1.02 ± 0.19 nM. Mean values \pm S.D. from triplicate determinations are shown.

C). Three sub-fragments F1, F2 and F3 spanning fragment LTBP-2 C(H) were produced and tested for FGF-2 binding as described under figure 3.2. LTBP-2C (H) (200 ng/well, 2.4 pmol) or sub-fragment (F1, F2 or F3) (66ng/well, 2.4 pmol) or BSA control was coated on the wells and incubated with FGF-2 (100 ng/ml). Strong specific binding of FGF-2 to sub-fragment LTBP-2C F2 was detected. Mean values \pm S.D. from triplicate wells are shown.

D). Subsequently binding curves were obtained for sub-fragments F1 (solid squares), F2 (open circles), F3 (solid circles) (35 ng/well, 1.2 pmol) coated on the wells and incubated with increasing concentrations of FGF-2 (0-30 ng / ml). Note specific FGF-2 binding to sub-fragment LTBP-2C F2 but no binding of fragments F1 and F3 above the BSA control (triangles). Mean values \pm S.D. from triplicate determinations are shown.

E). The Kd for the FGF-2 interaction with sub-fragment LTBP-2C F2 was calculated as 1.03 ± 0.10 nM which is similar to the Kds calculated for the interactions of FGF-2 with full-length LTBP-2 and fragment LTBP2C. Mean values \pm S.D. from triplicate determinations are shown.

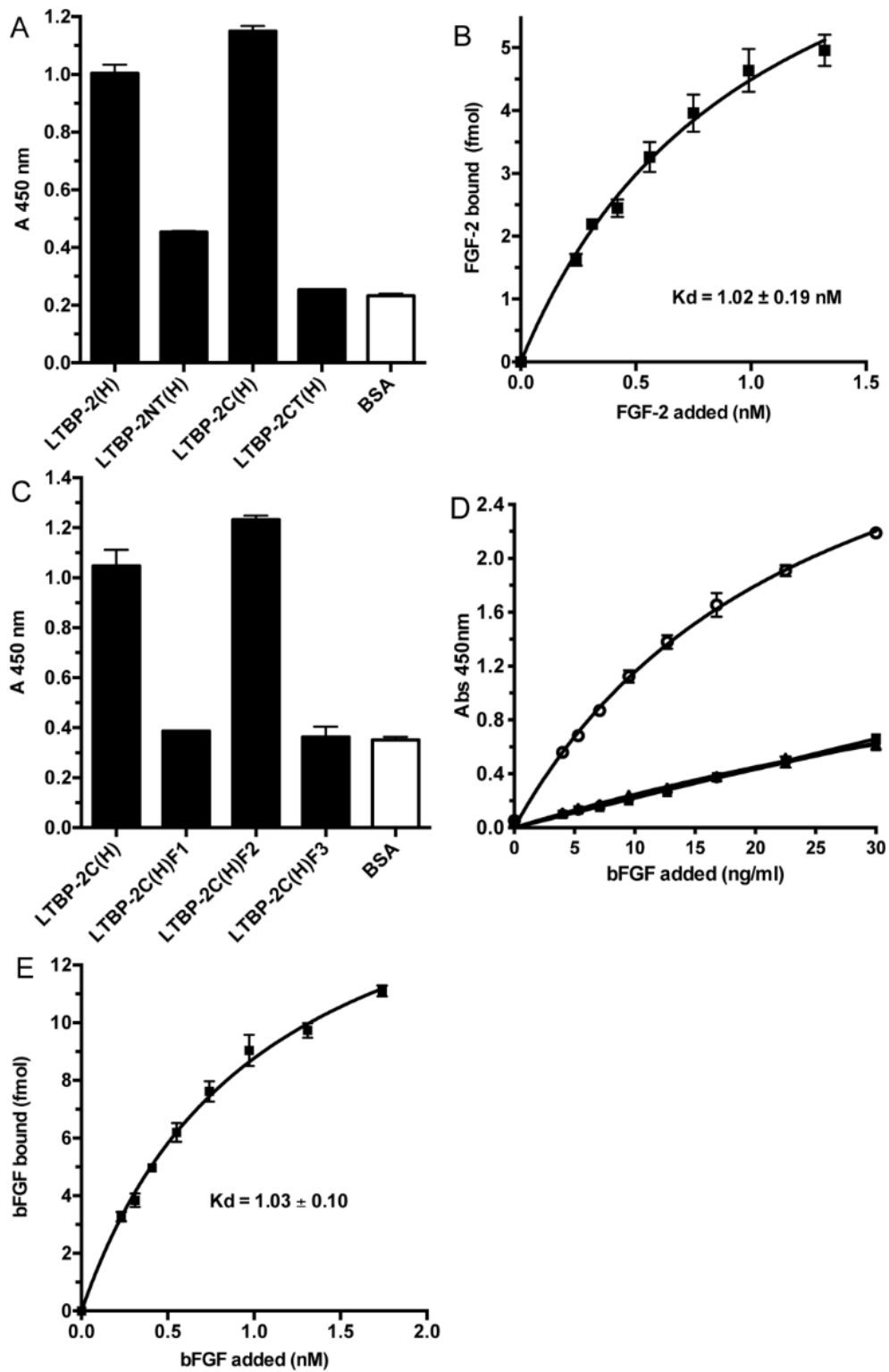


Figure 3.4 FGF-2 has a single binding domain in the central region of LTBP-2.

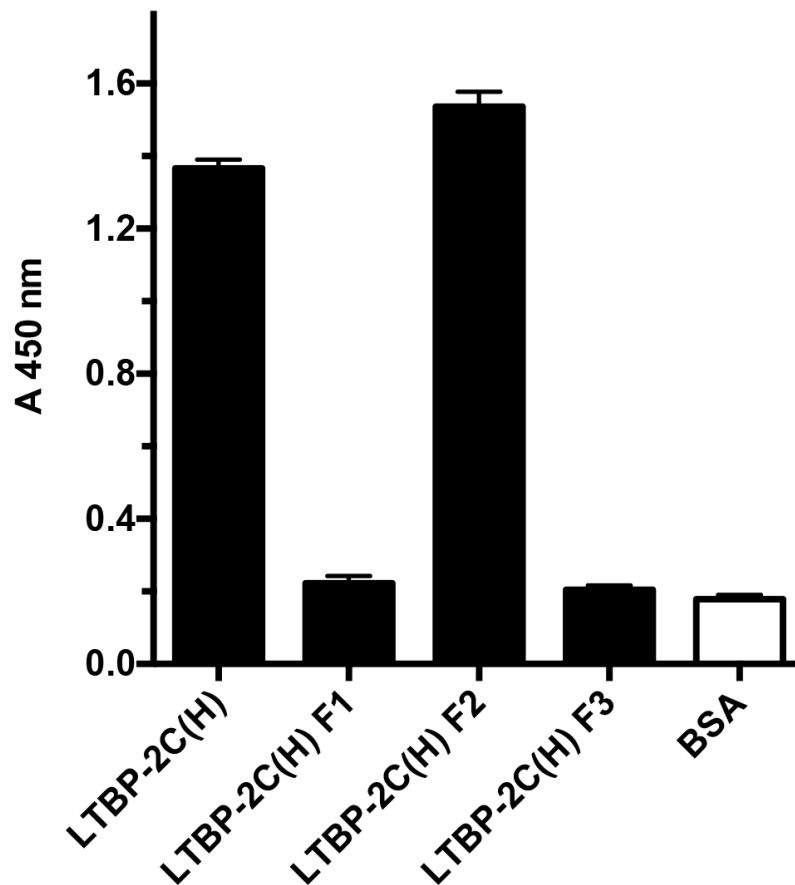


Figure 3.5 The FGF-2 binding site is close to the central heparin binding site on LTBP-2

In a previous study [32] we identified LTBP-2 C(H) as a heparin-binding fragment of LTBP-2. To further define the location of this heparin binding activity, the three sub-fragments F1, F2, F3 spanning LTBP-2 C(H), were assayed for heparin binding using a heparin-albumin conjugate (HAC). HAC or BSA control (400 ng) was coated on wells followed by incubation with equimolar concentrations (23.5 nM) of LTBP-2C(H) or sub-fragment F1, F2 or F3. Specific binding was detected using anti-His₄ antibody targeting the poly-His tag on each recombinant fragment. Fragment F2 showed strong specific binding to the heparin conjugate in contrast to F1 and F3 which showed no binding above background. Mean values \pm S.D. from triplicate wells are shown.

Figure 3.6 LTBP-2 blocks FGF-2-induced cell proliferation

A. The effect of LTBP-2 on the bio-activity of FGF-2 was tested in a cell proliferation assay (see experimental). Human foreskin fibroblasts were treated with FGF-2 with and without follistatin (white columns), or FGF-2 and follistatin pre-incubated with 5 or 10 fold molar excess of full length LTBP-2 or fragment LTBP-2C F2 (cross-hatched). Negative controls (black columns), included cells only and cells incubated with follistatin, LTBP-2 or fragment LTBP-2C F2. Mean values \pm S.D. from triplicate determinations. Note 5 fold molar excess of full-length LTBP-2 completely blocked FGF-2 induced cell proliferation ($p= 0.0001$) and 5-fold molar excess of fragment LTBP-2C F2 partially blocked the activity ($p= 0.0001$).

B. Immunoblot analysis FGF receptor (FGFR1) phosphorylation. Human foreskin fibroblasts were treated for 2 hours with FGF-2 (10 ng / ml) only or with FGF-2 plus 10-fold molar excess of full length LTBP-2 (LTBP-2 FL) or fragment F2 (LTBP-2C F2). Control cells had no FGF-2 or LTBP-2 added. Cellular proteins were extracted and duplicate samples were analysed by SDS-PAGE and immunoblotting with anti-phospho-FGFR1 antibody, and anti-total FGFR1 antibody. Bands were visualised using the LI-COR Odyssey Infrared Imaging System.

C. The band intensity was measured using ImageJ 1.48 software [National Institutes of Health (NIH), Bethesda, MD] and normalised to the internal β actin signal. The ratio of the phospho-FGFR1 to total FGFR1 value for each sample is expressed relative to the average FGF-2 only control value (= 100%). Note the strong FGFR1 activation by FGF-2 was substantially blocked by both LTBP-2 C and LTBP-2C F2 fragments. Mean values \pm S.D. of duplicate lanes.

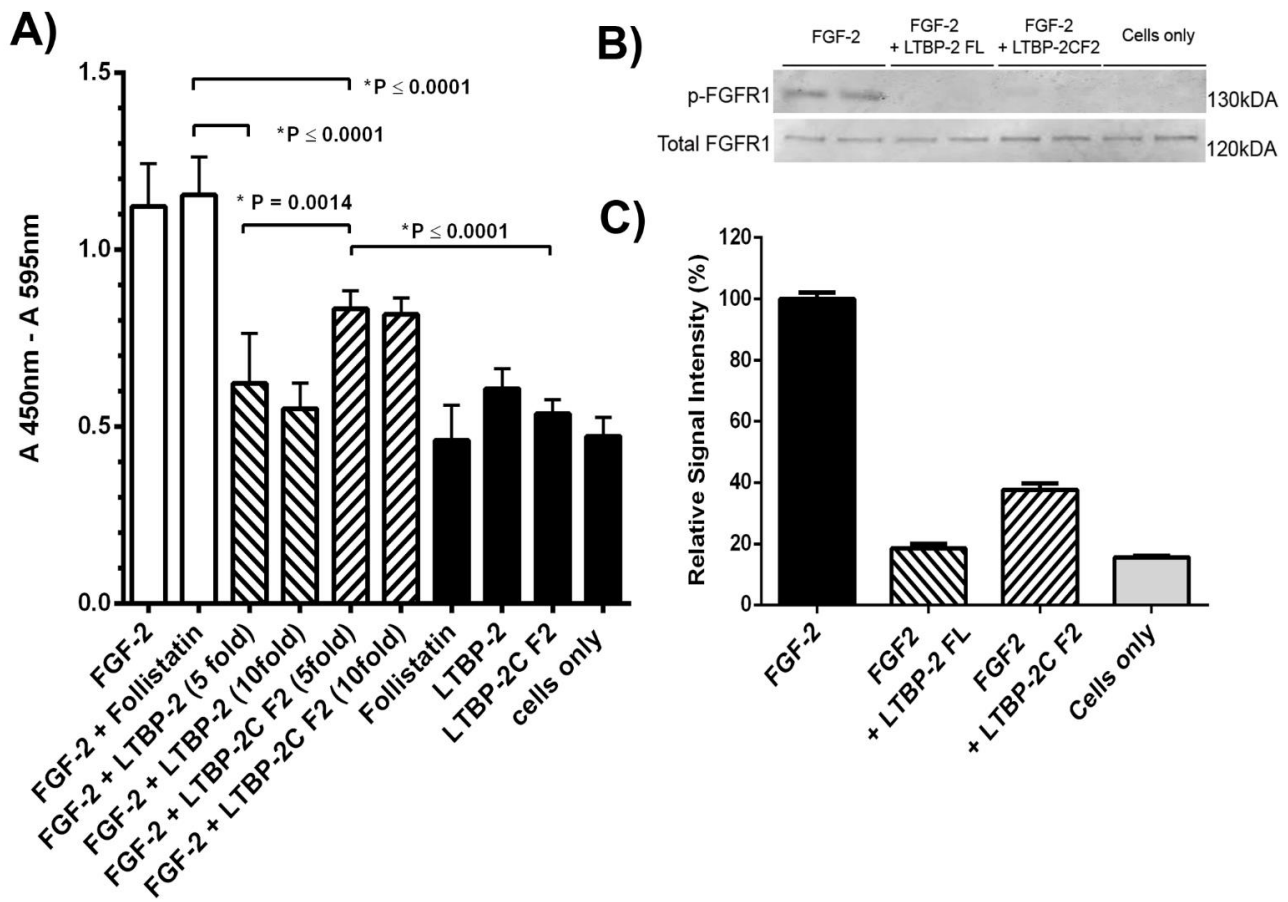


Figure 3.6 LTBP-2 blocks FGF-2-induced cell proliferation

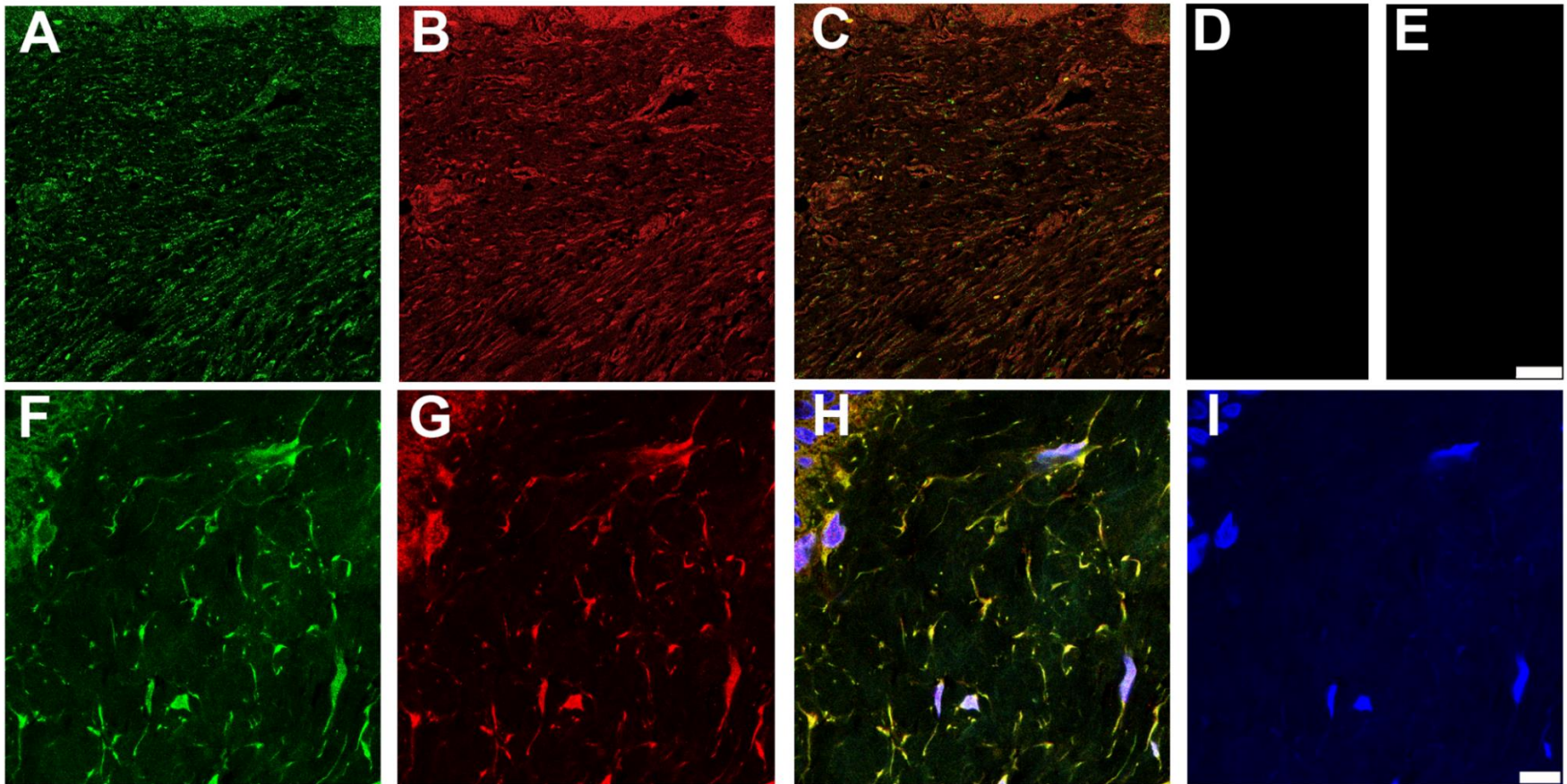


Figure 3.7 LTBP-2 and fibrillin-1 colocalize in fibrotic skin

Keloid tissue was prepared and analyzed by confocal microscopy as described in the methods section. **A and F**, polyclonal anti-[human LTBP-2 peptide] antibody 3504 (2 $\mu\text{g}/\text{ml}$) detected with anti-rabbit IgG antibody conjugated to fluor Alexa 488; **B and G**, monoclonal anti-[fibrillin-1] antibody MAB1919 (Merck millipore) (2.5 $\mu\text{g}/\text{ml}$) detected with anti-mouse IgG antibody conjugated to Alexa 594; **C**, A and B merged; **D**, rabbit IgG control (2 $\mu\text{g}/\text{ml}$); **E**, mouse IgG control (2.5 $\mu\text{g}/\text{ml}$); **H**, F, G and I merged; **I**, DAPI nuclear stain. Magnification: top row, Bar = 100 μm ; bottom row, Bar= 10 μm .

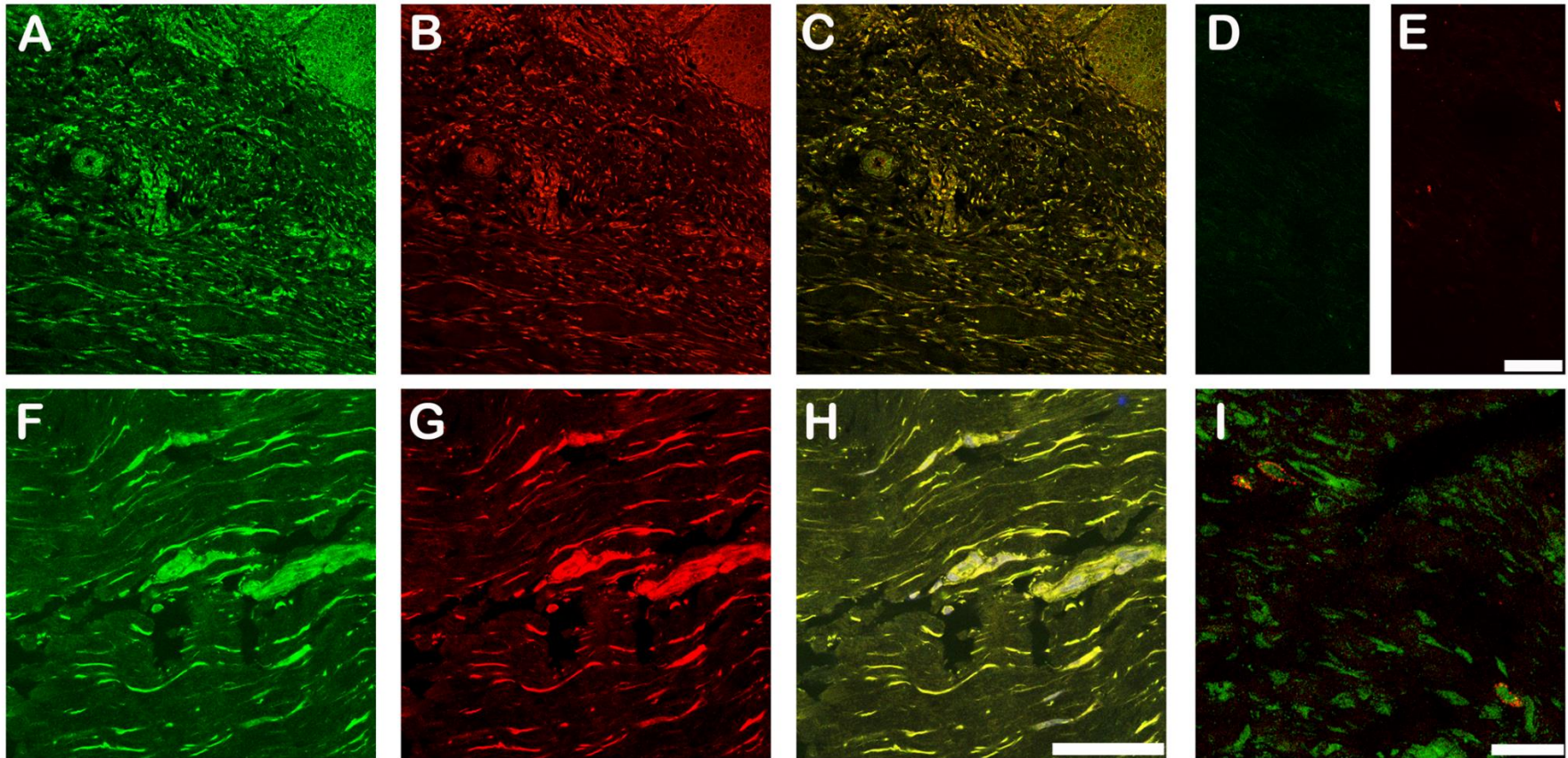


Figure 3.8 LTBP-2 and FGF-2 co-localize in keloid tissue

Keloid tissue was also analyzed for LTBP-2 and FGF-2 by confocal microscopy. **A and F**, polyclonal anti-[human LTBP-2 peptide] antibody 3504 (2 $\mu\text{g}/\text{ml}$) detected with anti-rabbit IgG antibody conjugated to fluor Alexa 488; **B and G**, monoclonal anti-[human FGF-2] antibody #61087 (BD Biosciences) (2.5 $\mu\text{g}/\text{ml}$) detected with anti-mouse IgG antibody conjugated to Alexa 594; **C**, A and B merged; **D**, rabbit IgG control (2 $\mu\text{g}/\text{ml}$); **E**, mouse IgG control (2.5 $\mu\text{g}/\text{ml}$); **H**, F, and G merged; **I**, Control confocal image showing distinct immunostaining patterns for VEGF (red) and LTBP-2 (green). Magnification: top row, Bar = 100 μm ; bottom row, Bar= 50 μm .

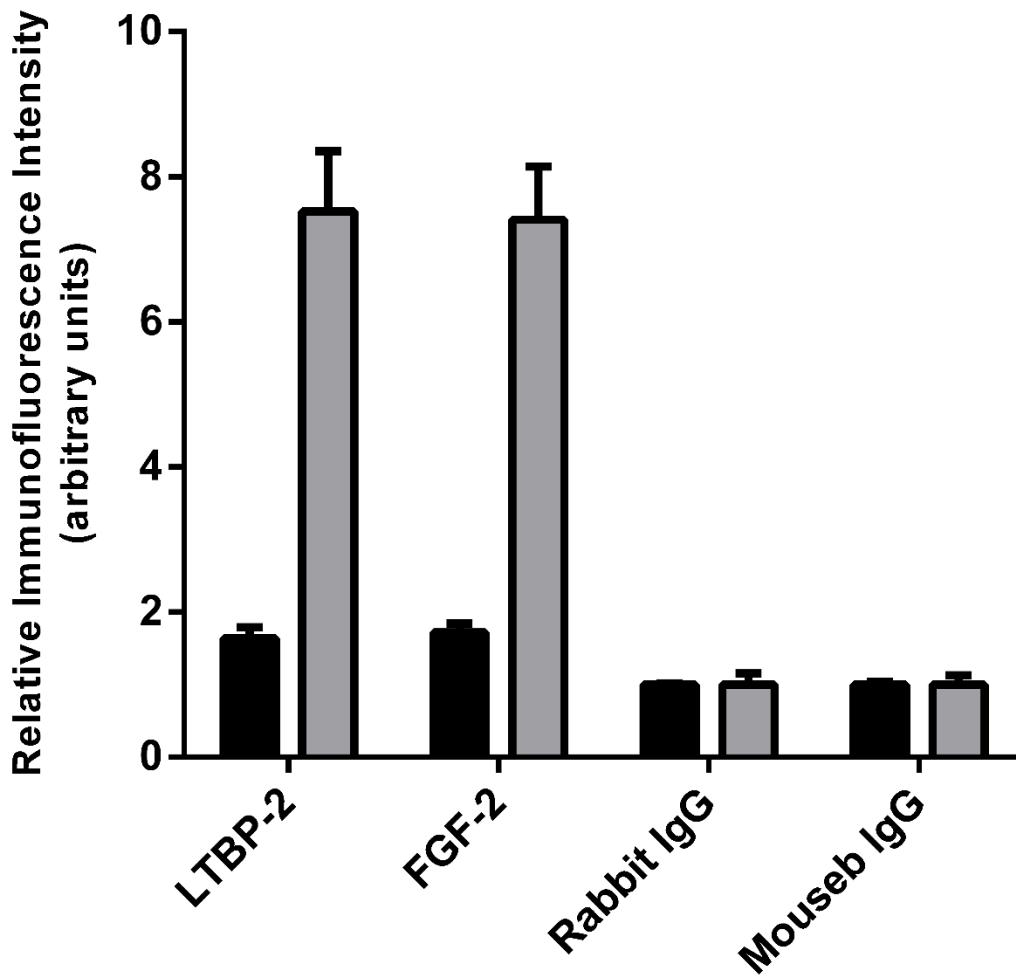


Figure 3.9 Quantitation of LTBP-2 and FGF-2 in normal skin and keloid.

The relative fluorescence intensities of LTBP-2 and FGF-2 staining (and appropriate IgG controls) in sections of normal human skin (black columns) and keloid (shaded columns) was quantitated from 3 random areas (each 0.038 mm²) per section using the AnalySIS software package (Soft-Imaging System, Munster, Germany). Values expressed relative to the background control signal (= 1 unit). Mean values \pm S.D. of triplicate determination are shown.

CHAPTER 4: Co-localization of LTBP-2 with FGF-2 in fibrotic human keloid and hypertrophic scar

Mohamed A. Sideek^{a,b}, Abdulrahman Teia^a, Zlatko Kopecki^c, Allison J. Cowin^c and Mark A. Gibson^{a*}

^a Discipline of Anatomy and Pathology, School of Medicine, University of Adelaide, Adelaide, South Australia 5005, Australia

^b Department of Biomedical Sciences, Kulliyah of Allied Health Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia

^c Regenerative Medicine, Mawson Institute, University of South Australia, Adelaide, South Australia 5095, Australia

* corresponding author. TEL: +61 88313 5337 Email address: mark.gibson@adelaide.edu.au

Key words

LTBP-2, FGF-2, fibrosis, keloid, hypertrophic scar

Received: 2 September 2015, Accepted: 25 November 2015, Published online: 7 December 2015

Journal of Molecular Histology DOI 10.1007/s10735-015-9645-0

Statement of Authorship

Title of Paper	Co-localization of LTBP-2 with FGF-2 in fibrotic human keloid and hypertrophic scar
Publication Status	<input checked="" type="checkbox"/> Published <input type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
Publication Details	Sideek, M.A., Teia, A., Kopecki, Z., Cowin, A.J., Gibson, M.A. (2015) 'Co-localization of LTBP-2 with FGF-2 in fibrotic human keloid and hypertrophic scar' Journal of Molecular Histology, DOI 10.1007/s10735-015-9645-0.

Principal Author

Name of Principal Author (Candidate)	Mohamed Arshad Sideek		
Contribution to the Paper	Performed all experiments Performed all data analyses Interpreted the data Wrote the manuscript		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/11/2015

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Abdulrahman Teia		
Contribution to the Paper	Helped with the experiments Manuscript evaluation and feedback.		
Signature		Date	30/11/2015

Name of Co-Author	Dr. Zlatko Kopecki		
Contribution to the Paper	Collection of tissue and experimental training in IHC. Helped with the experiments Manuscript evaluation and feedback.		
Signature		Date	26/11/2015

Name of Co-Author	Prof. Allison J. Cowin		
Contribution to the Paper	Supervised the development of work and data interpretation. Manuscript evaluation and feedback.		
Signature		Date	01/04/2016

Name of Co-Author	Dr. Mark A. Gibson		
Contribution to the Paper	Principal supervisor of candidate's PhD Supervise development and planning of the research Involved in data interpretation and manuscript evaluation		
Signature		Date	26/11/2015

Co-localization of LTBP-2 with FGF-2 in fibrotic human keloid and hypertrophic scar

Mohamed A. Sideek^{a,b}, Abdulrahman Teia^a, Zlatko Kopecki^c, Allison J. Cowin^c and Mark A. Gibson^{a*}

^a Discipline of Anatomy and Pathology, School of Medicine, University of Adelaide, Adelaide, South Australia 5005, Australia

^b Department of Biomedical Sciences, Kulliyah of Allied Health Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia

^c Regenerative Medicine, Mawson Institute, University of South Australia, Adelaide, South Australia 5095, Australia

* corresponding author. TEL: +61 88313 5337 Email address: mark.gibson@adelaide.edu.au

Key words

LTBP-2, FGF-2, fibrosis, keloid, hypertrophic scar

4.1 Abstract

We have recently shown that Latent transforming growth factor-beta 1 binding protein-2 (LTBP-2) has a single high-affinity binding site for fibroblast growth factor-2 (FGF-2) and that LTBP-2 blocks FGF-2 induced cell proliferation. Both proteins showed strong co-localisation within keloid skin from a single patient. In the current study, using confocal microscopy, we have investigated the distribution of the two proteins in normal and fibrotic skin samples including normal scar tissue, hypertrophic scars and keloids from multiple patients. Consistently, little staining for either protein was detected in normal adult skin and normal scar samples but extensive co-localisation of the two proteins was observed in multiple examples of hypertrophic scars and keloids. LTBP-2 and FGF-2 were co-localised to fine fibrous elements within the extracellular matrix identified as elastic fibres by immunostaining with anti-fibrillin-1 and anti-elastin antibodies. Furthermore, qPCR analysis of RNA samples from multiple patients confirmed dramatically increased expression of LTBP-2 and FGF-2, similar TGF-beta 1, in hypertrophic scar compared to normal skin and scar tissue. Overall the results suggest that elevated LTBP-2 may bind and sequester FGF-2 on elastic fibres in fibrotic tissues and modulate FGF-2's influence on the repair and healing processes.

4.2 Introduction

Fibrotic disorders are a major cause of morbidity and mortality worldwide since fibrosis is central to a range of severe life-threatening human diseases across multiple organ systems including kidney, liver, lung, skin and cardiovascular systems (Wynn 2004). In the United States, fibrotic disorders have been estimated to contribute to 45% of all-cause mortalities (Wynn 2004) and annual incidences of new cases of pulmonary fibrosis and fibrotic kidney disease have been estimated as 270/million/year (Raghu et al. 2006) and 63/million/year respectively (Rastegar and Kashgarian 1998). Despite advances in our understanding of the pathogenesis of fibrotic processes and the development of therapeutic agents that target known pro-fibrotic factors (Akhurst and Hata 2012), effective anti-scarring therapies and potential therapeutic targets are limited, and the vast majority of studies and clinical trials have failed (Brown and Wells 2008).

A persistent finding among all the fibrotic diseases in humans is the disruption of tissue architecture and progressive loss of organ function (Thannickal et al. 2014). Fibrosis is characterized by excessive, abnormal accumulation of extracellular matrix (ECM), particularly collagen fibres, following injury or repeated insult to a tissues or organ (Kissin and Korn 2003; de Vega et al. 2009). The mechanism of fibrosis is complex and poorly understood. Generally, following injury, damaged tissues undergo regeneration and repair whereby insulted epithelial or endothelial cells are replaced by activated fibroblasts that produce excess ECM components including collagen type I and type III, fibronectin and hyaluronic acid, resulting in matrix overgrowth and scarring (Wynn 2007). Persistently elevated TGF- β levels are a key element for progressive and idiopathic forms of pulmonary fibrosis; renal interstitial fibrosis; liver fibrosis; cardiac fibrosis in hypertrophy and heart failure; development of aberrant scars and scleroderma (Wynn 2008; Goodwin and Jenkins 2009; Pohlars et al. 2009). When inflammation subsides during the healing process, matrix synthesis in the post-inflammatory stages of fibro-genesis continues, stimulated by overexpression and aberrant TGF- β activity which results in massive progressive deposition of fibrous matrix (Strutz and Neilson 2003). In cultured human keloid fibroblasts, TGF- β up-regulates the expression of collagen type 1 and fibronectin with an approximately 25-fold increase compared to normal fibroblasts (Daian et al. 2003). Another important growth factor involved in fibrotic disorders is fibroblast growth factor-2 (FGF-2), a mesenchyme-derived growth factor that displays migratory, mitogenic, and

morphogenic properties and has functions in organ development, angiogenesis, organ regeneration, tissues remodelling and wound healing (Ono et al. 2007).

FGF-2 appears to have anti-fibrotic functions (Ortega et al. 1998; Ono et al. 2007; Xie et al. 2008; Eto et al. 2012; Shi et al. 2013). FGF-2 knockout mice exhibit a significant delay in the rate of healing of full-thickness excisional skin wounds (Ortega et al. 1998). In chronic wounds including hypertrophic scars (HTS) and keloids, post-operative administration of FGF-2 inhibits widening of the lesions without any serious side effects (Ono et al. 2007). In the rabbit ear model of wound healing, scars treated with FGF-2 show down-regulation of TGF- β induced collagen production and increased levels of collagen specific matrix metalloproteinase-1, compared with control scars (Xie et al. 2008; Eto et al. 2012; Shi et al. 2013).

Recently, we have identified a very strong interaction of FGF-2 with the matrix protein, latent TGF- β binding protein-2 (LTBP-2) with the binding site in a central cluster of six tandem epidermal growth factor (EGF)-like repeats (Menz et al. 2015). The function of LTBP-2 is unclear and it differs from other members of the LTBP family since it does not bind to TGF- β (Saharinen and Keski-Oja 2000). Previous in situ hybridisation and immunohistochemical studies showed that LTBP-2 was tightly associated with fibrillin microfibrils and elastic fibres in a variety of adult tissues; such as lung, heart maternal decidua, liver, spleen and skeletal muscles (Moren et al. 1994; Gibson et al. 1995). We have also demonstrated that exogenous LTBP-2 inhibits elastinogenesis in cultured ear cartilage chondrocyte indicating LTBP-2 may have a negative regulatory role during elastinogenesis (Sideek et al. 2014).

In vitro studies found that LTBP-2 competes with LTBP-1 for binding to fibrillin-1, suggesting that LTBP-2 may indirectly modulate storage and activation of TGF- β /LTBP-1 complex (LLC) (Hirani et al. 2007). An early study revealed that LTBP-2 mRNA and protein levels increased following tissues damage but the exact mechanism of how this occurs is unknown (Shi and Massague 2003). In addition, patients with LTBP-2 mutations develop a recessive form of Weill-Marchesani syndrome (WMS) which is characterized by brachydactyly, short stature, ectopia lentis and thick fibrotic skin suggestive of aberrant TGF- β signalling (Haji-Seyed-Javadi et al. 2012). LTBP-2 null humans develop primary congenital glaucoma from changes in the elastic fibre-rich ocular trabecular meshwork and ciliary processes (Ali et al. 2009). However some patients also have varied Marfan Syndrome-like phenotypes including abnormal bone over-growth, arachnodactyly, osteopenia, aortic stenosis,

and aortic valve problems (Ali et al. 2009). Our recent studies have shown that exogenous LTBP-2 was able to inhibit FGF-2-induced cell proliferation and that both proteins co-localised in keloid tissue from one patient (Menz et al. 2015). These findings suggest that LTBP-2 could act as a tissue store for FGF-2 released in fibrotic conditions and thus modulate FGF-2's influence in the repair process. The current study has investigated the expression and distribution of LTBP-2 and FGF-2 in fibrotic tissues from a range of patients and has found that both proteins consistently showed strong co-localisation in a range of fibrotic HTS and keloid skin samples.

4.3 Materials and Methods

4.3.1 Human tissue samples

Samples of normal human skin, normal scar tissue, HTS and keloid were obtained from the Calvary Hospital and Royal Adelaide Hospital with ethics approval from the Human Research Ethics Committee (11-CHREC-F007) and University of Adelaide (#H-16-2001) in accordance with the Declaration of Helsinki principles. Written consent was obtained from patients undergoing surgery to obtain excised tissue. Following ethics approval protocols de-identified samples were collected from male and female patients, ranging in age from 18-39 from various body sites including: arm, leg, back and ear. Skin and scar samples were bisected and fixed in 10% (v/v) neutral buffered formalin for histology/immunofluorescence or frozen in liquid nitrogen for biochemical analysis.

4.3.2 Antibodies

Rabbit anti-[human LTBP-2 peptide] antibody 3504 has been described previously (Hirani et al. 2007). Human monoclonal FGF-2 antibody (#610871) for immunohistochemistry was supplied by BD labs. Anti-fibrillin-1 antibody MAB1919 was purchased from Millipore Australia Ltd, Klisyth, Victoria. Mouse anti-human elastin monoclonal antibody BA4 was purchased from Sigma-Aldrich, St. Louis, MO. Immunoblotting to check for antibody cross-reactivity was performed as described previously (Menz et al. 2015).

4.3.3 Immunohistochemistry

a) Cell culture

Human foreskin fibroblasts (HFF) cells were cultured in DMEM for 21 days at an initial density of 4×10^5 /ml on microscope chamber slides (Lab-Tek NALGE NUNC). The cell layers were incubated with 3% para-formaldehyde in PBS for 10 min and fixed with cold acetone and methanol for 1 min and rehydrated in PBS for 5 min. The rehydrated sections were then incubated with 50mM of dithiothreitol (DTT) made up in 2M GuHCl/50mM Tris (100 μ l / chamber) for 5 min followed by addition of 1 M iodoacetamide (100 μ l / chamber) and incubation in the dark for 5 min. The cell layer was washed with TBS and blocked with 0.2 ml

1% ovalbumin in TBS for 15 min. After blocking, the cells were incubated overnight at 4 °C with affinity purified rabbit anti-LTBP-2 antibody 3504 and mouse monoclonal anti-FGF-2 antibody. Control sections were incubated with a matched concentration of rabbit IgG and mouse IgG. Sections were washed with TBS and incubated with the appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC) for 60 min at room temperature in the dark. Sections were washed and mounted in 90% glycerol / 10% TBS / methiolate containing anti-fade reagent p-phenylenediamine (Sigma-Aldrich, St. Louis, MO) prior to laser confocal microscopy. Labelled sections were viewed on a Leicia SP5 spectral scanning confocal microscope using a 488nm laser with emission settings at 496-533nm.

b) Tissue sections

Paraffin-embedded tissue sections from normal skin, normal scar, HTS and keloid were dewaxed in xylene for 30 minutes and dehydrated through a series of ethanol solutions (100% to 30%) followed by water and finally PBS. The sections were then placed in 15% target retrieval solution [Dako; S1700] in a decloaking chamber (Biocare Medical) for 60 minutes with initial temperature 90°C, dropping to 65°C (Kopecki et al. 2013). After washing with PBS, the sections were incubated with trypsin (0.025% w/v in PBS) at 37°C for 3 minutes and then blocked with 3% normal goat serum for 30 minutes. After washing in PBS, the tissues were incubated overnight at 4°C with primary antibodies or matched concentrations of rabbit and mouse IgG as negative controls. For verification of specific staining patterns, non-specific binding was determined by omitting primary or secondary antibodies. Sections were washed with PBS and incubated for 1 hour with a 1:200 dilution of appropriate secondary antibody (goat anti-rabbit IgG antibody conjugated to fluor Alexa 488 or goat anti-mouse IgG antibody conjugated to Alexa 594 [Life Technologies]. DAPI, (4',6-diamidino-2-phenylindole dihydrochloride) [Sigma;D9542] (1ng/μl) was added 20 seconds prior to mounting the sections with Dako fluorescence mounting medium and sealing under coverslip. The slides were examined sequentially using the Leica TCS SP5 microscope with the 488nm laser for Alexa 488 (emission window 496-533nm) and the 561nm laser (emission window 596-763nm) for Alexa 594. For quantitation, 3 random areas (each 0.038 mm²) per section (total of 16 sections) were analysed for fluorescence intensity using the AnalySIS software package (Soft Imaging System GmbH, Munster Germany).

4.3.4 Quantitative Polymerase Chain Reaction

Total RNA was extracted from frozen normal and fibrotic tissues with an UltraClear Tissue & Cells RNA Isolation Kit according to the manufacturer's instruction (Mo Bio Laboratories, Inc., Carlsbad; CA, USA). RNA concentration was determined at 260nm using the NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, USA). cDNA was prepared from reverse transcribed mRNA using the SuperScript III Reverse Transcriptase Synthesis System according to the manufacturer's instruction (Invitrogen, Mount Waverley, Vic., Australia). Briefly, to each 50µl RNA sample, 12.5µl of random hexamers (100µg/ml) and 12.5µl of 10mM dNTP mix (10mM each of dATP, dGTP, dCTP, dTTP) was added. The samples were incubated at 65°C for 5 minutes prior placing on ice for at least 60 seconds. The sample was made up to a volume of 100µl in 1x First-strand buffer, 0.01M DTT, and 100U of Superscript III. The samples were incubated at room temperature for 5 minutes, then at 50°C for 2 hours. The Superscript was inactivated by heating to 70°C for 15 minutes, and the resultant cDNA was stored at -80°C. Real-time PCR was then performed using the fluorescent dye SYBR green. Each 25µl reaction contained 400nM of each primer and template DNA (up to 1µg) in SYBR Green Master Mix (#11733-038, Invitrogen, Mount Waverley, Vic., Australia). Real-time PCR was performed on a Corbett Rotorgene 6000 (Qiagen) to measure the mRNA expression of LTBP-2, FGF-2, TGF-β and RNAPolIII control. Primer sequences used were as follows: LTBP-2 forward primer 5'-GGGCACCGCACCCACCTACACG-3', and reverse primer 5'-TCATCACACTCATTACATCTACG-3'; FGF-2 forward primer 5'-GCGGGAGGCTGGTGGGTGTG-3', and reverse primer 5'-CAAGGCGGGCAGCGTGGTG-3'; TGF-β forward primer 5'-CTCCGAGAAGCGGTACCTGAAC-3', and reverse primer 5'-CACTTGCAGTGTGTTATCCCT; RNAPolIII forward primer 5'-AGGGGCTAACAATGGACACC-3', and reverse primer 5'-CCGAAGATAAGGGGA ACTACT-3'. The primers were all obtained from GeneWorks, Adelaide, Australia. The PCR protocol was as follows, a hot start at 95°C for 10 minutes, followed by 40 cycles of 95 °C for 20 seconds, 60°C for 20 seconds, and 72 °C for 45 seconds, with fluorescence measurement at the end of each 72 °C amplification step. A melt curve was performed after 40 cycles to confirm the correct number of PCR products and screen for any contamination. Results were analysed using the $2(-\Delta\Delta C(T))$ method as described by Livak et al., 2001 (Livak and Schmittgen 2001). Briefly, the mRNA expression in each sample was

determined by comparing the cycle threshold (Ct) for the gene of the interest and the Ct for RNAPoIII.

4.3.5 Statistical analysis

All data are expressed as means \pm S.D. The quantitative immunostaining and qPCR were statistically analysed by 'Student's' t-test using GraphPad Prism 6 software. A p value ≤ 0.05 was regarded as statistically significant.

4.4 Results

4.4.1 LTBP-2 and FGF-2 do not co-localize in fibroblast cell culture

Our previous *in vitro* studies found that LTBP-2 has a single binding site for FGF-2 and is able to inhibit FGF-2 induced proliferation (Menz et al. 2015). To determine if the interaction of LTBP-2 and FGF-2 could have biological importance *in vivo*, we first compared immunolocalisation patterns in human skin fibroblast cell lines. Human foreskin fibroblast (HFF) cells were cultured for 21 days and stained with LTBP-2 and FGF-2 antibodies. LTBP-2 was strongly detected in the fibrous meshwork of the extracellular matrix (Fig. 4.1A and 4.1B) while the rabbit IgG controls showed minimal background staining (Fig. 4.1C). FGF-2 revealed a different staining pattern to LTBP-2 and was mainly localised intracellularly (Fig. 4.1D and 4.1E) while mouse IgG controls showed minimal non-specific staining (Fig. 4.1F). These findings are consistent with previous studies that suggest FGF-2 is stored intracellularly and only secreted from cells following tissue insult to become strongly bound to the GAG side-chains of HSPGs in the interstitial matrix and basement membranes (Kardami et al. 2007; Schultz and Wysocki 2009). Since the staining patterns of the two antibodies did not overlap, no co-localisation between LTBP-2 and FGF-2 was evident in the fibroblast cultures.

4.4.2 LTBP-2 and FGF-2 show extensive co-localizations in fibrotic skin lesions

Since the fibroblast cultures proved not to be a suitable model for studying the potential interaction of LTBP-2 and FGF-2, immunohistochemical analysis was undertaken in human fibrotic skin samples. To determine the distribution patterns of LTBP-2 and FGF-2, sections were prepared and immunostained with dual combinations of anti-(LTBP-2) antibody (Fig. 4.2A, 4.2F, 4.2K and 4.2P) and anti-(FGF-2) antibody (Fig. 4.2B, 4.2G, 4.2L and 4.2Q).

The staining patterns for both LTBP-2 and FGF-2 were dull and weak in normal skin tissues indicating only low levels of the two proteins were present (Fig. 4.2A and 4.2B). In normal scar (Fig. 4.2F and 4.2G), stronger staining intensity for both proteins was observed compared to normal skin. Interestingly, similar staining patterns for LTBP-2 and FGF-2 were found on irregular fibrous networks within the tissue matrix. The regions of co-localisation were confirmed from the merged images (Fig. 4.2C and 4.2H), visualized as yellow-orange. Examination of control sections showed negligible non-specific mouse and rabbit IgG staining

(Fig. 4.2D, 4.2E, 4.2I and 4.2J), thus confirming that the staining patterns for LTBP-2 and FGF-2 are specific. In HTS and keloid samples there was extensive staining for LTBP-2 (green) (Fig. 4.2K and 4.2Q) and FGF-2 (red) (Fig. 4.2L and 4.2R) with similar widespread, fibrous distributions. The merged images showed strong co-localisation visualised as yellow staining (Fig. 4.2M and 4.2S). There was also some intracellular staining adjacent to nuclei visualised by the DAPI staining (Fig. 4.2 N and 4.2T). Again, control sections treated with mouse or rabbit IgG showed negligible background signals (Fig. 4.2N, 4.2O, 4.2S and 4.2T). Additional controls were performed to ensure that the two primary antibodies were showing specific staining patterns. Firstly immunoblotting of purified LTBP-2 and FGF-2 with anti-LTBP-2 and anti-FGF-2 antibodies showed no cross-staining of the proteins. Moreover additional confocal controls included incubation of sections with one primary antibody together with both secondary antibodies. For each primary antibody, signal was only detected in the correct channel. This eliminated any possibility of the secondary antibodies cross-reacting with the reciprocal primary or secondary antibody, or of signal bleed through between the two detection channels. (supplementary data in Online Resource 1).

Quantitation of the relative immunofluorescence signals in normal and fibrotic sections was performed. For each category, a section from four different patients was stained, imaged and quantified (Fig. 4.3). All image processing and analyses were performed using the AnalySIS software package (Soft Imaging System GmbH, Munster Germany) which was kept consistent between images. Three random areas (each 0.038 mm²) were analysed per section, therefore for each category at least 12 areas were analysed. Representative confocal immunofluorescence images in Figure 4.3 showed approximately 4-fold increases in signal for both LTBP-2 and FGF-2 in HTS and keloid samples compared to normal skin. Signals in normal scar were only slightly higher than in normal skin. Overall, the overlay analysis of dual-labelled sections with LTBP-2 and FGF-2 indicated there is an extensive co-distribution of both proteins in HTS and keloid sections.

4.4.3 LTBP-2 shows similar distribution with elastin and fibrillin-1 in fibrotic skin

Since LTBP-2 has been documented to be associated with elastic fibres, HTS and keloid tissues were also dual stained for LTBP-2 and fibrillin-1 or elastin (Fig. 4.4). LTBP-2 showed extensive co-localization with fibrillin-1 and elastin in both tissue types (Fig. 4.4D, 4.4H, 4.4L and 4.4P). All three proteins showed staining of irregular fibres in the matrix between cells in

both HTS and keloid samples. Control sections showed a negligible signal (data not shown). These findings are consistent with previous studies showing LTBP-2 abundant association with fibrillin microfibrils in developing human foetal aorta and other tissues (Gibson et al. 1995). Since LTBP-2 showed almost complete co-localisation with elastin the results point to LTBP-2 being predominantly associated with elastic fibres rather than elastin-free fibrillin-microfibril bundles abundant in normal skin. Thus it is apparent that both LTBP-2 and FGF-2 have association with fibrillin-1-microfibrils of elastic fibres in fibrotic skin lesions.

4.4.4 Both LTBP-2 and FGF-2 mRNA expression levels are greatly elevated in hypertrophic scar

The relative expression of LTBP-2 and FGF-2 was compared to that of TGF- β in normal and fibrotic skin samples using real-time quantitative PCR (Fig. 4.5). The mRNA was harvested from the tissues and reverse transcribed into cDNA. Quantitative PCR was performed using primers specific to LTBP-2, FGF-2 and TGF- β , as well as primers for the housekeeping gene RNAPolIII. The mRNA levels for LTBP-2 and FGF-2 were elevated drastically in HTS (12-fold and 7-fold increases respectively) (black columns) compared to normal skin (cross-hatched). The mRNA levels of LTBP-2 and FGF-2 significantly increased in normal scars (white columns), however the increase is much less than that for HTS. In addition, mRNA levels of TGF- β were also elevated in HTS samples.

4.5 Discussion

The key mediator of tissue fibrosis is considered to be the activated fibroblast which produces an accumulation of ECM, mainly collagen, resulting in functional impairment and structural distortion of affected organs and tissues (Krenning et al. 2010). This process is regulated by a number of cytokines and growth factors but primarily by TGF- β (Meng et al. 2015). Another growth factor that contributes to fibrosis is FGF-2, which paradoxically appears to have an anti-fibrotic role in fibrotic skin conditions. The present study investigated the expression and localisation of the FGF-2 binding protein, LTBP-2, in a variety of fibrotic skin conditions. To the best of our knowledge, this is the first study showing consistent upregulation and co-localisation of LTBP-2 with FGF-2 in keloid and HTS, suggesting both proteins may play important interactive roles during tissue repair, fibrosis and scarring.

Our results showed that LTBP-2 and FGF-2 had no overlap in distribution in cultures of normal skin fibroblasts, LTBP-2 showing a fibrous staining pattern in the extracellular matrix and FGF-2 being largely intracellular. The result is consistent with studies showing FGF-2, which lacks a secretion signal (Yu et al. 2007), is stored intracellularly (Chua et al. 2004) and is only released at the time of injury or tissue remodelling (Suga et al. 2009). In normal skin, staining intensity was low for both LTBP-2 and FGF-2 whereas in normal scars slightly more intense signals were detected to dermal fibres suggesting possible direct interaction between LTBP-2 and FGF-2. To pursue this idea further, we investigated fibrotic skin lesions, namely HTS and keloid samples, where expression and extracellular localisation of FGF-2 were anticipated to be greater. Both HTS and keloid tissues represent abnormal wound healing resulting in increased fibrosis, including excessive collagen production by fibroblasts (Aarabi et al. 2007; Verhaegen et al. 2009). Both lesions can be firm, itchy, raised and painful, causing patient physical and psychological distress (Bayat et al. 2003; Bock et al. 2006). The main clinical differences between the two types of lesion are that HTS occur within 4-8 weeks after surgery or injury and remains confined within the boundaries of the original wound (Aarabi et al. 2007). In contrast, keloid scars develop several months after an initial trauma and the overgrowth of fibrosis extends beyond the boundaries of the original injury (Verhaegen et al. 2009). Aside from clinical features, HTS and keloids also can be distinguished by established histo-pathological criteria. HTS are generally characterized by the presence of nodular structures up-regulated by alpha-smooth muscle actin (α -SMA)-expressing myofibroblasts and fine collagen fibres that run parallel the epithelial surface. However in

keloids, (α -SMA)-expressing myofibroblasts are absent and collagen bundles are randomly orientated to the epithelial surface (Bayat et al. 2003; Bock et al. 2006; Aarabi et al. 2007; Verhaegen et al. 2009). In addition, keloids tend to indicate a greater genetic predisposition than HTS (Marneros et al. 2004; Nakashima et al. 2010; Shih and Bayat 2010). Interestingly, we found extensive expression and co-localization of both LTBP-2 and FGF-2 within HTS and keloid skin samples indicating high synthesis rates for both proteins during fibrosis. The close proximity of FGF-2 to LTBP-2 within the fibrotic tissues suggest the two proteins may directly interact during fibrosis and may be involved in mediating wound healing outcomes.

Our data in this study were in agreement with the reports that FGF-2 production is up-regulated during fibrosis and wound healing (Kurita et al. 1992; Floege et al. 1999; Strutz et al. 2000). FGF-2 is a potent mitogen of endothelial cells and fibroblasts, and has the ability to induce angiogenesis following tissue damage (Kardami et al. 2007). Moreover, FGF-2 acts as a survival factor in many models of cell and tissue injury (Bikfalvi et al. 1997). Suga et al. found that FGF-2 is released at the time of injury and reduces post-injury fibrogenesis (Suga et al. 2009). Administration of recombinant FGF-2 to skin wounds inhibits hypertrophy and widening of scars (Ono et al. 2007). Moreover, FGF-2 has been found to accelerate acute and chronic wound healing in both animal models and clinical use (Bikfalvi et al. 1997; Fu et al. 2000; Akita et al. 2008; Tan et al. 2008). In addition, healing of excisional skin wounds is delayed in FGF-2-knockout mice suggesting that FGF-2 is an essential component of wound healing and scar reduction (Ortega et al. 1998). Shi et al. demonstrated that FGF-2 promoted down-regulation of TGF- β 1 activity by inhibiting the SMAD2/SMAD3 signalling system of the TGF- β 1/SMAD-dependent pathway (Shi et al. 2013). Moreover, FGF-2 significantly reduced the expression of α -smooth muscle actin and increased apoptosis in granulation tissue cells (Eto et al. 2012; Shi et al. 2013). More recently, Kashpur et al demonstrated that FGF-2 activates signalling pathways that lead to upregulation of MMP1, the metalloproteinase responsible for cleaving collagen, and improving the healing process by reducing scar tissue formation (Kashpur et al. 2013). FGF-2 treatment also significantly reduces IL-6 levels, a cytokine that is known to increase production of collagen and excessive ECM production during fibrosis (Duncan and Berman 1991; Ray et al. 2013).

We have recently reported that LTBP-2 has a single binding site for FGF-2 and that it inhibits FGF-2 induced fibroblast proliferation *in vitro*. Moreover preliminary investigation of keloid tissue from a single patient showed strong co-localisation of the two proteins in the fibrotic matrix suggesting the two proteins may interact in tissues. The proteins gave a fibrous

distribution pattern which also co-stained for fibrillin-1 (Menz et al. 2015). We have previously shown that LTBP-2 has widespread association with fibrillin-1 and co-localizes with elastin-associated microfibrils during development in tissues such as aorta and elastic ligaments (Hirani et al. 2007). We have also demonstrated that exogenous LTBP-2 inhibits elastinogenesis in cultured ear cartilage chondrocyte indicating LTBP-2 may have a negative regulatory role during elastinogenesis (Sideek et al. 2014). The present study showed that in normal skin little LTBP-2 and FGF-2 were evident in the extracellular matrix. However both proteins were greatly upregulated in keloid tissue and consistently stained elastic fibres rather than elastin-free fibrillin-microfibrils in HTS and keloid (Amadeu et al. 2004; Sidgwick and Bayat 2012; Jumper et al. 2015). The results suggest that both LTBP-2 and FGF-2 are associated with the microfibril component of elastic fibres in these lesions. The findings are consistent with studies indicating that elastin is increased in fibrotic lesions whereas total fibrillin-1 (elastin-free plus elastin-associated) is reduced compared to normal skin. Fibrillin-1 gene mutations resulting in disrupted microfibril synthesis cause both Marfan syndrome and WMS and both phenotypes are believed to involve aberrant TGF- β signalling (Faivre et al. 2003; Haji-Seyed-Javadi et al. 2012). Interestingly, LTBP-2 mutations also cause WMS and Marfan-like traits have been observed in LTBP-2 null humans. Since LTBP-2 is the only component of elastic fibres known to bind FGF-2, upregulation of LTBP-2 in fibrotic disorders may influence the activity of FGF-2 in repair and disease processes (Ikeda et al. 2009). FGF-2 can influence TGF- β activity and depending on context, FGF-2 may activate signalling pathways leading to TGF- β elevation or it can inhibit TGF- β induced fibrosis (Phillips et al. 1997; Dhandapani et al. 2007; Shi et al. 2013). The LTBP-2 has also been reported as a pleiotropic tumour suppressor in nasopharyngeal carcinoma and as a marker for pulmonary deaths following acute dyspnea (Chan et al. 2011). A role for LTBP-2 -FGF-2 interactions in WMS and other diseases remains to be investigated.

In our previous study we found LTBP-2 blocked FGF-2-induced cell proliferation indicating LTBP-2 is a negative modulator of FGF-2 activity (Menz et al. 2015). Sinha et al. reported that LTBP-2 synthesis increased following injury in the arteries of porcine model suggesting role of LTBP-2 in tissue repair process (Sinha et al. 2002). Moreover, significant elevation of LTBP-2 has been observed in failing heart accompanied by obvious ECM remodelling and fibrosis, suggesting LTBP-2 may directly contribute to the regulation of cardiac ECM and be involved in fibrosis causing heart failure (Bai et al. 2012). The finding was consistent with our present studies where LTBP-2 is highly elevated in the matrix of HTS

and keloid tissues. Since FGF-2 exhibits repair/regenerative and anti-fibrotic effects, it is possible that elevated LTBP-2 exacerbates the fibrotic process by binding active FGF-2 in the repairing tissues, modulating FGF-2's contribution to wound repair. Following tissue injury, multiple FGF-2 molecules are released, by proteases and heparanases, complexed to individual heparan sulfate chains and these complexes bind with cell-surface FGF-receptors causing clustering of the FGFR molecules. This enhances intracellular signalling involving RAS/MAPK, PI3k/Akt, PLC- γ , and ERK1/2 pathways (Kardami et al. 2007; Yu et al. 2007; Schultz and Wysocki 2009). Interestingly, we have identified multiple high-affinity binding sites for heparin and HSPGs, syndecan-4 and perlecan on LTBP-2 (Parsi et al. 2010), including a site adjacent to the FGF-2 binding site (Menz et al. 2015). Thus it is possible that LTBP-2 may also attract and bind the heparin sulphate-bound FGF-2, affecting the anti-fibrotic effects of FGF-2-heparan sulphate complexes.

Recently a combined study using immunofluorescence and siRNA knockdown technology in cellular and animal models has identified multidrug resistance-associated protein 1 (MRP1) as a novel contributor to the fibrotic process in hypertrophic scar (Li et al 2015). A similar approach could be useful to define the mechanism of LTBP-2 and FGF-2 interactions in tissues and to determine the extent of their influence on wound healing and fibrosis.

In conclusion, this present study highlights consistent upregulation and co-localization of LTBP-2 and FGF-2 in fibrotic skin lesions from multiple patients. Since LTBP-2 strongly binds and inhibits FGF-2 activity *in vitro*, the present study suggests that LTBP-2 may bind and modulate FGF-2 effects in wound repair and contribute to fibrotic diseases.

4.6 References

- Aarabi S, Longaker MT, Gurtner GC (2007) Hypertrophic scar formation following burns and trauma: new approaches to treatment PLoS medicine 4:e234 doi:10.1371/journal.pmed.0040234
- Akhurst RJ, Hata A (2012) Targeting the TGFbeta signalling pathway in disease Nature reviews Drug discovery 11:790-811 doi:10.1038/nrd3810
- Akita S, Akino K, Imaizumi T, Hirano A (2008) Basic fibroblast growth factor accelerates and improves second-degree burn wound healing Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 16:635-641 doi:10.1111/j.1524-475X.2008.00414.x
- Ali M et al. (2009) Null mutations in LTBP2 cause primary congenital glaucoma Am J Hum Genet 84:664-671 doi:10.1016/j.ajhg.2009.03.017
- Amadeu TP, Braune AS, Porto LC, Desmouliere A, Costa AM (2004) Fibrillin-1 and elastin are differentially expressed in hypertrophic scars and keloids Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 12:169-174 doi:10.1111/j.1067-1927.2004.012209.x
- Bai Y, Zhang P, Zhang X, Huang J, Hu S, Wei Y (2012) LTBP-2 acts as a novel marker in human heart failure - a preliminary study Biomarkers : biochemical indicators of exposure, response, and susceptibility to chemicals 17:407-415 doi:10.3109/1354750X.2012.677860
- Bayat A, McGrouther DA, Ferguson MW (2003) Skin scarring BMJ (Clinical research ed) 326:88-92
- Bikfalvi A, Klein S, Pintucci G, Rifkin DB (1997) Biological roles of fibroblast growth factor-2 Endocr Rev 18:26-45 doi:10.1210/edrv.18.1.0292
- Bock O, Schmid-Ott G, Malewski P, Mrowietz U (2006) Quality of life of patients with keloid and hypertrophic scarring Archives of dermatological research 297:433-438 doi:10.1007/s00403-006-0651-7
- Brown KK, Wells AU (2008) Recent clinical trials in idiopathic pulmonary fibrosis and the BUILD-1 study European Respiratory Review 17:116-122 doi:10.1183/09059180.00010903

- Chan SH et al. (2011) The ECM protein LTBP-2 is a suppressor of esophageal squamous cell carcinoma tumor formation but higher tumor expression associates with poor patient outcome *Int J Cancer* 129:565-573 doi:10.1002/ijc.25698
- Chua CC, Rahimi N, Forsten-Williams K, Nugent MA (2004) Heparan sulfate proteoglycans function as receptors for fibroblast growth factor-2 activation of extracellular signal-regulated kinases 1 and 2 *Circulation research* 94:316-323 doi:10.1161/01.RES.0000112965.70691.AC
- Daian T et al. (2003) Insulin-Like Growth Factor-I Enhances Transforming Growth Factor-[beta]-Induced Extracellular Matrix Protein Production Through the P38/Activating Transcription Factor-2 Signaling Pathway in Keloid Fibroblasts *J Investig Dermatol* 120:956-962
- de Vega S, Iwamoto T, Yamada Y (2009) Fibulins: multiple roles in matrix structures and tissue functions *Cell Mol Life Sci* 66:1890-1902 doi:10.1007/s00018-009-8632-6
- Dhandapani KM, Khan MM, Wade FM, Wakade C, Mahesh VB, Brann DW (2007) Induction of transforming growth factor-beta1 by basic fibroblast growth factor in rat C6 glioma cells and astrocytes is mediated by MEK/ERK signaling and AP-1 activation *J Neurosci Res* 85:1033-1045 doi:10.1002/jnr.21182
- Duncan MR, Berman B (1991) Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6 *The Journal of investigative dermatology* 97:686-692
- Eto H et al. (2012) Therapeutic potential of fibroblast growth factor-2 for hypertrophic scars: upregulation of MMP-1 and HGF expression *Lab Invest* 92:214-223 doi:10.1038/labinvest.2011.127
- Faivre L et al. (2003) In frame fibrillin-1 gene deletion in autosomal dominant Weill-Marchesani syndrome *Journal of medical genetics* 40:34-36
- Floege J, Hudkins KL, Eitner F, Cui Y, Morrison RS, Schelling MA, Alpers CE (1999) Localization of fibroblast growth factor-2 (basic FGF) and FGF receptor-1 in adult human kidney1 *Kidney international* 56:883-897
- Fu X, Shen Z, Chen Y, Xie J, Guo Z, Zhang M, Sheng Z (2000) Recombinant bovine basic fibroblast growth factor accelerates wound healing in patients with burns, donor sites and chronic dermal ulcers *Chinese medical journal* 113:367-371
- Gibson MA, Hatzinikolas G, Davis EC, Baker E, Sutherland GR, Mecham RP (1995) Bovine latent transforming growth factor beta 1-binding protein 2: molecular cloning,

- identification of tissue isoforms, and immunolocalization to elastin-associated microfibrils *Mol Cell Biol* 15:6932-6942
- Goodwin A, Jenkins G (2009) Role of integrin-mediated TGFbeta activation in the pathogenesis of pulmonary fibrosis *Biochemical Society transactions* 37:849-854 doi:10.1042/BST0370849
- Haji-Seyed-Javadi R et al. (2012) LTBP2 mutations cause Weill-Marchesani and Weill-Marchesani-like syndrome and affect disruptions in the extracellular matrix *Hum Mutat* 33:1182-1187 doi:10.1002/humu.22105
- Hirani R, Hanssen E, Gibson MA (2007) LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein *Matrix biology : journal of the International Society for Matrix Biology* 26:213-223 doi:10.1016/j.matbio.2006.12.006
- Ikeda M et al. (2009) Elastic fiber assembly is disrupted by excessive accumulation of chondroitin sulfate in the human dermal fibrotic disease, keloid *Biochem Biophys Res Commun* 390:1221-1228 doi:10.1016/j.bbrc.2009.10.125
- Jumper N, Paus R, Bayat A (2015) Functional histopathology of keloid disease *Histology and histopathology* 30:1033-1057 doi:10.14670/HH-11-624
- Kardami E, Detillieux K, Ma X, Jiang Z, Santiago JJ, Jimenez SK, Cattini PA (2007) Fibroblast growth factor-2 and cardioprotection *Heart failure reviews* 12:267-277 doi:10.1007/s10741-007-9027-0
- Kashpur O, LaPointe D, Ambady S, Ryder EF, Dominko T (2013) FGF2-induced effects on transcriptome associated with regeneration competence in adult human fibroblasts *BMC genomics* 14:656 doi:10.1186/1471-2164-14-656
- Kissin EY, Korn JH (2003) Fibrosis in scleroderma *Rheum Dis Clin North Am* 29:351-369
- Kopecki Z et al. (2013) Topically applied flightless I neutralizing antibodies improve healing of blistered skin in a murine model of epidermolysis bullosa acquisita *J Invest Dermatol* 133:1008-1016 doi:10.1038/jid.2012.457
- Krenning G, Zeisberg EM, Kalluri R (2010) The origin of fibroblasts and mechanism of cardiac fibrosis *Journal of cellular physiology* 225:631-637 doi:10.1002/jcp.22322
- Kurita Y, Tsuboi R, Ueki R, Rifkin DB, Ogawa H (1992) Immunohistochemical localization of basic fibroblast growth factor in wound healing sites of mouse skin *Archives of dermatological research* 284:193-197

- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method *Methods* (San Diego, Calif) 25:402-408 doi:10.1006/meth.2001.1262
- Marneros AG, Norris JE, Watanabe S, Reichenberger E, Olsen BR (2004) Genome scans provide evidence for keloid susceptibility loci on chromosomes 2q23 and 7p11 *The Journal of investigative dermatology* 122:1126-1132 doi:10.1111/j.0022-202X.2004.22327.x
- Meng XM, Tang PM, Li J, Lan HY (2015) TGF-beta/Smad signaling in renal fibrosis *Frontiers in physiology* 6:82 doi:10.3389/fphys.2015.00082
- Menz C, Parsi MK, Adams JR, Sideek MA, Kopecki Z, Cowin AJ, Gibson MA (2015) LTBP-2 Has a Single High-Affinity Binding Site for FGF-2 and Blocks FGF-2-Induced Cell Proliferation *PloS one* 10:e0135577 doi:10.1371/journal.pone.0135577
- Moren A et al. (1994) Identification and characterization of LTBP-2, a novel latent transforming growth factor-beta-binding protein *J Biol Chem* 269:32469-32478
- Nakashima M et al. (2010) A genome-wide association study identifies four susceptibility loci for keloid in the Japanese population *Nature genetics* 42:768-771 doi:10.1038/ng.645
- Ono I, Akasaka Y, Kikuchi R, Sakemoto A, Kamiya T, Yamashita T, Jimbow K (2007) Basic fibroblast growth factor reduces scar formation in acute incisional wounds *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 15:617-623 doi:10.1111/j.1524-475X.2007.00293.x
- Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C (1998) Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2 *Proceedings of the National Academy of Sciences of the United States of America* 95:5672-5677
- Parsi MK, Adams JR, Whitelock J, Gibson MA (2010) LTBP-2 has multiple heparin/heparan sulfate binding sites *Matrix biology : journal of the International Society for Matrix Biology* 29:393-401 doi:10.1016/j.matbio.2010.03.005
- Phillips AO, Topley N, Morrissey K, Williams JD, Steadman R (1997) Basic fibroblast growth factor stimulates the release of preformed transforming growth factor beta 1 from human proximal tubular cells in the absence of de novo gene transcription or mRNA translation *Laboratory investigation; a journal of technical methods and pathology* 76:591-600
- Pohlers D et al. (2009) TGF-beta and fibrosis in different organs - molecular pathway imprints *Biochim Biophys Acta* 1792:746-756 doi:S0925-4439(09)00135-5 [pii] 10.1016/j.bbadis.2009.06.004

- Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G (2006) Incidence and prevalence of idiopathic pulmonary fibrosis *American journal of respiratory and critical care medicine* 174:810-816 doi:10.1164/rccm.200602-163OC
- Rastegar A, Kashgarian M (1998) The clinical spectrum of tubulointerstitial nephritis *Kidney international* 54:313-327 doi:10.1046/j.1523-1755.1998.00001.x
- Ray S, Ju X, Sun H, Finnerty CC, Herndon DN, Brasier AR (2013) The IL-6 trans-signaling-STAT3 pathway mediates ECM and cellular proliferation in fibroblasts from hypertrophic scar *The Journal of investigative dermatology* 133:1212-1220 doi:10.1038/jid.2012.499
- Saharinen J, Keski-Oja J (2000) Specific sequence motif of 8-Cys repeats of TGF-beta binding proteins, LTBP2, creates a hydrophobic interaction surface for binding of small latent TGF-beta *Mol Biol Cell* 11:2691-2704
- Schultz GS, Wysocki A (2009) Interactions between extracellular matrix and growth factors in wound healing *Wound Repair Regen* 17:153-162 doi:WRR466 [pii] 10.1111/j.1524-475X.2009.00466.x
- Shi HX et al. (2013) The anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo *PLoS One* 8:e59966 doi:10.1371/journal.pone.0059966
- Shi Y, Massague J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus *Cell* 113:685-700
- Shih B, Bayat A (2010) Genetics of keloid scarring *Archives of dermatological research* 302:319-339 doi:10.1007/s00403-009-1014-y
- Sideek MA, Menz C, Parsi MK, Gibson MA (2014) LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis *Matrix biology : journal of the International Society for Matrix Biology* 34:114-123 doi:10.1016/j.matbio.2013.10.007
- Sidgwick GP, Bayat A (2012) Extracellular matrix molecules implicated in hypertrophic and keloid scarring *Journal of the European Academy of Dermatology and Venereology : JEADV* 26:141-152 doi:10.1111/j.1468-3083.2011.04200.x
- Sinha S, Heagerty AM, Shuttleworth CA, Kielty CM (2002) Expression of latent TGF-beta binding proteins and association with TGF-beta 1 and fibrillin-1 following arterial injury *Cardiovascular research* 53:971-983
- Strutz F, Neilson EG (2003) New insights into mechanisms of fibrosis in immune renal injury *Springer Semin Immunopathol* 24:459-476 doi:10.1007/s00281-003-0123-5

- Strutz F, Zeisberg M, Hemmerlein B, Sattler B, Hummel K, Becker V, Muller GA (2000) Basic fibroblast growth factor expression is increased in human renal fibrogenesis and may mediate autocrine fibroblast proliferation *Kidney international* 57:1521-1538
- Suga H et al. (2009) IFATS collection: Fibroblast growth factor-2-induced hepatocyte growth factor secretion by adipose-derived stromal cells inhibits postinjury fibrogenesis through a c-Jun N-terminal kinase-dependent mechanism *Stem cells (Dayton, Ohio)* 27:238-249 doi:10.1634/stemcells.2008-0261
- Tan Y et al. (2008) Comparison of the Therapeutic Effects Recombinant Human Acidic and Basic Fibroblast Growth Factors in Wound Healing in Diabetic Patients *Journal of Health Science* 54:432-440 doi:10.1248/jhs.54.432
- Thannickal VJ, Zhou Y, Gaggar A, Duncan SR (2014) Fibrosis: ultimate and proximate causes *J Clin Invest* 124:4673-4677 doi:10.1172/JCI74368
- Verhaegen PD, van Zuijlen PP, Pennings NM, van Marle J, Niessen FB, van der Horst CM, Middelkoop E (2009) Differences in collagen architecture between keloid, hypertrophic scar, normotrophic scar, and normal skin: An objective histopathological analysis *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 17:649-656 doi:10.1111/j.1524-475X.2009.00533.x
- Wynn TA (2004) Fibrotic disease and the T(H)1/T(H)2 paradigm *Nature reviews Immunology* 4:583-594 doi:10.1038/nri1412
- Wynn TA (2007) Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases *J Clin Invest* 117:524-529 doi:10.1172/JCI31487
- Wynn TA (2008) Cellular and molecular mechanisms of fibrosis *J Pathol* 214:199-210 doi:10.1002/path.2277
- Xie JL et al. (2008) Basic fibroblast growth factor (bFGF) alleviates the scar of the rabbit ear model in wound healing *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 16:576-581 doi:10.1111/j.1524-475X.2008.00405.x
- Yu PJ, Ferrari G, Galloway AC, Mignatti P, Pintucci G (2007) Basic fibroblast growth factor (FGF-2): the high molecular weight forms come of age *J Cell Biochem* 100:1100-1108 doi:10.1002/jcb.21116

4.7 Figures

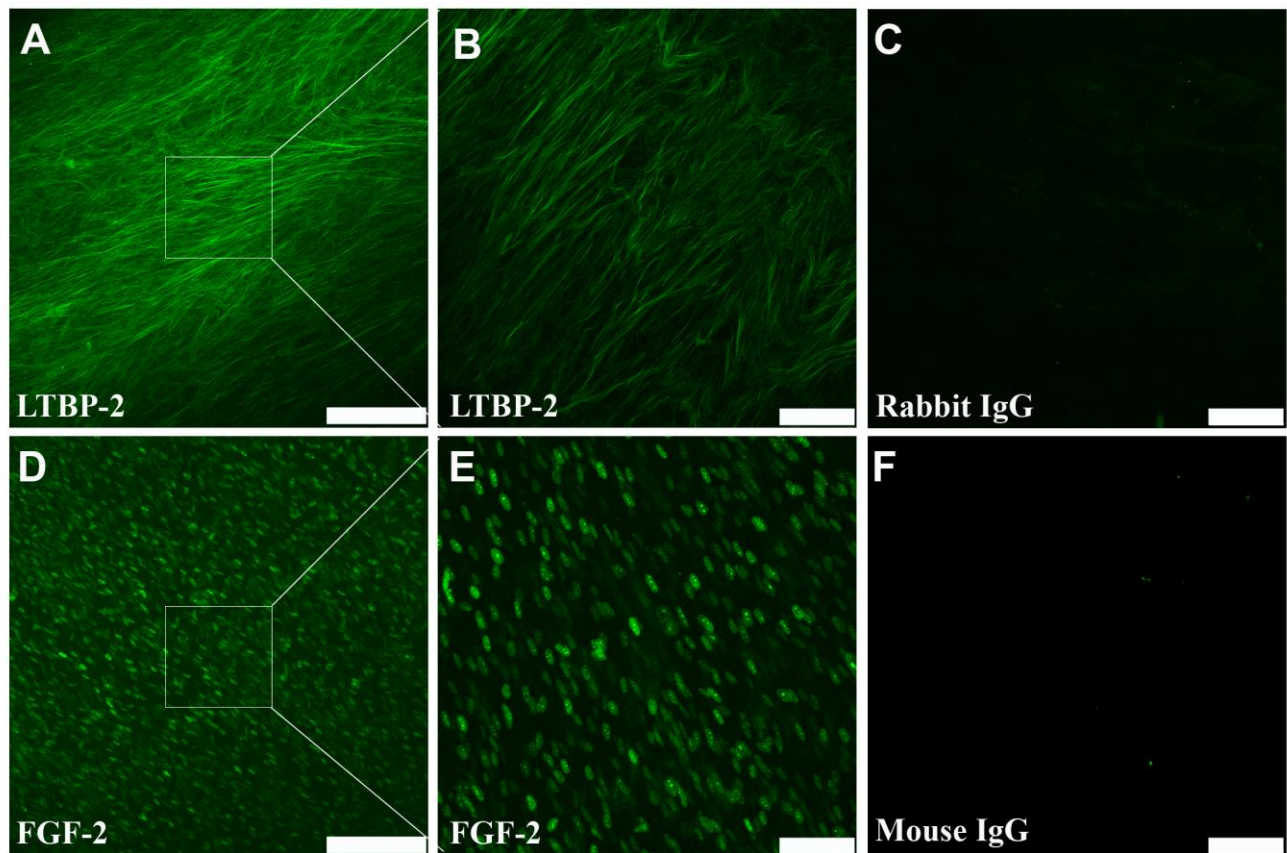


Figure 4.1 Immunofluorescence staining for LTBP-2 and FGF-2 in human foreskin fibroblast (HFF) cultures

HFFs were cultured for 21 days and the cell layer was fixed then incubated with antibodies to LTBP-2 or FGF-2 or with control IgG. Primary antibody was detected using an appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC) prior to analysis on the Leica SP5 spectral scanning confocal microscope (see methods section). **A and B**, anti-[human LTBP-2 peptide] antibody 3504 (2.08 $\mu\text{g/ml}$) **D and E**, anti-[human FGF-2] antibody (2.5 $\mu\text{g/ml}$) **C**, rabbit IgG control (2.08 $\mu\text{g/ml}$); **F**, mouse IgG control (2.5 $\mu\text{g/ml}$). Magnification: A and D, Bar = 200 μM ; B, C, E and F, Bar = 100 μM .

Figure 4.2 LTBP-2 and FGF-2 co-localize in fibrotic skin lesions

Tissue sections from normal adult skin (**A-E**), mature scar (**F-J**), HTS (**K-P**) and keloid (**Q-V**), were incubated with antibodies to LTBP-2 and FGF-2 and analysed by confocal microscopy as described in Methods. Tissue from 4 patients in each group was examined and a representative example from each group is shown. Normal skin; cranial skin, male 6 y.o.; Normal scar, abdominal skin (surgery) male 1 y.o.; HTS; hand (burn injury) female 27 y.o.; keloid; ear lobe (ear piercing), male 18 y.o.

A, F, K, and Q, polyclonal anti-[human LTBP-2 peptide] antibody 3504 (2.08 µg/ml) detected with anti-rabbit IgG antibody conjugated to fluor Alexa 488; **B, G, L and R**, monoclonal anti-[human FGF-2] antibody (2.5 µg/ml) detected with anti-mouse IgG antibody conjugated to Alexa 594; **C**, A and B merged; **H**, F and G merged; **M**, K, L and N merged; **S**, Q, R and T merged; **D, I, O and U**, rabbit IgG control (2.08 µg/ml); **E, J, P and V**, mouse IgG control (2.5 µg/ml). **N and T**, DAPI (1 ng/µl). Magnification: Bar = 25 µM.

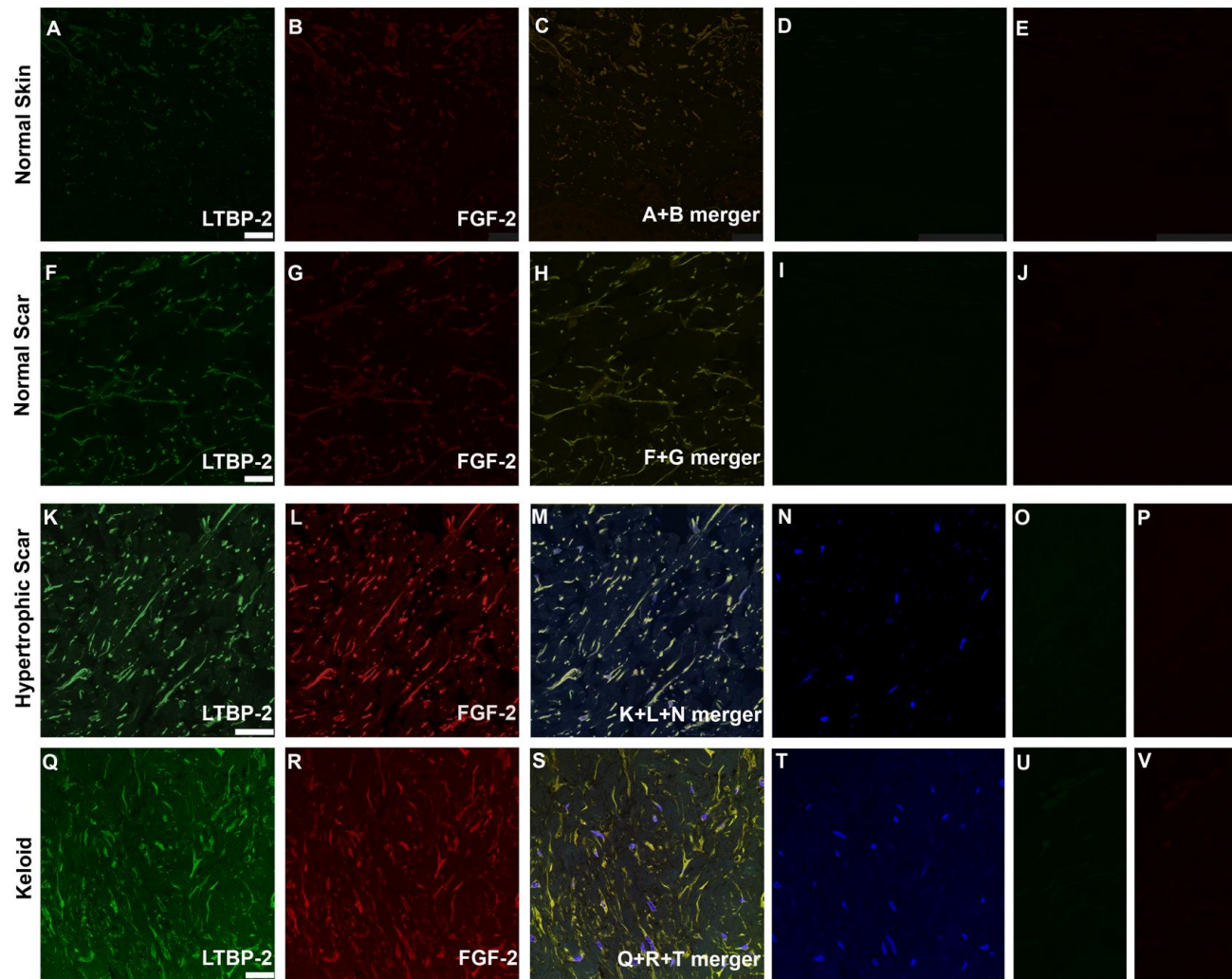


Figure 4.2 LTBP-2 and FGF-2 co-localize in fibrotic skin lesions

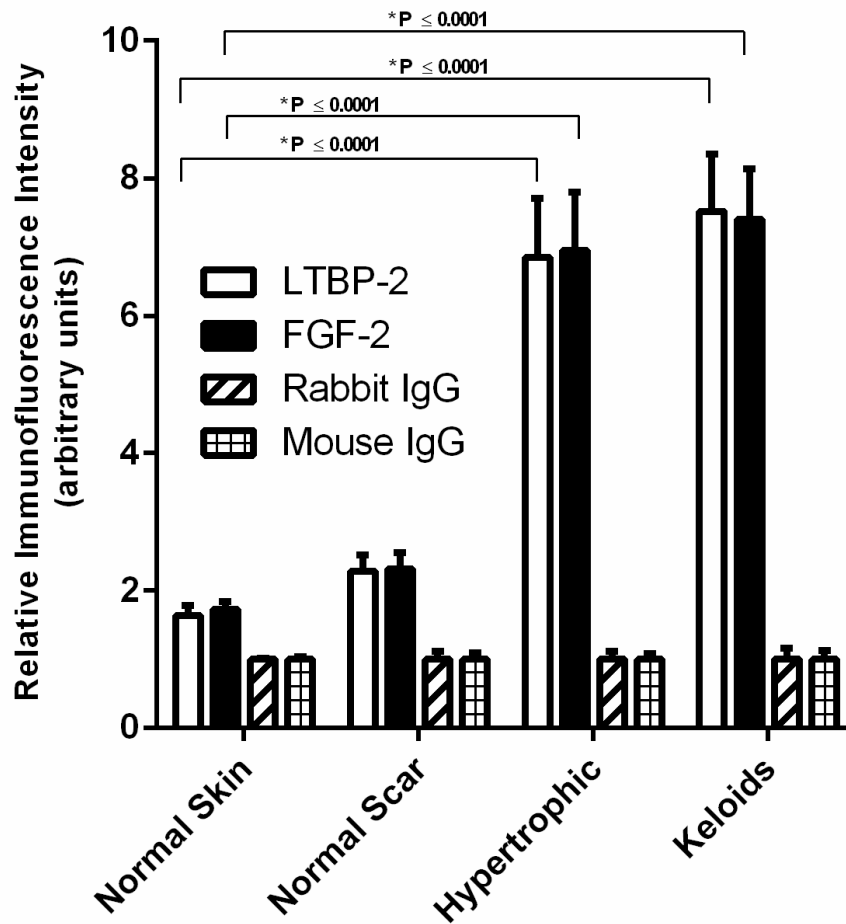


Figure 4.3 Quantitative immunofluorescence analysis of LTBP-2 and FGF-2 in normal skin, mature scar, hypertrophic scar and keloid tissue.

The relative fluorescence intensities were quantitated using the Analysis Software package (Soft-Imaging System, Munster, Germany) sampled from 3 random areas (each 0.038 mm²) per section from each of 4 patients per group. LTBP-2 (white columns), FGF-2 (black columns), rabbit IgG control (cross-hatched) and mouse IgG controls (grid) in sections of normal skin, normal scar, HTS and keloids. Values expressed relative to the background signal (=1 unit). Mean values ± S.D of 12 determinations are shown.

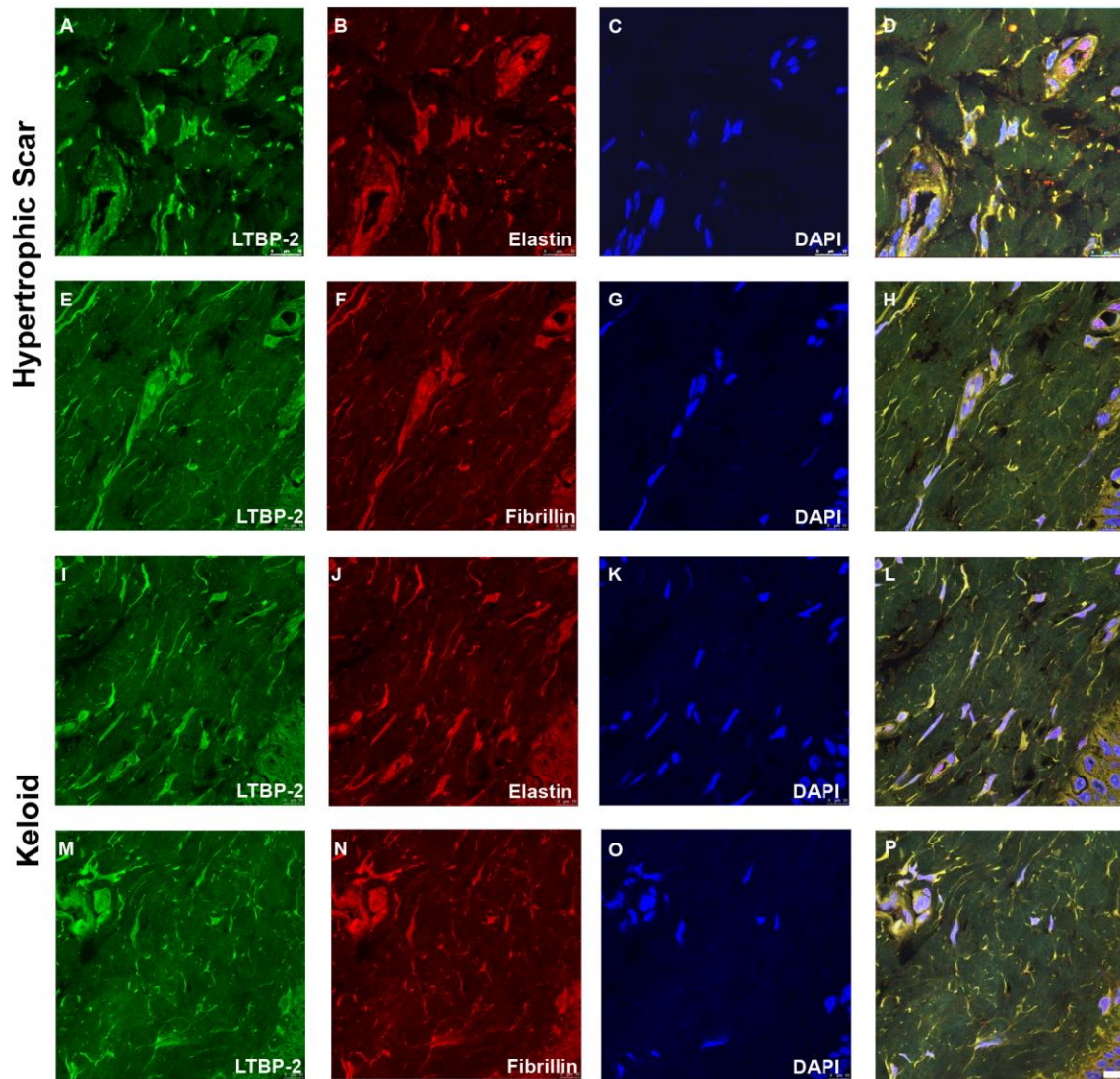


Figure 4.4 LTBP-2 co-localizes with fibrillin-1 and elastin in fibrotic skin

To identify the fibrous material immunostaining for LTBP-2 and FGF-2 in Fig 4.2, HTS (**A-H**) and keloid (**I-P**) tissues were also co-stained with LTBP-2 for fibrillin-1 and elastin by confocal microscopy. **A, E, I and M**; Anti-LTBP-2 antibody 3504 (2.08 $\mu\text{g/ml}$) detected with anti-rabbit IgG antibody conjugated to fluor Alexa 488; **B and J**, monoclonal anti-elastin antibody BA4 (0.96 $\mu\text{g/ml}$) detected with anti-mouse IgG antibody conjugated to Alexa 594; **F and N**, monoclonal anti-[fibrillin-1] antibody detected with anti-mouse IgG antibody conjugated to Alexa 594; **C, G, K and O**, DAPI nuclear stain. **D, A, B and C merged**; **H, E, F and G merged**; **L, I, J and K merged**, **P, M, N and O merged**. Magnification: Bar = 10 μM .

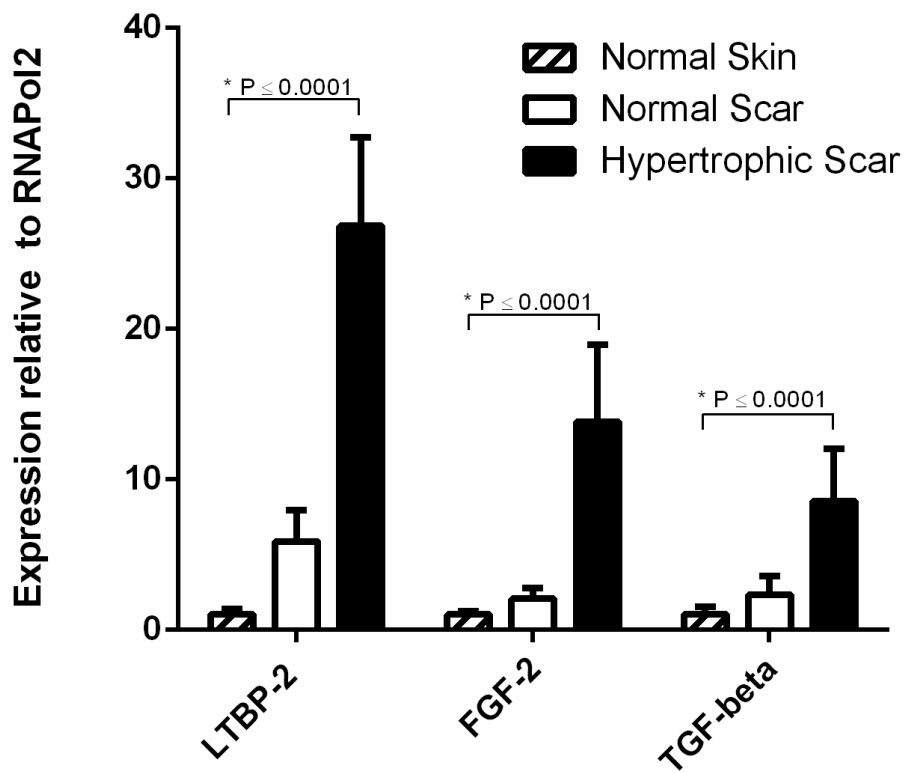


Figure 4.5 The expression levels of mRNA for LTBP-2, FGF-2 and TGF- β in fibrotic skin tissues

Total RNA was extracted from snap-frozen normal skin (cross-hatched), mature scars (white columns) and HTS (black columns) from 4 patients in each category, then reverse transcribed into cDNA for quantitation by qPCR as described in Methods. The LTBP-2, FGF-2 and TGF- β signals were normalised to the RNAPolIII control signal = 1. Mean values \pm S.D. of 12 determinations are shown.

4.8 Supplementary figures

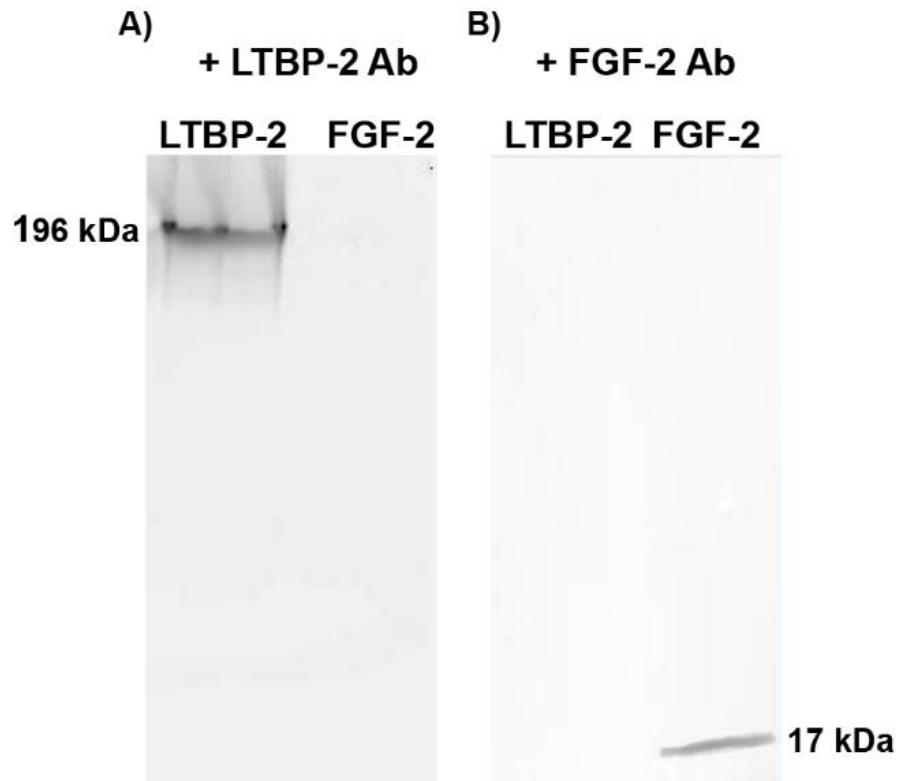


Figure 4.6 Specificity of LTBP-2 and FGF-2 antibodies.

Full length rLTBP-2 protein (1 $\mu\text{g}/\text{well}$) and rFGF-2 protein (1 $\mu\text{g}/\text{well}$) were analysed by 12 % SDS-PAGE and immunoblotting with rabbit anti-[LTBP-2 peptide] antibody 3504 (panel A) or mouse anti-[FGF-2] antibody (panel B). Bands were visualised with the anti-rabbit IgG or anti-mouse IgG antibodies conjugated with IR800 fluorescence dye using the LI-COR Odyssey Infrared Imaging System.

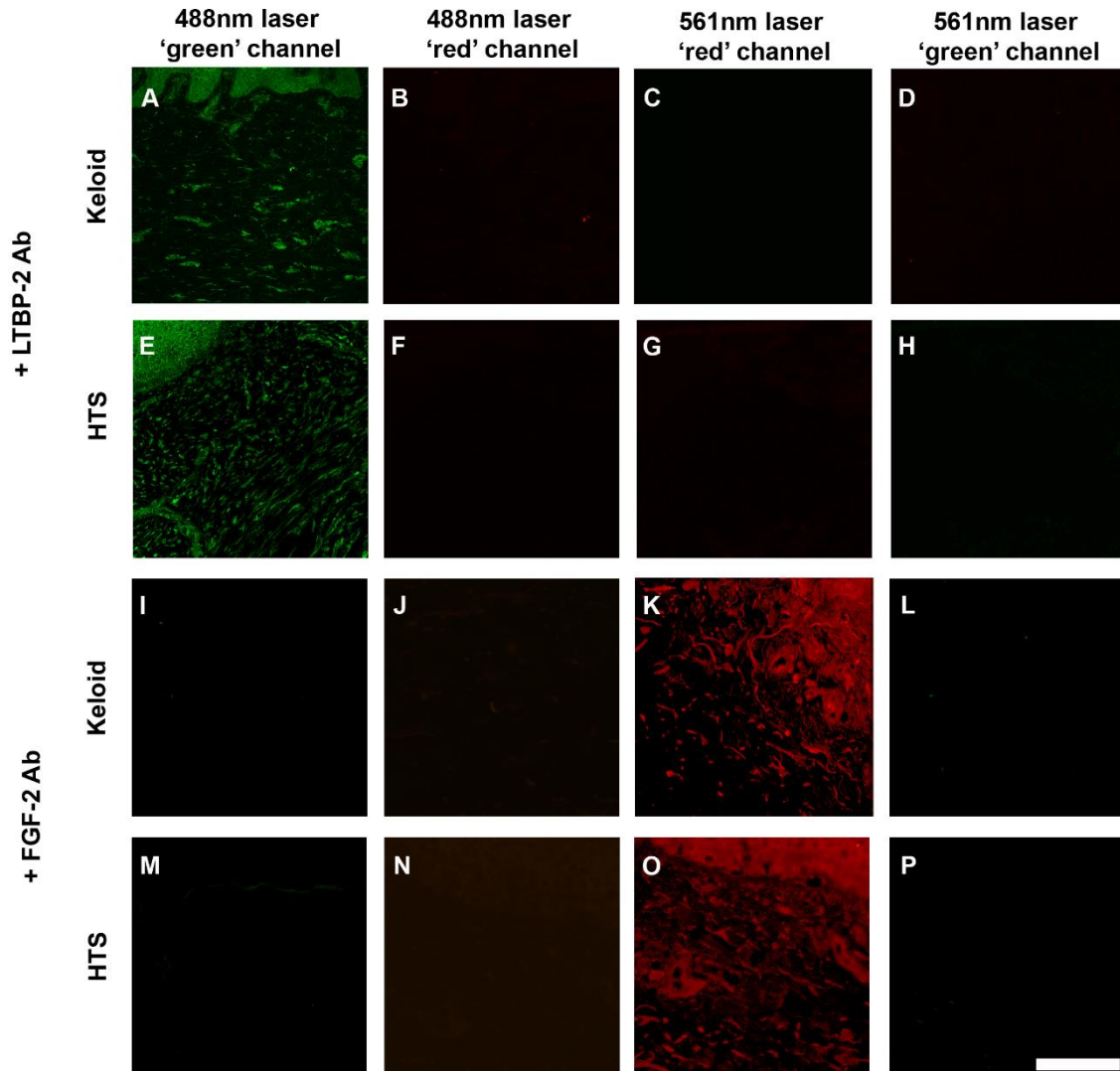


Figure 4.7 Specificity of secondary antibodies and laser channels.

Keloid tissues (A – D, I – L) and HTS tissues (E – H, M – P) was prepared and analysed by confocal microscopy as described in the methods section. The sections were treated with rabbit anti-[human LTBP-2 peptide] antibody 3504 (2 $\mu\text{g/ml}$) (A – H) or mouse anti-[human FGF-2] antibody (2.5 $\mu\text{g/ml}$) (I – P). All sections were then treated with a mixture of anti-rabbit IgG antibody conjugated to fluor Alexa 488 and anti-mouse IgG antibody conjugate to Alexa 594. After washing, the specificity of each secondary antibody was demonstrated by excitation in sequence with the 488nm and 561nm lasers and fluorescence measurement in both the 488 and 594 channels at each stage. Note that for both primary antibodies only specific fluorescence was detected with no bleed through between channels. Magnification, Bar = 50 μm .

CHAPTER 5: LTBP-2 stimulates the expression of TGF- β in human fibroblasts via Akt & p38 MAPK signalling pathways

Mohamed A. Sideek^{a,b}, Josh Smith^a, Clementine Menz^a, Julian R. J. Adams^a, Allison J. Cowin^c
and Mark A. Gibson^{a*}

^a Discipline of Anatomy and Pathology, School of Medicine, University of Adelaide, Adelaide,
South Australia 5005, Australia

^b Department of Biomedical Sciences, Kulliyah of Allied Health Sciences, International Islamic
University Malaysia, 25200 Kuantan, Pahang, Malaysia

^c Regenerative Medicine, Mawson Institute, University of South Australia, Adelaide, South
Australia 5095, Australia

* corresponding author. TEL: +61 88313 5337 Email address: mark.gibson@adelaide.edu.au

Key words

LTBP-2, TGF- β , fibroblast, p38, Akt, fibrosis

Statement of Authorship

Title of Paper	LTBP-2 stimulates the expression of TGF- β in human fibroblasts via Akt & p38 MAPK signalling pathways
Publication Status	<input type="checkbox"/> Published <input type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input checked="" type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
Publication Details	Mohamed A. Sideek, Josh Smith, Julian R. J. Adams, Allison J. Cowin, Mark A. Gibson

Principal Author

Name of Principal Author (Candidate)	Mohamed Arshad Sideek		
Contribution to the Paper	Performed most of the experiments. Performed all data analyses including ELISA assays, qPCR, and western blot. Interpreted data. Wrote manuscript.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/11/2015

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Josh Smith		
Contribution to the Paper	Performed TGF- β stimulation assays (Figure 5.1 and 5.2) Manuscript evaluation and feedback.		
Signature		Date	2/12/2015

Name of Co-Author	Clementine Menz		
Contribution to the Paper	Involved in recombinant fragment production Manuscript evaluation and feedback.		
Signature		Date	29/11/2015

Name of Co-Author	Julian R.J Adams		
Contribution to the Paper	Supervised development of the work. Manuscript evaluation and feedback.		
Signature		Date	29/11/2015

Name of Co-Author	Prof. Allison J. Cowin		
Contribution to the Paper	Supervised the development of work and data interpretation. Manuscript evaluation and feedback.		
Signature		Date	01/04/2016

Name of Co-Author	Dr. Mark A. Gibson		
Contribution to the Paper	Principal supervisor of candidate's PhD Supervise development and planning of the research Involved in data interpretation and manuscript evaluation		
Signature		Date	26/11/2015

LTBP-2 stimulates the expression of TGF- β in human fibroblasts via Akt & p38 MAPK signalling pathways

Mohamed A. Sideek^{a,b}, Josh Smith^a, Clementine Menz^a, Julian R. J. Adams^a, Allison J. Cowin^c and Mark A. Gibson^{a*}

^a Discipline of Anatomy and Pathology, School of Medicine, University of Adelaide, Adelaide, South Australia 5005, Australia

^b Department of Biomedical Sciences, Kulliyah of Allied Health Sciences, International Islamic University Malaysia, 25200 Kuantan, Pahang, Malaysia

^c Regenerative Medicine, Mawson Institute, University of South Australia, Adelaide, South Australia 5095, Australia

* corresponding author. TEL: +61 88313 5337 Email address: mark.gibson@adelaide.edu.au

Key words

LTBP-2, TGF- β , fibroblast, p38, Akt, fibrosis

5.1 Abstract

Latent transforming growth factor- β -1 binding protein-2 (LTBP-2) belongs to the LTBP-fibrillin superfamily of extracellular proteins. Unlike other LTBPs, LTBP-2 does not covalently bind TGF- β but LTBP-2 appears to be implicated in the regulation of TGF- β expression, secretion and activation, although the mechanisms are largely unknown. By using cell culture models, we found that addition of exogenous LTBP-2 to the medium of cultured human MSU 1.1 fibroblasts led to an increase in TGF- β levels in the conditioned medium and the secreted TGF- β was mainly in inactive form. The TGF- β increase was found to be via newly synthesised proteins produced by the cells rather than displacement of matrix-stored TGF- β . Time-course experiment showed the TGF- β concentration peaked at around 15 h. Using a series of recombinant LTBP-2 fragments, we have identified a central region of LTBP-2 consisting of an 8-cys motif flanked by pairs of EGF-like repeats (fragment LTBP-2C F3) responsible for the TGF- β stimulating activity. LTBP-2 stimulated the phosphorylation of Akt and p38 signalling proteins and specific inhibitors of activation of each protein blocked the expression of TGF- β . The search for the cell surface receptor for this LTBP-2 activity proved inconclusive. Inhibitory antibodies to β 1 integrin had no effect on LTBP-2 stimulation of TGF- β , but TGF- β upregulation was partially inhibited by anti- α V β 3 integrin antibodies suggestive of some role for this integrin in this process. Overall the study indicates that LTBP-2 can regulate TGF- β cellular expression and secretion and thus LTBP-2 may represent a novel therapeutic target for attenuation of TGF- β -induced pathologies.

5.2 Introduction

Transforming growth factor- β (TGF- β) is a multifunctional cytokine that exists as three mammalian isoforms (TGF- β 1, TGF- β 2 and TGF- β 3) and regulates numerous developmental and physiological processes, including cell growth, differentiation, adhesion, apoptosis, and formation and proteolytic degradation of the extracellular matrix (Annes et al., 2003; Clark and Coker, 1998; Roberts, 1998). The effect of TGF- β varies between tissues since TGF- β can act as a growth inhibitor in some cells, for example keratinocytes and lung epithelial cells, but can also act as a growth promoting factor in other cells, such as smooth muscle cells, mesodermal cells and fibroblasts (Branton and Kopp, 1999; Clark et al., 1997; Guo and Chen, 2012; Tatler and Jenkins, 2012). However, when this cytokine is over expressed it enhances pro-fibrotic characteristics including excess ECM component production, transdifferentiation of fibroblast into myofibroblast, and inhibition of apoptosis contributing to fibrotic diseases such as pulmonary fibrosis, liver cirrhosis, and keloid skin (Goodwin and Jenkins, 2009; Pohlers et al., 2009; Ruiz-Ortega et al., 2007). Moreover, disturbance of TGF- β pathways are also associated with numerous other pathological states, including tumor cell growth, cancer, and autoimmune diseases, indicative of a critical role for TGF- β in normal cellular function (Derynck et al., 2001; Gold, 1999). Knockout mice for all three TGF β isoforms die shortly after birth due to developmental abnormalities and excessive inflammatory response in the heart and lungs (Bottinger et al., 1997).

TGF- β is synthesized within cells as a latent form, noncovalently bound to its propeptide called latency-associated peptide (LAP). Dimers of the mature TGF- β and LAP form the small latent complex (SLC), which can bind covalently to LTBPs, forming the large latent complex (LLC) (Miyazono and Heldin, 1991). The LLC subsequently targets TGF- β to fibrillin-rich microfibrils in the extracellular matrix, thereby controlling the storage and activation of the growth factor (Rifkin, 2005). The LTBP/fibrillin superfamily consists of LTBPs 1-4 and fibrillins 1 and 2. The LTBPs share similar structural features with fibrillins since both proteins consist predominantly of tandem cbEGF-like 6-cysteine repeats interspersed with characteristic 8-cysteine (8-Cys) motifs which occur only within the LTBP/fibrillin superfamily (Rifkin, 2005; Sinha et al., 2002). In contrast to other LTBPs, LTBP-2 is unable to associate and bind covalently to latent TGF- β (Gibson et al., 1995; Saharinen and Keski-Oja, 2000). Similar to the 8-cys motifs of

fibrillins, the third 8-Cys domain of LTBP-2 lacks the required latent TGF- β binding consensus sequence (Gibson et al., 1995; Saharinen and Keski-Oja, 2000). Currently, the function of LTBP-2 is poorly understood although *in situ* hybridisation and immunohistochemical studies have shown LTBP-2 is expressed abundantly in tissues such as lung, heart, aorta, placenta, liver and skeletal muscle (Hirani et al., 2007; Moren et al., 1994). For some years, LTBP-2 null mice were reported to die during embryo implantation and this hampered progress in our understanding of LTBP-2 biology (Shipley et al., 2000). However, a LTBP-2 null mouse strain has recently been reported that survives to adulthood with lens luxation caused by ciliary zonule defects from abnormal formation of fibrillin microfibril bundles which are a major component this tissue (Inoue et al., 2014). The phenotype is consistent with LTBP-2 gene mutations in humans which cause mild phenotypes mainly involving the ocular system (Ali et al., 2009; Desir et al., 2010). Studies from our laboratory have demonstrated that LTBP-2 can negatively regulate elastinogenesis in cell culture by inhibiting tropoelastin interaction with fibulin-5 and heparan sulfate proteoglycan (Sideek et al., 2014). In addition we have identified several binding sites for heparin/heparan sulfate in LTBP-2, and have shown that LTBP-2 partially co-localizes with HSPGs perlecan and syndecan-4 in some tissues (Parsi et al., 2010). LTBP-2 also strongly binds FGF-2 and blocks its activity in vitro (Menz et al., 2015). Both proteins are upregulated and co-localise to fibrillin microfibrils in fibrotic skin lesions hypertrophic scar and keloid suggesting LTBP-2 may modulate FGF-2 activity in fibrosis (Menz et al., 2015; Sideek et al., 2015).

Although LTBP-2 does not bind latent TGF- β , multiple recent studies indicate that LTBP-2 may be involved in regulation of latent TGF- β storage and activation in the ECM. In a rat model, treatment of astrocytes with antisense LTBP-2 oligonucleotides resulted in downregulation of both LTBP-2 mRNA expression and TGF- β activity, strongly suggesting LTBP-2 may regulate TGF- β activation (Krohn, 1999). Hirani et al. reported that LTBP-2 specifically binds to fibrillin-1 rather than fibrillin-2 and that a C-terminal fragment of LTBP-2 blocks the interaction of LTBP-1 with fibrillin-1 in vitro (Hirani et al., 2007). These findings lead to the hypothesis that LTBP-2 might indirectly regulate TGF- β bioavailability by releasing LTBP-1 from microfibrils through competitive binding for fibrillin-1. More recently, LTBP-2 gene mutations have been linked to Weill-Marchesani syndrome (WMS) which is characterized by ocular and skeletal deformities and thick fibrotic skin, (Haji-Seyed-Javadi et al., 2012). The skin from patient with WMS had disrupted

and unorganized fibrillin-1 microfibrillar networks compared to normal individuals. Fibrillin-1 gene mutations also cause WMS (Cain et al., 2012) indicating that the disease mechanism involves both proteins. Fibrillin-1 modulation of TGF- β storage and activation is well documented (Annes, 2003; Dallas et al., 2000; Massam-Wu et al., 2010) and thus it is now evident that LTBP-2 may also influence TGF- β bioavailability. Moreover, this idea is supported by observations that some individuals with LTBP-2 mutations exhibit Marfan-Syndrome-like characteristics which are considered to be directly caused by aberrant TGF- β upregulation (Ali et al., 2009; Desir et al., 2010).

Given the above evidence, it seems clear that LTBP-2 plays some role in the regulation of TGF- β bioactivity but the mechanism is unclear. In this paper, we report that a central bioactive region of LTBP-2 can up-regulate TGF- β expression in fibroblasts and that the mechanism involves both Akt and p38 signalling pathways. The cell surface receptor(s) involved in the upregulation remains unclear but may involve α V β 3 integrin.

5.3 Materials and Methods

5.3.1 Reagents

Recombinant human LTBP-2 and fragments were produced using the pCEP4 expression vector in 293EBNA cells as described previously (Hirani et al., 2007; Parsi et al., 2010). The primary antibodies against p-Akt1/2/3 (Ser473), Akt1/2/3 (H-136), p-p38 (D-8), p38 (H-147), p-ERK (E-4), ERK (MK1) and β -actin (C4) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-mouse IgG and anti-rabbit IgG fluorescence labelled with Dylight 800 were obtained from Thermo Scientific, Waltham, MA, USA. Rabbit anti-[human LTBP-2 peptide] antibody 3504 has been described previously (Hirani et al., 2007). Inhibitors for specific signalling molecules; SB202190 and VX-702 for p38, and GSK690693 and AZD5363 for Akt were purchased from Selleckchem, Houston, USA. Integrin blocking antibodies, anti- β 1 integrin (P4C10) and anti- α V β 3 integrin (CD51/61) were purchased from Millipore (CA, USA); anti- α V β 5 integrin (P1F6) was from Abcam (Cambridge, UK.)

5.3.2 Expression and purification of recombinant LTBP-2 and fragments

His₆-tagged full-length r-LTBP-2 and contiguous fragments NT(H), C(H) and CT(H) including sub-fragments of LTBP-2C(H), entitled F1,F2, and F3, were produced in human embryo kidney, 293-EBNA cells and expressed as described previously (Hirani et al., 2007; Menz et al., 2015; Parsi et al., 2010). The recombinant proteins were purified from serum-free culture medium by using chelating Ni-Sepharose as previously described (Hirani et al., 2007). Purified recombinant proteins were dialyzed into TBS-0.5M NaCl and analysed by SDS-PAGE and immunoblotting to confirm size and purity.

5.3.3 Cell culture and treatment

MSU1.1 fibroblast cells were grown for a range of times (1 day to 3 weeks post-confluence) in 6 well plates in DMEM plus 10% FCS, non-essential amino acids (NEAA), and penicillin-streptomycin. Full length human recombinant LTBP-2 or fragment in serum-free medium was

added to give a final concentration of 2.5 - 12.5 µg/ml, and the cells were incubated at 37°C, for various times (3 h – 24 h). For control wells, BSA (10 µg/ml) was added instead of LTBP-2. In some cases, protein synthesis was inhibited by addition of 10µg/ml cycloheximide (SIGMA) to wells with or without added LTBP-2. In signalling pathway studies, Akt or p38 inhibitors were added at 10mM to the medium and cells were pre-incubated for 2 hours prior to incubation with LTBP-2. In integrin receptor studies, integrin blocking antibodies (anti-integrin β1, αVβ3, or αVβ5) were added at 10 or 20 µg/ml and the cells were pre-incubated for 2 hours prior to incubation with LTBP-2.

Samples of conditioned medium were taken for analysis of TGFβ content by ELISA. The cell layer was washed with PBS then scraped from the wells and resuspended in 1 ml PBS, and treated with 200 µl of 1M HCl to liberate and activate any matrix-bound latent TGFβ. After 10 min, 200 µl of 1.2M NaOH/0.5M HEPES pH 14 was added to neutralise the samples. The cell layer suspension was centrifuged for 5 min at 350 g, and the supernatant analysed for TGFβ content.

5.3.4 Detection and quantitation of TGF-β

Samples of conditioned medium were assayed for TGFβ content using the DuoSet® ELISA Development System for human TGF-β1 (RnD Systems), following the manufacturers instructions. Briefly, the wells of a 96 well plate were coated with 200 ng of Capture Antibody (mouse anti-TGF-β1 in PBS) and incubated for 18 h at RT. Each well was then washed three times with 300 µl of wash buffer (0.05% Tween 20 in PBS) followed by incubation with 300 µl of block buffer (5% Tween20 in PBS) for at least 1 h at RT. After washing, wells were incubated with 100 µl of conditioned medium for 2 h at RT. Control wells were incubated with fresh medium or PBS. For standard curves, wells were incubated with 100 µl of TGFβ1 standard at a range of concentrations (31.3 pg/ml – 2000 pg/ml) in reagent diluent (1.4% BSA in PBS, RnD Systems). Following another wash step, wells were incubated with 100 µl of Detection Antibody (biotinylated chicken anti-human TGF-β1, 300 ng/ml in reagent diluent) for 2 h at RT. Wells were washed again, then incubated with 100 µl of Streptavidin-HRP (horseradish peroxidase) for 20 min at RT, under reduced lighting. Wells were washed three times then incubated with 100 µl of

Substrate Solution (RnD Systems) for up to 20 min, in reduced lighting. The colour reaction was stopped by adding 50 μ l of 1M H₂SO₄ per well. The absorbance at 450nm for each well was immediately measured using a Titertek Multiscan microplate reader (Flow Laboratories).

5.3.5 Real-time PCR

Total RNA was extracted from cell using trizol (Invitrogen), according to the manufactures instructions. To remove any DNA contaminants, RNA samples were treated with DNase 1 (RQ1 DNase, Promega) followed by recovery using the RNEasy Mini Kit (Qiagen). A total of 1 μ g of total RNA was subjected to reverse transcription using a Superscript III RNA PCR Kit (Invitrogen, CA, USA). The cDNA produced was PCR-amplified using a SYBR-Green kit (Qiagen). Primer sequences used were as follows: TGF- β forward primer: 5'-CTCCGAGAAGCGGTACCTGAAC-3', and reverse primer 5'-CACTTGCAGTGTGTTATCCCT-3'; LTBP-2 forward primer: 5'-GGGCACCGCACCACCTACACG-3' and reverse primer 5'-TCATCACAATCATTACATCTACG-3'; RNAPolIII forward primer 5'-AGGGGCTAACAATGGACACC-3', and reverse primer 5'-CCGAAGATAAGGGGAACTACT-3'. The housekeeping gene RNAPolIII was used as the internal standard. PCR was performed with hot start at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 45 s.

5.3.6 Measurement of signal phosphorylation

MSU1.1 fibroblast cells (4 x 10⁵ cells/well) were treated with LTBP-2 (10 μ g/ml) for 30 min. Cell were then lysed using lysis buffer (containing 50 mM Tris (pH 6.8), 0.5% SDS, 2 mM EDTA and cocktails of phosphatase and protease inhibitors [#04906837001 and #11836153001, Roche, Germany]). The protein concentrations in cell extracts were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, VIC, Australia). A total of 50 μ g of extracted protein was subjected to analysis by SDS-PAGE on 12% gels and transferred to nitrocellulose membranes (Pall Corporation, USA). The membrane was blocked with 10% milk powder in TBS for at least 1hr at RT, with shaking. The membrane was then incubated with a primary antibody to phosphorylated or total signalling molecule (AKT, P-38, or ERK) in 2% milk in TBS at 4°C overnight. The

membrane was washed three times in TBS for 5min, then incubated with the appropriate secondary anti-rabbit IgG or anti-mouse IgG antibodies conjugated with IR800 fluorescence dye, at a concentration of 1:5000, in 3 % milk in TBS for at least 1 h at RT. Membranes were imaged with the LI-COR Odyssey Infrared Imaging System. Bands were quantitated using ImageJ 1.48 software [National Institutes of Health (NIH), Bethesda, MD] and normalised to the internal β -actin signal or total protein. For comparison of the phospho- and total signal for each molecule, the ratio of the normalised phospho-protein signal to the total protein signal was expressed as an arbitrary unit relative to the average value from cells incubated without LTBP-2 treatment (equalling 1).

5.3.7 Statistical analysis

The data from *in vitro* TGF- β stimulation assays, cell count assays, western blot quantitation and qPCR were statistically analysed using paired Student's t-test with GraphPad Prism 6 software. A p value ≤ 0.05 was regarded as statistically significant.

5.4 Results

5.4.1 Exogenous LTBP-2 stimulates expression and secretion of TGF- β 1 in MSU 1.1 fibroblasts

To study the potential role of LTBP-2 in modulation of TGF- β matrix storage and activation, the effect of the addition of exogenous LTBP-2 to human MSU 1.1 fibroblast cultures was investigated. MSU 1.1 cells were selected since preliminary data showed that these cells have high levels of expression of fibrillin-1, LTBP-1 and TGF- β 1 but low expression of LTBP-2 (data not shown). The cells form an extensive fibrillin microfibril network (Kettle et al., 2000). The initial experiment was designed to determine if LTBP-2 could displace LTBP-1/TGF- β 1 complexes from their attachment to fibrillin microfibrils. MSU1.1 cells were cultured to 3 weeks post-confluence, to allow formation of an extensive extracellular matrix rich in fibrillin-1 microfibrils. After incubation with exogenous LTBP-2 for 16 h, TGF- β 1 was significantly elevated in the culture medium with an approximately 3-fold increase compared to control cells incubated with BSA. (Fig 5.1A). However, the TGF- β 1 content of the cell layer showed no significant difference between treated and control cultures. This result indicated that the increase in TGF- β 1 may represent newly synthesised protein rather than protein displaced from the microfibrils in the matrix.

To test this idea, exogenous LTBP-2 was added to MSU1.1 cells grown to only 24 h post-confluence such that only minimal fibrillin microfibrils would be present in the matrix (Kielty and Shuttleworth, 1993) (Fig 5.1A). Again TGF- β 1 was highly elevated in the medium relative to control cultures. In contrast there was little difference in cell layer TGF- β 1 levels between the treated and control cells. However there was generally less TGF- β 1 in the cell layers at 24 hours post-confluence compared to the 3 week post-confluence cultures. Analysis of the TGF- β released into the medium of the 3 week post-confluence cultures indicated that approximately 70% of the TGF- β 1 was in latent (complexed) form (Fig 5.1B). Similar results were obtained for the 24 h post confluence cultures treated with LTBP-2 (data not shown). To confirm that the increased levels of TGF- β in the medium are due to new synthesis of the protein, the addition of exogenous LTBP-2 to 24 h post-confluence cells was repeated in the presence of cycloheximide, a well-documented inhibitor of protein synthesis (Baliga et al., 1969; McKeehan and Hardesty, 1969)

(Fig 5.2A). As was previously demonstrated (Fig 5.1), positive control cultures treated with exogenous LTBP-2 in the absence of cycloheximide showed a dramatic increase in TGF- β 1 in conditioned medium, compared to BSA controls. However when the cells were incubated with LTBP-2 in the presence of cycloheximide, there is no significant increase in TGF- β in the medium above BSA controls (Fig 5.2A).

Similar results were obtained with MG-63 osteosarcoma cells (data not shown). The results indicated that the observed increase in TGF- β levels in the conditioned medium involved new protein synthesis. Thus the results were not due to a simple displacement interaction of exogenous LTBP-2 with matrix fibrillin-microfibrils, as was first hypothesised. Quantitative PCR for TGF- β expression was also performed on RNA extracted from MSU 1.1 cells after 15 h incubation with exogenous LTBP-2 or BSA control (Fig 5.2B). The LTBP-2 treated cells showed a threefold elevation in TGF- β mRNA compared to the BSA treated controls, indicating that LTBP-2 greatly stimulated TGF- β expression in this fibroblast cell line. Taken together the results indicate that LTBP-2 greatly upregulated release of TGF- β into the culture medium, but the mechanism primarily involved upregulation of TGF- β gene expression rather than increased secretion of existing TGF- β or displacement of existing latent TGF- β complexes from fibrillin-microfibrils.

5.4.2 LTBP-2 stimulation of TGF- β synthesis and secretion is dose-dependent.

To determine the optimal concentration of exogenous LTBP-2 needed to stimulate increased TGF- β synthesis and secretion by MSU 1.1 cells, a dose response curve was produced for LTBP-2 concentrations from 0.5 μ g/ml to 10 μ g/ml (Fig 5.3). A LTBP-2 concentration of 0.5 μ g/ml showed a significant 2-fold increase in TGF- β which rose to around 4-fold at 10 μ g/ml LTBP-2. No further significant increase was observed above 10 μ g/ml, suggesting a possible saturation level at this LTBP-2 concentration. Overall the results indicated that the increase in TGF- β synthesis in response to exogenous LTBP-2 showed dose-dependent characteristics.

5.4.3 Time course for LTBP-2 stimulation of TGF- β upregulation

A time course experiment was conducted to determine the duration and peak of TGF- β secretion and the time required for an increase to be first detected following LTBP-2 stimulation. The TGF- β 1 content in the conditioned medium was measured across several time points in the range 0 – 24 h following addition of exogenous LTBP-2 (Fig 5.4A). The TGF- β content of the medium peaked at the 15 h time point with 3-fold increase compared to the BSA treated control cells. No significant increase above controls was observed at 3 h and 6 h after LTBP-2 addition. However, significantly elevated TGF- β secretion was evident at the 9 h time point, and at the 12 h time point the LTBP-2-treated cells showed more than a twofold increase above control cells. No significant difference was observed between 24 h and 15 h time point. However TGF- β secretion was significantly higher by control cells at 24 h compare to 15 h, suggesting that LTBP-2 stimulation of TGF- β production peaked at around 15 h, and showed a declining trend thereafter. To determine if any of the increase above controls cells was due to increased cell numbers, cell counts were conducted at several time points (Fig 5.4B). No significant differences were found between the cell number in cultures treated with LTBP-2 and untreated controls, although cell number in all cultures doubled after 24 h of incubation. Thus the LTBP-2-induced stimulation of TGF- β secretion by MSU1.1 cells was not due to a greater increase in cell number above the control cultures.

5.4.4 A short exposure to exogenous LTBP-2 is sufficient to stimulate TGF- β upregulation

The experiments described above involved continuous incubation of MSU 1.1 fibroblasts with exogenous LTBP-2 for 9-24 hours before any increase in TGF- β levels in the medium could be detected. This raised the possibility that, rather than a direct effect of LTBP-2, the raise in TGF- β secretion was a secondary effect stimulated by other agent(s) modulated by addition of exogenous LTBP-2. Firstly we determined if a short incubation with LTBP-2 was sufficient to raise TGF- β levels in the conditioned medium. MSU1.1 cells were incubated with exogenous LTBP-2 for range of short time periods (10 mins – 30 mins) before the LTBP-2 was removed by changing the medium. After 15 h the conditioned medium was collected and analysed for TGF- β content (Fig 5.5A). Interestingly exposure of cells to LTBP-2 for as little as 10 min caused a small but significant increase in TGF- β in the conditioned medium after 15 h suggesting that the effect was

mediated by direct interaction of LTBP-2 with the cell surface. Exposure of the cells to LTBP-2 for 30 min and 60 min further elevated TGF- β levels in the medium, with a 60 min exposure to LTBP-2 causing around 50% of the increase resulting from incubation with LTBP-2 for 15 h (see Fig 5.4A).

To determine if the LTBP-2 stimulated increase in TGF- β secretion correlated with increased cellular expression of TGF- β , TGF- β 1 mRNA levels in MSU 1.1 fibroblasts were measured at 15 h after short exposure times to LTBP-2. To quantify the TGF- β increase at the mRNA level, the cell layer was harvested after 15h and total RNA was extracted and reverse transcribed into cDNA for use in qPCR (Fig 5.5B). Exposure of the cells to LTBP-2 for as little as 10 min LTBP-2 resulted in significant elevation of TGF- β mRNA above the BSA controls measured after 15 h. Incubation with LTBP-2 for 30 and 60 mins caused additional increases in TGF- β mRNA levels, with the 60 min exposure causing an approximately fourfold increase in TGF- β above control cells. These results indicate that a short exposure of the MSU 1.1 cells to exogenous LTBP-2 is sufficient to stimulate TGF- β expression and secretion, strongly suggesting that the process is a directly mediated by the interaction of LTBP-2 with a cell surface receptor(s) to trigger intracellular signalling pathways.

5.4.5 The TGF- β stimulating activity maps to a central region of LTBP-2 consisting of an eight -cys motif flanked by pairs of EGF-like repeats.

Cellular interactions of LTBP-2 via integrin receptors have previously been documented (Vehvilainen et al., 2003). To determine if the TGF- β upregulation by LTBP-2 involves known or novel cellular interactions, a series of fragments spanning the LTBP-2 molecule were used to identify the relevant bioactive region(s). Initially, three recombinant fragments, designated LTBP-2NT(H), LTBP-2C(H) and LTBP-2CT(H) (Hirani et al., 2007) spanning the LTBP-2 molecule were tested in place of full length LTBP-2 in exogenous addition experiments (Fig 5.6A). After 16 h the TGF- β content of medium from cells treated with fragments LTBP-2NT(H) or LTBP-2CT(H) was similar to the BSA treated control cells, while the central fragment LTBP-2C(H) caused a significant increase in TGF- β , similar to full length LTBP-2 (Fig 5.6B). This result indicated that the relevant bioactive region of LTBP-2 occurs somewhere in the centre of the

molecule and away from known integrin binding activity in the N-terminal region (Vehvilainen et al., 2003).

To further specify the region that contained TGF- β stimulating activity, three sub-fragments F1, F2 and F3, spanning fragment LTBP-2C(H), were produced and tested in the TGF- β bioassay. Only fragment F3 caused a significant increase in TGF- β secretion above control cells indicating it contained the TGF- β stimulating activity (Fig 5.6C). Fragment F3 consists of an 8-cys motif flanked by 2 pairs of EGF-like repeats (Fig 5.6A). This region has no known cellular or molecular interactions although it is adjacent to a region containing heparin and FGF-2 binding activities (Fragment F2) (Menz et al., 2015; Parsi et al., 2010).

5.4.6 Induction of Akt and p38 phosphorylation by LTBP-2

We firstly examined the effects of LTBP-2 on the phosphorylation of serine, threonine, cJUN, cFOS, AKT1/2/3, ERK and p38 by Western blot analyses using specific antibodies to phosphorylated forms of the amino acids and proteins. Figure 5.7A shows that the treatment of MSU1.1 cells with full length LTBP-2 (1 μ g/ml) for 30 mins increased the phosphorylation of serine, threonine, cJUN, Akt1/2/3 and p38. However we observed no increase in threonine, cFOS and ERK phosphorylation. Thus, LTBP-2 seems to specifically activate signalling molecules containing phospho-serine and threonine, and signalling pathways involving AKT and p38 (Fig 5.7A).

The experiment was repeated including bioactive fragment LTBP-2C and sub-fragment LTBP- 2C F3. Phosphorylation of each signal molecule was quantitated relative to the no LTBP-2 control after normalisation to total signal molecule present (Fig 5.7B and 5.7C). Results showed both bioactive fragments also activated Akt and p38 signalling but not ERK. This lead to the lead to the hypothesis that LTBP-2 may stimulate TGF- β expression by activating these signalling pathways.

5.4.7 LTBP-2 stimulates the expression of TGF- β via Akt and p38 MAPK signalling pathways

To determine whether the LTBP-2-stimulated upregulation of TGF- β correlated with the phosphorylation of AKT1/2/3 or p38, specific signal inhibitors were tested for inhibition of TGF- β upregulation by LTBP-2. MSU1.1 cells treated with or without LTBP-2 were cultured in the presence of an Akt phosphorylation inhibitor, GSK690693 or AZD5363 or a p38 phosphorylation inhibitor, SB202190 or VX-702 and the TGF- β in the medium was measured by ELISA after 16 h (Fig 5.8). The LTBP-2 induced upregulation of TGF- β was totally (GSK690693) or partially (AZD536310) blocked by the AKT phosphorylation inhibitors and totally blocked by one p38 inhibitor (SB202190). The other p38 inhibitor VX-702 showed no reduction in TGF- β stimulation. SB202190 blocks phosphorylation of both α and β forms of p38 whereas VX-702 blocks only the α isoform (Damjanov et al., 2009). Thus it appears that LTBP-2 induced up-regulation of TGF- β 1 involves AKT1/2/3 and the β form only of p38. It also seems that blocking one of these signalling molecules also blocks the signal from the other, indicative of sequential rather than parallel signalling transmission.

5.4.8 Blocking of integrin α V β 3 receptors partially attenuates TGF- β production by LTBP-2

The integrin family of cell surface receptors mediate many cell-matrix and cell-cell interactions and play essential cellular roles including adhesion, migration, growth and survival important for biological processes such as organ development and wound healing (Giancotti and Ruoslahti, 1999; Hynes, 1992). Integrins are also involved in many pathological conditions such as angiogenesis, inflammation, invasion of cancer cells and fibrosis (Gerber et al., 2013; Liu et al., 2008; Margadant and Sonnenberg, 2010; Philippe, 2015). Integrins mediate many interactions with matrix proteins and thus an unidentified integrin was considered a prime candidate for the mediation of LTBP-2 upregulation of TGF- β . LTBP-2 is known to contain an integrin binding region in its N-terminal domain which is not present in either bioactive fragment LTBP-2C or LTBP-2C F3. To determine if an integrin was involved in upregulation of TGF- β , a range of established blocking anti-integrin antibodies were tested in the LTBP-2 stimulation assay (Fig

5.9A). The anti- $\beta 1$ integrin antibody had no effect on LTBP-2 upregulation of TGF- β indicating that the large family of $\beta 1$ integrins was not involved in the process. Antibody to $\alpha V\beta 5$ integrin also had no effect but intriguingly the anti- $\alpha V\beta 3$ integrin antibody (10 $\mu\text{g/ml}$) showed partial inhibition of the TGF- β upregulation. Doubling of the anti- $\alpha V\beta 3$ antibody concentration and reducing the concentration of LTBP-2 F3 10-fold showed only a very minor further attenuation suggesting that the inhibitory effect of the antibody was close to saturation (Fig 5.9B). Even under these conditions LTBP-2 elevated TGF- β levels to double the amount in control cultures suggesting that an unknown primary receptor might be involved in conjunction with $\alpha V\beta 3$ to mediate TGF- β up-regulation by LTBP-2.

5.5 Discussion

Recently, mutations in the LTBP-2 gene have been found to cause Weill-Marchesani syndrome, which can also result from fibrillin-1 gene mutations, linking LTBP-2 to fibrillin biology and TGF- β signalling aberrations (Haji-Seyed-Javadi et al., 2012). Several other studies reported the potential of LTBP-2 in regulation of growth factor storage and activation especially TGF- β , but little is known about the mechanism of action. To our knowledge, the current study is the first to investigate the mechanism of TGF- β regulation by LTBP-2.

Our initial hypothesis that excess LTBP-2 would compete with LTBP-1 for binding and release the TGF- β complex from its storage sites in the matrix was initially supported by TGF- β stimulation assays where incubation of exogenous LTBP-2 for 16 h with 3-week post-confluent MSU1.1 cells resulted in significantly increased levels of latent TGF- β in the culture medium. This possibility of an extracellular matrix protein stimulating release of TGF- β complexes is not unique. A study by Chaudhry *et al.*, 2007 found that fragment of fibrillin-1 caused a significant increase in the bioavailability of TGF- β by inhibiting the binding of LTBP-1 to fibrillin microfibrils. However, our results showed that the TGF- β increase in the medium did not correlate with a similar decrease within the cell layer suggesting an alternative mechanism was responsible for the elevation of TGF- β in the medium. The experiment was repeated with confluent cell cultures containing very limited matrix but there was a similar increase in TGF- β in the medium after incubation with LTBP-2. Moreover, LTBP-2 incubation in the presence of cycloheximide completely inhibited the observed increase in TGF- β . Analysis of RNA extracted from the cells showed threefold increase in TGF- β mRNA in response to LTBP-2 addition. These results confirmed that the increase in TGF- β in the medium was due to increased expression and secretion of the TGF- β 1 rather than release of matrix-stored TGF- β . Furthermore, a time course experiment showed TGF- β started to increase after incubation with LTBP-2 for 9 h and reached maximum levels after 15 h. These results contrast with the mechanism of TGF- β increased by liberation from matrix storage where fibrillin-1 fragments causing TGF- β increase after as little as 10 min (Chaudhry et al., 2007).

The longer time frame involved indicates that LTBP-2 is causing a cascade effect that results in new TGF- β synthesis and release into the medium. We found that incubation with LTBP-2 for as little as 10 minutes was sufficient to stimulate significantly increased TGF- β expression after 15 h. Moreover, the mRNA from the cells incubated with LTBP-2 for short-time span (10 – 60 mins) showed significantly higher expression of TGF- β compared to the control cells. The results suggested that LTBP-2 may be interacting directly with cells via a surface receptor, such as an integrin, to cause immediate activation of intracellular signalling pathways. This activation may lead to TGF- β mRNA upregulation, rather than it eliciting an indirect effect involving interactions of the cells with other molecules displaced by LTBP-2 such as growth factors or cytokines.

Since LTBP-2 has known integrin-binding activity (Vehvilainen et al., 2003), it was important to delineate the TGF- β upregulation from known LTBP-2-cell interactions. The only known integrin binding region, (binding integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$) is in a cysteine free region situated towards the N-terminus (see fig 5.6A) (Vehvilainen et al., 2003). In contrast, the bioactivity was found to be principally confined to a short region in the centre of LTBP-2 consisting of an 8-cys motif flanked on each side by a pair of EGF-like repeats (fragment F3).

Since it was now evident that LTBP-2 may be acting directly on fibroblasts it was of interest to determine which signalling pathway(s) it activated and which of these pathway(s) resulted in TGF- β upregulation. We found that exogenous LTBP-2 increased phosphorylation of intracellular proteins at serine and threonine but not at tyrosine residues after 30 mins. Such post-translational modifications are involved in cell signalling pathways resulting gene expression modulation governing a diversity of cellular processes including development and differentiation, cell cycle progression, migration, proliferation and apoptosis (Sarbasov et al., 2005). Further experiment revealed that LTBP-2 increased phosphorylation of p38 MAPK and Akt1/2/3 (but not ERK) suggesting these molecules may be involved in the pathway(s) leading to stimulation of TGF- β expression by LTBP-2. p38 α was initially identified as a 38 kDa polypeptide and three additional isoform of the p38 family have been identified: p38 β , p38 γ (also called SAPK3 and ERK6) and p38 δ (also called SAPK4) (Cuadrado and Nebreda, 2010). P38 is strongly activated by a variety of extracellular stimuli including environmental stress, inflammatory cytokines, serum

and growth factors (Cuadrado and Nebreda, 2010). P38 MAPK is also involved in several pathological conditions such as inflammation, rheumatoid arthritis, cancer, cardiac hypertrophy, neurodegenerative disorders and fibrosis (Roux and Blenis, 2004; Sui et al., 2014). Akt (also known as protein kinase B) is a serine/threonine-specific protein kinase that plays key roles in the regulation of cell survival, cell cycle progression and cellular growth (Song et al., 2005). More recently Akt signalling pathway components have been shown to be frequently altered in human cancers and fibrosis (Fresno Vara et al., 2004; Lan and Du, 2015). Studies by Li et al., 2013 demonstrated that activation of Akt pathway promoted collagen production and scar formation in the acute contusion of skeletal muscle (Li et al., 2013). Moreover, Akt pathway activation has been reported to promote lung fibroblast proliferation and pulmonary fibrosis by enhancing macrophage survival via modulation of the mevalonate pathway (Larson-Casey et al., 2014).

Interestingly, the key bioactive fragment of LTBP-2 (LTBP-2C F3) which contains the TGF- β stimulating activity did not increase p38 and Akt1/2/3 phosphorylation to the extent of full length LTBP-2 or the larger LTBP-2C fragment suggesting other adjacent motifs within LTBP-2C may contribute for full stimulation of the signal pathways. Inhibition of Akt1/2/3 and p38 β signalling pathway by specific inhibitors resulted in the suppression of TGF- β production stimulated by LTBP-2. Interestingly, GSK690693 inhibitor completely blocked the up-regulation of TGF- β by AKT phosphorylation compared to AZD536310 inhibitor which partially blocked TGF- β stimulation. It has been shown that GSK690693 has an IC₅₀ of 2nM for Akt1 which indicates stronger inhibition than AZD5363 (IC₅₀ of 3 nM for Akt1) suggesting the possibility that Akt1 acts as the main Akt isoform involved in the up-regulation of TGF- β . Akt1 is one of the most frequently activated protein kinases in human cancers and associated with altered cell invasion and migration in several mammalian systems (Altomare and Testa, 2005). Furthermore, Akt1 has been demonstrated to further enhance the invasion capability of cancer cell cultures and is considered a potential therapeutic target for several cancer types (Arboleda et al., 2003; Kim et al., 2001; Zhang et al., 2016). Akt1 also caused cardiac fibrosis by promoting inflammatory response in the early stage of hypertensive heart disease (Miao et al., 2013). Moreover, GSK690693 also potentially inhibits AMPK and DAPK3 from the CAMK family and PAK4, 5 and 6 from the STE family indicating involvement of other signalling molecules may contribute to the stimulation of TGF- β .

It appears that only the β form of p38 is involved in the cascade since VX-702 which is specific inhibitor for p38 α showed no reduction in TGF- β stimulation (Kuliopulos et al., 2004). P38 β MAPK has been demonstrated to act as a survival protein by inducing a potent antiapoptotic factor induced in the synovial membrane of rheumatoid arthritis patients (Kim et al., 2005). Several studies have elucidated a role for p38 β MAPK activation in anti-inflammatory, anti-proliferative, and anti-apoptotic effects of carbon monoxide in various models (Otterbein et al., 2000). Sustaining cell survival is considered a main feature of p38 β MAPK activation (Nemoto et al., 1998).

Our findings suggested both Akt and p38 β pathways were essential for TGF- β upregulation by LTBP-2. Previous studies have shown blockade of the Ras/PI3K/Akt pathways reduce the expression of TGF- β as angiogenic factor in mouse osteosarcoma (Tsubaki et al., 2011). Moreover, inhibition of PI3K/Akt signalling pathway result in the suppression of TGF- β 1 production by macrophages treated with PS-liposomes (Otsuka et al., 2007). In addition, Xiao et al. 2014 showed that apoptotic cell up-regulate TGF- β mRNA expression and protein translation p38 MAPK and Akt signalling pathways (Xiao et al., 2008).

From the above results it is apparent that LTBP-2 may elicit the TGF- β upregulation via interaction with a specific cell surface receptor complex. Since the activity is mainly confined to one small region (LTBP-2C F3), it is independent of known LTBP-2-integrin interactions. However, it was still possible that the LTBP-2 upregulation of TGF- β involved unknown integrin(s) interactions since integrins are the major receptor family involved in mediating cell-matrix signalling process regulating a wide variety of cellular functions. We tested blocking anti-integrin antibodies in the TGF- β stimulation assay and found blocking the integrin α V β 3 receptor partially inhibited the upregulation of TGF- β by LTBP-2 whereas blocking α V β 5 and the β 1 family of integrins had no effect. Doubling the anti- α V β 3 antibody concentration and reducing the LTBP-2 concentrations 10 fold caused no significant further attenuation of the TGF- β upregulation. The results suggested that the LTBP-2 bioactivity involved active α V β 3 integrin but that other receptor(s) were also involved. Integrins can stimulate signalling pathways independently, but more often they act synergistically with other receptors to elicit a full signalling response (Alam

et al., 2007; Assoian and Schwartz, 2001). Specific Integrins can interact for instance with several growth factor receptors (including the VEGF receptor, TGF- β receptor, insulin receptor, EGF receptor and platelet-derived growth factor- β receptor (PDFGR)) or with cell surface HSPGs. For example, PDFGR which is potent stimulator of cell motility, has been shown to be modulated by α V β 3 integrin and the interaction between PDFGR and α V β 3 integrin appears to have an antagonistic effect in the reactive oxygen species production at focal adhesion sites (Heldin et al., 1998; Lin et al., 2013; Woodard et al., 1998).

In addition, syndecan-4 HSPG requires interaction with α 5 β 1 integrin to regulate matrix structure and cell adhesion during all stages of embryonic development and in most adult tissues (Woods and Couchman, 2001). In the case of LTBP-2 F3, there is a possibility that integrin α V β 3 is part of complex with an unknown main receptor or co-receptor for LTBP-2. Binding of blocking antibody to the α V β 3 might partially obscure the binding of LTBP-2 to its main receptor without LTBP-2 being a direct ligand for α V β 3. One candidate group for an unknown LTBP-2 receptor is cell surface HSPGs. Various HSPGs are found at the cell surface and/or the extracellular matrix and play important roles as cell receptors, co-receptors, co-factors, and can act in conjunction with integrins to modulate cell behaviour and signalling outcomes, often through growth factor receptor signalling pathways (Alam et al., 2007; Kim et al., 2011; Soares et al., 2015). Interestingly, recent studies revealed that LTBP-2 binds strongly to HSPGs, including basement membrane perlecan and cell-signalling, transmembrane syndecan-4, with multiple binding sites within the N-terminal region of LTBP-2 and one site (in fragment LTBP-2 F2) adjacent to the TGF- β stimulating region (fragment LTBP-2 F3). Binding to HSPG via the adjacent site may explain the stronger activation and phosphorylation of the Akt and p38 MAPK signalling molecules caused by the interaction of HSPGs with full length LTBP-2 and LTBP-2C than with F3 which lacks a HSPG binding site. However, HSPG binding is not required for TGF- β upregulation by LTBP-2 as the LTBP-2C F3 fragment lacks a HSPGs binding site. Overall it is evident that the interaction of fibroblasts with the bioactive region of LTBP-2 contained in fragment LTBP-2 C F3 is a complex process perhaps involving a complex of an unidentified main receptor with α V β 3 integrin and enhanced by interaction of cell surface HSPGs with an adjacent binding site in fragment LTBP-2 F2. The concepts are illustrated schematically in Fig 5.10. Further research is needed to elucidate the full nature of LTBP 2 interaction(s) with the cell surface required for TGF- β upregulation.

We have recently shown that LTBP-2 is highly expressed in fibrotic skin conditions keloid and hypertrophic scar (Menz et al., 2015; Sideek et al., 2015). Moreover LTBP-2 co-localised in these tissues with another growth factor FGF-2. LTBP-2 has a high affinity binding site for FGF-2 in LTBP-2C F2 fragment and is a potent inhibitor of FGF-2 activity (Menz et al., 2015; Sideek et al., 2015). It is possible that elevated LTBP-2 might that may influence FGF-2 bioactivity during wound repair and healing processes. TGF- β also plays a key role in the proliferative phase of wound healing (Finnson et al., 2013; Pakyari et al., 2013). The present study has now shown that LTBP-2 can increase fibroblast output of TGF- β , and therefore this effect may contribute to the exacerbation of the fibrotic process in diseases such as hypertrophic scar and keloid. In conclusion, we have identified a new bioactive region on LTBP-2 which triggers fibroblast upregulation of TGF- β expression and secretion and identified cell surface and intracellular signalling molecules involved in this stimulatory pathway(s) including α V β 3 integrin, and Akt and p38 intracellular signalling cascades. These concepts are represented schematically in Fig 5.10. Further elucidation of this pathway may eventually lead to novel therapy strategies to treat fibrosis and other diseases associated with TGF- β dysregulation.

5.6 References

- Alam, N., Goel, H.L., Zarif, M.J., Butterfield, J.E., Perkins, H.M., Sansoucy, B.G., Sawyer, T.K., Languino, L.R., 2007. The integrin-growth factor receptor duet. *J Cell Physiol* 213, 649-653.
- Ali, M., McKibbin, M., Booth, A., Parry, D.A., Jain, P., Riazuddin, S.A., Hejtmancik, J.F., Khan, S.N., Firasat, S., Shires, M., Gilmour, D.F., Towns, K., Murphy, A.L., Azmanov, D., Tournev, I., Cherninkova, S., Jafri, H., Raashid, Y., Toomes, C., Craig, J., Mackey, D.A., Kalaydjieva, L., Riazuddin, S., Inglehearn, C.F., 2009. Null mutations in LTBP2 cause primary congenital glaucoma. *Am J Hum Genet* 84, 664-671.
- Altomare, D.A., Testa, J.R., 2005. Perturbations of the AKT signaling pathway in human cancer. *Oncogene* 24, 7455-7464.
- Annes, J.P., 2003. Making sense of latent TGF- β activation. *Journal of Cell Science* 116, 217-224.
- Annes, J.P., Munger, J.S., Rifkin, D.B., 2003. Making sense of latent TGF- β activation. *J Cell Sci* 116, 217-224.
- Arboleda, M.J., Lyons, J.F., Kabbinavar, F.F., Bray, M.R., Snow, B.E., Ayala, R., Danino, M., Karlan, B.Y., Slamon, D.J., 2003. Overexpression of AKT2/protein kinase B β leads to up-regulation of β 1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. *Cancer Res* 63, 196-206.
- Assoian, R.K., Schwartz, M.A., 2001. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. *Curr Opin Genet Dev* 11, 48-53.
- Baliga, B.S., Pronczuk, A.W., Munro, H.N., 1969. Mechanism of cycloheximide inhibition of protein synthesis in a cell-free system prepared from rat liver. *J Biol Chem* 244, 4480-4489.
- Bottinger, E.P., Letterio, J.J., Roberts, A.B., 1997. Biology of TGF- β in knockout and transgenic mouse models. *Kidney Int* 51, 1355-1360.
- Branton, M.H., Kopp, J.B., 1999. TGF- β and fibrosis. *Microbes Infect* 1, 1349-1365.
- Cain, S.A., McGovern, A., Baldwin, A.K., Baldock, C., Kielty, C.M., 2012. Fibrillin-1 mutations causing Weill-Marchesani syndrome and acromicric and geleophysic dysplasias disrupt heparan sulfate interactions. *PLoS One* 7, e48634.

- Chaudhry, S.S., Cain, S.A., Morgan, A., Dallas, S.L., Shuttleworth, C.A., Kielty, C.M., 2007. Fibrillin-1 regulates the bioavailability of TGF- β 1. *J Cell Biol* 176, 355-367.
- Clark, D.A., Coker, R., 1998. Transforming growth factor- β (TGF- β). *Int J Biochem Cell Biol* 30, 293-298.
- Clark, R.A., McCoy, G.A., Folkvord, J.M., McPherson, J.M., 1997. TGF- β 1 stimulates cultured human fibroblasts to proliferate and produce tissue-like fibroplasia: a fibronectin matrix-dependent event. *J Cell Physiol* 170, 69-80.
- Cuadrado, A., Nebreda, A.R., 2010. Mechanisms and functions of p38 MAPK signalling. *Biochem J* 429, 403-417.
- Dallas, S.L., Keene, D.R., Bruder, S.P., Saharinen, J., Sakai, L.Y., Mundy, G.R., Bonewald, L.F., 2000. Role of the latent transforming growth factor β binding protein 1 in fibrillin-containing microfibrils in bone cells in vitro and in vivo. *J Bone Miner Res* 15, 68-81.
- Damjanov, N., Kauffman, R.S., Spencer-Green, G.T., 2009. Efficacy, pharmacodynamics, and safety of VX-702, a novel p38 MAPK inhibitor, in rheumatoid arthritis: results of two randomized, double-blind, placebo-controlled clinical studies. *Arthritis Rheum* 60, 1232-1241.
- Derynck, R., Akhurst, R.J., Balmain, A., 2001. TGF- β signaling in tumor suppression and cancer progression. *Nat Genet* 29, 117-129.
- Desir, J., Sznajder, Y., Depasse, F., Roulez, F., Schrooyen, M., Meire, F., Abramowicz, M., 2010. LTBP2 null mutations in an autosomal recessive ocular syndrome with megalocornea, spherophakia, and secondary glaucoma. *Eur J Hum Genet* 18, 761-767.
- Finnson, K.W., McLean, S., Di Guglielmo, G.M., Philip, A., 2013. Dynamics of Transforming Growth Factor β Signaling in Wound Healing and Scarring. *Adv Wound Care (New Rochelle)* 2, 195-214.
- Fresno Vara, J.A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., Gonzalez-Baron, M., 2004. PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* 30, 193-204.
- Gerber, E.E., Gallo, E.M., Fontana, S.C., Davis, E.C., Wigley, F.M., Huso, D.L., Dietz, H.C., 2013. Integrin-modulating therapy prevents fibrosis and autoimmunity in mouse models of scleroderma. *Nature* 503, 126-130.

- Giancotti, F.G., Ruoslahti, E., 1999. Integrin signaling. *Science* 285, 1028-1032.
- Gibson, M.A., Hatzinikolas, G., Davis, E.C., Baker, E., Sutherland, G.R., Mecham, R.P., 1995. Bovine latent transforming growth factor β 1-binding protein 2: molecular cloning, identification of tissue isoforms, and immunolocalization to elastin-associated microfibrils. *Mol Cell Biol* 15, 6932-6942.
- Gold, L.I., 1999. The role for transforming growth factor- β (TGF- β) in human cancer. *Crit Rev Oncog* 10, 303-360.
- Goodwin, A., Jenkins, G., 2009. Role of integrin-mediated TGF- β activation in the pathogenesis of pulmonary fibrosis. *Biochem Soc Trans* 37, 849-854.
- Guo, X., Chen, S.Y., 2012. Transforming growth factor- β and smooth muscle differentiation. *World J Biol Chem* 3, 41-52.
- Haji-Seyed-Javadi, R., Jelodari-Mamaghani, S., Paylakhi, S.H., Yazdani, S., Nilforushan, N., Fan, J.B., Klotzle, B., Mahmoudi, M.J., Ebrahimian, M.J., Chelich, N., Taghiabadi, E., Kamyab, K., Boileau, C., Paisan-Ruiz, C., Ronaghi, M., Elahi, E., 2012. LTBP2 mutations cause Weill-Marchesani and Weill-Marchesani-like syndrome and affect disruptions in the extracellular matrix. *Hum Mutat* 33, 1182-1187.
- Heldin, C.H., Ostman, A., Ronnstrand, L., 1998. Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta* 1378, F79-113.
- Hirani, R., Hanssen, E., Gibson, M.A., 2007. LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein. *Matrix Biol* 26, 213-223.
- Hynes, R.O., 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69, 11-25.
- Inoue, T., Ohbayashi, T., Fujikawa, Y., Yoshida, H., Akama, T.O., Noda, K., Horiguchi, M., Kameyama, K., Hata, Y., Takahashi, K., Kusumoto, K., Nakamura, T., 2014. Latent TGF- β binding protein-2 is essential for the development of ciliary zonule microfibrils. *Hum Mol Genet* 23, 5672-5682.
- Kettle, S., Card, C.M., Hutchinson, S., Sykes, B., Handford, P.A., 2000. Characterisation of fibrillin-1 cDNA clones in a human fibroblast cell line that assembles microfibrils. *Int J Biochem Cell Biol* 32, 201-214.

- Kielty, C.M., Shuttleworth, C.A., 1993. Synthesis and assembly of fibrillin by fibroblasts and smooth muscle cells. *J Cell Sci* 106 (Pt 1), 167-173.
- Kim, D., Kim, S., Koh, H., Yoon, S.O., Chung, A.S., Cho, K.S., Chung, J., 2001. Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production. *FASEB J* 15, 1953-1962.
- Kim, H.P., Wang, X., Zhang, J., Suh, G.Y., Benjamin, I.J., Ryter, S.W., Choi, A.M., 2005. Heat shock protein-70 mediates the cytoprotective effect of carbon monoxide: involvement of p38 β MAPK and heat shock factor-1. *J Immunol* 175, 2622-2629.
- Kim, S.H., Turnbull, J., Guimond, S., 2011. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *J Endocrinol* 209, 139-151.
- Krohn, K., 1999. TGF- β 1-dependent differential expression of a rat homolog for latent TGF- β binding protein in astrocytes and C6 glioma cells. *Glia* 25, 332-342.
- Kuliopulos, A., Mohanlal, R., Covic, L., 2004. Effect of selective inhibition of the p38 MAP kinase pathway on platelet aggregation. *Thromb Haemost* 92, 1387-1393.
- Lan, A., Du, J., 2015. Potential role of Akt signaling in chronic kidney disease. *Nephrol Dial Transplant* 30, 385-394.
- Larson-Casey, J.L., Murthy, S., Ryan, A.J., Carter, A.B., 2014. Modulation of the mevalonate pathway by akt regulates macrophage survival and development of pulmonary fibrosis. *J Biol Chem* 289, 36204-36219.
- Li, H.Y., Zhang, Q.G., Chen, J.W., Chen, S.Q., Chen, S.Y., 2013. The fibrotic role of phosphatidylinositol-3-kinase/Akt pathway in injured skeletal muscle after acute contusion. *Int J Sports Med* 34, 789-794.
- Lin, L.J., Grimme, J.M., Sun, J., Lu, S., Gai, L., Cropek, D.M., Wang, Y., 2013. The antagonistic roles of PDGF and integrin α V β 3 in regulating ROS production at focal adhesions. *Biomaterials* 34, 3807-3815.
- Liu, Z., Wang, F., Chen, X., 2008. Integrin α V β 3 -Targeted Cancer Therapy. *Drug Dev Res* 69, 329-339.
- Margadant, C., Sonnenberg, A., 2010. Integrin-TGF- β crosstalk in fibrosis, cancer and wound healing. *EMBO Rep* 11, 97-105.

- Massam-Wu, T., Chiu, M., Choudhury, R., Chaudhry, S.S., Baldwin, A.K., McGovern, A., Baldock, C., Shuttleworth, C.A., Kielty, C.M., 2010. Assembly of fibrillin microfibrils governs extracellular deposition of latent TGF- β . *J Cell Sci* 123, 3006-3018.
- McKeehan, W., Hardesty, B., 1969. The mechanism of cycloheximide inhibition of protein synthesis in rabbit reticulocytes. *Biochem Biophys Res Commun* 36, 625-630.
- Menz, C., Parsi, M.K., Adams, J.R., Sideek, M.A., Kopecki, Z., Cowin, A.J., Gibson, M.A., 2015. LTBP-2 Has a Single High-Affinity Binding Site for FGF-2 and Blocks FGF-2-Induced Cell Proliferation. *PLoS One* 10, e0135577.
- Miao, Y.j., Yang, M., Zheng, J., Wang, L.y., Du, J., 2013. Role of Akt1 in Cardiac Fibrosis Induced by Angiotensin II. *American Journal of Hypertension* 26, 1635-1635.
- Miyazono, K., Heldin, C.H., 1991. Latent forms of TGF- β : molecular structure and mechanisms of activation. *Ciba Found Symp* 157, 81-89; discussion 89-92.
- Moren, A., Olofsson, A., Stenman, G., Sahlin, P., Kanzaki, T., Claesson-Welsh, L., ten Dijke, P., Miyazono, K., Heldin, C.H., 1994. Identification and characterization of LTBP-2, a novel latent transforming growth factor- β -binding protein. *J Biol Chem* 269, 32469-32478.
- Nemoto, S., Xiang, J., Huang, S., Lin, A., 1998. Induction of apoptosis by SB202190 through inhibition of p38 β mitogen-activated protein kinase. *J Biol Chem* 273, 16415-16420.
- Otsuka, M., Negishi, Y., Aramaki, Y., 2007. Involvement of phosphatidylinositol-3-kinase and ERK pathways in the production of TGF- β 1 by macrophages treated with liposomes composed of phosphatidylserine. *FEBS Lett* 581, 325-330.
- Otterbein, L.E., Bach, F.H., Alam, J., Soares, M., Tao Lu, H., Wysk, M., Davis, R.J., Flavell, R.A., Choi, A.M., 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 6, 422-428.
- Pakyari, M., Farrokhi, A., Maharlooei, M.K., Ghahary, A., 2013. Critical Role of Transforming Growth Factor β in Different Phases of Wound Healing. *Adv Wound Care (New Rochelle)* 2, 215-224.
- Parsi, M.K., Adams, J.R., Whitelock, J., Gibson, M.A., 2010. LTBP-2 has multiple heparin/heparan sulfate binding sites. *Matrix Biol* 29, 393-401.

- Philippe, C.L., 2015. Therapeutic value of an Integrin antagonist in prostate cancer. *Curr Drug Targets*.
- Pohlers, D., Brenmoehl, J., Loffler, I., Muller, C.K., Leipner, C., Schultze-Mosgau, S., Stallmach, A., Kinne, R.W., Wolf, G., 2009. TGF- β and fibrosis in different organs - molecular pathway imprints. *Biochim Biophys Acta* 1792, 746-756.
- Rifkin, D.B., 2005. Latent transforming growth factor- β (TGF- β) binding proteins: orchestrators of TGF- β availability. *J Biol Chem* 280, 7409-7412.
- Roberts, A.B., 1998. Molecular and cell biology of TGF- β . *Miner Electrolyte Metab* 24, 111-119.
- Roux, P.P., Blenis, J., 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 68, 320-344.
- Ruiz-Ortega, M., Rodriguez-Vita, J., Sanchez-Lopez, E., Carvajal, G., Egido, J., 2007. TGF- β signaling in vascular fibrosis. *Cardiovasc Res* 74, 196-206.
- Saharinen, J., Keski-Oja, J., 2000. Specific sequence motif of 8-Cys repeats of TGF- β binding proteins, LTBPs, creates a hydrophobic interaction surface for binding of small latent TGF- β . *Mol Biol Cell* 11, 2691-2704.
- Sarbassov, D.D., Guertin, D.A., Ali, S.M., Sabatini, D.M., 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307, 1098-1101.
- Shiple, J.M., Mecham, R.P., Maus, E., Bonadio, J., Rosenbloom, J., McCarthy, R.T., Baumann, M.L., Frankfater, C., Segade, F., Shapiro, S.D., 2000. Developmental expression of latent transforming growth factor β binding protein 2 and its requirement early in mouse development. *Mol Cell Biol* 20, 4879-4887.
- Sideek, M.A., Menz, C., Parsi, M.K., Gibson, M.A., 2014. LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis. *Matrix Biol* 34, 114-123.
- Sideek, M.A., Teia, A., Kopecki, Z., Cowin, A.J., Gibson, M.A., 2015. Co-localization of LTBP-2 with FGF-2 in fibrotic human keloid and hypertrophic scar. *J Mol Histol*.
- Sinha, S., Heagerty, A.M., Shuttleworth, C.A., Kielty, C.M., 2002. Expression of latent TGF- β binding proteins and association with TGF- β 1 and fibrillin-1 following arterial injury. *Cardiovasc Res* 53, 971-983.
- Soares, M.A., Teixeira, F.C., Fontes, M., Areas, A.L., Leal, M.G., Pavao, M.S., Stelling, M.P., 2015. Heparan Sulfate Proteoglycans May Promote or Inhibit Cancer Progression by

- Interacting with Integrins and Affecting Cell Migration. *Biomed Res Int* 2015, 453801.
- Song, G., Ouyang, G., Bao, S., 2005. The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* 9, 59-71.
- Sui, X., Kong, N., Ye, L., Han, W., Zhou, J., Zhang, Q., He, C., Pan, H., 2014. p38 and JNK MAPK pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents. *Cancer Lett* 344, 174-179.
- Tatler, A.L., Jenkins, G., 2012. TGF- β activation and lung fibrosis. *Proc Am Thorac Soc* 9, 130-136.
- Tsubaki, M., Yamazoe, Y., Yanae, M., Satou, T., Itoh, T., Kaneko, J., Kidera, Y., Moriyama, K., Nishida, S., 2011. Blockade of the Ras/MEK/ERK and Ras/PI3K/Akt pathways by statins reduces the expression of bFGF, HGF, and TGF- β as angiogenic factors in mouse osteosarcoma. *Cytokine* 54, 100-107.
- Vehvilainen, P., Hyytiainen, M., Keski-Oja, J., 2003. Latent transforming growth factor- β -binding protein 2 is an adhesion protein for melanoma cells. *J Biol Chem* 278, 24705-24713.
- Woodard, A.S., Garcia-Cardena, G., Leong, M., Madri, J.A., Sessa, W.C., Languino, L.R., 1998. The synergistic activity of α V β 3 integrin and PDGF receptor increases cell migration. *J Cell Sci* 111 (Pt 4), 469-478.
- Woods, A., Couchman, J.R., 2001. Syndecan-4 and focal adhesion function. *Curr Opin Cell Biol* 13, 578-583.
- Xiao, Y.Q., Freire-de-Lima, C.G., Schiemann, W.P., Bratton, D.L., Vandivier, R.W., Henson, P.M., 2008. Transcriptional and Translational Regulation of Transforming Growth Factor- β Production in Response to Apoptotic Cells. *J. Immunol* 181, 3575-3585.
- Zhang, G., Liu, Z., Xu, H., Yang, Q., 2016. miR-409-3p suppresses breast cancer cell growth and invasion by targeting Akt1. *Biochem Biophys Res Commun* 469, 189-195.

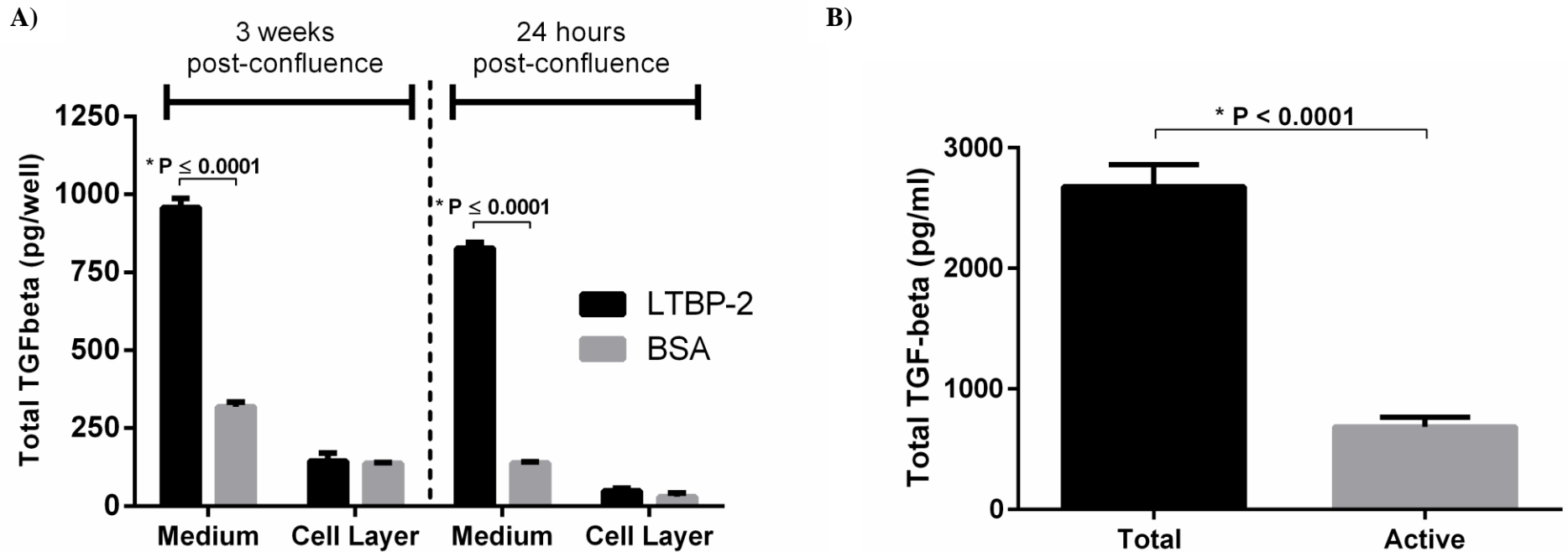


Figure 5.1 Exogenous LTBP-2 increases TGF- β in conditioned medium which is independent of extracellular matrix.

A) MSU1.1 cells were cultured for 24 hours or 3 weeks post-confluence then incubated in serum-free medium overnight containing 10 $\mu\text{g/ml}$ LTBP-2 (black columns) or BSA control (grey columns) prior to TGF- β assay. Total TGF- β in the conditioned medium and cell layer was measured by ELISA (see material and methods). LTBP-2 caused a highly significant increase in TGF- β without the presence of extensive extracellular matrix. **B)** The secreted TGF- β is mainly in inactive form. The medium from 3-week post-confluence LTBP-2-treated cultures was analysed with and without acid treatment for total and active TGF- β respectively. Approximately 70% of the TGF- β in the conditioned medium was inactive. Mean values \pm S.D. from triplicate wells are shown.

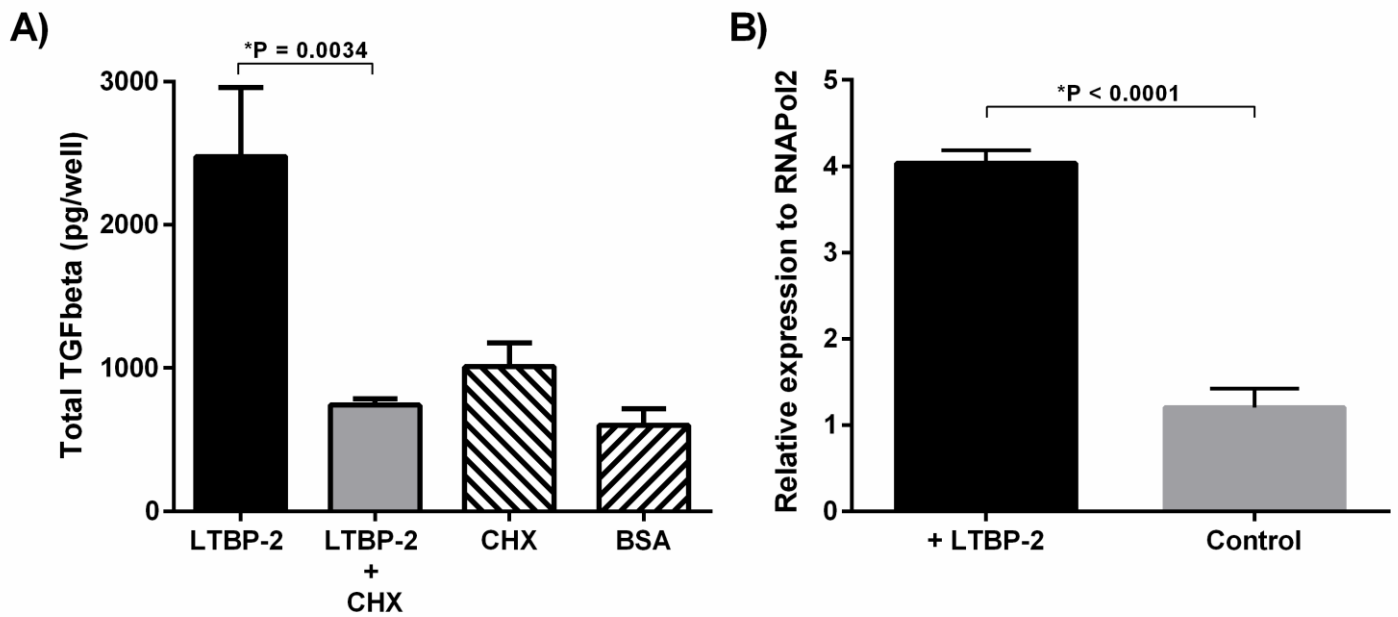


Figure 5.2 LTBP-2 upregulates TGF-β expression in MSU 1.1 cells

A). MSU1.1 cells were cultured for 24 h post-confluence, then incubated for 16 h in serum-free media with 10 μg/ml LTBP-2, or both LTBP-2 and 10 μg/ml cycloheximide (CHX). The negative controls included 10 μg/ml BSA, or CHX only. The conditioned media was analysed for TGF-β content (see material and methods). Mean values ± S.D. from triplicate wells are shown. **B)** MSU1.1 cells were grown to 24 h post-confluence, then incubated for 16 h with 10 μg/ml full length LTBP-2 or 10 μg/ml BSA control. Total RNA was harvested from the cells, then reverse transcribed into cDNA for use in qPCR. The cDNA was analysed for TGFβ, and normalised to RNAPolIII. LTBP-2 stimulated a threefold increase in the levels of TGFβ mRNA, compared to the BSA control. Mean values ± S.D. from triplicate wells are shown.

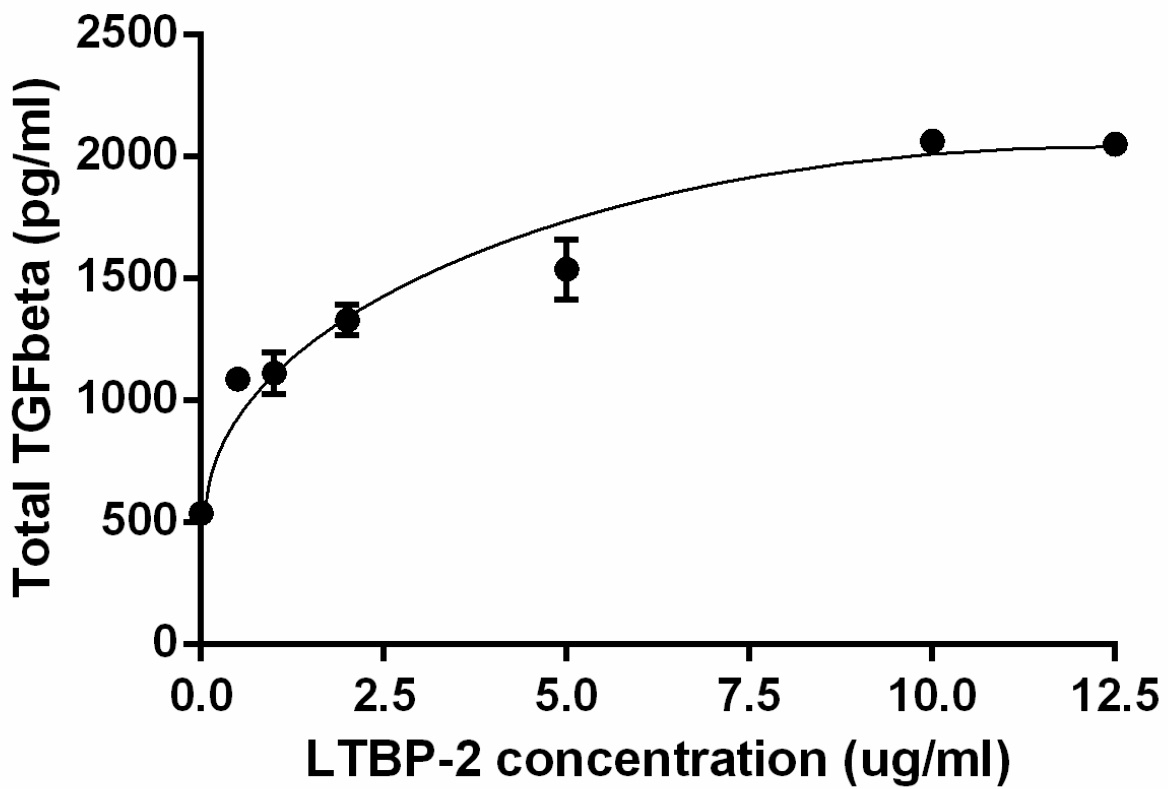


Figure 5.3 LTBP-2 upregulates TGF- β in a dose-dependent manner.

Confluent cultures of MSU1.1 cells were incubated with LTBP-2 at a range of concentrations (0 – 12.5 $\mu\text{g/ml}$). After 16 h the medium was analysed for TGF- β content (see material and methods). Mean values \pm S.D. from triplicate wells are shown.

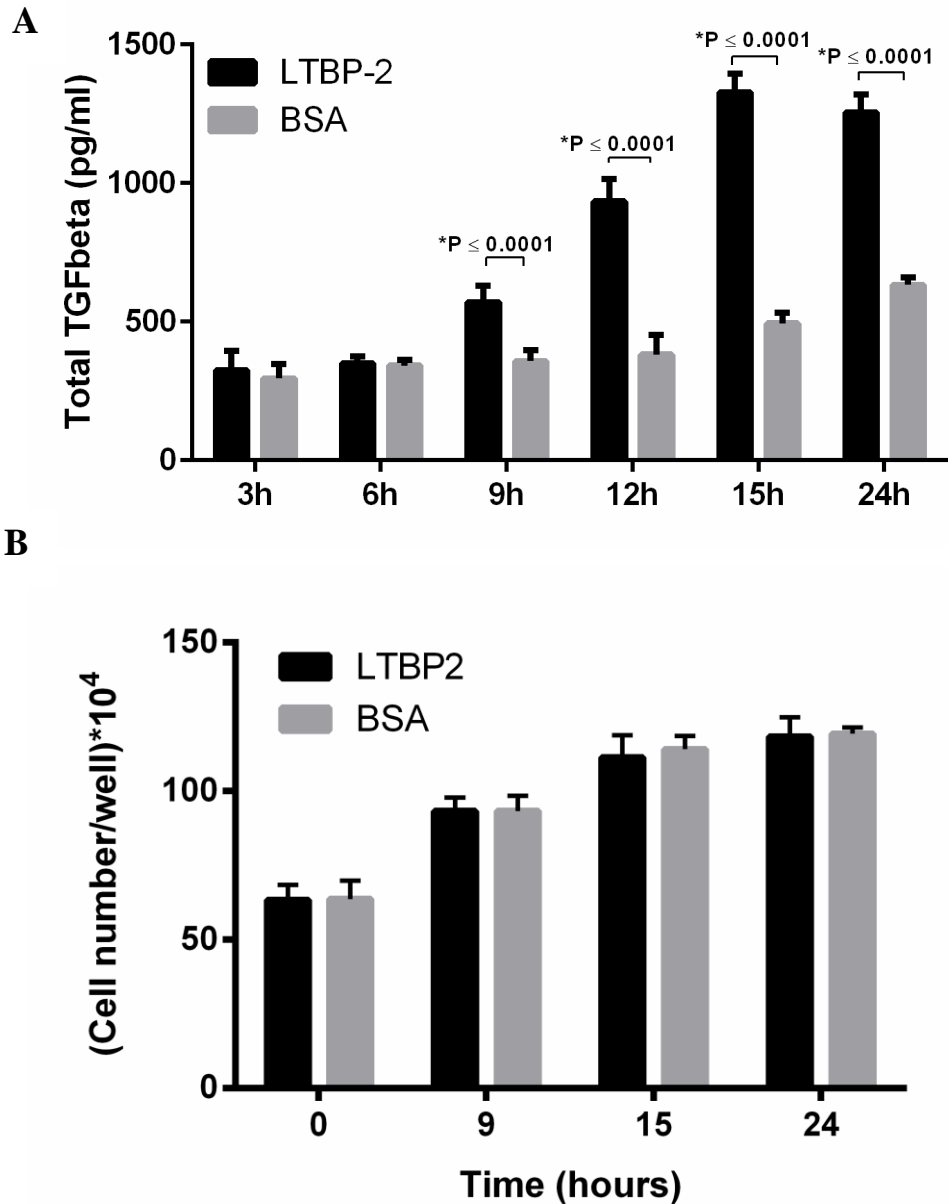


Figure 5.4 Time course for LTBP-2 stimulation of TGF- β production.

MSU1.1 cells were grown on 12 well plates to 24 h post-confluence and incubated with LTBP-2 (10 μ g/ml) for various times (3-24 h). BSA at the same concentration was added to control wells. **A.** TGF- β accumulation in the medium was measured by ELISA as described in materials and methods. The TGF- β concentration peaked at around 15 h. **B.** At the end of each incubation, the cells were counted. Note LTBP-2 had no effect on cell proliferation above the BSA control. Mean values \pm S.D. from triplicate wells are shown.

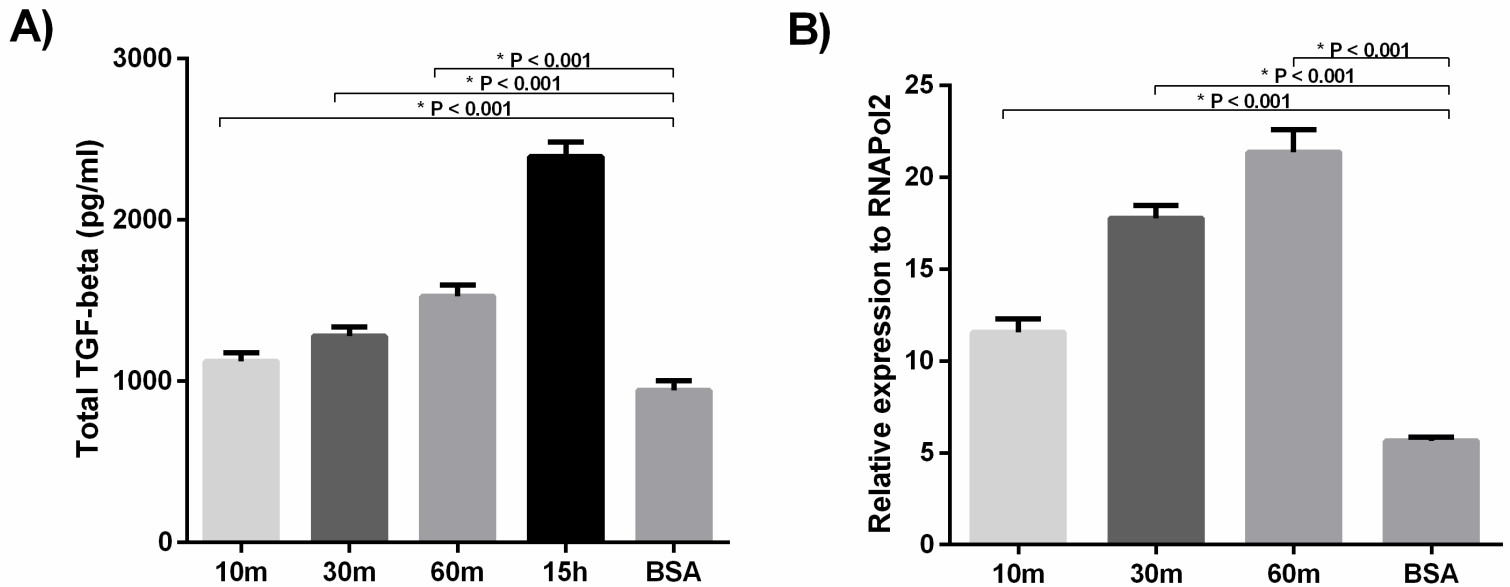


Figure 5.5 A short incubation of MSU 1.1 cells with LTBP-2 is sufficient to upregulate TGF- β expression and secretion.

A) Quantitation of TGF- β in the medium. MSU1.1 cells were grown to 24 h post-confluence, then incubated in serum-free medium with full length LTBP-2 (10 μ g/ml) for various time periods (10 mins - 60 min). After each incubation period, the medium were discarded and replaced with fresh serum-free medium lacking LTBP-2. The conditioned medium was collected at 15 h and analysed for TGF- β content (see material and methods). The results were compared to cells incubated for 15h with LTBP-2 or BSA control. Significant increase in TGF- β was detected with as little as 10 minutes of LTBP-2 exposure ($p < 0.001$).

B) Quantitation of TGF- β mRNA. At the end of each 15 h incubation above, total RNA was harvested from the cells, then reverse transcribed into cDNA. Quantitative PCR was performed to determine cellular TGF- β mRNA levels as described in methods. Values were expressed relative to RNAPolIII housekeeping gene. The control consisted of cells exposed to BSA instead of LTBP-2 for 15 h. Significant increase in TGF- β mRNA was detected after 15 h with as little as 10 minutes of LTBP-2 exposure ($p < 0.001$). Mean values \pm S.D. from triplicate wells are shown.

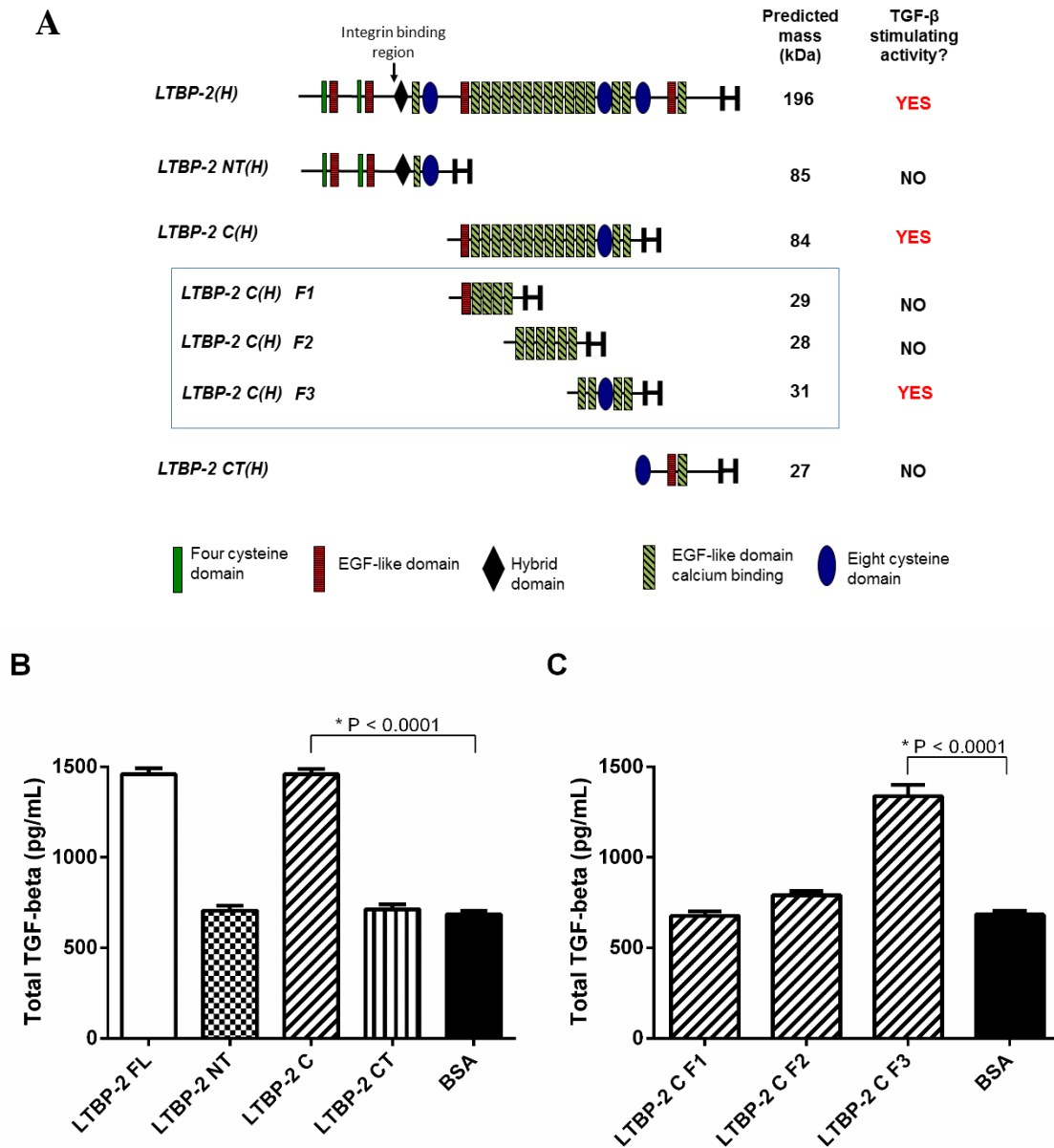


Figure 5.6 A central region of LTBP-2 consisting of an 8-cys motif flanked by pairs of EGF-like repeats (fragment LTBP-2C F3) contains the stimulatory activity.

A) Schematic diagram of recombinant LTBP-2 fragments. Protein fragments produced specifically for this study (LTBP-2C(H) F1, F2 and F3) are highlighted within the blue box.

B) MSU1.1 cells were grown to 24 h post-confluence, then incubated for 16 h with full-length LTBP-2 (10 μ g/ml), each of three fragments spanning LTBP-2 (LTBP-2 NT, LTBP-2 C, LTBP-2 CT) or BSA control.

C) Fresh cells were subsequently incubated with each of three sub-fragments F1, F2 and F3 spanning central fragment LTBP-2 C(H). In both experiments the conditioned medium was analysed for TGF- β content (see material and methods). Mean values \pm S.D. from triplicate wells are shown.

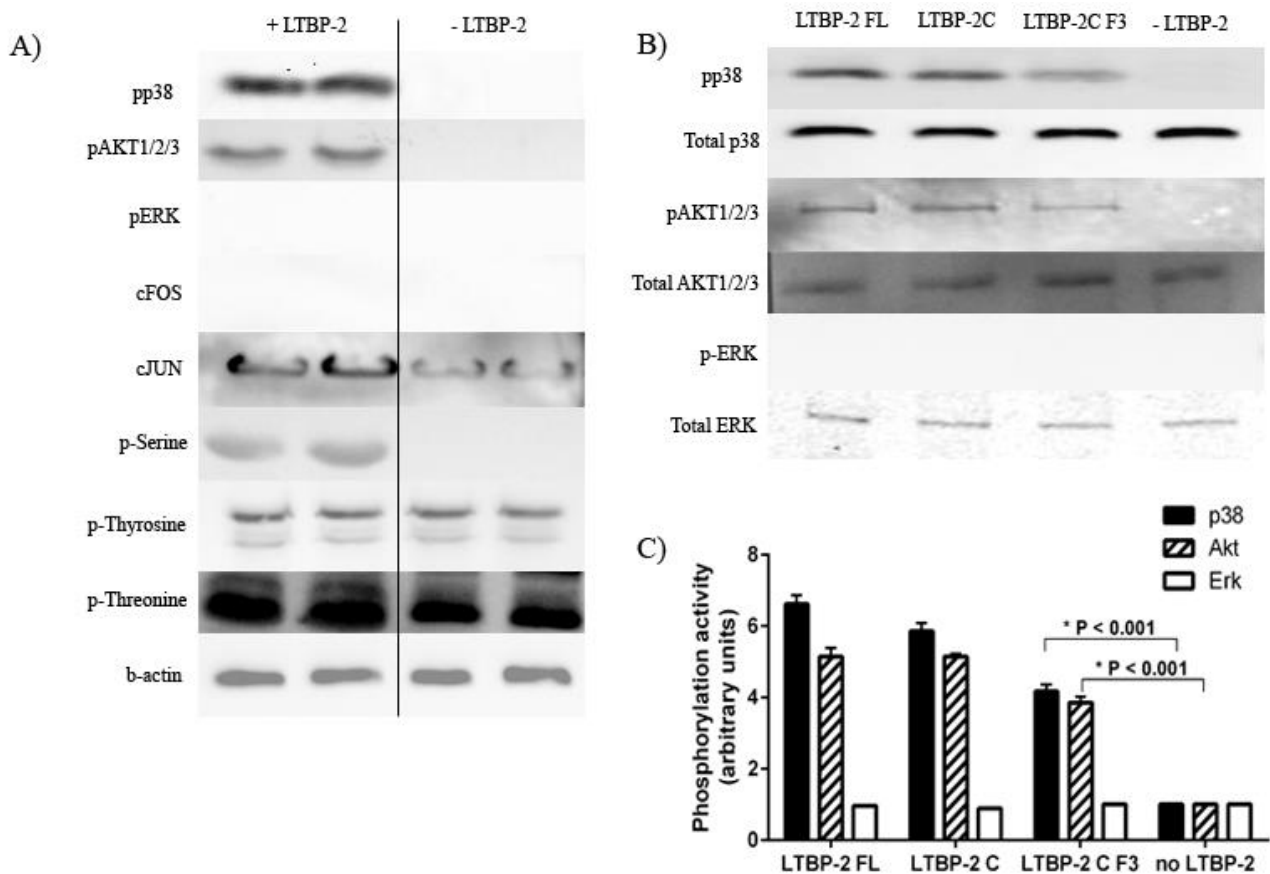


Figure 5.7 Exogenous LTBP-2 stimulates phosphorylation of AKT and p38 MAPK in human fibroblasts

A) MSU 1.1 cells (1×10^5 cells/well) were treated with or without LTBP-2 (10 μg/ml) for 30 minutes. Total cell lysates were immunoblotted for phosphorylation of candidate signalling molecules including phospho-serine, phospho-thyrosine, phospho-threonine, phospho-p38, phospho-Akt1/2/3, phospho-ERK, c-FOS, c-JUN, and for β-actin internal control as described in methods. Note major phosphorylation of p38 and AKT1/2/3 but no stimulation of ERK or cFOS.

B) Cell were treated for 30 minutes with full length LTBP-2 and fragments LTBP-2 C(H) and LTBP-2 C(H) F3 containing TGF-β stimulating activity. Cell lysates were immunoblotted for total and phosphorylated p38, Akt1/2/3, and ERK.

C) The ratio of phospho-protein to total protein for each signal molecule from each treatment is expressed relative to the average value from no LTBP-2 control cells (given an arbitrary value of 1.0). Mean values ± S.D. from duplicate lanes.

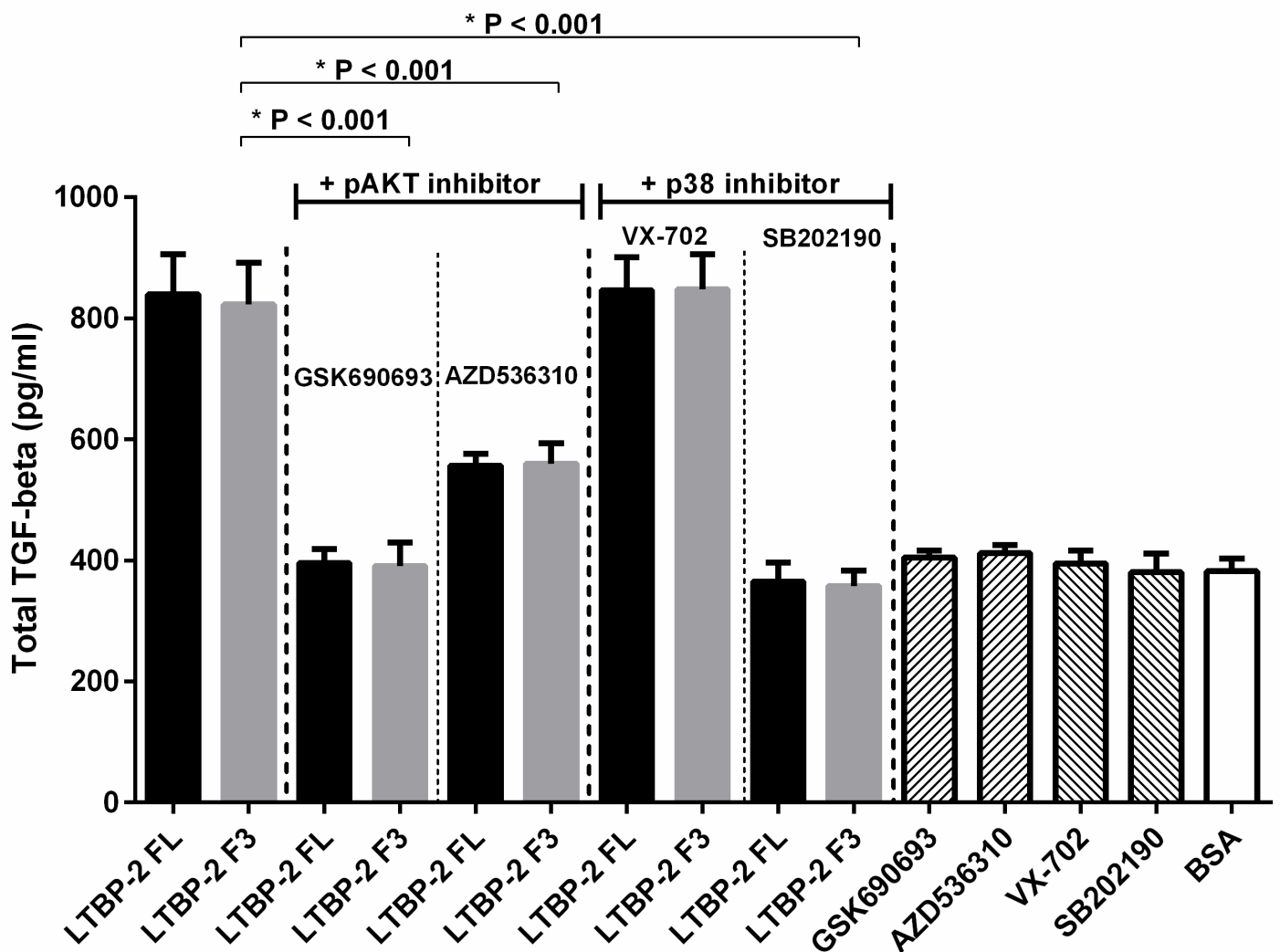


Figure 5.8 LTBP-2 stimulation of TGF- β upregulation involves Akt and p38 MAPK signalling pathways.

MSU1.1 cells were grown to 24 h post-confluence, then incubated in serum-free medium with or without inhibitor (10 μ M) of Akt (GSK690693 or AZD536310) or p38 (VX-702 or SB202190) for 2 h, followed by addition of full length LTBP-2 or bioactive fragment LTBP-2C F3 to a final concentration of 10 μ g/ml. Incubation was continued for a further 15 h and the conditioned medium was analysed for TGF- β content (see material and methods). Controls involved incubations with individual inhibitors or BSA in the absence of LTBP-2. Mean values \pm S.D. from triplicate wells are shown.

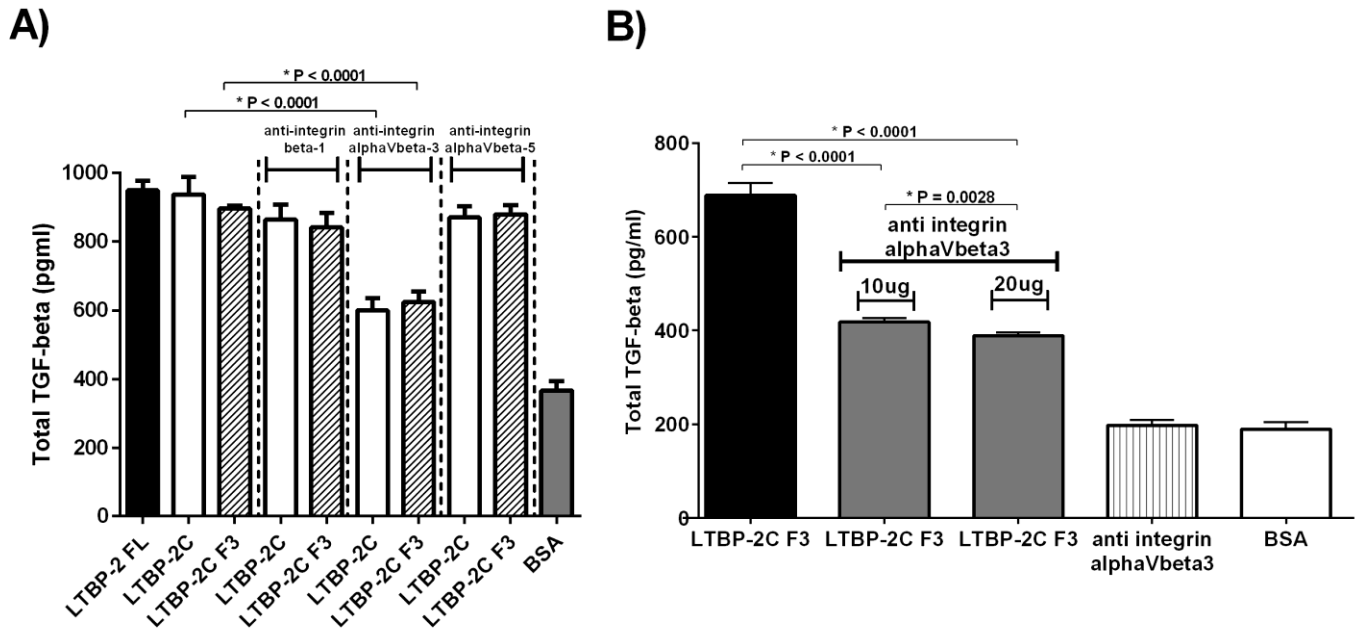


Figure 5.9 Blocking of integrin $\alpha V\beta 3$ receptors partially attenuates TGF- β production induced by LTBP-2.

A) Effects of blocking antibodies to integrins on LTBP-2 stimulated TGF- β expression. MSU1.1 cells were pre-treated with blocking antibody for integrins β -1 (10 μ g/ml), $\alpha V\beta 3$ (10 μ g/ml) and $\alpha V\beta 5$ (10 μ g/ml) for 2 h. The cells were then incubated with bioactive LTBP-2 fragment (LTBP-2 C(H) or LTBP-2 C(H) F3) (concentration equimolar to 50nM rLTBP-2) for 15 h and the conditioned medium was analysed for TGF- β content (see material and methods). Control incubation included LTBP-2 or bioactive fragment without antibody and BSA in the absence of LTBP-2. Mean values \pm S.D. from triplicate wells are shown.

B) Increasing the concentration of anti-integrin $\alpha V\beta 3$ antibody caused little further attenuation of LTBP-2 induced TGF- β upregulation. The above experiment was repeated using two concentrations of anti-integrin $\alpha V\beta 3$ (10 μ g/ml and 20 μ g/ml) prior incubation with LTBP-2 C F3 fragment (equimolar concentration of 5nM rLTBP-2). The conditioned medium was collected at 15 h and analysed for TGF- β content (see material and methods). Controls involved incubations with anti-integrin antibody, or BSA in the absence of LTBP-2. Mean values \pm S.D. from triplicate wells are shown. Doubling of the anti avb3 antibody concentration caused only a very slight (although significant) additional reduction in TGF- β stimulation suggesting the blocking effect was close to saturation.

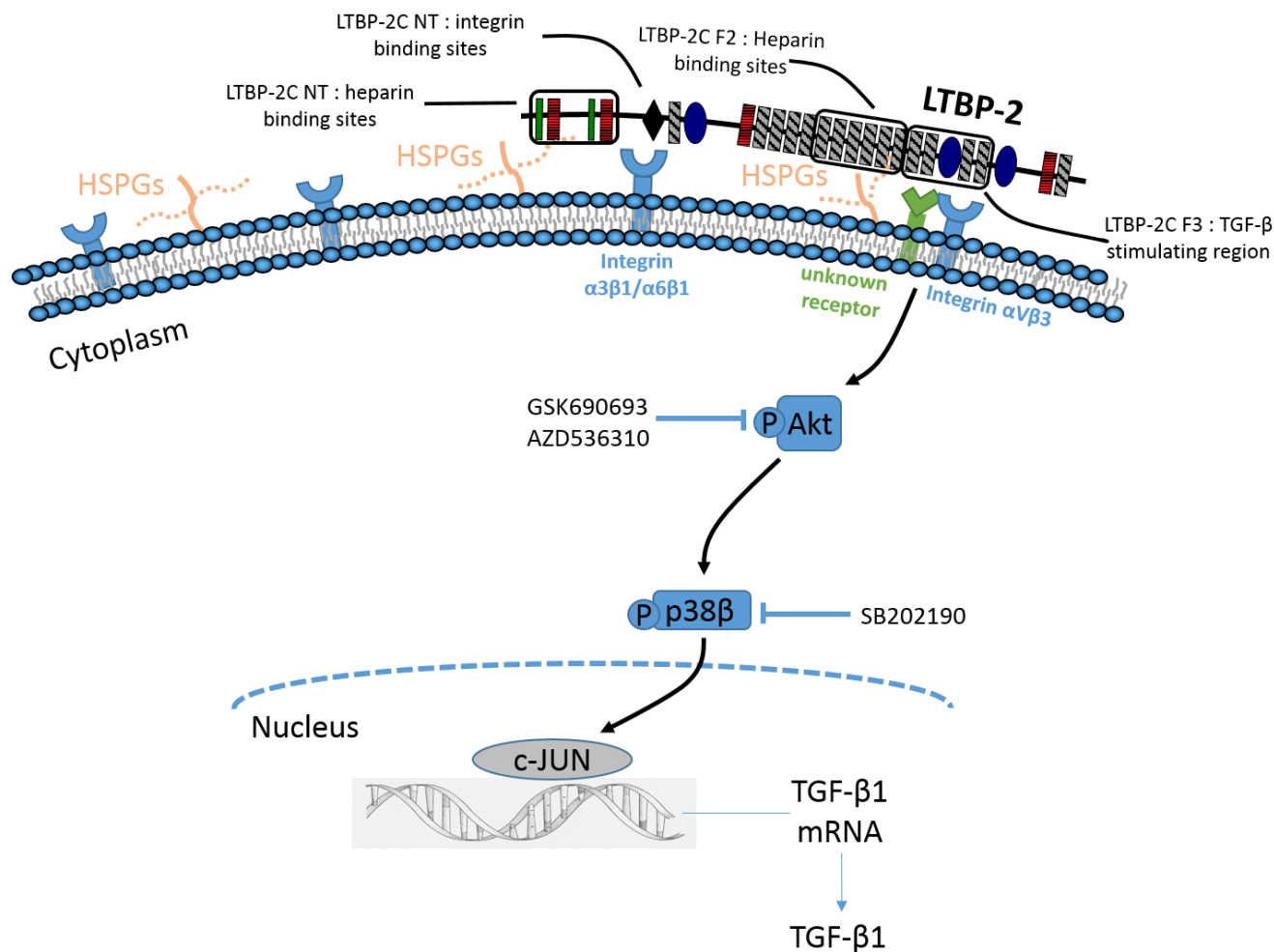


Figure 5.10. Schematic representation of possible signalling pathways involved in the production of TGF- $\beta 1$ by LTBP-2.

LTBP-2 binds to an unidentified receptor (perhaps complexed with alpha V beta 3 integrin) and induces sequential phosphorylation of Akt and p38 β pathways. This triggers c-JUN phosphorylation and TGF- $\beta 1$ mRNA transcription. Arrows indicate positive (stimulatory) effects. Inhibitors used to specifically block respective signalling molecules (pathways) are also depicted.

CHAPTER 6: Discussion

6.1 LTBP-2 plays an important role in elastinogenesis and elastic fibre assembly

The research project presented in this thesis was aimed to increase our knowledge of the functions of LTBP-2 in elastic fibre structure and assembly, growth factor storage and upregulation, and disease processes. The present study showed (Chapter 2) that LTBP-2 blocks rather than enhances the critical interaction of tropoelastin with fibulin-5, presumably by competing with tropoelastin for binding to fibulin-5. Therefore it seems unlikely LTBP-2 forms tertiary complexes as suggested by Hirai *et al.* (2007) since its binding site on fibulin-5 will be blocked by tropoelastin. We suggested that LTBP-2 might be involved in release of elastin globules from complexes with fibulin-5 during the deposition process. However, the N-terminal fragment of LTBP-2 (LTBP-2 NT(H)) inhibits the fibulin-5 interaction with tropoelastin to a lesser extent than the full-length molecule although all fibulin-5 binding activity is contained in this fragment. The cause for this difference is uncertain but it may be due to a second tropoelastin binding site at the N-terminal end of the fibulin-5 which is not completely blocked by fibulin-5/LTBP-2 NT(H) interaction but can be blocked indirectly by the larger full-length LTBP-2 molecules. These results imply that LTBP-2 may function not only as a structural protein, but also as a regulatory protein that modulates other elastic fibre components during elastic fibre assembly.

Recent studies from our lab revealed that LTBP-2 has a strong affinity for heparin/heparin sulphate and has three binding sites located in the N-terminal region of the molecules which is adjacent to the documented fibulin-5 binding site and at least one site in the central region as shown in fig 7.1 (Parsi *et al.*, 2010). Unidentified HSPGs appear to play a critical role in the formation of the fibrillin microfibrils and in elastin deposition during elastic fibre assembly (Tiedemann *et al.*, 2001; Cain *et al.*, 2005; Cain *et al.*, 2008). HSPGs have been shown to assist aggregation of tropoelastin secreted from cells to form elastin microassemblies via coacervation at the cell surface (Clarke *et al.*, 2006; Cain *et al.*, 2008). Since LTBP-2 has a very strong affinity for heparin/heparin sulphate and is associated with elastic fibre assembly, it was important to investigate the effect of LTBP-2 on the interaction of tropoelastin with heparin. Interestingly, we found excess LTBP-2

partially inhibited (up to approximately 65%) the interaction of tropoelastin with heparin. Even though the inhibition was incomplete, the finding indicates LTBP-2 may be able to compete with some but not all tropoelastin binding sites on the heparin molecule. Our findings suggest LTBP-2 may assist the release of elastin-microassemblies from the cell by competing for binding with cell surface HSPGs. The mechanism of how elastin microassemblies are released from cell surface to be deposited on to fibrillin-microfibril is still unclear and the subject of much research (Wagenseil and Mecham, 2007). Confocal microscopy demonstrated extensive co-distribution of LTBP-2 with fibulin-5, fibrillin-1 and fibronectin in the matrix elaborated by fibroblasts in culture suggestive of a molecular interaction between LTBP-2 and fibulin-5 on the surface of the microfibrils (Chapter 2). In addition, LTBP-2 partially localized with perlecan and syndecan-4 HSPGs suggestive of LTBP-2 interactions with HSPGs at cell surface and within the ECM (Sideek *et al.*, 2014). A similar study by other researchers (Hayes *et al.*, 2014) has demonstrated strong co-localisation of LTBP-2 with perlecan in the medial layer of the developing foetal aorta and foetal human intervertebral discs (IVDs) suggesting LTBP-2 could potentially modulate some of HS-dependent activities by competitively occupying of HS binding sites on perlecan. In addition, LTBP-2 could play a role in the stabilization and organization of the interaction between fibrillin-1 and the core protein of perlecan via HS-chains of perlecan. Further studies on the interaction of LTBP-2 with HSPGs and other proteoglycan may provide major insights into elastic fibre assembly.

Our hypothesis that LTBP-2 can be a negative modulator of elastinogenesis was confirmed when we showed that addition of recombinant LTBP-2 to ear cartilage chondrocytes cultures blocked elastinogenesis in a concentration-dependent manner. Interestingly, fibrillin-1 deposition into the matrix was only affected at much higher concentrations of LTBP-2 than those required to block elastinogenesis. This indicated the elastin deposition is the major step inhibited by LTBP-2 rather than microfibril assembly. The exact mechanism(s) of LTBP-2 inhibition is unclear but these studies point to two major interactions important in the elastinogenic process inhibited by LTBP-2 in vitro, the binding of tropoelastin to a) heparan sulphate and b) fibulin-5. It is possible that exogenous LTBP-2 may disrupt binding of tropoelastin to cell surface proteoglycans inhibiting assembly of micro-aggregates and/or preventing assembly of tropoelastin-fibulin-5 complexes, inhibiting the normal deposition of elastin onto the fibrillin-microfibril scaffold

(Halper and Kjaer, 2014). The influence of LTBP-2 during microfibril assembly remains unclear. A recent study showed LTBP-2 and fibulin-5 localisation on "oxytalan" fibres, which are essentially pure bundles of fibrillin-microfibrils. Fibulin-5 was found to control the rate of formation and essential for coalescence of these fibres (Nakashima *et al.*, 2009). Interestingly, LTBP-2 also controls the maturation of oxytalan fibres by negatively influencing the function of fibulin-5 during coalescence of microfibrils and oxytalan fibres produced by cultured fibroblasts (Tsuruga *et al.*, 2012) which is consistent with our findings. Moreover, TGF- β signaling appears to be involved in the oxytalan fibre coalescence (Tsuruga *et al.*, 2012) and our work with bioactive LTBP-2 fragments indicates LTBP-2 can elevate TGF- β in fibroblast cultures suggesting LTBP-2 could be directly involved during the coalescence of these fibres. Studies by Langton *et al.* (2011) showed significant upregulation of LTBP-2 in epidermal skin with increasing age. Aged skin has less elasticity and becomes more lax and wrinkled caused by disintegration of the normal elastic fibre networks. Up-regulation of LTBP-2 in aged skin may be linked to reduction in its elasticity. Overall our studies provided key evidence that LTBP-2 can have a negative regulatory role during elastic fiber assembly.

Recent studies by Inoue *et al.* (2014) using an LTBP-2 null mouse model showed that LTBP-2 is an essential component for the formation of microfibril bundles in ciliary zonules of the eye. Even though the LTBP-2 knockout mice survive to adulthood, lens luxation is present caused by compromised ciliary zonule formation. Fibrillin microfibrillar bundles were almost absent from the ciliary zonules indicating LTBP-2 may have a role in the aggregation of fibrillin microfibrils into bundles that provides the mechanical strength for the zonules (Inoue *et al.*, 2014). In addition, recombinant LTBP-2 promoted the formation of microfibril meshworks and restored unfragmented and bundled ciliary zonules in organ cultures of LTBP-2 null mice eyes (Inoue *et al.*, 2014). Remarkably, the complete absence of LTBP-2 does not appear to affect elastic fibre assembly in all tissues, since LTBP-2 null humans and mice develop normal elastic fibres except for those of ocular tissues (Ali *et al.*, 2009; Narooie-Nejad *et al.*, 2009; Desir *et al.*, 2010; Kumar *et al.*, 2010; Inoue *et al.*, 2014). These phenotypes suggest that LTBP-2 may specifically control the fine organization of the nascent elastic fibres in particular tissues. Formation of elastic fibres may be functional in the absence of LTBP-2 except where fine, specialized elastic fibre architecture is required, such as the ocular trabecular network. Alternatively, other matrix proteins

may compensate for the lack of LTBP-2 in the other tissue where LTBP-2 is normally highly expressed such as lung, aorta, and skin. Candidate proteins LTBP-1 and LTBP-3 are expressed in the ciliary body, but they do not compensate for the lack of LTBP-2 suggesting that LTBP-2 has an essential, specific function in ocular tissue. Further work is necessary to investigate fully the role of LTBP-2 in other tissues where it is strongly expressed including fetal aorta and lungs. Overall, the above studies support our findings in Chapter 2 that LTBP-2 can inhibit elastinogenesis and reveal a potentially important role in elastic fibre biology.

6.2 Role of LTBP-2 in the regulation of growth factor activities

Several studies have now documented LTBP-2 involvement in several chronic genetic and acquired diseases. Interestingly, a recent study reported a patient with Weill-Marchesani syndrome (WMS) linked to a mutation in the LTBP-2 gene. Some affected patients also had varied Marfan-like characteristics including tall structure, arachnodactyly, joint hyper mobility, osteopenia, aortic stenosis and aortic valve problem and many of the phenotypes of WMS and Marfan syndrome caused by altered TGF- β levels and signaling in these patients (Haji-Seyed-Javadi *et al.*, 2012). Since fibrillin-1 mutations also cause WMS and Marfan syndrome, this clearly confirms a functional link between LTBP-2 and fibrillin-1 biology, especially relating to abnormal TGF- β signaling. WMS is characterized by skeletal, cardiovascular and ocular defects as well as exhibiting thickened fibrotic skin suggesting a connection between LTBP-2 and fibrosis. Moreover, we also have evidence from our lab that LTBP-2 upregulates TGF- β in fibroblasts (Chapter 5), a key mediator of fibrosis (Mauviel, 2005). We therefore investigated the relationship of LTBP-2 to fibrosis (Haji-Seyed-Javadi *et al.*, 2012) by determining LTBP-2 localisation and expression in keloid and hypertrophic scar tissues. Both keloid and HTS tissues represent fibrotic conditions caused by abnormal wound healing including excessive collagen production by fibroblasts (Aarabi *et al.*, 2007; Verhaegen *et al.*, 2009). Previously, LTBP-2 synthesis has been shown to increase following induced injury to arteries in a porcine model suggesting the involvement of LTBP-2 in tissue repair processes and modulation of growth factor storage and activity (Sinha *et al.*, 2002). In addition, a significant increase of LTBP-2 mRNA and protein level was detected in end-stage heart failure in human patients and in a mice model accompanied by obvious cardiac ECM remodelling and fibrosis (Gabrielsen *et al.*, 2007; Wei *et al.*, 2008; Bai *et*

al., 2012). Thus LTBP-2 appears to be directly involved in the pathophysiology of heart failure particularly with the development of cardiac remodelling and fibrosis. Interestingly, we found LTBP-2 expression was greatly upregulated in HTS and keloid tissue compared to normal skin and LTBP-2 consistently matched the distribution of fibrillin-1 microfibrils and elastin. This result is consistent with previous studies showing that LTBP-2 is strongly co-localized with fibrillin microfibrils and developing elastic fibres in fetal tissues and in tissue remodelling (Gibson *et al.*, 1995; Sinha *et al.*, 2002). The findings support the idea that LTBP-2 has roles in elastic fibre formation and regulation of microfibrils-associated growth factors.

6.3 Co-localization of LTBP-2 and FGF-2 in fibrotic human tissues

Given the above evidence, it seems clear that LTBP-2 has some as yet unidentified role in modulation of growth factor biology, storage and activity in fibrosis. Initially, we screened major cytokines involved in the pathogenesis of fibrotic disorders that could potentially interact with LTBP-2. We identified a very strong interaction of LTBP-2 with FGF-2, a well-known protein that has important roles in range of processes including angiogenesis, tissue remodeling and wound healing (Agasse *et al.*, 2007; Mikroulis *et al.*, 2007; Akita *et al.*, 2008). Interestingly, proliferation assays revealed that LTBP-2 inhibits FGF-2-induced fibroblast proliferation in vitro suggesting LTBP-2 could inhibit anti-fibrotic functions of FGF-2 during fibrosis and wound healing. FGF-2 promotes differentiation, migration and proliferation in fibroblasts and has an influence on tissue remodelling and wound healing (Ortega *et al.*, 1998; Govindraj *et al.*, 2006; Kardami *et al.*, 2007). More importantly, FGF-2 has been shown to have anti-fibrotic functions and promote healing in many models of cell and tissue injury (Bikfalvi *et al.*, 1997; Fu *et al.*, 2000; Akita *et al.*, 2008; Tan *et al.*, 2008). Studies by Ono *et al.* demonstrated that post-operative administration of FGF-2 inhibits hypertrophy widening of the lesion in chronic wound including HTS and keloid (Ono *et al.*, 2007). In addition, FGF-2 can attenuate fibrosis and promote healing by inhibiting the SMAD2/SMAD3 intracellular signalling system of the TGF- β 1/SMAD-dependent pathway (Shi *et al.*, 2013). FGF-2 treatment also causes the down-regulation of TGF- β -induced collagen production (Xie *et al.*, 2008; Shi *et al.*, 2013), upregulation of MMP1, the metalloproteinase responsible for cleaving collagen (Eto *et al.*, 2012), and reduction in IL-6 levels, a cytokine that increases production of collagen and fibrosis in fibrosis (Duncan and Berman, 1991; Ray *et al.*,

2013). Following tissue injury, multiple FGF-2 molecules are released by proteases and heparanases activity and complexed to HS chains and subsequent interaction with cell surface FGF-receptors causing clustering of the FGFR molecules. This stimulates and enhances several major signaling pathways including RAS/MAPK, PI3k/Akt, PLC- γ , and ERK1/2 pathways (Kardami et al., 2007; Yu et al., 2007; Schultz and Wysocki, 2009). Interestingly, both the central heparin binding region and the FGF-2 binding site on LTBP-2 are present within six calcium-binding EGF-like motifs (EGFs 9-14) (Menz *et al.*, 2015) suggesting LTBP-2 could modulate FGF-2 release and activation via inhibiting FGF-2 complexing with HS chains. Perlecan is a candidate HSPG for such an interaction. It is involved in cellular differentiation and proliferation, angiogenesis, osteogenesis and chondrogenesis (Gomes Jr *et al.*, 2004; Knox and Whitelock, 2006). Many of these processes depend on the interaction with a large number of components through HS side chains of perlecan which sequester growth factors including FGF-2, BMPs, VEGF and PDGF (Whitelock *et al.*, 1999; Zoeller *et al.*, 2009; Lord *et al.*, 2014). The HS chains on perlecan have been shown to sequester and present FGF-2 to its cognate cell surface receptors. Our binding studies suggested LTBP-2 could potentially modulate FGF-2 storage and activity by binding to perlecan and FGF-2, blocking the activity of FGF-2-heparan sulphate complexes.

The fibroblast cultures proved not to be a suitable model to study the interaction of LTBP-2 and FGF-2 since FGF-2 is stored intracellularly and only secreted from cells following tissue insult, immunohistochemical analysis was undertaken in human fibrotic skin samples (Schultz and Wysocki, 2009). Interestingly, FGF-2 was also found to be strongly co-localized with LTBP-2 in both fibrotic HTS and keloid skin from several patients (Chapter 4) suggesting that synthesis rate for both proteins was greatly up-regulated in the fibrotic condition. Importantly, these studies also indicated that the two proteins might directly interact and influence the biological activity of each other *in vivo*. Since LTBP-2 depends on fibrillin-1 microfibrils for incorporation into the ECM, loss of microfibril structure is likely to disrupt matrix sequestration of LTBP-2 and any attached protein such as FGF-2 (Vehvilainen *et al.*, 2009). It maybe that elevated LTBP-2 exacerbates the fibrotic process by removing active FGF-2 from the repairing tissue, inhibiting its contribution to wound repair. The study suggested that LTBP-2 might act as a matrix store of latent FGF-2 or as a mechanism for removal of excess active FGF-2 during tissue repair and remodelling. Alternatively, LTBP-2 could bind FGF-2 and direct it to the wound area and modulate the release

and activation of FGF-2 depending on the stage of wound healing process. It remains unclear which stages of fibrosis show elevated expression of LTBP-2 and FGF-2. Further research using cellular and animal models of fibrosis will be useful to elucidate the exact role of LTBP-2 and its influence on FGF-2 in the various stages of the process.

6.4 LTBP-2 stimulates the expression and secretion of TGF- β

For some years, knowledge regarding the role of LTBP-2 role in TGF- β modulation remained enigmatic and very limited. Studies from our lab demonstrated that LTBP-2 specifically binds to fibrillin-1 rather than fibrillin-2 and competes with LTBP-1 to bind with fibrillin-1. This led to our hypothesis that LTBP-2 may indirectly increase TGF- β bioavailability by releasing LTBP-1 from microfibrils through competitive binding. However, our studies in Chapter 5 revealed that addition of exogenous LTBP-2 to the medium of cultured human MSU 1.1 fibroblasts led to an increase in TGF- β levels in the conditioned medium but the increase in TGF- β was found to be fresh synthesis by the cells rather than displacement of matrix-stored TGF- β . We also identified a region consisting of an eight-cys motif (2nd domain) flanked by pairs of EGF-like repeats (14-17 EGF-like domain) in the central region of LTBP-2 (LTBP-2C F3) which contains the TGF- β activating site (see figure 7.1). This finding fits with other research suggesting LTBP-2 may be involved in regulation of TGF- β expression, storage and activation within tissues (Krohn, 1999; Hirani *et al.*, 2007; Haji-Seyed-Javadi *et al.*, 2012). Both LTBP-2 and fibrillin-1 mutations cause MWS and Marfan-like traits and both phenotypes are believed to involve aberrant TGF- β signaling (Faivre *et al.*, 2003; Blyth *et al.*, 2008; Haji-Seyed-Javadi *et al.*, 2012). In a rat model, reduction of LTBP-2 mRNA inhibits TGF- β activity strongly suggesting LTBP-2 as a positive regulator of TGF- β activation (Krohn, 1999). However the exact mechanism involved in the regulation of TGF- β by LTBP-2 remains unresolved and our studies are the first to address the direct association between LTBP-2 and TGF- β . Our studies showing that LTBP-2 directly up-regulates TGF- β expression provides the best explanation of the function of LTBP-2 in regulation of this growth factor. It is well documented that increased levels of TGF- β are correlated with tumor progression and metastasis by inducing tumor angiogenesis and providing immunosuppressive effects in a wide variety of cancers (Gold, 1999; Katsuno *et al.*, 2013; Gao *et al.*, 2014; Yang *et al.*, 2014; Chen *et al.*, 2015). Could LTBP-2 be involved in this process?

Interestingly Immunohistochemical studies have demonstrated that among esophageal carcinoma patients with distant metastatic tumors, those with low expression of LTBP-2 had longer survival times (Chan *et al.*, 2011). Taken together with our studies, the evidence suggests that that LTBP-2 may stimulate TGF- β levels directly in such tumors, enhancing their proliferation and invasive properties. This provides a possible explanation for the inverse correlation of high LTBP-2 and survival in esophageal carcinoma patients (Chan *et al.*, 2011). An alternative explanation is that LTBP-2 also upregulates macrophage chemoattractant protein-1 (MCP1), a proangiogenic chemokine that regulates migration and infiltration of macrophages into tumor sites. Remarkably, an increase of LTBP-2 expression also has been observed in various tumor cells from liver, lung, ovarian and esophageal cancers. For example, LTBP-2 was found to be significantly expressed in a large group of pancreas ductal adenocarcinomas and in mouse and human tumor stroma samples (Turtoi *et al.*, 2011). (Vehvilainen *et al.*, 2003; Yoshihara *et al.*, 2009; Chan *et al.*, 2011; Ren *et al.*, 2015). LTBP-2 is significantly overexpressed in cervical adenocarcinoma compared to normal cervix and its expression is associated to clinical stage, invasion depth, cervical tumor size, and cervical lymph node metastasis stroma suggesting LTBP-2 may participate in the pathogenesis of cervical cancers (Ren *et al.*, 2015). Moreover, knockdown of LTBP-2 expression can inhibit proliferation and invasive ability of human cervical cancer cells (HeLa cells) further supporting the notion that LTBP-2 may promote the growth and progression of cervical cancer cells. Thus LTBP-2 is a potential prognostic marker in the clinical evaluation of patients with cervical adenocarcinoma and other cancer types (Ren *et al.*, 2015). In addition, LTBP-2 levels in the blood were raised in patients dying from pulmonary causes and the protein may be a novel predictor of all-cause mortality (Breidhardt *et al.*, 2012). Thus further investigation of LTBP-2 in cancer and other life threatening diseases is warranted.

TGF- β regulation is complex and can involve several stages, including transcription, translation, secretion and activation of the molecule. Moreover, activation of the same signalling pathways by TGF- β can lead to different outcomes depending on the context of the cell. For example, administration of apoptotic cells onto inflammatory sites induced immediate release of TGF- β caused by its liberation from the cells rather than new protein synthesis (Huynh *et al.*, 2002). To determine if LTBP-2 upregulation of TGF- β expression in fibroblasts was the result of a direct interaction of LTBP-2 with the cells, we initiated a study of the intracellular signalling

pathways upregulated after 30 mins of exposure to LTBP-2. We detected phosphorylation of Akt and p38 but not ERK. Although ERK was not activated in our tests the involvement of ERK and other MAPKs in the up-regulation of TGF- β by LTBP-2 cannot be ruled out since we only observed the phosphorylation of these molecules at a single time-point. Involvement of the Akt and p38 pathways was confirmed using specific inhibitors of these major signalling pathways that resulted in suppression of LTBP-2 induced TGF- β upregulation. It was evident that stimulation of both pathways was essential for the increase in TGF- β production. The findings are consistent with other studies showing blockade of Ras/PI3K/Akt pathways reduces the expression of TGF- β in mouse osteosarcoma cells (Tsubaki *et al.*, 2011) and macrophages treated with liposomes composed of phosphatidylserine (PS-liposomes) (Otsuka *et al.*, 2007). Xiao *et al.* (2008) demonstrated that apoptotic cells up-regulate TGF- β mRNA expression through p38 MAPK, ERK and JNK pathways, and enhance protein translation through RhoA/PI3K/Akt/mTOR/eIF4E pathways. However, the identity of the cell surface receptor(s) responsible for triggering these cascades is not clear (Xiao *et al.*, 2008). Interestingly in our studies, we found involvement of p38 MAPK and Akt signaling pathways in up-regulation of TGF- β by LTBP-2. Further research is needed to determine the detail mechanism of the activation of other MAPK pathways possibility by measuring the phosphorylation level and specific inhibition of these pathways at different time points following stimulation by LTBP-2. Changes in some important tumor-related genes, including the MAPKs pathway (p38 MAPK, ERK and JNK), PI3K-Akt signalling pathways and receptor tyrosine kinase pathways (VEGF, EGFR, FGFR1) have been observed after knockdown of LTBP-2 in HeLa cells, suggesting LTBP-2 could influence the invasive and metastatic potential of cancer cells by modulating these signaling pathways (Ren *et al.*, 2015). Elevated levels of LTBP-2 could potentially disrupt the normal network of these pathways leading to upregulation of cytokines and growth factors, especially TGF- β . However, more studies are needed to explore the underlying mechanisms mediating the effects of LTBP-2 on cell signaling in different cell types.

The results of our signalling experiments suggested that LTBP-2 mediated TGF- β upregulation could involve direct binding of LTBP-2 to a cell surface receptor such as an integrin. Vehvilainen *et al.* (2003) showed that LTBP-2 supports melanoma cell adhesion and migration via $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins. However generally LTBP-2 acts as an anti-adhesive matrix component

for other cells such as embryonic lung fibroblasts, and LTBP-2 is linked to the modulation of fibroblast migration in the lung and other tissues. (Hyytiainen and Keski-Oja, 2003; Vehvilainen *et al.*, 2003). The only known integrin binding region is in a cysteine-free region situated towards the N-terminal domain which is not present in either bioactive fragment LTBP-2C or LTBP-2C F3. However, the involvement of an unknown integrin(s) interaction with LTBP-2C F3 was a strong possibility since integrins are the major receptor family involved in mediating cell-matrix signaling processes regulating a wide variety of cellular functions (Hynes, 1992; Giancotti and Ruoslahti, 1999). Thus, it was interesting to test whether an integrin receptor was involved in LTBP-2 activation of signalling pathways causing elevation of TGF- β . By using blocking anti-integrin antibodies in the TGF- β stimulation assays, we found the integrin α V β 3 receptor partially inhibited the expression of TGF- β -induced by bio-active fragment LTBP-2 F3. On the other hand, the whole family of β 1 integrins was ruled out. The integrin α V β 3 has been the focus of intensive research because of its major role in several biological process and diseases, including macrophage migration and activation, angiogenesis and tumor metastasis (Brooks *et al.*, 1994; Felding-Habermann *et al.*, 2001). A study by Cheresh and Stupack (2002) demonstrated that cell anchorage to ECM through α V β 3 integrin is critical for driving survival signals whereas the unbound integrin leads to apoptosis indicating this integrin, in addition to an adhesive function, has a role in regulation of cell survival and proliferation. Several studies have reported that over-expression of integrin α V β 3 significantly enhances tumor progression and metastatic activities of cancer cells, and the integrin is a proposed target to restrain cancer proliferation and progression (Drake *et al.*, 1995; Stucci *et al.*, 2015). Mice lacking integrin α V β 3 develop defective mammary remodeling during pregnancy (Seguin *et al.*, 2015). Moreover, blocking α V β 3 integrin has been shown to restrain tumor progression in mice through the inhibition of angiogenesis (Desgrosellier and Cheresh, 2010; Weis and Cheresh, 2011).

Since doubling the blocking antibody concentration and reducing LTBP-2 concentration caused no significant further inhibition of the LTBP-2 stimulation, there is a possibility that another(s) main receptor is also involved in the activation of the signal which leads to TGF- β stimulation. Some integrins including α V β 3 may be act as a non-essential enhancer and can stimulate signalling pathways independently, but more often they act synergistically to elicit a full signalling response by modulating the binding of growth factors or cytokines to their receptors

(Assoian and Schwartz, 2001; Alam *et al.*, 2007; Mori *et al.*, 2015). Integrin $\alpha V\beta 3$ also could serve to amplify signal-generating capacity by clustering with growth factor receptors and binding partners directly or indirectly (Seguin *et al.*, 2014; Seguin *et al.*, 2015). Integrin $\alpha V\beta 3$ can up-regulate several growth factor receptors (including FGFR1 and FGFR2 and receptors for VEGF, TGF- β , insulin, EGF, and platelet-derived growth factor- β) and interact with cell surface HSPGs, signifying the functional activities of these growth factor receptors are synergistically and dynamically controlled by $\alpha V\beta 3$ integrin (Tsou and Isik, 2001; Wilder, 2002; Kim *et al.*, 2011). The increased expression of these receptors is blocked by exposure to anti- $\alpha V\beta 3$ integrin antibodies suggesting critical roles for the integrin in regulating or clustering of important cell surface molecules involved in cell survival, growth, invasion and migration through the ECM (Tsou and Isik, 2001). For example, the interaction between PDGFR and $\alpha V\beta 3$ integrin is important for cell motility and it appears to have an antagonistic effect on reactive oxygen species production which is important in regulating various cellular functions (Heldin *et al.*, 1998; Woodard *et al.*, 1998; Lin *et al.*, 2013). Studied by Schneller *et al.* (1997) have demonstrated the effects of PDGF on downstream signaling were significantly enhanced when cells were cultured in the $\alpha V\beta 3$ ligand vitronectin. A potential main receptor for the LTBP-2 interaction with cells leading to TGF- β stimulation could be vascular endothelial growth factor receptor-1/2 (VEGFR1/2) since it is highly expressed in fibroblast cells and can complex with $\alpha V\beta 3$ integrin in an interaction that is crucial for a number of cellular functions (Somanath *et al.*, 2009). The $\alpha V\beta 3$ integrin and VEGFR2 form a complex wherein VEGFR2 activates the $\alpha V\beta 3$ integrin and the activated integrin sustains VEGFR2 signalling demonstrating the important of synergy between the two cell surface receptors systems in the functional responses of cells including cell migration and survival (Mahabeleshwar *et al.*, 2007; Rapraeger *et al.*, 2013). Interestingly, blocking of VEGFR reduces the pathophysiological of allergic airway disease including reduced airway hyper-responsiveness, numbers of inflammatory cells and peribronchial fibrosis caused by increased levels TGF- β (Lee *et al.*, 2008). Moreover, inhibition of VEGFR attenuates peribronchial fibrosis and TGF- β production through a PI3K/Akt signaling pathway. These findings are consistent with our studies and others that activation of the Akt pathway is important for TGF- β expression (Li *et al.*, 2005). In addition, p38 MAPK is activated by VEGFR2 in shear stress-induced angiogenesis and p38 phosphorylation by VEGFR2 is necessary for in vitro endothelial cell tube formation (Gee *et al.*, 2010). The evidence strongly supports the idea that

LTBP-2 elevates of TGF- β expression via the p38 signalling pathway through a complex of AVB3 integrin with an unidentified main receptor possibly from a growth factor receptor family. α V β 3 integrin may enhance the signaling event initiated by the LTBP-2/receptor complex leading to increased expression of TGF- β . Further research possibly using co-immunoprecipitation techniques will be useful to elucidate the specific binding complex of LTBP-2 with the elusive cell surface receptor.

There is also a possibility that cell surface HSPGs act to enhance the activation of TGF- β upregulation pathway via interaction with LTBP-2 and potentially a α V β 3 integrin/receptor complex. A study by Gao and Brigstock (2004) found that connective tissue growth factor (CTGF) induced adhesion of hepatic stellate cells by binding to α V β 3 integrin and HSPG. HSPGs has also been shown to mediate the stimulatory activity of thrombospondin-1 on α V β 3 integrin to promote melanoma cell spreading and focal adhesion kinase phosphorylation (Sipes *et al.*, 1999). Interaction between HSPGs and integrins are crucial in various diseases including metastatic cancer where epidermal growth factor receptor 2 promotes tumor cell survival in vitro via interaction with α 6 β 4 integrin and syndecan-1 (Wang *et al.*, 2010; Soares *et al.*, 2015). Interaction between CTGF and HSPG/integrin receptors is essential for the development of fibrosis and blocking this interaction could lead to development of anti-fibrotic therapies (Leask, 2011). Interestingly, full length LTBP-2 and LTBP-2C fragment showed stronger activation and phosphorylation of the Akt and p38 MAPK signaling molecules compared to LTBP-2C F3 which lacks heparin binding activity. This finding suggests that HSPGs is not essential for, but may enhance, the interaction between LTBP-2 and the cell surface necessary for activation of signaling pathways causing TGF- β upregulation. Further research is required to reveal the full nature of the LTBP-2 interaction(s) with the cell surface responsible for stimulating TGF- β expression and secretion.

The potential role of LTBP-2 in fibrotic disease pathogenesis

Overall, our findings demonstrate a complex but potentially important relationship between LTBP-2 and TGF- β regulation and bioactivity. TGF- β is normally stored on fibrillin microfibril and mutations in the FBN1 gene, which encodes fibrillin-1, causes excessive TGF- β

signaling contributing to the pathogenesis of many diseases including MFS, WMS and acromioclavicular dysplasia (Robinson and Godfrey, 2000; Kaartinen and Warburton, 2003; Neptune *et al.*, 2003; Le Goff *et al.*, 2011; Benke *et al.*, 2013; Tjeldhorn *et al.*, 2015; Zeyer and Reinhardt, 2015). Interestingly, fibrillin-1 knock-down does not affect the ECM association of LTBP-1, which is part of the large latent TGF- β complex involved in matrix sequestering of TGF- β suggesting fibrillin-1 is not needed for LTBP-1 matrix deposition (Dallas *et al.*, 2005; Vehvilainen *et al.*, 2009). In contrast, recent studies demonstrated that the incorporation of LTBP-2 into the matrix of cultured human fibroblasts is dependent on fibrillin-1, although LTBP-2 binds to the same site in fibrillin-1 as LTBP-1 (Hirani *et al.*, 2007; Vehvilainen *et al.*, 2009). Since LTBP-1 binds to fibrillin-2 whereas LTBP-2 does not, it is possible fibrillin-2 mediates the deposition of LTBP-1 in the ECM when fibrillin-1 is not present (Isogai *et al.*, 2003; Hirani *et al.*, 2007). However, since LTBP-2 does not bind fibrillin-2 (Hirani *et al.*, 2007) it is incapable of compensating for the loss of fibrillin-1 in the processes of LTBP-2 deposition and storage. Taken together, our results suggest that disruption of LTBP-2 deposition due to lack of fibrillin-1 microfibrils may allow free LTBP-2 or bioactive fragments to stimulate cells causing upregulation of TGF- β synthesis and secretion. This process may in turn contribute to the aberrant TGF- β signalling characteristic of microfibrilopathies MFS and WMS, other TGF- β -associated diseases, and fibrosis (Roberts *et al.*, 1986; Gold, 1999; Harradine and Akhurst, 2006).

TGF- β is extensively involved in the development of fibrosis in various organs (Broekelmann *et al.*, 1991; Border and Noble, 1994; Branton and Kopp, 1999; Ruiz-Ortega *et al.*, 2007; Pohlars *et al.*, 2009). Persistently elevated TGF- β levels is a key element for the pathogenesis of tissue fibrosis in most organs causing chronic diseases including pulmonary, liver, cardiac and renal fibrosis, and development of aberrant scars and scleroderma (Wynn, 2008; Goodwin and Jenkins, 2009; Pohlars *et al.*, 2009). In fibrosis, aberrant TGF- β activity increases fibrogenesis and results in massive progressive deposition of fibrous matrix causing functional impairment and structural distortion of affected organs and tissues. However, there has been little study of the influence and function of LTBP-2 in abnormal wound healing and fibrosis. To the best of our knowledge, the present study is the first to relate LTBP-2 to fibrosis and wound healing. Our studies demonstrated that LTBP-2 highly expressed in fibrotic tissues, especially in fibrotic keloid skin and hypertrophic scars suggesting the involvement of LTBP-2 in fibrogenesis.

Interestingly, we found LTBP-2 completely blocked the proliferation of fibroblast stimulated by FGF-2 supports our hypothesis that LTBP-2 could potentially modulate FGF-2 bioactivity during fibrosis. This idea was strengthened by examination of fibrotic tissues where LTBP-2 colocalises strongly with FGF-2 suggesting LTBP-2 may contribute to the fibrosis by binding and inhibiting FGF-2 activity during tissue repair process. Interestingly, recent findings demonstrated FGF-2 can inhibit TGF- β induced fibrosis via downregulation of TGF- β 1 activity by inhibiting the SMAD2/SMAD3 signalling system of the TGF- β 1/SMAD dependent pathway (Shi *et al.*, 2013). Thus LTBP-2 may also indirectly up-regulate TGF- β by binding and inactivating FGF-2. Since LTBP-2 has also now been shown to directly increase in TGF- β expression within fibroblasts (Chapter 5), it seems likely LTBP-2 could also directly increase TGF- β levels in fibrotic conditions. It is worth noting that there is significant increase of both LTBP-2 and TGF- β mRNA expression in human hypertrophic scar compared to normal skin (Chapter 4). Therefore these findings provide evidence supporting our hypothesis that LTBP-2 may play a significant role in fibrogenesis and tissue repair by inhibiting FGF-2 activity and increase TGF- β expression, which may worsen the fibrotic condition.

6.5 The domain organization of the human LTBP-2 protein

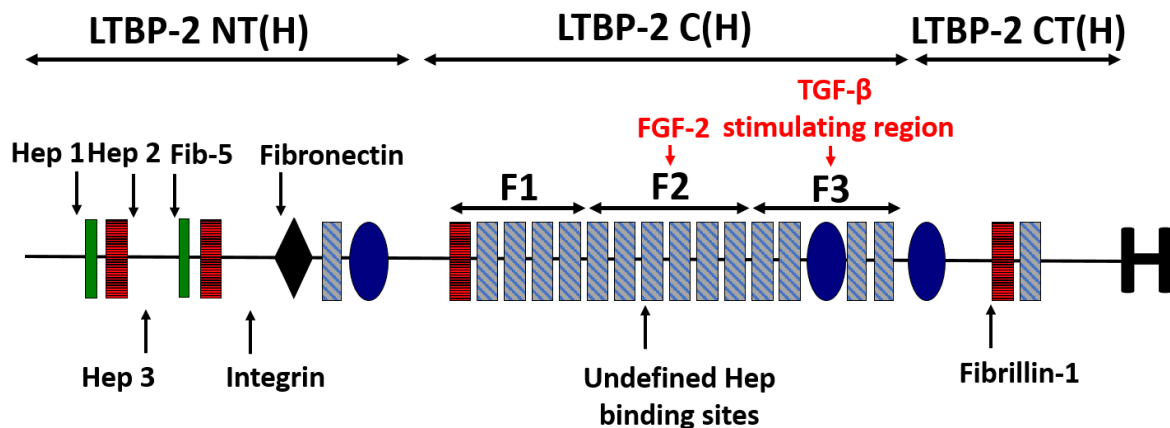


Fig 7.1 Domain structures of LTBP-2, showing molecular interaction sites identified in vitro. The LTBP-2 gene encodes a 195-240kDa protein containing 20 cbEGF repeats, three 8-Cys motifs, and one hybrid 8-Cys motif interspread with proline-rich regions.

In the present study, we have added new binding sites and molecular activities to the molecular map of LTBP-2 map as illustrated in Fig 7.1 (red). The N-terminal region of LTBP-2 contains the binding site for fibulin-5 which appears to be a negative modulator of elastic fibre assembly by interacting with fibulin-5 and inhibiting its interaction with elastin. Moreover, the N-terminal region also contains three major binding sites for heparin/HSPGs, adjacent to the fibulin-5 binding site. LTBP-2 shows strong interaction with the cell surface HSPG, syndecan-4 and basement membrane HSPG, perlecan. Our findings provide evidence that LTBP-2 may compete with tropoelastin for binding to cell surface HSPG and control the release of elastin micro-assemblies from the cell surface. Using a series of recombinant LTBP-2 fragments, a single binding sites for FGF-2 was identified in a central region of LTBP-2 consisting of six EGF-like motifs (LTBP-2C F2) (Fig 7.1). We found LTBP-2 also inhibited FGF-2-induced fibroblast proliferation and LTBP-2 and FGF-2 showed strong co-localisation in fibrotic keloid skin, suggesting LTBP-2 may inactivate FGF-2, inhibiting its contribution to wound healing and perpetuating the fibrotic process. We found exogenous LTBP-2 stimulates expression and secretion of TGF- β 1 in MSU 1.1 fibroblasts and this stimulating activity maps to a central region of LTBP-2 consisting of an eight -cys motif flanked by pairs of EGF-like repeats (LTBP-2C F3) (Fig 7.1). This finding has major implications and significance in understanding the role of LTBP-2 in TGF- β modulation which has been unresolved until now. Interestingly, the LTBP-2C F3 region on LTBP-2 is the same region that binds latent TGF- β on LTBP-1. This leads us to suggest that LTBP-2 may function as positive regulator in the control of expression of new TGF- β , where LTBP-1 modulates the activity of TGF- β in ECM. There is also possibility of binding sites for integrin α V β 3 in complex with an unknown major receptor on this fragment which activates the signaling pathway leading to TGF- β stimulation. In addition, our studies proposed that HSPGs binding sites on adjacent fragment (LTBP-2C F2; see fig 7.1) may enhance the interaction of LTBP-2 with the cell surface receptor complex and the activation of signaling pathways. Together these studies strengthen the idea that LTBP-2 may play important roles in the regulation of growth factors especially TGF- β and FGF-2 and provides essential information on how LTBP-2 may influence TGF- β and FGF-2 dysregulation in severe fibrotic disorders which have no effective treatment. Thus the findings from this study may eventually lead to the development of novel therapeutic targets for attenuation of fibrotic disorders and enhancement of tissue repair mechanisms.

6.6 References

- Aarabi, S., Longaker, M. T. and Gurtner, G. C. (2007) 'Hypertrophic scar formation following burns and trauma: new approaches to treatment', *PLoS Med*, 4(9), p. e234.
- Agasse, F., Nicoleau, C., Petit, J., Jaber, M., Roger, M., Benzakour, O. and Coronas, V. (2007) 'Evidence for a major role of endogenous fibroblast growth factor-2 in apoptotic cortex-induced subventricular zone cell proliferation', *Eur J Neurosci*, 26(11), pp. 3036-42.
- Akita, S., Akino, K., Imaizumi, T. and Hirano, A. (2008) 'Basic fibroblast growth factor accelerates and improves second-degree burn wound healing', *Wound Repair Regen*, 16(5), pp. 635-41.
- Alam, N., Goel, H. L., Zarif, M. J., Butterfield, J. E., Perkins, H. M., Sansoucy, B. G., Sawyer, T. K. and Languino, L. R. (2007) 'The integrin-growth factor receptor duet', *J Cell Physiol*, 213(3), pp. 649-53.
- Ali, M., McKibbin, M., Booth, A., Parry, D. A., Jain, P., Riazuddin, S. A., Hejtmancik, J. F., Khan, S. N., Firasat, S., Shires, M., Gilmour, D. F., Towns, K., Murphy, A. L., Azmanov, D., Tournev, I., Cherninkova, S., Jafri, H., Raashid, Y., Toomes, C., Craig, J., Mackey, D. A., Kalaydjieva, L., Riazuddin, S. and Inglehearn, C. F. (2009) 'Null mutations in LTBP2 cause primary congenital glaucoma', *Am J Hum Genet*, 84(5), pp. 664-71.
- Assoian, R. K. and Schwartz, M. A. (2001) 'Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression', *Curr Opin Genet Dev*, 11(1), pp. 48-53.
- Bai, Y., Zhang, P., Zhang, X., Huang, J., Hu, S. and Wei, Y. (2012) 'LTBP-2 acts as a novel marker in human heart failure - a preliminary study', *Biomarkers*, 17(5), pp. 407-15.
- Benke, K., Agg, B., Szilveszter, B., Tarr, F., Nagy, Z. B., Polos, M., Daroczi, L., Merkely, B. and Szabolcs, Z. (2013) 'The role of transforming growth factor-beta in Marfan syndrome', *Cardiol J*, 20(3), pp. 227-34.
- Bikfalvi, A., Klein, S., Pintucci, G. and Rifkin, D. B. (1997) 'Biological roles of fibroblast growth factor-2', *Endocr Rev*, 18(1), pp. 26-45.
- Blyth, M., Foulds, N., Turner, C. and Bunyan, D. (2008) 'Severe Marfan syndrome due to FBN1 exon deletions', *Am J Med Genet A*, 146A(10), pp. 1320-4.

- Border, W. A. and Noble (1994) 'Transforming growth factor beta in tissue fibrosis', *N Engl J Med*, 331, pp. 1286-1292.
- Branton, M. H. and Kopp, J. B. (1999) 'TGF- β and fibrosis', *Microbes Infect*, 1(15), pp. 1349-65.
- Breidthardt, T., Vanpoucke, G., Potocki, M., Mosimann, T., Ziller, R., Thomas, G., Laroy, W., Moerman, P., Socrates, T., Drexler, B., Mebazaa, A., Kas, K. and Mueller, C. (2012) 'The novel marker LTBP2 predicts all-cause and pulmonary death in patients with acute dyspnoea', *Clin Sci (Lond)*, 123(9), pp. 557-66.
- Broekelmann, T. J., Limper, A. H., Colby, T. V. and McDonald, J. A. (1991) 'Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis', *Proc Natl Acad Sci U S A*, 88(15), pp. 6642-6.
- Brooks, P. C., Clark, R. A. and Cheresh, D. A. (1994) 'Requirement of vascular integrin alpha v beta 3 for angiogenesis', *Science*, 264(5158), pp. 569-71.
- Cain, S. A., Baldock, C., Gallagher, J., Morgan, A., Bax, D. V., Weiss, A. S., Shuttleworth, C. A. and Kielty, C. M. (2005) 'Fibrillin-1 interactions with heparin. Implications for microfibril and elastic fiber assembly', *J Biol Chem*, 280(34), pp. 30526-37.
- Cain, S. A., Baldwin, A. K., Mahalingam, Y., Raynal, B., Jowitt, T. A., Shuttleworth, C. A., Couchman, J. R. and Kielty, C. M. (2008) 'Heparan sulfate regulates fibrillin-1 N- and C-terminal interactions', *J Biol Chem*, 283(40), pp. 27017-27.
- Chan, S. H., Yee Ko, J. M., Chan, K. W., Chan, Y. P., Tao, Q., Hyytiainen, M., Keski-Oja, J., Law, S., Srivastava, G., Tang, J., Tsao, S. W., Chen, H., Stanbridge, E. J. and Lung, M. L. (2011) 'The ECM protein LTBP-2 is a suppressor of esophageal squamous cell carcinoma tumor formation but higher tumor expression associates with poor patient outcome', *Int J Cancer*, 129(3), pp. 565-73.
- Chen, Z. Y., Wang, P. W., Shieh, D. B., Chiu, K. Y. and Liou, Y. M. (2015) 'Involvement of gelsolin in TGF-beta 1 induced epithelial to mesenchymal transition in breast cancer cells', *J Biomed Sci*, 22(1), p. 90.
- Cheresh, D. A. and Stupack, D. G. (2002) 'Integrin-mediated death: an explanation of the integrin-knockout phenotype?', *Nat Med*, 8(3), pp. 193-4.
- Clarke, A. W., Arnsperg, E. C., Mithieux, S. M., Korkmaz, E., Braet, F. and Weiss, A. S. (2006) 'Tropoelastin massively associates during coacervation to form quantized protein spheres', *Biochemistry*, 45(33), pp. 9989-96.

- Dallas, S. L., Sivakumar, P., Jones, C. J., Chen, Q., Peters, D. M., Mosher, D. F., Humphries, M. J. and Kielty, C. M. (2005) 'Fibronectin regulates latent transforming growth factor-beta (TGF beta) by controlling matrix assembly of latent TGF beta-binding protein-1', *J Biol Chem*, 280(19), pp. 18871-80.
- Desgrosellier, J. S. and Cheresch, D. A. (2010) 'Integrins in cancer: biological implications and therapeutic opportunities', *Nature reviews. Cancer*, 10(1), pp. 9-22.
- Desir, J., Sznajder, Y., Depasse, F., Roulez, F., Schrooyen, M., Meire, F. and Abramowicz, M. (2010) 'LTBP2 null mutations in an autosomal recessive ocular syndrome with megalocornea, spherophakia, and secondary glaucoma', *Eur J Hum Genet*, 18(7), pp. 761-7.
- Drake, C. J., Cheresch, D. A. and Little, C. D. (1995) 'An antagonist of integrin alpha v beta 3 prevents maturation of blood vessels during embryonic neovascularization', *J Cell Sci*, 108 (Pt 7), pp. 2655-61.
- Duncan, M. R. and Berman, B. (1991) 'Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6', *J Invest Dermatol*, 97(4), pp. 686-92.
- Eto, H., Suga, H., Aoi, N., Kato, H., Doi, K., Kuno, S., Tabata, Y. and Yoshimura, K. (2012) 'Therapeutic potential of fibroblast growth factor-2 for hypertrophic scars: upregulation of MMP-1 and HGF expression', *Lab Invest*, 92(2), pp. 214-23.
- Faivre, L., Gorlin, R. J., Wirtz, M. K., Godfrey, M., Dagonneau, N., Samples, J. R., Le Merrer, M., Collod-Beroud, G., Boileau, C., Munnich, A. and Cormier-Daire, V. (2003) 'In frame fibrillin-1 gene deletion in autosomal dominant Weill-Marchesani syndrome', *J Med Genet*, 40(1), pp. 34-6.
- Felding-Habermann, B., O'Toole, T. E., Smith, J. W., Fransvea, E., Ruggeri, Z. M., Ginsberg, M. H., Hughes, P. E., Pampori, N., Shattil, S. J., Saven, A. and Mueller, B. M. (2001) 'Integrin activation controls metastasis in human breast cancer', *Proc Natl Acad Sci U S A*, 98(4), pp. 1853-8.
- Fu, X., Shen, Z., Chen, Y., Xie, J., Guo, Z., Zhang, M. and Sheng, Z. (2000) 'Recombinant bovine basic fibroblast growth factor accelerates wound healing in patients with burns, donor sites and chronic dermal ulcers', *Chin Med J (Engl)*, 113(4), pp. 367-71.

- Gabrielsen, A., Lawler, P. R., Yongzhong, W., Steinbruchel, D., Blagoja, D., Paulsson-Berne, G., Kastrup, J. and Hansson, G. K. (2007) 'Gene expression signals involved in ischemic injury, extracellular matrix composition and fibrosis defined by global mRNA profiling of the human left ventricular myocardium', *J Mol Cell Cardiol*, 42(4), pp. 870-83.
- Gao, J., Zhu, Y., Nilsson, M. and Sundfeldt, K. (2014) 'TGF-beta isoforms induce EMT independent migration of ovarian cancer cells', *Cancer Cell Int*, 14(1), p. 72.
- Gao, R. and Brigstock, D. R. (2004) 'Connective Tissue Growth Factor (CCN2) Induces Adhesion of Rat Activated Hepatic Stellate Cells by Binding of Its C-terminal Domain to Integrin $\alpha\beta 3$ and Heparan Sulfate Proteoglycan', *Journal of Biological Chemistry*, 279(10), pp. 8848-8855.
- Gee, E., Milkiewicz, M. and Haas, T. L. (2010) 'p38 MAPK is Activated by Vascular endothelial growth factor receptor 2 and is essential for shear stress-induced angiogenesis', *Journal of cellular physiology*, 222(1), pp. 120-126.
- Giancotti, F. G. and Ruoslahti, E. (1999) 'Integrin signaling', *Science*, 285(5430), pp. 1028-32.
- Gibson, M. A., Hatzinikolas, G., Davis, E. C., Baker, E., Sutherland, G. R. and Mecham, R. P. (1995) 'Bovine latent transforming growth factor β 1-binding protein 2: molecular cloning, identification of tissue isoforms, and immunolocalization to elastin-associated microfibrils', *Mol Cell Biol*, 15(12), pp. 6932-42.
- Gold, L. I. (1999) 'The role for transforming growth factor-beta (TGF-beta) in human cancer', *Crit Rev Oncog*, 10(4), pp. 303-60.
- Gomes Jr, R. R., Farach-Carson, M. C. and Carson, D. D. (2004) 'Perlecan Functions in Chondrogenesis: Insights from in vitro and in vivo Models', *Cells Tissues Organs*, 176(1-3), pp. 79-86.
- Goodwin, A. and Jenkins, G. (2009) 'Role of integrin-mediated TGF- β activation in the pathogenesis of pulmonary fibrosis', *Biochem Soc Trans*, 37(Pt 4), pp. 849-54.
- Govindraj, P., West, L., Smith, S. and Hassell, J. R. (2006) 'Modulation of FGF-2 binding to chondrocytes from the developing growth plate by perlecan', *Matrix Biol*, 25(4), pp. 232-9.
- Haji-Seyed-Javadi, R., Jelodari-Mamaghani, S., Paylakhi, S. H., Yazdani, S., Nilforushan, N., Fan, J. B., Klotzle, B., Mahmoudi, M. J., Ebrahimian, M. J., Chelich, N., Taghiabadi, E., Kamyab, K., Boileau, C., Paisan-Ruiz, C., Ronaghi, M. and Elahi, E. (2012) 'LTBP2

- mutations cause Weill-Marchesani and Weill-Marchesani-like syndrome and affect disruptions in the extracellular matrix', *Hum Mutat*, 33(8), pp. 1182-7.
- Halper, J. and Kjaer, M. (2014) 'Basic components of connective tissues and extracellular matrix: elastin, fibrillin, fibulins, fibrinogen, fibronectin, laminin, tenascins and thrombospondins', *Adv Exp Med Biol*, 802, pp. 31-47.
- Harradine, K. A. and Akhurst, R. J. (2006) 'Mutations of TGFbeta signaling molecules in human disease', *Ann Med*, 38(6), pp. 403-14.
- Hayes, A. J., Gibson, M. A., Shu, C. and Melrose, J. (2014) 'Confocal microscopy demonstrates association of LTBP-2 in fibrillin-1 microfibrils and colocalisation with perlecan in the disc cell pericellular matrix', *Tissue Cell*, 46(3), pp. 185-97.
- Heldin, C. H., Ostman, A. and Ronnstrand, L. (1998) 'Signal transduction via platelet-derived growth factor receptors', *Biochim Biophys Acta*, 1378(1), pp. F79-113.
- Hirai, M., Horiguchi, M., Ohbayashi, T., Kita, T., Chien, K. R. and Nakamura, T. (2007) 'Latent TGF-beta-binding protein 2 binds to DANCE/fibulin-5 and regulates elastic fiber assembly', *EMBO J*, 26(14), pp. 3283-95.
- Hirani, R., Hanssen, E. and Gibson, M. A. (2007) 'LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein', *Matrix Biol*, 26(4), pp. 213-23.
- Huynh, M. L., Fadok, V. A. and Henson, P. M. (2002) 'Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation', *J Clin Invest*, 109(1), pp. 41-50.
- Hynes, R. O. (1992) 'Integrins: versatility, modulation, and signaling in cell adhesion', *Cell*, 69(1), pp. 11-25.
- Hyytiainen, M. and Keski-Oja, J. (2003) 'Latent TGF-beta binding protein LTBP-2 decreases fibroblast adhesion to fibronectin', *J Cell Biol*, 163(6), pp. 1363-74.
- Inoue, T., Ohbayashi, T., Fujikawa, Y., Yoshida, H., Akama, T. O., Noda, K., Horiguchi, M., Kameyama, K., Hata, Y., Takahashi, K., Kusumoto, K. and Nakamura, T. (2014) 'Latent TGF- β binding protein-2 is essential for the development of ciliary zonule microfibrils', *Hum Mol Genet*, 23(21), pp. 5672-82.
- Isogai, Z., Ono, R. N., Ushiro, S., Keene, D. R., Chen, Y., Mazzieri, R., Charbonneau, N. L., Reinhardt, D. P., Rifkin, D. B. and Sakai, L. Y. (2003) 'Latent transforming growth

- factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein', *J Biol Chem*, 278(4), pp. 2750-7.
- Kaartinen, V. and Warburton, D. (2003) 'Fibrillin controls TGF-[beta] activation', *Nat Genet*, 33(3), pp. 331-332.
- Kardami, E., Detillieux, K., Ma, X., Jiang, Z., Santiago, J. J., Jimenez, S. K. and Cattini, P. A. (2007) 'Fibroblast growth factor-2 and cardioprotection', *Heart Fail Rev*, 12(3-4), pp. 267-77.
- Katsuno, Y., Lamouille, S. and Derynck, R. (2013) 'TGF-beta signaling and epithelial-mesenchymal transition in cancer progression', *Curr Opin Oncol*, 25(1), pp. 76-84.
- Kim, S. H., Turnbull, J. and Guimond, S. (2011) 'Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor', *J Endocrinol*, 209(2), pp. 139-51.
- Knox, S. M. and Whitelock, J. M. (2006) 'Perlecan: how does one molecule do so many things?', *Cellular and Molecular Life Sciences CMLS*, 63(21), pp. 2435-2445.
- Krohn, K. (1999) 'TGF- β 1-dependent differential expression of a rat homolog for latent TGF- β binding protein in astrocytes and C6 glioma cells', *Glia*, 25(4), pp. 332-342.
- Kumar, A., Duvvari, M. R., Prabhakaran, V. C., Shetty, J. S., Murthy, G. J. and Blanton, S. H. (2010) 'A homozygous mutation in LTBP2 causes isolated microspherophakia', *Hum Genet*, 128(4), pp. 365-71.
- Langton, A. K., Sherratt, M. J., Griffiths, C. E. and Watson, R. E. (2011) 'Differential expression of elastic fibre components in intrinsically aged skin', *Biogerontology*.
- Le Goff, C., Mahaut, C., Wang, L. W., Allali, S., Abhyankar, A., Jensen, S., Zylberberg, L., Collod-Beroud, G., Bonnet, D., Alanay, Y., Brady, A. F., Cordier, M. P., Devriendt, K., Genevieve, D., Kiper, P. O., Kitoh, H., Krakow, D., Lynch, S. A., Le Merrer, M., Megarbane, A., Mortier, G., Odent, S., Polak, M., Rohrbach, M., Sillence, D., Stolte-Dijkstra, I., Superti-Furga, A., Rimoin, D. L., Topouchian, V., Unger, S., Zabel, B., Bole-Feysot, C., Nitschke, P., Handford, P., Casanova, J. L., Boileau, C., Apte, S. S., Munnich, A. and Cormier-Daire, V. (2011) 'Mutations in the TGFbeta binding-protein-like domain 5 of FBN1 are responsible for acromicric and geleophysic dysplasias', *Am J Hum Genet*, 89(1), pp. 7-14.

- Leask, A. (2011) 'CCN2/decorin interactions: a novel approach to combating fibrosis?', *Journal of Cell Communication and Signaling*, 5(3), pp. 249-250.
- Lee, K. S., Park, S. J., Kim, S. R., Min, K. H., Lee, K. Y., Choe, Y. H., Hong, S. H., Lee, Y. R., Kim, J. S., Hong, S. J. and Lee, Y. C. (2008) 'Inhibition of VEGF blocks TGF-beta1 production through a PI3K/Akt signalling pathway', *Eur Respir J*, 31(3), pp. 523-31.
- Li, Z. D., Bork, J. P., Krueger, B., Patsenker, E., Schulze-Krebs, A., Hahn, E. G. and Schuppan, D. (2005) 'VEGF induces proliferation, migration, and TGF-beta1 expression in mouse glomerular endothelial cells via mitogen-activated protein kinase and phosphatidylinositol 3-kinase', *Biochem Biophys Res Commun*, 334(4), pp. 1049-60.
- Lin, L. J., Grimme, J. M., Sun, J., Lu, S., Gai, L., Cropek, D. M. and Wang, Y. (2013) 'The antagonistic roles of PDGF and integrin $\alpha V\beta 3$ in regulating ROS production at focal adhesions', *Biomaterials*, 34(15), pp. 3807-15.
- Lord, M. S., Chuang, C. Y., Melrose, J., Davies, M. J., Iozzo, R. V. and Whitelock, J. M. (2014) 'The role of vascular-derived perlecan in modulating cell adhesion, proliferation and growth factor signaling', *Matrix Biol*, 35, pp. 112-122.
- Mahabeleshwar, G. H., Feng, W., Reddy, K., Plow, E. F. and Byzova, T. V. (2007) 'Mechanisms of integrin-vascular endothelial growth factor receptor cross-activation in angiogenesis', *Circ Res*, 101(6), pp. 570-80.
- Mauviel, A. (2005) 'Transforming growth factor-beta: a key mediator of fibrosis', *Methods Mol Med*, 117, pp. 69-80.
- Menz, C., Parsi, M. K., Adams, J. R., Sideek, M. A., Kopecki, Z., Cowin, A. J. and Gibson, M. A. (2015) 'LTBP-2 Has a Single High-Affinity Binding Site for FGF-2 and Blocks FGF-2-Induced Cell Proliferation', *PLoS One*, 10(8), p. e0135577.
- Mikroulis, D., Papanas, N., Maltezos, E. and Bougioukas, G. (2007) 'Angiogenic growth factors in the treatment of peripheral arterial disease', *Curr Vasc Pharmacol*, 5(3), pp. 195-209.
- Mori, S., Kodaira, M., Ito, A., Okazaki, M., Kawaguchi, N., Hamada, Y., Takada, Y. and Matsuura, N. (2015) 'Enhanced Expression of Integrin $\alpha V\beta 3$ Induced by TGF- β Is Required for the Enhancing Effect of Fibroblast Growth Factor 1 (FGF1) in TGF- β -Induced Epithelial-Mesenchymal Transition (EMT) in Mammary Epithelial Cells', *PLoS ONE*, 10(9), p. e0137486.

- Nakashima, K., Tsuruga, E., Hisanaga, Y., Ishikawa, H. and Sawa, Y. (2009) 'Stretching stimulates fibulin-5 expression and controls microfibril bundles in human periodontal ligament cells', *J Periodontal Res*, 44(5), pp. 622-7.
- Narooie-Nejad, M., Paylakhi, S. H., Shojaee, S., Fazlali, Z., Rezaei Kanavi, M., Nilforushan, N., Yazdani, S., Babrzadeh, F., Suri, F., Ronaghi, M., Elahi, E. and Paisan-Ruiz, C. (2009) 'Loss of function mutations in the gene encoding latent transforming growth factor beta binding protein 2, LTBP2, cause primary congenital glaucoma', *Hum Mol Genet*, 18(20), pp. 3969-77.
- Neptune, E. R., Frischmeyer, P. A., Arking, D. E., Myers, L., Bunton, T. E., Gayraud, B., Ramirez, F., Sakai, L. Y. and Dietz, H. C. (2003) 'Dysregulation of TGF-[beta] activation contributes to pathogenesis in Marfan syndrome', *Nat Genet*, 33(3), pp. 407-411.
- Ono, I., Akasaka, Y., Kikuchi, R., Sakemoto, A., Kamiya, T., Yamashita, T. and Jimbow, K. (2007) 'Basic fibroblast growth factor reduces scar formation in acute incisional wounds', *Wound Repair Regen*, 15(5), pp. 617-23.
- Ortega, S., Ittmann, M., Tsang, S. H., Ehrlich, M. and Basilico, C. (1998) 'Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2', *Proc Natl Acad Sci U S A*, 95(10), pp. 5672-7.
- Otsuka, M., Negishi, Y. and Aramaki, Y. (2007) 'Involvement of phosphatidylinositol-3-kinase and ERK pathways in the production of TGF- β 1 by macrophages treated with liposomes composed of phosphatidylserine', *FEBS Lett*, 581(2), pp. 325-30.
- Parsi, M. K., Adams, J. R., Whitelock, J. and Gibson, M. A. (2010) 'LTBP-2 has multiple heparin/heparan sulfate binding sites', *Matrix Biol*, 29(5), pp. 393-401.
- Pohlers, D., Brenmoehl, J., Loffler, I., Muller, C. K., Leipner, C., Schultze-Mosgau, S., Stallmach, A., Kinne, R. W. and Wolf, G. (2009) 'TGF-beta and fibrosis in different organs - molecular pathway imprints', *Biochim Biophys Acta*, 1792(8), pp. 746-56.
- Rapraeger, A. C., Ell, B. J., Roy, M., Li, X., Morrison, O. R., Thomas, G. M. and Beauvais, D. M. (2013) 'Vascular endothelial-cadherin stimulates syndecan-1-coupled insulin-like growth factor-1 receptor and cross-talk between α V β 3 integrin and vascular endothelial growth factor receptor 2 at the onset of endothelial cell dissemination during angiogenesis', *FEBS Journal*, 280(10), pp. 2194-2206.

- Ray, S., Ju, X., Sun, H., Finnerty, C. C., Herndon, D. N. and Brasier, A. R. (2013) 'The IL-6 trans-signaling-STAT3 pathway mediates ECM and cellular proliferation in fibroblasts from hypertrophic scar', *J Invest Dermatol*, 133(5), pp. 1212-20.
- Ren, Y., Lu, H., Zhao, D., Ou, Y., Yu, K., Gu, J., Wang, L., Jiang, S., Chen, M., Wang, J., Zhang, R. and Xu, C. (2015) 'LTPB2 acts as a prognostic factor and promotes progression of cervical adenocarcinoma', *Am J Transl Res*, 7(6), pp. 1095-105.
- Roberts, A. B., Sporn, M. B., Assoian, R. K., Smith, J. M., Roche, N. S., Wakefield, L. M., Heine, U. I., Liotta, L. A., Falanga, V. and Kehrl, J. H. (1986) 'Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro', *Proceedings of the National Academy of Sciences*, 83(12), pp. 4167-4171.
- Robinson, P. N. and Godfrey, M. (2000) 'The molecular genetics of Marfan syndrome and related microfibrillopathies', *J Med Genet*, 37(1), pp. 9-25.
- Ruiz-Ortega, M., Rodriguez-Vita, J., Sanchez-Lopez, E., Carvajal, G. and Egido, J. (2007) 'TGF- β signaling in vascular fibrosis', *Cardiovasc Res*, 74(2), pp. 196-206.
- Schneller, M., Vuori, K. and Ruoslahti, E. (1997) 'Alphavbeta3 integrin associates with activated insulin and PDGFbeta receptors and potentiates the biological activity of PDGF', *The EMBO Journal*, 16(18), pp. 5600-5607.
- Schultz, G. S. and Wysocki, A. (2009) 'Interactions between extracellular matrix and growth factors in wound healing', *Wound Repair Regen*, 17(2), pp. 153-62.
- Seguin, L., Desgrosellier, J. S., Weis, S. M. and Cheresh, D. A. (2015) 'Integrins and cancer: regulators of cancer stemness, metastasis, and drug resistance', *Trends in Cell Biology*, 25(4), pp. 234-240.
- Seguin, L., Kato, S., Franovic, A., Camargo, M. F., Lesperance, J., Elliott, K. C., Yebra, M., Mielgo, A., Lowy, A. M., Husain, H., Cascone, T., Diao, L., Wang, J., Wistuba, I. I., Heymach, J. V., Lippman, S. M., Desgrosellier, J. S., Anand, S., Weis, S. M. and Cheresh, D. A. (2014) 'An integrin β 3-KRAS-RalB complex drives tumour stemness and resistance to EGFR inhibition', *Nat Cell Biol*, 16(5), pp. 457-468.
- Shi, H. X., Lin, C., Lin, B. B., Wang, Z. G., Zhang, H. Y., Wu, F. Z., Cheng, Y., Xiang, L. J., Guo, D. J., Luo, X., Zhang, G. Y., Fu, X. B., Bellusci, S., Li, X. K. and Xiao, J. (2013) 'The

- anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo', *PLoS One*, 8(4), p. e59966.
- Sideek, M. A., Menz, C., Parsi, M. K. and Gibson, M. A. (2014) 'LTBP-2 competes with tropoelastin for binding to fibulin-5 and heparin, and is a negative modulator of elastinogenesis', *Matrix Biol*, 34, pp. 114-23.
- Sinha, S., Heagerty, A. M., Shuttleworth, C. A. and Kielty, C. M. (2002) 'Expression of latent TGF- β binding proteins and association with TGF- β 1 and fibrillin-1 following arterial injury', *Cardiovasc Res*, 53(4), pp. 971-83.
- Sipes, J. M., Krutzsch, H. C., Lawler, J. and Roberts, D. D. (1999) 'Cooperation between Thrombospondin-1 Type 1 Repeat Peptides and $\alpha v \beta 3$ Integrin Ligands to Promote Melanoma Cell Spreading and Focal Adhesion Kinase Phosphorylation', *Journal of Biological Chemistry*, 274(32), pp. 22755-22762.
- Soares, M. A., Teixeira, F. C., Fontes, M., Areas, A. L., Leal, M. G., Pavao, M. S. and Stelling, M. P. (2015) 'Heparan Sulfate Proteoglycans May Promote or Inhibit Cancer Progression by Interacting with Integrins and Affecting Cell Migration', *Biomed Res Int*, 2015, p. 453801.
- Somanath, P. R., Malinin, N. L. and Byzova, T. V. (2009) 'Cooperation between integrin $\alpha(v)\beta(3)$ and VEGFR2 in angiogenesis', *Angiogenesis*, 12(2), pp. 177-185.
- Stucci, S., Tucci, M., Passarelli, A. and Silvestris, F. (2015) 'Avbeta3 integrin: Pathogenetic role in osteotropic tumors', *Crit Rev Oncol Hematol*, 96(1), pp. 183-93.
- Tan, Y., Xiao, J., Huang, Z., Xiao, Y., Lin, S., Jin, L., Feng, W., Cai, L. and Li, X. (2008) 'Comparison of the Therapeutic Effects Recombinant Human Acidic and Basic Fibroblast Growth Factors in Wound Healing in Diabetic Patients', *J Health Sci*, 54(4), pp. 432-440.
- Tiedemann, K., Batge, B., Muller, P. K. and Reinhardt, D. P. (2001) 'Interactions of fibrillin-1 with heparin/heparan sulfate, implications for microfibrillar assembly', *J Biol Chem*, 276(38), pp. 36035-42.
- Tjeldhorn, L., Amundsen, S. S., Baroy, T., Rand-Hendriksen, S., Geiran, O., Frengen, E. and Paus, B. (2015) 'Qualitative and quantitative analysis of FBN1 mRNA from 16 patients with Marfan Syndrome', *BMC Med Genet*, 16(1), p. 113.

- Tsou, R. and Isik, F. F. (2001) 'Integrin activation is required for VEGF and FGF receptor protein presence on human microvascular endothelial cells', *Molecular and Cellular Biochemistry*, 224(1-2), pp. 81-89.
- Tsubaki, M., Yamazoe, Y., Yanae, M., Satou, T., Itoh, T., Kaneko, J., Kidera, Y., Moriyama, K. and Nishida, S. (2011) 'Blockade of the Ras/MEK/ERK and Ras/PI3K/Akt pathways by statins reduces the expression of bFGF, HGF, and TGF- β as angiogenic factors in mouse osteosarcoma', *Cytokine*, 54(1), pp. 100-7.
- Tsuruga, E., Oka, K., Hatakeyama, Y., Isokawa, K. and Sawa, Y. (2012) 'Latent transforming growth factor-beta binding protein 2 negatively regulates coalescence of oxytalan fibers induced by stretching stress', *Connect Tissue Res*, 53(6), pp. 521-7.
- Turtoi, A., Musmeci, D., Wang, Y., Dumont, B., Somja, J., Bevilacqua, G., De Pauw, E., Delvenne, P. and Castronovo, V. (2011) 'Identification of novel accessible proteins bearing diagnostic and therapeutic potential in human pancreatic ductal adenocarcinoma', *J Proteome Res*, 10(9), pp. 4302-13.
- Vehvilainen, P., Hyytiainen, M. and Keski-Oja, J. (2003) 'Latent transforming growth factor- β -binding protein 2 is an adhesion protein for melanoma cells', *J Biol Chem*, 278(27), pp. 24705-13.
- Vehvilainen, P., Hyytiainen, M. and Keski-Oja, J. (2009) 'Matrix association of latent TGF-beta binding protein-2 (LTBP-2) is dependent on fibrillin-1', *J Cell Physiol*, 221(3), pp. 586-93.
- Verhaegen, P. D., van Zuijlen, P. P., Pennings, N. M., van Marle, J., Niessen, F. B., van der Horst, C. M. and Middelkoop, E. (2009) 'Differences in collagen architecture between keloid, hypertrophic scar, normotrophic scar, and normal skin: An objective histopathological analysis', *Wound Repair Regen*, 17(5), pp. 649-56.
- Wagenseil, J. E. and Mecham, R. P. (2007) 'New insights into elastic fiber assembly', *Birth Defects Res C Embryo Today*, 81(4), pp. 229-40.
- Wang, H., Leavitt, L., Ramaswamy, R. and Rapraeger, A. C. (2010) 'Interaction of Syndecan and $\alpha 6 \beta 4$ Integrin Cytoplasmic Domains: Regulation Of ErbB2-mediated Integrin Activation', *J. Biol. Chem*, 285(18), pp. 13569-13579.

- Wei, Y. J., Huang, Y. X., Zhang, X. L., Li, J., Huang, J., Zhang, H. and Hu, S. S. (2008) 'Apolipoprotein D as a novel marker in human end-stage heart failure: a preliminary study', *Biomarkers*, 13(5), pp. 535-48.
- Weis, S. M. and Cheresh, D. A. (2011) 'αv Integrins in Angiogenesis and Cancer', *Cold Spring Harbor Perspectives in Medicine*., 1(1), p. a006478.
- Whitelock, J. M., Graham, L. D., Melrose, J., Murdoch, A. D., Iozzo, R. V. and Anne Underwood, P. (1999) 'Human perlecan immunopurified from different endothelial cell sources has different adhesive properties for vascular cells', *Matrix Biol*, 18(2), pp. 163-178.
- Wilder, R. L. (2002) 'Integrin alpha V beta 3 as a target for treatment of rheumatoid arthritis and related rheumatic diseases', *Ann Rheum Dis*, 61 Suppl 2, pp. ii96-9.
- Woodard, A. S., Garcia-Cardena, G., Leong, M., Madri, J. A., Sessa, W. C. and Languino, L. R. (1998) 'The synergistic activity of αVβ3 integrin and PDGF receptor increases cell migration', *J Cell Sci*, 111 (Pt 4), pp. 469-78.
- Wynn, T. A. (2008) 'Cellular and molecular mechanisms of fibrosis', *J Pathol*, 214(2), pp. 199-210.
- Xiao, Y. Q., Freire-de-Lima, C. G., Schiemann, W. P., Bratton, D. L., Vandivier, R. W. and Henson, P. M. (2008) 'Transcriptional and Translational Regulation of Transforming Growth Factor-β Production in Response to Apoptotic Cells', *J. Immunol*, 181(5), pp. 3575-3585.
- Xie, J. L., Bian, H. N., Qi, S. H., Chen, H. D., Li, H. D., Xu, Y. B., Li, T. Z., Liu, X. S., Liang, H. Z., Xin, B. R. and Huan, Y. (2008) 'Basic fibroblast growth factor (bFGF) alleviates the scar of the rabbit ear model in wound healing', *Wound Repair Regen*, 16(4), pp. 576-81.
- Yang, F., Chen, Y., Shen, T., Guo, D., Dakhova, O., Ittmann, M. M., Creighton, C. J., Zhang, Y., Dang, T. D. and Rowley, D. R. (2014) 'Stromal TGF-beta signaling induces AR activation in prostate cancer', *Oncotarget*, 5(21), pp. 10854-69.
- Yoshihara, K., Tajima, A., Komata, D., Yamamoto, T., Kodama, S., Fujiwara, H., Suzuki, M., Onishi, Y., Hatae, M., Sueyoshi, K., Fujiwara, H., Kudo, Y., Inoue, I. and Tanaka, K. (2009) 'Gene expression profiling of advanced-stage serous ovarian cancers distinguishes novel subclasses and implicates ZEB2 in tumor progression and prognosis', *Cancer Sci*, 100(8), pp. 1421-8.

- Yu, P. J., Ferrari, G., Galloway, A. C., Mignatti, P. and Pintucci, G. (2007) 'Basic fibroblast growth factor (FGF-2): the high molecular weight forms come of age', *J Cell Biochem*, 100(5), pp. 1100-8.
- Zeyer, K. A. and Reinhardt, D. P. (2015) 'Engineered mutations in fibrillin-1 leading to Marfan syndrome act at the protein, cellular and organismal levels', *Mutat Res Rev Mutat Res*, 765, pp. 7-18.
- Zoeller, J. J., Whitelock, J. M. and Iozzo, R. V. (2009) 'Perlecan regulates developmental angiogenesis by modulating the VEGF-VEGFR2 axis', *Matrix Biol*, 28(5), pp. 284-291.

CHAPTER 7: Conclusions and Future Directions

7.1 Conclusion

Overall, the findings presented within this thesis presented LTBP-2 as an important matrix protein in elastinogenesis and in modulating growth factor expression and activity. Since LTBP-2 does not bind covalently to latent TGF- β in contrast to other LTBPs in family, the potential role of LTBP-2 in growth factor biology has been largely ignored. However, our findings demonstrated LTBP-2 could have a more crucial role in regulating TGF- β bioactivity than initially anticipated. Our studies provide important information on how LTBP-2 may influence TGF- β and FGF-2 bioactivity in fibrosis and thus have some role in severe fibrotic disorders of kidney, lung, cardiovascular and skin which have no effective treatment. Elucidation of the role of LTBP-2 in elastic fibres and wound healing will provide insight into repair mechanisms and why LTBP-2-deficient patients exhibit osteopenia. Further studies revealing the full spectrum of LTBP-2 functions may eventually lead to the development of novel therapeutic targets for attenuation of elastic fibres associated disorders and enhancement of tissue repair mechanisms.

7.2 Future directions

The research presented in this thesis leaves many promising opportunities open for future research. These following future directions of investigation on LTBP-2 will certainly strengthen our understanding of full spectrum of biological and structural function of LTBP-2:

1. In Chapter 1 we have discovered the potential function of LTBP-2 as negative modulator of elastinogenesis. However, the specific fragment/sites on LTBP-2 that are responsible for the inhibitory effects on elastinogenesis remain unclear. Thus it would be interesting to map specific LTBP-2 regions important for elastinogenesis by repeating the cell culture assembly experiment in the presences of series of fragments spanning the LTBP-2 molecule. The interaction between LTBP-2 and cell HSPGs during elastinogenesis still not fully elucidated. Our studies in vitro indicate that LTBP-

2 could be involved in displacing elastin-HSPGs complexes from the cell surface and LTBP-2 could negatively modulate the function of fibulin-5. Thus it would be valuable to repeat the elastinogenesis experiments in chondrocyte cultures in the presence or absence of LTBP-2, fibulin-5 and heparin and perhaps other elastic fibre associated molecules including MAGPs and EMILIN-1 to understand the full picture of elastic fibre assembly. It also will be interesting to study the role of LTBP-2 in elastic fibre assembly using LTBP-2 $-/-$ mouse cells in culture by investigating the elastinogenesis and fibrillogenesis assembly compared to normal cells. Moreover, it would be also useful to investigate gene expression of collagens, elastin, fibrillins, microfibril-associated proteins and relevant growth factors including TGF- β and FGF-2. The experiments above may lead to new therapies for elastic tissue disorders such as emphysema and vascular aneurysms.

2. Our findings in Chapter 3,4, and 5 have provided evidence that LTBP-2 might be involved in fibrosis by modulating the expression and bioactivity of TGF- β and FGF-2. It will be interesting to investigate the influences of LTBP-2 in fibrosis and wound healing using fibrotic mouse models where LTBP-2 can be administered to the model at different points during the fibrotic process. Further experiment using LTBP-2 null mice could elucidate the effect of LTBP-2 on fibrotic diseases and wound healing. It would be useful to determine the resistance to development of fibrotic diseases and wound healing impairment in LTBP-2 $-/-$ mice especially in lung fibrosis and scleroderma.
3. Due to the time limitations, the elevation of TGF- β levels was only studied in normal fibroblast cells. It will be worthwhile to determine if exogenous LTBP-2 or its fibrogenic fragments elevates TGF- β expression and secretion in fibrotic skin cells in the same way as normal cells. In addition, the expression and matrix deposition of LTBP-2, FGF-2, TGF- β and fibrotic marker genes, collagen I and III and smooth muscle actin can be compared between the fibrotic skin cell cultures and normal skin cells. Furthermore, by using specific siRNA to knock down a target gene, the effects

of LTBP-2 and/or FGF-2 gene knock downs on fibrotic marker genes and collagen deposition could be measured and compared in cellular models of fibrosis.

4. Since our studies have shown that an unknown main receptor(s) could be involved in activation of signalling pathways leading to TGF-beta upregulation, it would be useful to identify the cell surface receptor(s) interacting with the bioactive fragment of LTBP-2 possibly by using combination of cross-linkers and tandem affinity purification. In addition, inhibitors of candidate receptors such as VEGFR could be tested to identify the cell surface LTBP-2 receptor by directly blocking its activity. Once the surface receptor(s) is identified, inhibitory peptide or antibody could be used to determine the effect of fibrosis markers of blocking that receptor. Because of the time limitations, the effect of LTBP-2 on phosphorylation of signalling molecules only were measured on single time point (30 minutes); therefore, the signalling pathways profile over time following LTBP-2 treatment needs to be further evaluated. Moreover, future studies should investigate the involvement of other potential signalling molecules including RAS, RAF, MEK1, TAK1, JNK1/2 and pSMAD regulatory systems to elucidate the full map of LTBP-2 signalling pathways. The above studies may also identify new ligands for LTBP-2 and discover novel matrix related LTBP-2 activities which could lead to potential therapeutic targets for fibrotic diseases.