Cellular and Molecular Mechanisms Involved in Bony Tissue Repair of Injured Growth Plate Cartilage in Rats

A THESIS SUBMITTED IN TOTAL FULFILMENT
OF THE REQUIREMENTS OF
THE DEGREE OF DOCTOR OF PHILOSOPHY

BY
Rosa Chung

Discipline of Physiology,
School of Medical Sciences, University of Adelaide

Bone Growth and Repair Research Group
Department of Orthopaedic Surgery,
Women's and Children's Hospital, North Adelaide, SA
School of Pharmacy and Medical Sciences,
University of South Australia, Adelaide, SA

MAY 2011

TABLE OF CONTENTS

DECLARATION

This work contains no material which has been accepted for the award of any other degrees or diplomas in any university or other tertiary institution to Rosa Chung 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.

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 (as listed below) resides with the copyright holder(s) of those works.

Published works are:

Chung, R., Foster, B.K., Zannettino, A.C.W., Xian, C.J. (2009), *Potential roles of growth factor PDGF-BB in the bony repair of injured growth plate*, Bone, Vol 44 (5), pp. 878- 885

Chung, R., Foster, B.K, Xian, CJ (2011), *Injury responses and repair mechanisms of the injured growth plate*. Front Biosci (Schol Ed), 1;3, pp. 117-125

14th February 2011

ACKNOWLEDGMENTS

This PhD project was funded by the Bone Growth foundation, The University of South Australia (UniSA) and the NHMRC. The University of Adelaide, Faculty of Science has provided my PhD Scholarship and UniSA Bone Growth and Repair Research Group/Sansom Institute have provided me with a Top-up PhD Scholarship upon completion of my initial funds.

I would first like to thank my primary supervisor Professor Cory Xian who has been the best possible supervisor and mentor any student can ask for. Thankyou so much for you kind patient attitude, encouragement, guidance and amazing opportunities that you have provided me throughout my years with you. I have learnt so much from you. Also, a special thanks to my co-supervisor A/Prof Bruce Foster for his invaluable support and advice throughout my candidature.

I would also like to send a special thanks to the original Bone Lab crew – Chiaming Fan, Carmen Macsai, Tristan King, Kristen Georgiou, Rethi Nadhanan and Laura Fong. Thankyou for the support, experimental advice, company and amazingly funny conversations and giggles we have had. I will treasure your friendships for many years to come. Also thanks to the newer members of the Lab – Alice Lee, Long, Derick, Michelle and Yeap it has been a real pleasure getting to know you and working with you. Thankyou.

I would also like to thank my beautiful family –Agnes, Esther, Dan & Toffee "T-man" with special thanks to Mum and Dad. They are the heart and soul of everything happy in my life. Words can not explain the gratitude I have for them. Last but more importantly, a big thankyou to Thong for his support and encouragement - thankyou for always being there for me and for listening to me complain about how evil a PhD can be. You have kept me sane (sort of).

ABBREVIATIONS

%	Percentage
ABC	Advidin -Biotin Complex
ALK-1	Activin A Receptor Type II-like kinase- 1
ALK-3	Activin A Receptor Type II-like kinase- 3
ALK-5	Activin A Receptor Type II-like kinase- 5
ALP	Alkaline Phosphatase
BM MSC	Bone Marrow Mesenchymal Stem Cells
BMP	Bone morphogenic Protein
BMPR-1a	Bone Morphogenic Protein Receptor-1a
BrdU	Bromodeoxyuridine (5-bromo-2-deoxyuridine)
cbf-α1	Core Binding Factor Alpha-1
cDNA	complementary DNA from mRNA
CD	Cell adhesion molecule
CINC-1	Cytokine-induced neutrophil chemoattractant-1
col-IIa	Collagen- IIa
COX-2	cyclo-oxygenase 2
CT	Cycle Threshold
DAB	3,3-diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
FACs	Fluorescence-Activated Cell Sorter
FBS	Fetal Bovine Serum
FGF-2	Fibrogenic Growth Factor

g,mg,µg,ng	Grams, milli Grams, micro Grams, nano Grams
H&E	Haematoxylin & Eosin
HGF	hepatocyte growth factor
I-B4	Isolectin- B4
IGF-I	Insulin-like Growth Factor
IgG	Immunoglobulin G
Ihh	Indian Hedgehog
iNOS	Inducible Nitric Oxide Synthase
IVD	Intervertebral disk disease
M, mM, nM	Molar, milli Molar, nano Molar
MAPK	mitogen activated protein kinase
M-CSF	Macrophage Colony-Stimulating Factor.
Micro-CT	micro computed tomography
ml, µl	Milli Litre, micro Litre
mm, µm	Milli Metre, micro Metre
MMP	Matrix metalloproteinases
mRNA	Messenger RiboNucleic Acid
MSCs	Mesenchymal stem cells
^{o}C	Degrees Celcius
OCN	Osteocalcin
OCT	Optimal cutting temperature
OP-1	osteogenic protein-1
Osx	Osterix
PBS	Phosphate buffered solution
PBS/BSA	Phosphate buffered solution/ Bovine Serum Albumin

PCR	Polymerase Chain Reaction
PDGF-BB	Platelet Derived Growth Factor-BB
Pen/ Strep	Penicillin:streptomycin
PKD	Protein Kinase D
PPARγ2	Peroxisome proliferator-activated receptor gamma
RNA	RiboNucleic Acid
rpm	Rotations per minute
RT	Reverse Transcriptase
Runx2	runt-related transcription factor 2
SCF	Stem cell Factor
SEM	Standard Error of Mean
Sox-9	Sex determining region box containing gene 9 protein
TGF-β1	Transforming Growth Factor-beta1
TNF-α	Tumor Necrosis Factor- Alpha
TRAP	Tartrate Resistant Acid Phosphatase
VEGFa	Vascular Endothelial Growth Factor-a
vWf	von Willebrand Factor
αMEM	Alpha Minimum Essential Media
αSMA	Alpha smooth muscle actin

THESIS ABSTRACT

Being cartilage, the growth plate is often injury prone. This remains to be a significant problem particularly in children where, due to the dynamic nature of their skeletal growth, injury to the growth plate can result in orthopaedic problems including limb-length discrepancy and angulation deformity. Previous studies have identified these problems as a direct result of formation of bony repair tissue at the injury site. Although the sequential post-injury responses (namely the inflammatory, fibrogenic, osteogenic and remodelling phases) have been previously well documented histologically, the molecular and cellular events underlying the bony repair remain unclear. Using a well established rat growth plate injury model, this PhD project characterised presence of possible stromal progenitor cells within the mesenchymal infiltrate, roles of chemotactic growth factor PDGF-BB and protein kinase-D (PKD) in the fibrogenic response and subsequent bony repair events. Immunohistochemical analysis of tibial growth plates at different time points post-injury revealed cells immunopositive for alpha-smooth muscle-actin (αSMA) or Activin-A Receptor Type II-like kinase- 3 (ALK-3) within the mesenchymal infiltrate, suggesting the potential presence of mesenchymal stem cell (MSC)-like cells. In addition, positive immunostaining of MSC-negative but endothelial cell-positive marker, von Willebrand Factor (vWF), also indicated that not all the cells within the infiltrate were MSC-like cells. Further analysis revealed that a portion of cells were immunopositive for osteogenic transcription factor core-binding factor-alpha 1 (cbf-α1) or chondrogenesis marker collagen-IIa, suggesting osteogenic and chondrogenic progenitors may also exist, respectively. Further studies are required for confirmation of MSC-like and progenitor cell existence within the infiltrate and their involvement in the bony repair.

While the importance of the fibrogenic phase of repair is evident, the factors responsible for this cell influx are poorly studied. Previous studies have shown upregulation of the known key chemoattractant, PDGF-BB just prior to and during fibrogenic response. Studies in this project

revealed that inhibition of PDGF signalling resulted in a significant delay in the healing responses in rats. Also *in vitro* studies found that PDGF-BB increased bone marrow stromal cell migration into an artificial "wound" site ($P<0.005$), which can be suppressed by the PDGF receptor inhibitor. These results suggest that PDGF signalling contributes to growth plate injury repair by promoting mesenchymal progenitor cell infiltration and subsequent tissue repair.

Fibrogenic cells within the injury site can differentiate into bone or cartilage cells. However, what signals/ factors underlie these cell differentiation processes and bony repair remain unexplored. While osterix is one known important transcriptional factor for osteoblast maturation, and PKD is known to be involved in transcription of osterix, their potential roles in growth plate bony repair are unknown and were investigated in this project. Micro-CT and histology analysis of injury sites in rats treated with PKD inhibitor revealed significantly lower amount of bone formed after inhibiting PKD signalling ($P<0.05$). Consistently, inhibitor-treated animals showed decreased mRNA expression of bone-related genes (osterix and osteocalcin) and increased levels of cartilage-related genes (collagen-IIa and Sox9). In support, in *vitro* experiments showed that addition of PKD inhibitor during chondrogenic differentiation of rat primary bone marrow stromal progenitor cells resulted in a significant increase in collagen-IIa expression ($P<0.05$). These results suggest that PKD is an important factor for growth plate bony repair and blocking PKD activity after growth plate injury may result in partial suppression of osterix, less bone formation and potentially more desirable cartilage repair.