CHANGING THE FACE OF CRANIOSYNOSTOSIS

The role of RBP4 in osteogenesis and suture fusion

Victoria Dawn Leitch
Bachelor of Science (Biomedical Science), Bachelor of Science (Honours)

A thesis submitted for the degree of Doctor of Philosophy in Medicine
School of Paediatrics and Reproductive Health
Faculty of Health Sciences
University of Adelaide
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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 quantitive 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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ABBREVIATIONS

α-MEM	minimal essential media alpha modification
µl	microlitre
µm	micrometre
µM	micromolar
+/-	heterozygous knockout
-/-	homozygous knockout
3D	three dimensional
°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
CO_2	carbon dioxide

CRABP	cellular retinoic acid binding protein
CRBP	cellular retinoid binding protein
Ct	cycle threshold
CT	computed tomography
CYPA	cyclophillin 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
E.coli	*Escherichia coli*
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
H_2O	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
$MgCl_2$	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- quantative 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