

CHANGING THE FACE OF CRANIOSYNOSTOSIS

The role of RBP4 in osteogenesis and suture fusion

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November 2010



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ABSTRACT

Craniosynostosis is the premature fusion of cranial sutures and results in the compensatory malformation of the skull to accommodate the rapid growth of the brain during early childhood. This PhD thesis aims to look at the molecular mechanisms at play during this premature fusion; in particular it follows on from a recent microarray study of craniosynostosis tissue conducted in this laboratory. This study showed a 37x down regulation of RBP4 in fused sutures in humans. RBP4 is a retinol binding protein whose function is to transport retinol in the blood to target tissue, where it is metabolised to retinoic acid. This is of interest as retinoic acid is known to have an influence on bone growth. In this PhD project we have used animal and cell culture models to assess the levels of RBP4 during suture fusion and osteogenesis and its possible role in this process.

Expression of *Rbp4*, *Stra6* and other markers of osteogenesis were assessed using quantitative PCR in a mouse model of Saethre-Chotzen craniosynostosis syndrome (*Twist1*^{+/-}). This demonstrated an initial correlation between suture fusion and *Rbp4* down regulation as well as an inverse relationship between *Rbp4* and *Stra6* expression. However, sutures that did not fuse and parietal bone also displayed downregulation of *Rbp4* at later timepoints. Histology showed that this might be related to parietal bone thickening. Multiple cell culture models were trialed, but proved unsuitable for RBP4 studies in osteogenesis. The commonly used mouse pre-osteoblastic cell line, MC3T3-E1, mineralised but did not express *Rbp4*. Primary coronal suture cells were isolated from mice, which expressed *Rbp4*, but failed to mineralise. Subsequently, primary cell cultures from human sutures were tested in osteogenesis assays and showed a decrease in *RBP4* levels during mineralisation.

Immunocytochemistry was used to determine the localisation of RBP4 in suture cells compared to Huh7 cells, a liver carcinoma cell line with known secretion of RBP4. Results showed that RBP4 is localised to the endoplasmic reticulum in suture cells, differing to the localization seen in Huh7 cells. Western blot analysis also demonstrated that unlike liver cells, human suture cells do not secrete detectable levels of RBP4. Finally, functional studies to analyse the role of RBP4 in osteogenesis using a lentiviral delivery system for over expression of RBP4 showed no effect on the ability of human suture cells to mineralise. A high level of overexpression was achieved however there were issues with infection efficiency which may have affected the outcome of these experiments.

These studies demonstrate some unique characteristics of RBP4 in suture cells and extend its role beyond a simple serum transporter of retinol. In addition to a role in suture fusion, these results could be a reflection of a broader function of RBP4 in normal bone growth and osteogenesis.

KEYWORDS: RBP4, retinoic acid, Saethre-Chotzen, suture fusion, craniosynostosis, osteogenesis.

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 Victoria Dawn Leitch 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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Research completed during the candidature of this PhD was generously funded by

Hansen Yuncken



Australian Rotary Health



Women's and Children's Health Research Institute



The University of Adelaide



National Health and Medical Research Council

Women's and Children's Hospital Foundation

Australian Cranio Maxillofacial foundation

ACKNOWLEDGEMENTS

When deciding to begin my PhD, I did not truly understand how significant an undertaking I was about to face. During this PhD, I have had the benefit of both technical and emotional support from friends, family and colleagues. Without the help of these people, I would not have been able to complete this project with my sanity intact! In particular, there are a few people that I would like to thank for their help. Firstly, I would like to thank Dr. David Loebel for the gift of the *TwistI*^{+/-} mice and Dr. Maya Kansara, Dr. Michael Cunningham, Dr. Cheryl Shoubridge and A/Prof Don Anson for the gift of cell lines used in cell culture experiments. Furthermore, I would like to thank Don for the gift of the lentiviral vectors and protocol, in addition to significant technical assistance. I would also like to thank Dr. Peter Self for teaching me to use microCT and helping me with trouble shooting of the CT scans. Thank you to Lynn, Lesley and Tash in the animal house. Without the animal house ladies I couldn't have completed half the work that I have for this thesis. For the collection of human patient samples for both this study and the original study on which this one is based, I would also like to thank Professor David David and the staff at the Australian Craniofacial Unit.

I would also like to thank my PhD supervisors, Barry and Peter. Firstly, thank you for the opportunity. I have thoroughly enjoyed this project and it has given me direction for my future career. Barry, thank you for letting me run with ideas and for the generous way in which you run your lab. Peter, thank you for always being so enthusiastic. It is very rewarding to know that someone is excited about my research!

The work I performed here was following on from the PhD of Dr. Anna Coussens. I would

like to thank Anna for both her work which has allowed me this project and for her time in our lab where she taught me many of the tricks of the trade. When I started in this lab it was a very small bunch of people and would have been very overwhelming if it weren't for Rino. Thanks Rino for all the pointers, both unofficial and official and for all the chats about cats! Ads, thanks for the help with PCRs, westerns, thesis writing, supervisor approaching and most importantly, friendship. My time at WCHRI wouldn't have been the same without you! Also thanks to Emma and Selena for understanding me more than most. Thank you to the members of the craniofacial research group that have come and gone. You have made my time an adventure, both good and bad! In particular I would like to thank Susan. Su-san, your knowledge is truly invaluable. Thank you for letting me pick your brains on many occasions. Coffee breaks with you were, sometimes sneaked, but always needed and always enjoyed. Melissa, the never-ending smile machine. Thank you for your friendship, your housemate-ship, your kitty sitting and skyping. Your support has been constant and no matter what disasters I have been facing, whether technical, emotional or volcano induced, you have always lent me your ear for a bitch and a whinge and it means a lot to me.

Finally, I would like to thank my family. Meghy and Tim, thank you for always asking. Even if what I was saying made no sense to you at all, you have always been interested in my project and my career. Thank you for all the job suggestions, even if they all do seem to correlate to somewhere Meghy wants to go shopping.... Mum and Dad, thank you for everything. Thank you for helping me, guiding me and letting me make mistakes. I may have taken the long way there but I have made it there in the end. I could not have done it without you.

ABBREVIATIONS

α-MEM	minimal essential media alpha modification
μl	microlitre
μm	micrometre
μM	micromolar
+/-	heterozygous knockout
-/-	homozygous knockout
3D	three dimensional
$^{\circ}$C	degrees celsius
A (nucleic)	adenine
A (amino acid)	alanine
ALP	alkaline phosphatase
Arg	arginine
ATCC	American type culture collection
BCO	bone chip outgrowth
BMP	bone morphogenetic protein
BSA	bovine serum albumin
C (nucleic)	cytosine
C (amino acid)	cysteine
CANX	calnexin
cDNA	complementary DNA
cm	centimetre
CO₂	carbon dioxide

CRABP	cellular retinoic acid binding protein
CRBP	cellular retinoid binding protein
Ct	cycle threshold
CT	computed tomography
CYPA	cyclophilin A
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
Dex	dexamethasone
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E	glutamic acid
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
EF1α	elongation factor 1-alpha 1
EFNA4	ephrin A4
ER	endoplasmic reticulum
eYFP	enhanced yellow fluorescent protein
F	phenylalanine
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FD	full digestion
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor

g	grams
G (nucleic)	guanine
G (amino acid)	glycine
G (lentiviral vector)	phCMV-G
gagpol	phCMV-gagpol
GFP	green fluorescent protein
H	histidine
H&E	haematoxylin and eosin
HCl	hydrochloric acid
HPLC	high pressure/performance liquid chromatography
HPRT	hypoxanthine-guanine phosphoribosyltransferase
hRBP4	human RBP4
HRP	horse radish peroxidase
I	isoleucine
H₂O	water
IgG	immunoglobulin
IHH	Indian hedgehog
iLBP	intracellular lipid binding protein
IRES	internal ribosome entry site
kb	kilobase
LB broth	Luria Bertani broth
LN₂	liquid nitrogen
LV	lentivirus
M	molar (concentration)

M (amino acid)	methionine
MgCl₂	magnesium chloride
microCT	Microcomputerised tomography
ml	millilitre
mM	millimolar (concentration)
MSX2	muscle segment homeobox 2
mRNA	messenger RNA
NaCl	sodium chloride
ng	nanograms
nM	nanomolar (concentration)
OC	osteocalcin
OG	osteogenic
O/N	overnight
P	proline
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	partial digestion
pen/strep	penicillin and streptomycin
PF	posterior frontal
PFA	paraformaldehyde
Pro	proline
Q	glutamine
R	arginine
RA	retinoic acid

RARE	retinoic acid response element
RBP4	retinol binding protein 4
Rev	phCMV-Rev/whv
RIPA	radioimmunoprecipitation buffer
RNA	ribonucleic acid
RR	retinoid receptor
RT	room temperature
RT-qPCR	Real time- quantitative polymerase chain reaction
RUNX2	runt related transcription factor 2
S	serine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SHH	Sonic Hedgehog
STRA6	stimulated by retinoic acid gene homolog 6
T (nucleic)	thymine
T (amino acid)	threonine
Tat	pcDNA3.1Tat
TBS	tris buffered saline
TGF-β	transforming growth factor β
Tris	tris(hydroxymethyl)aminomethane
TTBS	tween20 tris buffered saline
TTR	transthyretin
V (amino acid)	valine

V	volts
v/v	volume per volume
W	tryptophan
WCHRI	Women's and Children's Health Research Institute
WT	wildtype
w/v	weight per volume
Y	tyrosine