

CELLULAR AND MOLECULAR MECHANISMS INVOLVED
IN THE REPAIR OF THE INJURED GROWTH PLATE
IN YOUNG RATS

A THESIS SUBMITTED IN TOTAL FULFILMENT
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BY

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THESIS SUMMARY

The growth plate cartilage, which is located at the ends of children's long bones, is responsible for longitudinal growth of the skeleton. However, due to its cartilaginous composition and its location, the growth plate is commonly injured, mostly through fractures. An undesirable outcome to growth plate fracture is the bony repair of the injured cartilage at the fractured area. Consequently, children often incur skeletal angular deformities and growth arrest. Current corrective surgical treatments for these outcomes are highly invasive, and therapeutic interventions are not possible as little is known about the mechanisms and pathways that lead to bone bridge formation.

Using a rat model, previous studies have shown sequential inflammatory, fibrogenic, osteogenic and bone maturation responses involved in the bony repair of the injured growth plate. However, structural changes in the growth plate, at both the injury site and at the non-injured area, have not been closely examined previously, and little is known about the molecular mechanisms underlying the bony repair. Therefore, this PhD study, using a rat tibial growth plate injury model, aimed to examine the effects of growth plate injury on the structure and composition of the injured growth plate in a longitudinal study using micro-CT and histology. Microarray analysis of the injury site only, collected using laser capture microdissection was used to identify potential cellular and molecular mechanisms involved in bone bridge formation. In addition, Real Time RT-PCR on adjacent uninjured growth plate was used to examine potential cellular/molecular changes at the uninjured area and on whole growth plate scrapes to examine the potential involvement of Wnt signalling in bone bridge formation.

Micro-CT analysis revealed a significant increase in bone material within the injury site (when compared to normal) at 14 and 60 days post-injury, where 12% and 40% of the injury site was replaced by bone, respectively. Interestingly, although there were no changes in growth plate thickness between injured and normal rats at either day 14 or 60, at day 60, many small bone tethers formed at the adjacent growth plate outside the injury site but none were found in normal aged-matched control rats. Histological studies revealed decreased proliferation but increased apoptosis of chondrocytes at the adjacent growth plate cartilage, and RT-PCR analysis revealed differential expression of apoptosis-regulatory genes Bcl-2 and FasL (compared to normal), confirming the increase in apoptosis in the adjacent uninjured growth plate. Down-regulation of Sox-9 and IGF-1 on days 7 and 14 suggests that growth plate injury may slow down the rate of longitudinal growth by decreasing chondrocyte proliferation and/or differentiation soon after injury. Lastly, bone matrix protein osteocalcin was increased on day 60, suggesting degeneration and bone formation at the adjacent uninjured area.

Microarray analysis identified changes in several key BMP and Wnt signalling components across the time-course of bone bridge formation, including BMP-2, BMP-6, BMP-7, chordin, chordin-like 2, and Id-1, and β -catenin, Csnk2a1, SFRP-1 and SFRP-4, respectively, in early stages of bone bridge formation (day 4 and day 8). Osteocalcin expression was also prominent at day 8, supportive of osteoblast development and bone formation. During later stages (day 14), active bone formation and remodelling was prominent and was largely regulated by the BMP signalling pathway (increased BMP-1 and BMP-6 but decreased inhibitor chordin), as well as by Traf6, Fgfr1, osteopontin, Mmp9 and

Wnt signalling, where several genes were up and down-regulated. Expression levels of Wnt signalling inhibitors (SFRP-1, SFRP-4 and Wisp1) were increased at days 8 and 14 and may be negatively regulating bone formation during the osteogenic phases of the repair of the growth plate injury site. Findings were also suggestive of an overall increase in the canonical Wnt signalling pathway at days 4 and 14, supported by increased expression of β -catenin and decreased expression of Wnt inhibitors, and decreased expression of Fzd1 and Fzd2 and increased Lef1 expression, respectively. Overall, this study found a complex balance between the canonical and non-canonical Wnt pathways as well as an association with BMP signalling over the time-course of bone bridge formation.

Lastly, Real-Time PCR on Wnt signalling components revealed significant changes in gene expression of Wnt genes, receptors and inhibitors, but were inconclusive as the method of tissue isolation was not specific enough to represent true changes in gene expression.

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 Carmen Elizabeth Macsai 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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Published works are:

Carmen E. Macsai, Bruce K. Foster, Cory J. Xian (2008). Roles of Wnt signalling in bone growth, remodelling, skeletal disorders and fracture repair. *J Cell Physiol.* Jun; 215(3):578-87.

C.E. Macsai, B. Hopwood, R. Chung, B.K. Foster, C.J. Xian (2011). Structural and molecular analyses of bone bridge formation within the growth plate injury site and cartilage degeneration at the adjacent uninjured area. *Bone.* Oct; 49(4):904-12.

Carmen E. Macsai, Kristen R. Georgiou, Bruce K. Foster, Andrew C.W. Zannettino, Cory J. Xian (2012). Microarray expression analysis of genes and pathways involved in growth plate cartilage injury responses and bony repair. *Bone.* 50:1081-1091.

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ABBREVIATIONS

| <u>Abbreviation</u> | <u>Full name</u> |
|----------------------------|---|
| ALP | Alkaline phosphatase |
| APC | Adenomatous polyposis coli |
| ATF4 | Activating transcription factor 4 |
| Bglap2 | Bone gamma-carboxyglutamate protein 2 |
| BMPs | Bone morphogenic proteins |
| BMUs | Basic multicellular units |
| BSA | Bovine serum albumin |
| BSP | Bone sialoprotein |
| Camk2 | Calmodulin dependent protein kinase 2 |
| Cbfa1 | Core-binding factor alpha 1 |
| CBP | Cyclic AMP response element-binding protein |
| cDNA | complementary DNA |
| Ck1A1 | Casein kinase 1, alpha 1 |
| COX-2 | Cyclo-oxygenase 2 |
| cRNA | complementary RiboNucleic Acid |
| DMSO | Dimethyl sulfoxide |
| DNA | DeoxyriboNucleic Acid |
| DSH | Dishevelled |
| EDTA | Ethylenediaminetetraacetic acid |
| FGF | Fibroblast growth factor |
| FHL2 | Four and half LIM domain 2 |

| | |
|--------------|--|
| Fzd | Frizzled |
| GH | Growth hormone |
| GSK-3 | Glycogen synthase kinase-3 |
| H&E | Haematoxylin & Eosin |
| HGF | Hepatocyte growth factor |
| HSCs | Hematopoietic stem cells |
| 5-HT | Serotonin/5-HydroxyTryptamine |
| IGFBPs | IGF binding proteins |
| Ihh | Indian hedgehog |
| iNos | inducible Nitric oxide synthase |
| Id-1 | Inhibitor of DNA binding-1 |
| LCM | Laser capture microdissection |
| LEF1 | Lymphoid enhancer-binding factor 1 |
| LRP-5/-6 | Low density lipoprotein receptor-related protein-5/6 |
| Ltbp2 | Latent transforming growth factor beta binding protein 2 |
| MAPK | Mitogen activated protein kinase |
| M-CSF | Macrophage colony-stimulating factor |
| MES | 2-(<i>N</i> -Morpholino) EthaneSulfonic Acid |
| mm, μ m | millimetre, micrometre |
| Mmps | Matrix metalloproteases |
| MSCs | Mesenchymal stem cells |
| NFAT | Nuclear factor of activated T cells |
| $^{\circ}$ C | Degrees Celcius |

| | |
|---------------|--|
| OCT | Optimal cutting temperature |
| OPG | Osteoprotegerin |
| Osx | Osterix |
| PCP | Planar cell polarity pathway |
| PCR | Polymerase chain reaction |
| PDGF | Platelet derived growth factor |
| Ptc | Patched |
| PTHrP | Parathyroid hormone (PTH)-related peptide |
| % | Percent |
| RANK | Receptor activator of NF- κ B |
| RANKL | Receptor activator of nuclear factor-kappaB ligand |
| RNA | RiboNucleic Acid |
| rpm | revolutions per minute |
| RT | Reverse transcription |
| Runx2 | Runt-related transcription factor 2 |
| SFRP | Secreted frizzled related protein |
| Shh | Sonic hedgehog |
| Sox | SRY (sex determining region Y)-box |
| TCF | T-cell factor |
| TGF- β | Transforming growth factor- β |
| TH | Thyroid hormone |
| TNF- α | Transforming nuclear factor- α |
| Tph1 | Tryptophan hydroxylase 1 |

| | |
|---------------------|---|
| Traf6 | Tumour necrosis factor receptor-associated factor 6 |
| TRAP | Tartrate resistant acid phosphatase |
| g, mg, μ g, ng, | gram, milligram, microgram, nanogram |
| ml, μ l | millilitre, microlitre, |
| M, mM | Mole, milliMolar |
| VEGF | Vascular endothelial growth factor |
| Wif-1 | Wnt inhibitory factor-1 |
| Wnt | Wingless-Int |
| Wisp | Wnt1 inducible signalling pathway protein |