

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

A THESIS SUBMITTED IN TOTAL FULFILMENT  
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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

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## 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.

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## **ABBREVIATIONS**

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

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



<b>PCR</b>	Polymerase Chain Reaction
<b>PDGF-BB</b>	Platelet Derived Growth Factor-BB
<b>Pen/ Strep</b>	Penicillin:streptomycin
<b>PKD</b>	Protein Kinase D
<b>PPAR<math>\gamma</math>2</b>	Peroxisome proliferator-activated receptor gamma
<b>RNA</b>	RiboNucleic Acid
<b>rpm</b>	Rotations per minute
<b>RT</b>	Reverse Transcriptase
<b>Runx2</b>	runt-related transcription factor 2
<b>SCF</b>	Stem cell Factor
<b>SEM</b>	Standard Error of Mean
<b>Sox-9</b>	Sex determining region box containing gene 9 protein
<b>TGF-<math>\beta</math>1</b>	Transforming Growth Factor-beta1
<b>TNF-<math>\alpha</math></b>	Tumor Necrosis Factor- Alpha
<b>TRAP</b>	Tartrate Resistant Acid Phosphatase
<b>VEGFa</b>	Vascular Endothelial Growth Factor-a
<b>vWf</b>	von Willebrand Factor
<b><math>\alpha</math>MEM</b>	Alpha Minimum Essential Media
<b><math>\alpha</math>SMA</b>	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 ( $\alpha$ SMA) 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- $\alpha$ 1) 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.