Effect of Flightless protein on skin architecture, cellular responses and Epidermolysis Bullosa

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ABSTRACT

Wound healing is an area of largely unmet medical need with patients often relying on wound management practice rather than specific therapies. Recent research in our laboratory has identified a cytoskeletal protein Flightless (Flii) as a negative regulator of wound healing. This highly conserved protein is important in development and has a unique structure allowing it to act as a multifunctional protein. Flii expression increases in response to wounding, inhibiting cellular migration and proliferation while its deficiency improves wound healing. The aim of this study was to investigate the effect of differential Flii expression on skin architecture, cellular responses during wound healing, adhesion-mediated cell signaling and skin blistering associated with the genetic skin disorder Epidermolysis Bullosa (EB).

Chapter 3 of this thesis describes the effect of differential Flii expression on skin architecture and formation of hemidesmosomes which anchor the skin layers. Using primary fibroblasts and keratinocytes with varying Flii expression this study investigated the effect of Flii expression on cellular adhesion, spreading and migration on different extracellular matrix substrates. The results presented in Chapter 3 also describe the effect of Flii neutralising antibodies on primary keratinocyte proliferation illustrating improved proliferation in response to decreased Flii expression.

In Chapter 4 an incisional wound healing model was used to investigate the effect of differential Flii expression on different components of hemidesmosomes. Flightless was shown to regulate hemidesmosome formation through its effects on integrin-mediated cellular adhesion and migration. Using immunoprecipitation studies, Flii association with structural and signaling proteins present at the dermal-epidermal junction was investigated. Flii was found to form a cytoskeletal complex with talin, vinculin and paxillin suggesting its role in downstream signaling. The association of Flii with paxillin was further investigated in Chapter 5 where the effect of Flii over-expression on fibroblast adhesion and formation of adhesion structures was examined. Flii over-expression inhibited paxillin activation and the turnover of adhesion structures through down regulation of signaling proteins involved in cell adhesion signaling pathways.

Chapter 6 of this thesis summarises the effect of Flii in skin blistering by utilizing both human samples and two mouse models of Epidermolysis Bullosa. Flii expression is significantly increased in response to skin blistering and its effects on integrin mediated cellular adhesion, migration and type VII collagen expression make Flii a negative contributor to blister formation. Decreasing Flii expression genetically or using neutralizing antibodies reduces skin blistering, improves cellular adhesion and decreases TGF-β mediated collagen contraction.

In summary, Flii adversely affects skin strength and blister formation. Using a multidimensional approach of both in vitro and in vivo methodologies, human tissue and animal models this thesis reveals several novel findings and contributes to better

understanding the involvement of Flii in both maintaining skin homeostasis and regulating wound repair. Flii is a novel target for development of mechanistic based therapy for improved wound healing. Findings presented in this thesis may open doors to significant changes in clinical practice and contribute to better therapeutic design by which would healing of blisters in patients with Epidermolysis Bullosa might be improved.

DECLARATION

"This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to **Zlatko Kopecki** and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text"

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PUBLICATIONS ARRISING FROM WORK IN THIS THESIS

Kopecki Z, Cowin AJ. Flightless I: an actin-remodelling protein and an important negative regulator of wound repair. *Int J Biochem Cell Biol*. 2008;40(8):1415-9. **IF 4.804 RIF 39/156.**

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NATIONAL AND INTERNATIONAL SCIENTIFIC MEETINGS AND AWARDS ARISING FROM WORK PRESENTED IN THIS THESIS

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Kopecki Z, Adams D, Arkell R, Powell B.C and Cowin, A.J. Flightless I affects hemidesmosome formation and integrin mediated cellular adhesion and migration during wound repair. **Australian Wound Management Association Conference – Darwin 2008.**

Kopecki Z, Adams D, Arkell R, Powell B.C and Cowin, A.J. Flightless I affects hemidesmosome formation and integrin mediated cellular adhesion and migration during wound repair. **Australian Society for Medical Research, oral presentation – South Australia Annual Conference 2008.**

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Kopecki Z, Adams D, Arkell R, Powell B.C and Cowin, A.J. Flightless I, a negative regulator of wound healing, affects cellular adhesion and blister formation in Epidermolysis Bullosa – Abstract presentation, **Wound Healing Society and European Tissue Repair Society conference, Limoge, France 2009.**

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LIST OF ABREVIATIONS

A	absorbance
α	alpha
AD	autosomal dominant
AF	collagen VII anchoring fibrils
AP	anchoring plaque
APS	ammonium persulphate
AR	autosomal recessive
Arp 2/3	actin related protein
AF	anchoring fibrils
α-SMA	Alpha Smooth Muscle Actin
AP-1	activating protein-1
APS	ammonium persulfate
B	blister
β	beta
β-tubulin	beta tubulin
BP	bullous phemphigoid
bp	base pair
BCA	bicinchoninic acid
BSA	bovine serum albumin
°C	degrees Celsius
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cm	centimetre

CaMK-II	calcium/calmodium dependent protein kinase type II
c-DNA	complementary deoxyribonucleic acid
CISK	cytoline-inderpendent survival kinase
CO_2	carbon dioxide
ColVII	type VII Collagen protein
COL7A1	human type VII alpha-1 collagen gene
COL17A1	human type XVII alpha-1 collagen gene
d	dermis
dATP	deoxtadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEB	Dystrophic Epidermolysis Bullosa
DDEB	Dominant Dystrophic Epidermolysis Bullosa
DEJ	dermal-epidermal junction
dGTP	deoxyguanosine triphosphate
dTTP	deoxythyamine triphosphate
d0	day 0 post-wounding
d3	day 3 post-wounding
d7	day 7 post-wounding
d14	day 14 post-wounding
d21	day 21 post-wounding
DAPI	4',6 – Diamidino-2-phenylindole dihydrochloride
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's media
DNA	deoxyribonucleic acid
e	epidermis
EB	Epidermolysis Bullosa

EBS	Epidermolysis Bullosa Simplex
ECL	enhanced chemical luminescence
ECM	extracellular matrix
EDTA	ethylediaminetetraacetic acid
EGF	epidermal growth factor
EM	emission wavelength
EX	excitation wavelength
FA	focal adhesion
FAK	focal adhesion kinase
FC	focal complex
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
Flii	Flightless I protein
Flii	Flightless I gene
$Flii^{+/-}$	Flightless heterozygous
$Flii^{Tg/+}$	Flightless transgenic (contains $Flii^{+/+}$; $Flii^{Tg/+}$)
$Flii^{Tg/Tg}$	Flightless transgenic (contains $Flii^{+/+}$; $Flii^{Tg/Tg}$)
fli-1	Flightless I homolog
FLAP-1	Flightless associated protein-1
FLAP-2	Flightless associated protein-2
FnAb	Flightless neutralising antibodies
Fp	filopodium
FRT	Flp recombinase target
x g	times the force of gravity
γ	gamma

g	grams
GFP	
HaCaTs	human keratinocytes
HD	hemidesmosome
H&E	Haematoxylin and Eosin
HFF	human foreskin fibroblasts
hr	hour
HRP	horse radish peroxidise
IgG	immunoglobulin-G
IL-1	interlucin-1
IP	inner plaque
ITG	integrin gene
JEB	Junctional Epidermolysis Bullosa
K5	Keratin-5
K15	Keratin-15
KCl	potassium chloride
kDa	kilo Daltons
KIND1	kindler gene
L	litre
LAMA	laminin gene
LD	lamina densa
LL	lamina lucida
LLGL1	Lethal Giant Larve homolog
LM	lamella
LRR	leucine rich repeat
M	molar

MAPK	mitogen activated protein kinase
$MgCl_2$	magnesium chloride
min	minutes
ml	millilitre
mm^2	millimetre square
mM	milimolar
MMP	matrix metalloproteinases
MMP7	matrilysin
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NHS	normal horse serum
NF-κB	Nuclear Factor Kb
nm	wavelength
NR	nuclear receptor
nt	nuceotide
OP	outher plaque
PAGE	polyacrylamide
PBS	phosphate buffered saline
PCR	Polymerase Chain Raction
PCNA	proliferating cell nuclear antigen
PCT	progenitor cell trageted
PDGF	platelet derived growth factor
PGKNeo	phosphoglycerate kinase promoter–driven neomycin phosphotransferase expression
PIP_2	phosphatidylinositol 4,5-biphosphate
PLEC1	plectin 1 gene

PM	plasma membrane
rFlii	recombinant Flii protein
RAI1	retinoic acid induced 1
RDEB	Recessive Dystrophic Epidermolysis Bullosa
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT-qPCR	real time-quantitative polymerase chain reaction
sbdp	hemidesmosomal sub-basal plate
SCC	Squamous Cell Carcinoma
SDS	sodium dodecylsulphate polyacrylamide gel electrophoresis
sec	seconds
SEM	standard error of mean
SF	stress fiber
SMCR7	Smith Magenis Syndrome chromosome region candidate homolog
SMS	Smith Magenis Syndrome
TEMED	N,N,N’,N’-tetramethylethylenediamine
TIR	Toll/IL-1 receptor domain
TLR	Toll-like receptor
TGF-β	transforming growth factor-beta
TGF-β1	transforming growth factor-beta one
TGF-β2	transforming growth factor-beta two
TGF-β3	transforming growth factor-beta three
TNF-α	tumor necrosis factor-alpha
TOPA3A	Topoisomerase DNA III alpha
Tris	tri(hydroxymethyl)methylamine

TRS	target retrieval solution
µg	microgram
µl	microlitre
µm	micrometer
μm^2	micrometer square
µM	micro molar
WT	wild-type
WST-1	2-(4-iodophenyl)-3-(4-nitrophenly)-2H-tetrazolium
x	times
%	percent
+/+	homozygous
+/-	heterozygous
<	smaller than