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

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MAY 2011

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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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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.

CHAPTER 1

Literature review and project aims

NOTE:
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INTRODUCTION

Situated at the ends of all long bones, the growth plate is solely responsible for the lengthening of long bones. However, being of a cartilaginous nature, the growth plate is often highly susceptible to physical injuries. Whilst the injury themselves are able to undergo a healing response, rather than the original cartilage structure, growth plate injuries are often repaired by bony tissue (referred also as bone bridge formation) resulting in orthopaedic conditions such as limb length discrepancies and bone angulation deformities. Since the current methods of correcting growth plate injury-induced bone growth defects are mainly surgically based, they are highly invasive and not always successful – often requiring multiple treatments in growing adolescents. Consequently an increasing interest has been shown towards other potential ways of developing a biological therapy with the aim of prevention rather than correction. Subsequently gaining a better understanding about the molecular and cellular events occurring after growth plate injury is necessary for gaining an insight to why this bony repair tissue occurs and how this process maybe altered or stopped. In a bid to avoid invasive surgical techniques and increase successful cartilage regeneration, current research for cartilage regeneration is being focussed on types of tissue engineering with a particular interest towards multipotent mesenchymal stem cells (MSC)- based approaches. Thus far, while the majority of cartilage repair research has mostly been aimed towards articular cartilage, some work has been published on growth plate cartilage repair using animal models. This review has discussed the major molecular and cellular events involved with the growth plate injury responses and bony repair. This review has also briefly summarised previous and current research investigating potential biological therapeutics (including utilising multipotent MSCs) for cartilage regeneration.

1.1. BONE GROWTH AND THE STRUCTURE AND FUNCTION OF THE GROWTH PLATE

Children's long bones contain a cartilaginous region known as the growth plate (epiphyseal plate) at either end, which is solely responsible for the longitudinal growth of that long bone by producing a mineralised cartilaginous template for bone formation (a process called endochondral ossification) [1-3]. The area of this cartilaginous region significantly decreases as the person gets older and the maximum growth of the long bone is achieved. During endochondral ossification, chondrocytes move progressively through three distinguishable zones of the growth plate (Fig.1): the resting (reserve) zone, the proliferative zone and the hypertrophic zone [1, 4, 5] to form bone within the metaphyseal region.

1.1.1. The Resting zone

Previously, the resting zone has been thought to play no direct roles in longitudinal growth of bones as the cells within the zone (pre-chondrocytes) proliferate very slowly or do not proliferate at all [6]. Histologically, the resting zone is characterised by the sparse distribution of either singular or coupled round cells that are abundant in lipid and cytoplasmic vacuoles within the matrix, indicative of its proposed role as a storehouse for nutrients [1, 6, 7]. Even though the resting zone possesses the ability to produce a cartilaginous matrix, it remains relatively inactive in both cell and matrix turnover [1], with very low rates of proteoglycan and collagen-IIa production [8]. On the other hand, research has suggested that the cells within the resting zone act as a pool of stem cell-like cells, producing proliferative chondrocytes for the proliferative zone [9, 10]. In addition, Abad *et al* (2002) reported that, by producing an unknown growth plate orienting morphogen, the resting zone may be

responsible for influencing the columnar directional arrangement of growth plate chondrocytes within the proliferative zone [10].

1.2.2. The Proliferative zone

There are two main functions at the proliferative zone, matrix production and cellular division, which are vital contributions to the longitudinal growth of long bones [1]. Histologically, the proliferative zone is characterised by longitudinal columns of slightly flattened chondrocytes. These columns are separated from each other via the surrounding cartilage matrix, which is enriched in collagen-IIa [6]. The extent of total longitudinal growth can be determined by the thickness of the proliferative zone, with a greater number of cells present representing a greater potential of longitudinal growth [6]. At the end of the proliferative zone, the chondrocytes no longer proliferate and instead begin to undergo hypertrophy as they enter into the hypertrophic zone.

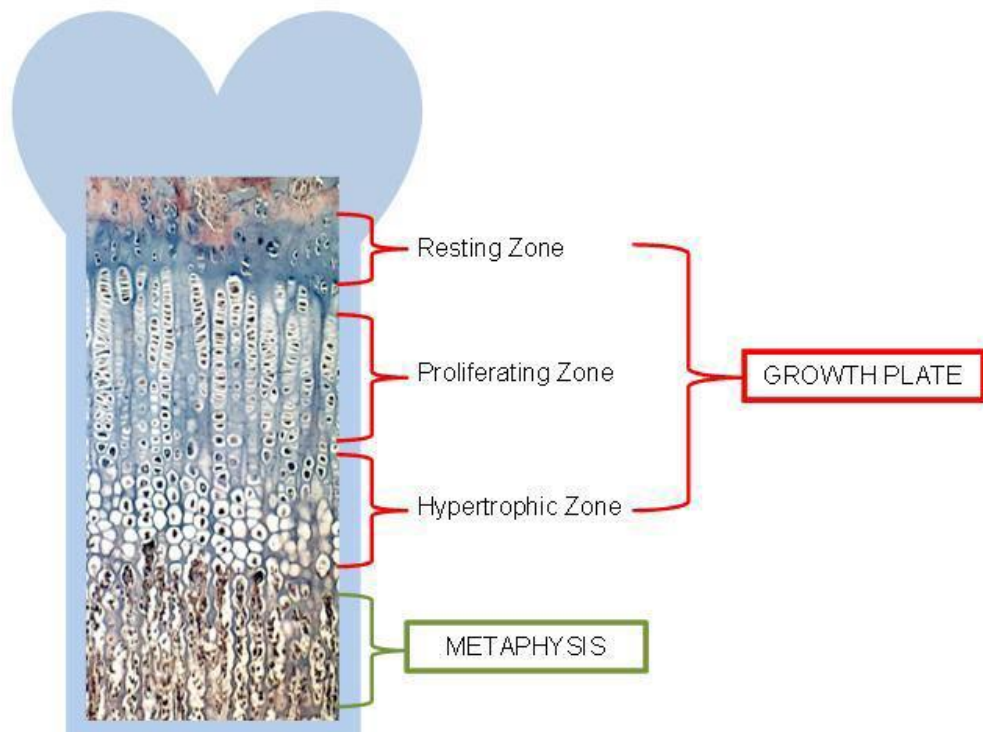

Figure 1. The structure of growth plate and metaphysis. The growth plate is a cartilaginous structure situated at the end of long bones and made up of three distinct zones, namely the resting, proliferative and hypertrophic zones. During the process of endochondral ossification, the hypertrophic cartilage structure calcifies and acts as a template for formation of trabecular bone within the metaphyseal region.

1.2.3. The Hypertrophic zone

Histologically, the cells within the hypertrophic zone are 5 to 10 times greater in size than those in the proliferative zone. Producing collagen-X and alkaline phosphatase, the hypertrophic zone is involved with matrix mineralization. More specifically, along with the production of vascular endothelial growth factor (VEGFa), mineralisation and angiogenesis occur, resulting inchondrocytes within the lower hypertrophic zone particularly at the chondro-osseo junction to undergo apoptotic cell death, hence causing calcified tissue/bone resorbing cells (osteoclasts or chondroclasts) to zone in and dissolve the calcified cartilage [7]. The influx of bone building cells (osteoblasts) deposits bone matrix to replace the previously resorbed tissue to form trabecular bone [1, 4, 11, 12]. Therefore, with vascularisation and coordinated action of osteoclasts/chondroclasts and osteoblasts, the calcified hypertrophic cartilage is modelled and remodelled into the metaphyseal trabecular bone, in which mineralised growth plate cartilage is first being replaced by primary woven bone (primary spongiosa) and then further modelled and remodelled into more mature laminar trabecular bone (secondary spongiosa) [4]. In mature bone, the metaphysis is where the epiphysis and diaphysis meet. Since the hypertrophic chondrocytes are larger in size and this relatively thicker zone of calcified cartilage serves as a template for bone deposition, the hypertrophic zone is the principal engine of longitudinal bone growth, and thus the variation in the rate at which the hypertrophic zone increases in thickness has been regarded as the major reason behind the differences in growth rate in different parts of the body [6].

1.2. GROWTH PLATE INJURIES, INJURY RESPONSES AND REPAIR MECHANISMS

1.3.1. Growth plate injuries, their classifications, and effects on bone growth

Due to accidents in sports and play, skeletal fractures are common in children. Since the growth plate is the least rigid region of the long bone, its injuries are common, and it has been estimated that around 20% of bone fractures involve the growth plate [13]. The Salter-Harris classification system has been used to distinguish the different types of growth plate injuries and relationship between the characteristics of the fractures and their prognoses (Fig.2) [1, 14-16]. Current literature indicates the most common types of fractures occurring in the distal tibias of younger children is type I (around 40%), which in most cases has a reasonably good prognosis as the cells responsible for interstitial growth of the growth plate as well as the epiphyseal blood supply remain undisturbed [16-18]. Similarly, the prognoses for future growth in type II fractures are also quite good. Other types of fractures, types III, IV, and V, however, may/will all result in bony formation at the injured site [19]. It has been estimated that in up to 30% of all children with growth plate-related injuries, undesirable formation of bony tissue and bone bridge at the injury site hinders normal growth of the developing long bone in the affected limb [20, 21], which results in significant orthopaedic problems such as limb length discrepancy and bone angulation deformity [21, 22].

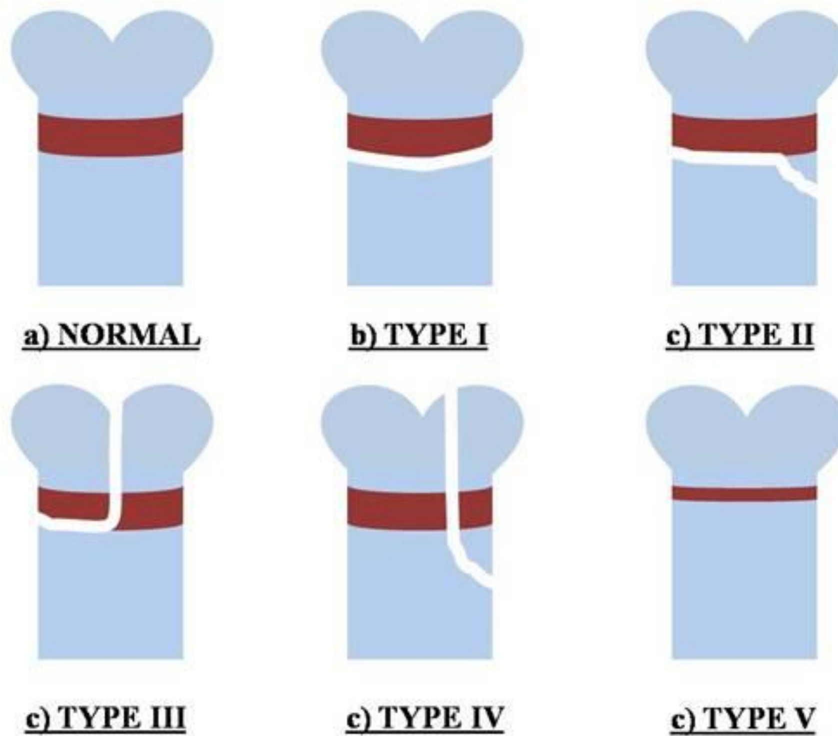

Figure 2. The Salter-Harris classification system. Type I and II fractures do not affect the epiphyseal blood supply. On the other hand, type III, IV and V do disrupt the blood supply and will more than often result in undesirable bony repair tissue- causing problems of angulation and growth arrest.

1.3.2. Injury responses after a growth plate fracture

The cellular and molecular mechanisms for the bony repair of the injured growth plate remain largely unknown. An earlier study identified four different phases of injury responses in a rat growth plate injury model [23] - the inflammatory, fibrogenic, osteogenic and bone bridge maturation remodelling responses occurring during days 1-3, 3-7, 7-14, 10-25, respectively. Similarly, this pattern of growth plate injury repair was demonstrated in a murine growth plate injury model [24]. In addition, similar injury responses were also observed in various growth plate injury models including mice, rabbits, pigs and sheep [24-27]. Following from these studies, there have been some additional *in vivo* mechanistic studies using a rat tibial growth plate injury model [28-31].

1.3.2.1. *Inflammatory phase*

Common to bone fractures and soft tissue injuries, the first response after a growth plate injury is the inflammatory phase [23, 31, 32]. During this initial phase there is an influx of inflammatory cells - predominately neutrophils together with macrophages/monocytes and lymphocytes entering into the growth plate injury site. This rapid influx of inflammatory cells has been shown to commence approximately 8 hours after the injury in a rat growth plate injury model, peaking at day 1 and gradually subsiding by day 3. Consistent with the abundant numbers of neutrophils seen within the infiltrate, the gene expression of rat neutrophil chemokine CINC-1 (similar to human interleukin-8) was shown to be significantly increased during the peak of the inflammatory phase (day 1) [29]. By the end of the inflammatory phase (day 4) the levels of CINC-1 had decreased back to almost basal levels. Along with the influx of inflammatory cells entering the injury site, the infiltrate also secretes a myriad of growth factors and cytokines that are thought to regulate further downstream responses

during growth plate injury repair. Pro-inflammatory cytokines tumour necrosis factor-alpha (TNF-alpha) and interleukin-1 (IL-1beta), which are known regulators of inflammation after tissue injury and bone fractures, were seen in their mRNA expression levels during the inflammatory phase - peaking between 8 hours to day 1 post injury [33] (Fig.3). Follow-up studies also showed a significant increase of these cytokines at day 1 post injury in a rat growth plate injury model [28, 29]. Growth factors insulin-like growth factor (IGF-I) and transforming growth factor (TGF-beta) were also found to be upregulated during this early phase of injury repair [33].

Previous studies have examined the potential role of the inflammatory phase in mediating the cascade of downstream events leading to the bony bridge formation after growth plate injury. As one of the key regulators of the inflammatory response, p38 mitogen activated protein kinase (MAPK) has been shown to be increased in activation at the injured growth plate [31] (Fig.3). Furthermore, Zhou *et al* (2006) found that TNF-alpha was needed for the activation of p38 at the injured growth plate as p38 activation was blocked in rats treated with a TNF-alpha antagonist [31]. TNF-alpha inhibition also resulted in a reduced mesenchymal infiltration, proliferation as well as a reduced expression of FGF-2, indicating the potential role of TNF-alpha in mesenchymal infiltration and proliferation within the growth plate injury site [31]. Similarly, Gerstenfeld *et al* (2001) found that in bone fractures, blocking TNF-alpha signalling resulted in a significant delay in bone callus formation [34]. The role of TNF-alpha has also been studied in other types of tissue repair. Consistent with the finding of TNF-alpha role in mesenchymal cell infiltration into growth plate injury site [31], Fu *et al* (2009) reported that TNF-alpha had a strong chemotaxis role for mesenchymal stem cell migration during wound repair [35], and thus abrogation of TNF-alpha resulted in an obvious delay in MSC migration and wound healing. Overall, these studies highlight the importance of TNF-alpha during tissue repair.

Since the major inflammatory cells involved with the inflammatory phase are found to be neutrophils [23], a follow-up study examined the role of the neutrophil-mediated inflammatory response in growth plate injury repair by utilising an anti-serum to deplete the majority of neutrophils [29]. As a result of the depletion, an increase in expression of osteogenesis genes such as osteocalcin and Runt-related transcription factor 2 (Runx2 or also commonly referred to as core binding factor alpha-1 or cbf alpha-1) was seen. In addition, neutrophil depletion also decreased the expression of chondrogenesis-related genes such as Sox-9 and collagen-IIa [29]. This study indicates that neutrophils play a role of initiating the growth plate injury response and consequently may enhance chondrogenic differentiation. During both soft tissue and bone healing repair, neutrophil recruitment has also been found to be vital, as they play an active role in the clearance of undesirable bacteria and microdebris within the injured zone [36, 37].

Furthermore, one previous study observed significant upregulated gene expression of injury-induced key inflammatory mediators cyclo-oxygenase-2 enzyme (COX-2) and inducible nitric oxide synthase (iNOS) during the inflammatory phase at the injured growth plate (Fig.3) and found that inhibition of COX-2 or iNOS by specific inhibitors caused an increased proportion of undifferentiated mesenchymal tissue but a decrease in chondrogenic differentiation within the injury site [28]. This study confirms that the injury-induced inflammatory response in general at the growth plate injury site is necessary for enhancing the chondrogenic differentiation of mesenchymal cells. Overall, these studies suggest that the injury-induced inflammatory response has an important role early in regulating growth plate injury repair as it initiates and regulates a cascade of downstream events which lead to the bony repair at the growth plate injury site. Similarly during bone fracture healing, these two inflammatory mediators (COX-2 and iNOS) have been found to be important for triggering

the cascade of events leading to tissue repair. More specifically, numerous bone fracture studies have demonstrated that inhibiting COX-2 resulted in a delay in bone formation and fracture healing [38-41], highlighting the importance of injury-induced inflammatory response and COX-2 enzyme during tissue repair.

Other studies have also shown that during the inflammatory phase there were increases in the levels of several members of the bone morphogenic protein (BMP) family. The BMPs have been known for having roles in chondrogenic and osteoblastogenic differentiation as well as encouraging mesenchymal cell proliferation and migration [42, 43]. Ngo *et al* (2006) observed the presence and upregulation of BMP-3 and BMP-4 within the growth plate injury sites of young rats [44] (Fig.3). BMP-4 also appeared to be produced by inflammatory cells- indicating their role in mediating the initial inflammatory event in regulating mesenchymal cell migration and differentiation [44]. BMP-4's proposed role in regulating mesenchymal cell migration and differentiation during skeletal repair was also echoed in another earlier study which examined BMP-4's potential role and level of expression in regenerating tissue of a rabbit leg-lengthening model [45].

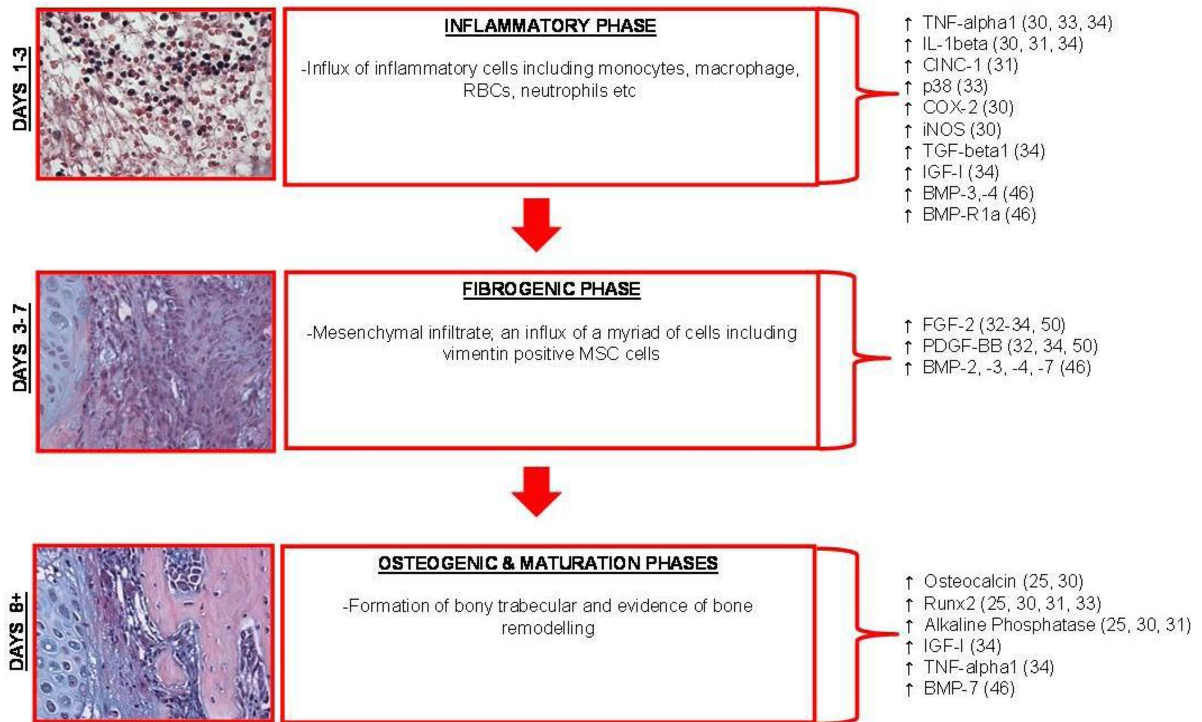

Figure 3. Growth plate injury repair responses and gene expression. In a rat growth plate injury model four distinct phases of injury repair were observed, namely the inflammatory, fibrogenic and osteogenic and maturation phases on days 1-3, 3-7 and 8 onwards, respectively, which are accompanied by elevated levels of mRNA expression of specific cytokines, inflammatory mediators, and growth factors during each phase.

1.3.2.2. *Fibrogenic phase*

Following the initial inflammatory phase in the rat growth plate injury model is the fibrogenic phase - occurring during days 3-7 post injury [23]. The fibrogenic response involves the influx of fibrous vimentin-immunopositive mesenchymal cells into the injury site [23]. This response was also observed in mice, whereby approximately 7 days post injury, there was presence of undifferentiated, spindle-shaped cells near the superior and inferior areas of the growth plate injury site [24]. Although it is yet to be confirmed, previous findings of osteogenesis as well as chondrogenesis from these infiltrated cells [23, 28-30] suggest that these filtrating cells may contain pre-determined chondroprogenitor and osteoprogenitor cells as well as multipotent mesenchymal stem cells. The infiltration of such stromal progenitor cells (originating from periosteum, the circulation as well as from the bone marrow) following the inflammatory response has been confirmed in bone fractures, which is critical for the formation of the bridging "soft callus" as the next stage of the fracture repair process [25, 32, 46].

During the influx of fibrogenic cells in both injured growth plate and bone, mRNA levels of growth factors FGF-2 and PDGF-BB have been found to be significantly upregulated, indicating the possible involvement of both growth factors in regulating this mesenchymal reaction phase in both bone or growth plate injury repair (Fig.3) [33, 47]. FGF-2 has functions in various biological responses such as cell proliferation, differentiation and migration [48]. During bone fracture healing, various cells such as monocytes, macrophages, mesenchymal cells, osteoblasts and chondrocytes produce FGF-2 [49]. Along with its well known roles in mesenchymal cell migration and proliferation [50, 51], FGF-2 has been found to inhibit chondrocyte differentiation (55), alkaline phosphastase activity [52, 53] as

well as stimulating bone resorption *in vitro* [54, 55], suggesting its role in suppressing skeletal cell differentiation during bone fracture repair. Interestingly, a more recent *in vitro* study has shown that FGF-2 was able to increase the osteogenic and chondrogenic differentiation potentials of mesenchymal cells via suppression of key signalling from TGF-beta [51, 56]. However, although it has been suggested that FGF-2 may play a possible role in mesenchymal and osteoprogenitor cell proliferation, migration and differentiation, further studies are required to investigate the functions of the upregulated FGF-2 at the injured growth plate during the fibrogenic phase [31, 33].

PDGFs have been documented to have many different roles including cell migration, cell proliferation and angiogenesis in wound healing [57-60]. In particular, it is also a potent chemotactant for fibroblasts and smooth muscle cells [61]. During bone fracture repair, PDGFs have been found to be essential for triggering the initial events leading to the migration and proliferation of fibroblasts and osteoblasts [49]. Similarly, Zhou *et al* (2004) found that gene expression levels of PDGF-BB were significantly upregulated following the inflammatory phase in a rat growth plate injury model [33],suggesting a potentially critical role of PDGF in the fibrogenic phase of growth plate repair [30]

1.3.2.3. *Osteogenic and maturation phases*

Following the fibrogenic phase, the subsequent osteogenic response involves some bone cell differentiation among some of the infiltrated mesenchymal cells, as indicated by positive immunohistochemical staining of Runx-2 and alkaline phosphatase (markers of osteoblastic differentiation and maturation, respectively) [23, 28-30]. Furthermore, the presence of active bone deposition containing bone matrix protein osteocalcin on the new trabecular bone surface within the

growth plate injury site is indicative of the bony tissue formation [23, 28]. During the remodelling and maturation of the bony bridge, bone trabeculae are found to be separated by abundant marrow cells, and surrounded by flattened spindle-like inactive osteoblasts in resting phase - producing little or no osteocalcin which is characteristic of inactive bone formation [23]. In addition, resorptive cells osteoclasts are sometimes seen on some areas of newly formed trabeculae at the injury site [30], suggesting that osteoclastic bone resorption is involved in the bone bridge matuation phase at the injured growth plate. While the molecular mechanisms regulating this maturation phase remains unclear, upregulation of TNF-alpha, IGF-I and BMP-7 at the injured growth plate (Fig.3) suggest their involvement in the bony bridge remodelling [33, 44]. Consistently, TNF-alpha upregulation has been observed during the remodelling phase in bone fracture repair [62], and TNF-alpha has been shown to be important in regulating bone remodelling by promoting differentiation of bone resorptive osteoclasts [63]. Similarly, BMP-7 upregulation is known to be important for bone formation and remodelling at the bone fracture sites [64]. Further studies are required to characterise the molecular and cellular mechanisms regulating the bony bridge maturation/remodelling at the growth plate injured site.

1.3.2.4. *Effects of injuries on the adjacent non-injured growth plate tissue*

While most growth plate injury studies have focused and looked at the events occurring purely within the injury site, very few have investigated the potential effects of injuries on adjacent growth plate chondrocytes. An early study looking at the effects of growth plate trauma observed the intrusion of growth plate cartilage tissue into the metaphyseal region, and found that these islands of trapped cartilage disrupt the continuing bone growth of the surrounding tissue [65]. Consequently, there were abnormal widening and irregularities of the remaining growth plate, hence potentially resulting in the

deformities and discrepancies seen in many patients as a result from their growth plate-related injuries [65]. More recently, Coleman *et al* (2010) utilised micro-CT imaging to characterise changes occurring within the injured growth plates of rats as well as the effect on the whole tibial bone itself [66]. Interestingly, Coleman *et al* (2010) observed that bone volume present within the injury site did not directly correlate with overall reduced bone growth by 35 days after the injury. Furthermore, using micro-CT imaging, by the time a bone bridge has formed, significant damage could already be detected in the remaining non-injured growth plate, including cellular disorganisation as well as a significant decrease in overall growth plate thickness and volume. Coleman *et al* (2010) also observed that tethers, which usually form with age as the growth plate begins to close [67], were present earlier in the adjacent growth plate after injury [66]. These studies highlight the potential involvement of the adjacent remaining growth plate during growth plate injury repair and its contribution to limb length discrepancies and bone angulation deformities that form after growth plate injuries [65, 66]. Further mechanistic studies are required to gain a better understanding of how the bone bridge formation within the injury site and changes in the adjacent non-injured growth plate tissue contribute to the final undesirable bony repair and bone growth defects after a growth plate injury.

1.3.3. Mechanisms of bony repair of injured growth plate cartilage

Studies in both murine and rat growth plate injury models by Lee *et al* (2000) and Xian *et al* (2004) respectively showed that the bony bridge formation occurring after injury was a result from direct bone formation mainly via intramembranous ossification [23, 24]. In support, Lee *et al* (2000) saw no changes in the levels of endochondral ossification-related molecules including collagen-IIa, Indian hedgehog (Ihh) and vascular endothelial growth factor (VEGF) at the time points examined [24], and

Xian *et al* revealed $Runx2^{+}$ osteoblastic differentiation and bony trabecular formation from infiltrated mesenchymal cells [23]. Similarly, Zhou *et al* (2004) reported no up-regulation of chondrogenic transcription factor Sox-9 and cartilage matrix protein collagen-IIa at the injured growth plate in this rat model [33]. However, more recent studies in the rat growth plate injury model (some with different post-injury time points examined) have found that apart from direct bone formation being as the major bony repair mechanism present, endochondral ossification, despite to a lesser extent, also occurred as a potential mechanism underlying the bony repair. Arasapam *et al* (2006) found increased expression of some cartilage related genes including collagen-IIa, collagen-X and Sox-9 together with increased levels of some bone related genes [28]. This indicates the presence of the formation of both cartilage and bone within the growth plate injury site and hence involvement of both endochondral and intramembranous ossification mechanisms during the bony repair. Similarly, Chung *et al* also found endochondral ossification involvement during bone bridge formation showing presence of cartilage-related molecules, Sox-9 and collagen-IIa and -X at the growth plate injury site and positive immunostaining of both collagen-IIa and -X in cartilage-like tissue derived from infiltrated mesenchymal cells [29]. Further mechanistic studies are required to understand the bone formation pathways underlying the bony repair of injured growth plate.

1.3.4. Molecular control of osteoblast or chondrocyte differentiation

While the molecular mechanisms for the bony and cartilage repair occurring at the growth plate injury site are currently unclear, it is assumed that the general mechanisms regulating osteoblast and chondrocyte differentiation from mesenchymal stem cells (MSC) or progenitor cells could also apply to the differentiation events at the injured growth plate.

MSCs are abundant and have been successfully isolated from many sources including bone marrow [68, 69], periosteum [70-72], trabecular bone [73, 74], adipose tissue [75-77], skeletal muscle [78, 79] and synovium [80-82]. Due to their pluripotency, abundance and accessibility, bone marrow derived MSCs have made a particularly attractive source for use in articular and growth plate cartilage regeneration [5, 68, 83, 84].

Bone marrow is the major source for the MSC [68, 69] - with approximately 0.001- 0.01% of the total nucleated cells being MSC within the bone marrow [85-87]. MSCs residing within the bone marrow interact closely with haematopoietic stem cells to maintain bone marrow homeostasis and play an important role in maintaining bone mass and tissue regeneration. In current literature, although there are no known specific markers to identify true MSCs, an array of different positive and negative markers have been used to identify potential MSC. Some the reported positive cell surface markers of MSC include STRO-1, CD105, CD90, CD73, CD29, CD44, CD166 and CD146 [84, 87-91]. Furthermore, MSCs lack the expression of CD45 and CD31 which are markers specific for haematopoietic and endothelial cells, respectively. Apart from surface antigens, previous studies have also reported that immunohistologically MSCs were alpha smooth muscle actin (αSMA)-positive and negative for the endothelial marker, von willibrand factor (vWF) [88]. Apart from using immunohistochemistry and flow cytometry to detect and isolate true MSC, various other known characteristics of MSCs are also commonly observed, including the ability to adhere to plastic surfaces [92] as well as an ability to proliferate and differentiate (under specific media conditions) into chondrocytes, adipocytes and osteoblasts *in vitro* [93].

A plethora of bone marrow derived MSC related studies have demonstrated the ability of MSCs to differentiate *in vitro* into multiple lineages depending on defined culture conditions including differentiation into chondrocytes [69, 84, 94]. In addition, MSC have also been documented as possessing unique immuno- suppressive properties which are advantageous during procedures such as transplantation [95, 96]. Chen *et al* (2003) successfully transplanted periosteum- derived MSCs into a rabbit growth plate injury model, concluding that the high proliferation rate of MSCs made them an excellent source for donor cells [97]. Additionally, an *in vivo* study done by Park *et al* (2006) showed that MSCs derived from bone marrow and perichondrium/periosteum were more successful at forming hyaline cartilage than from those MSCs derived from other sources such as adipose tissue [98].

While Sox-9 is the master transcription factor of chondrogenesis in endochondral ossification during bone development and postnatal growth (see above), Sox-9 also plays an important role in the chondrogenesis of MSC [99]. In addition, cbfα-1 (core-binding factor- alpha1), also commonly referred to as Runx2 (runt-related transcription-factor 2), is an important transcription factor in chondrocyte maturation and hypertrophy [2, 100, 101]. While cbfα-1 is known as a master regulator of osteogenesis [102] regulated by bone morphogenic proteins 2 and 7 (BMP-2 and -7) [6], it was found that once committed to the osteogenic lineage, cbfα-1 in MSCs suppressed maturation and hence maintaining the pre-osteoblasts in an immature stage [103], suggesting another or other regulatory factor(s) is required for osteoblastic maturation. Osterix is a zinc-finger containing transcription factor that is reportedly a key regulator in osteoblastic differentiation [104-106]. Osterix acts downstream of cbfα-1 and is regulated by similar anabolic signals like BMP-2 and IGF-I [102] and functions to regulate the expression of osteoblastic genes such as osteocalcin, osteopontin, collagen-I and bone sialoprotein [107]. The importance of osterix in osteoblastic differentiation and bone formation was highlighted in a

genetic study where osterix deficient mice formed normal shaped skeletons composed of cartilage only with the clear absence of osteoblasts or mineralized matrix [102]. In addition, Kaback *et al* (2008) showed that osterix is also involved in inhibiting chondrocyte differentiation [108].

Interestingly, Nakashima *et al* (2002) also found that osteoprogenitor precursor cells in osterix null mice still expressed cartilage-related genes and transcriptional factors such as Col-IIa and Sox-9, respectively [109]. In support, Rodda *et al* (2006) showed that with the absence of certain osteogenic promoting signals such as Indian hedgehog (Ihh) and Wnt signalling, cbfα-1 and osterix-positive preosteoblasts were able to switch to differentiating into chondrocytes rather than osteoblasts [99]. Previous studies have shown that Wnt signalling acts downstream from Ihh [110]. Together with transcriptional factor osterix, the Wnt/β-catenin signalling activity is essential for the formation and the maturation of osteoblasts [111]. One study found that with the transfection of a mesenchymal progenitor cell line with β–catenin saw a significant 4-fold increase in alkaline phosphatase activity in comparison to the BMP-2 alone control, demonstrating the nessecity of β–catenin expression during osteoblastogenesis. Conversely, the loss of β-catenin activity caused the blocking of osterix and as a result cells obtained a chondrogenic phenotype [112]. This bi-potential phenomenon of osteoprogenitor cells and regulation by the key signalling pathways (Fig.4) represent a promising opportunity for intervention for cartilage tissue engineering and repair including growth plate regeneration where chondrogenesis instead of osteogenesis is encouraged.

Furthermore, BMP-2 and -7 have been shown to increase expression of osteogenic transcriptional factor cbfα1 and BMP-2 to induce osterix in both mice and human MSCs/progenitor cells [6, 109, 113].

Several BMPs are heavily involved in chondrocyte differentiation and formation. More specifically, BMP-4, -5, -7, -12 to -14 have been shown to induce chondroprogenitor formation in MSCs [114], promote cellular recruitment and chondrocyte differentiation [115], and up-regulate chondrocyte metabolism and protein synthesis *in vitro* and *in vivo* [116]. Furthermore, many studies have reported that use of FGF-2 as a potent chemoattractant for MSC infiltration into cartilage defects [117, 118]. In addition, the use of FGF-2 as a supplement in media resulted in smaller but rapidly proliferating stem cells *in vitro* than compared to control, indicating FGF-2 role in promoting the proliferation and differentiation of MSCs [119].

NOTE:
This figure is included on page 31 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4. Mesenchymal stem cells derived from the bone marrow are multipotential and able to differentiate into fat, cartilage and bone cells. Under the influence of PPARγ2, BMMSCs differentiate into adipocytes. Osteo-chondroprogenitor cells possess the ability to become cartilage or bone tissue under influences of key transcriptional and growth factors such as sox-9 and runx2, respectively. Furthermore, during the pre-osteoblast stage of osteoblastic differentiation, the cells are able to switch and express chondrogenic-related transcriptional factors and proteins to undergo chondrogenic differentiation rather than osteogenic differentiation (figured modified from Rodda *et al* (2006) [99]).

1.3. PREVIOUS AND CURRENT RESEARCH ON BIOLOGICAL TREATMENTS FOR GROWTH PLATE REPAIR

1.3.1. Current Surgical Treatments for Injured Growth Plate

Due to the significant orthopaedic problems resulting from growth plate injuries, many previous studies have looked at different ways of correcting growth plate injury-induced defects as well as preventing the bony repair [120]. The type of treatment for growth plate injuries is largely dependent on the age of the patient as well as the severity and type of injury sustained [121]. Surgical intervention is usually needed only if the patient is quite young and significant growth remains. If the injury only results in a very slight length discrepancy, it is often fixed through the use of a shoe lift and in most cases, the patient must cease using the affected limb for a period of time in order to prevent orthopaedic problems such as angular deformity from occurring. An already established angular deformity is commonly corrected with a wedge osteotomy [122-124]. On the other hand, larger limb length discrepancies require bone lengthening or bone shortening procedures [5, 125, 126]. The most common way of correcting larger limb length discrepancies is through a lengthening procedure which surgically creates a fracture at the diaphysis and then gradually lengthens the injured limb to match the growth of the unaffected limb using a large external frame (*Ilizarov* frame) placed around the affected limb [123, 125, 127]. As effective as this method of treatment is, the downside is that the procedure is highly invasive, painful and lengthy as it can take as long as 6 months or longer. As only a limited amount of lengthening can be done at a time, the patient often requires the procedure repeated several times throughout adolescence until skeletal maturity is reached. Furthermore, complications arising from pin site infections, further fractures, dislocation and compartment syndromes make this procedure even more difficult and not practical [128]. More recently, another technique has been introduced which can be used only for adolescents who have reached maximal growth. This technique lengthens the affected

limb by using an implantable and programmable distraction nail known as "Fitbone". [129]. Fitbone eliminates the need for an external fixator and hence has the potential to reduce pain and the risk of infections occurring within the treatment site. However, this "Fitbone" procedure cannot be used in younger children with growth plate injuries as the procedure will disrupt the growth plate.

Sometimes, an established bone-bridge can be surgically removed for correction of growth defects. In order to prevent growth arrest and angulaiton deformity from recurring, the defect site can be filled with transplanted fat, muscle, polymeric silicone, bone wax and bone cement as interposition materials [120]. This procedure is called the Langenskiold method [130]. However, all of these available treatments so far are extremely invasive, time consuming and often results are not as successful as desired. Currently, much interest has been drawn in finding a better treatment (particularly by a preventative biological approach) to prevent and/or correct problems associated with bony bridge formation. In particular, in more recent times, more research has focussed on utilising tissue engineering and the use of stem cells for the regeneration of growth plate cartilage.

1.3.2. Earlier studies on transplantation of tissues or chondrocytes

This void or deficiency of a biological treatment for growth plate injuries has instigated many medical scientists and clinicians to find a potential biological therapy which is able to prevent the bony repair at the injured growth plate and hence thwart the serious orthopaedic problems associated with this condition. Ideally, a successful therapy would have the ability to regenerate the growth plate cartilage so that the long bone is able to grow with minimal disruption- minimising any angulation and/or growth arrest of the affected limb. However, as with any cartilaginous structures the trans-physeal growth plate injuries are very hard to fix as chondrocytes are very difficult to regenerate [93, 131].

Allogeneic and autogenous chondrocytic transplantations are one potential therapy to overcome this problem, and both methods of chondrocytic transplantation have previously been utilised or trialled for articular cartilage or growth plate repair studies. Allogeneic chondrocytic transplantation involves the removal of healthy chondrocytes from one source followed by the *ex vivo* expansion and finally the replantation of the healthy chondrocytes into another source (of the same species) [132]. However the disadvantages of this procedure involve the risk of disease transmission between the two sources. Alternatively, autogenous chondrocytic transplantation involves the direct harvest of healthy chondrocytes (often from the knee) which are then cultured and expanded *ex vivo*; unlike the allogeneic approach, the chondrocytes are implanted back into the patient at the location of the defect, therefore, eliminating any risks of disease transmission [133]. Nevertheless, the disadvantage of this method is the time frame taken to collect, expand and reimplant which is an estimated 3 weeks [134] - by which time, in the case of growth plate injury a bone bridge has already started to form, thus eliminating this autogenous chondrocytic transplantation approach being feasible for growth plate regeneration.

Although there have been many successful studies which have used these methods for cartilage regeneration of articular cartilage, not many studies have been performed on growth plate injury models. One early study by Bentley and Greer (1971) found success when allogeneic chondrocytes (collected from the growth plate) were delivered into the growth plate injury site of White New Zealand rabbits. This study reported that chondrocytes filled the defect and were able to form columns, and although there were signs of endochondral ossification at the base of the injury site, no rejection of the implanted chondrocytes occurred [135]. However, one study, using a large animal (sheep) model of tibial growth plate injury, attempted transplanting allogeneic chondrocytes directly

into the growth plate injury site and did not produce any successful outcomes in preventing the bony bridge formation [136]. However, due to the risk of disease transmission, time consumption, limited amount of harvestable chondrocytes, and poor and limited ability to regenerate the injured growth plate [121], the chondrocytic transplantation approach is not a clinically viable option for the treatment of growth plate cartilage damage, and therefore a better source of cells is much needed.

1.3.3. Growth factor - based treatments

Chondrocytes within the body are embedded within an enriched matrix, offering support and being an ideal environment for these cells. Therefore, in order to encourage integration and maintenance of implanted chondrocytes, the use of scaffolding made from various natural and synthetic materials such as collagen gels, agarose gels, polyglycolic acid have been previously studied. Gringolo *et al* found that chondrocytes which were embedded in a hyaluronic acid based scaffold were unable to redifferentiate and maintain their original phenotype [137]. In addition, they found that there was a reduction in the production of factors involved in cartilage degradation such as MMP-13 and caspases- hence resulting in a lower percentage of apoptotic cells [137]. As well as scaffolds, previous studies have also added various growth factors *in vitro* and *in vivo* to promote and maintain chondrogenesis including FGF-2, TGF-β 1&3, BMPs and IGF-I [138-141]. For example, transforming growth factor TGF-β1 was shown to increase gene expression of typical cartilage related genes (Sox9, aggrecan, and collagen-IIa) *in vitro* in human mesenchymal cells [138], and addition of TGF-β1 to a chondrocyte gel construct was able to encourage proliferation of chondrocytes within the scaffold [142]. In addition, TGF-β was shown to be able to facilitate cartilage regeneration [143, 144]. Similarly, bone morphogenic protein (BMP), the other member of TGF-β superfamily, has also been

established as a potent chondro-inducing protein which stimulates mitosis and matrix production in chondrocytes as well as the chondrogenic differentiation of mesenchymal stem cells (MSCs) [145-149].

Although there have been only a few studies showing positive results in support of direct injection of growth factors, many articular cartilage related studies have reported the lack of cell growth within the site of degeneration. One of the reasons may be related to a relatively small molecular mass and high solubility of the growth factors and thus their short half life. For example, the half life of FGF-2 is around 1.5 minutes and that of TGF-β1 is around 111-160 minutes [150]. The development of scaffolds and controlled releasing microspheres have enabled a more stable and sustained method of delivery for these chondrogenic growth factors into the their desired site of injury. For example, Holland *et al* (2005) developed a microsphere system to deliver IGF-I and TGF-β1 over a sustained period of time to promote optimal cartilage repair [142]. One study has suggested that implantable scaffold and growth factor combinations would allow the undamaged existing chondrocytes from adjacent areas to be able to migrate into the affected site and form repair tissue- using the scaffolding as bearing [151]. Furthermore, Kim *et al* (2006) observed that incorporation of TGF-β1-containing microspheres in a chitosan-based scaffold was able to successfully promote chondrocyte proliferation and matrix synthesis *in vitro* [152] and to promote cartilage regeneration *in vitro* [153]. One of the few growth factor-based treatment studies carried out specifically for growth plate cartilage was conducted in a sheep model. Thomas *et al* (2005) utilized a combination of the Langenskiöld method (whereby an interpositional material is inserted in to the region where the bone bridge was previously surgically removed) with the addition of osteogenic protein-1 (OP-1) (commonly also referred to as BMP-7) [154, 155]. OP-1 has been well documented for its osteogenic as well as chondrogenic potential [156-158]. It

was found that placement of OP-1 resulted in an increased chondrogenic response in the adjacent growth plate cartilage; however, the addition of OP-1 also resulted in an accelerated osteogenic response with increased expression of bone formation related genes Type I collagen, osteopontin and decorin [154, 155].

Although the potential of TGF-β1 as an effective inductor of chondrogenesis is well known [134, 159, 160], sometimes the use of this particular growth factor can result in some side effects such as inflammation, effusions and osteophyte formation within the joint cavities and subsynovial connective tissue [161, 162]. Therefore, a study in an adult miniature pig osteoarthritic model has identified ideal concentrations of TGF-β1 to exert optimal chemoattractant and mitogenic properties as approximately 200-1000ng/ml [163] - an appropriate dose to maximize effect and minimize adverse effects, Although there are several different growth factors such as TGF-β1, IGF-I or FGF-2 which have shown some potential in encouraging new cartilage formation in various affected areas for disorders including rheumatoid arthritis and intervertebral disc disease (IVD), unfortunately many of these studies have not found successful and desirable cartilage repair outcomes. Overall, the majority of studies so far have been based on articular cartilage repair with an obvious lack of studies regarding the regenerating of growth plate cartilage. Hence, the search for the ultimate biological therapy for encouraging cartilage formation during growth plate injury repair will require further work and possibly a different approach other than growth factor-based.

1.3.4. Mesenchymal stem cell-based treatments

Due to the limitations associated with chondrocytic transplantation including instability during expansion as well as donor tissue availability [164], an alternative cell source was thought to be necessary. Being of an undifferentiated type, embryonic stem cells hold great potential in differentiation and successful

tissue engineering, however the myriad of ethical and potential health risks and dilemmas involved with their use deem them almost inaccessible [165]. On the other hand, adult mesenchymal stem cells (MSC) are renewable multipotent cells which possess the potential to proliferate and differentiate into osteoblasts, chondrocytes, adipocytes and some other cell types [103, 166]. Due to these properties and their ability to be expanded *in vitro*, many studies have now shown their potential in regenerative medicine including cartilage repair [84, 87, 167, 168].

Similar to chondrocytic transplantation, two potential methods of positioning MSCs into the desired area of injury is via autogenous or allogeneic transplantation. Autogenous transplantation of MSCs involves the harvesting of patients own MSCs and then reimplantation after *in vitro* expansion. On the other hand allogeneic transplantation of MSCs involves the use of MSCs taken directly from a cell bank. Using a growth plate injury model in rabbits, Planka *et al* (2008) compared the difference between autogenous and allogeneic mesenchymal transplantation. The study concluded that there were no major differences in the effect of these implanted MSCs on tibia length and potential angular deformities [169]. Furthermore, the implantation of these cells saw the formation of hyaline chondrocytes with the growth plate injury site [169]. This result was also seen when allogeneic MSCs were transplanted into the site of growth plate injury in a guinea pig model [170]. Up to now, the majority of studies have been done on smaller animal models however more recently, McCarty *et al* (2010) performed a similar transplantation using autologous bone marrow MSCs into the larger ovine model of growth plate cartilage injury [171]. Unfortunately, following the transplantation of the BMMSCs which were embedded into a gelatin sponge, no cartilage regeneration was observed. Alternatively however, transplantation resulted in a fibrous formation with a noticeably reduction in undesirable bone formation and more importantly no acceleration of bony repair resulting from the implant [171].

Therefore, these studies implicate MSCs as potential and viable therapeutic options for growth plate regeneration although further translational research particularly with large animal models of growth plate injury repair is required to define whether MSCs can indeed be used for promoting growth plate regeneration.

In studies other than those involving the growth plate, directly injected MSCs resulted in signs of medial meniscus regeneration in a goat osteo-arthritic model. In addition, further analysis also showed that the injected MSC were found incorporated into the newly formed tissue [172]. A similar study was also done in a mini pig articular cartilage knee defect model. Direct injection of MSCs dispersed in a hyaluronic acid solution improved cartilage healing when comparing to untreated controls. However, the authors of this study highlight that the use of hyaluronic acid may have enhanced the cartilage repair of the treated animals by improving MSC migration and adherence [173]. Aside from growth plate and articular cartilage repair, direct MSC application has also been applied to a rat degenerative IVD model. Jeong *et al* (2009) found an increase in cell numbers as well as an increase in levels of extracellular matrix, disc height and signaling activity in rats treated with MSCs. Unfortunately, on the downside, the MSCs were only able to survive for approximately two weeks after injection [174].

1.3.5. Endogenous MSCs

Transplantation of both allogeneic and autologous MSCs offered many advantages in cartilage repair. However a major problem associated with the use of *ex vivo* expanded MSCs is the need for fetal calf serum during ex *vitro* growing and expansion. In addition, the costs and time associated with such expansion and growth of MSCs are also major disadvantages of this technique. Another disadvantage involves the risk of disease transmission particularly during allogeneic transplantation - whereby cells

from one person are transferred into another. However, it has been shown the MSCs can migrate into site of injury and contribute to repair. Ideally, utilizing endogenous MSCs would eliminate some of the issues related with transplantation of cells including immunoreactivity, efficiency as well as the need for repeated surgical procedures [175]. Using a rabbit fracture model, Shirley *et al* (2005) showed systemic movement of fluorescently labeled MSCs/osteoprogenitor cells infiltrating into the fracture during repair with some migrating out from remote bone marrow cavities [176]. In addition to bone fractures, mobilisation of MSCs during tissue repair after injury has also been observed in lung injury [177], liver injury [178] as well as in brain injuries [179]. Importantly, it is evident in previous studies that bone marrow-derived MSCs or chondroprogenitor cells migrate and differentiate into chondrocytes or osteoblasts, thus contributing to the phase of cartilaginous callus formation during growth plate bony repair [5, 23, 28, 29]. Hence, there might be a possibility that endogenous multipotential MSCs located in the bone marrow or around the injury site can be accessed for cartilage repair. The use of endogenous cells would be advantageous as no surgery would be needed and all the cells involved would belong to the patient. However the main problem with accessing these endogenous cells for example from the bone marrow is that they may not be present in a density large enough to support adequate cartilage regeneration. Hence, to overcome this problem a recent study has suggested that selected growth factors are needed to stimulate and enhance MSC migration and accumulation into the cartilage injury site [180]. More specifically, Zhao *et al* (2008) found that dose dependently the addition of hepatocyte growth factor (HGF) *in vitro* increased the amount of migrating MSCs. The existence of functional stem cells within the joint environment and bone marrow represents an opportunity to get around the limitations surrounding MSC transplantation achieving cartilage tissue engineering *in situ* and cartilage regeneration by enhancing the local reparative mechanisms and mobilizing the endogenous MSCs [81].

1.4. CONCLUSIONS

Being of cartilaginous nature the growth plate is highly prone to injury which can result in undesirable bony repair tissue. As a consequence, this type of repair often leads to orthopaedic problems such as limb length discrepancy and bone angulation deformity. Current methods to correct and treat these conditions are mostly highly invasive surgery based, not always effective, often compounded by problems such as infections - often requiring repeated procedures to keep up with the dynamic nature of long bones in young adolescent patients. Although the different phases of growth plate injury repair responses (namely the inflammatory, fibrogenic, osteogenic and remodelling phases) have been established in numerous publications, not a lot is known about the molecular and cellular functions of these events which lead to this unwanted bone repair. Further research and investigations are necessary to develop a clearer idea of the cascade of cellular and molecular events occurring during growth plate injury repair. This knowledge will hopefully aid in developing that elusive biological therapy to prevent and eliminate orthopaedic problems from occurring after growth plate injury by stopping bony repair tissue and encouraging appropriate cartilage formation. In more recent times of cartilage repair research, more interest has been shown towards developing a biological therapy via tissue engineering, with a particular focus on multipotent MSCs. Since MSCs are likely to be involved directly during growth plate injury repair, both endogenous MSCs or *ex vivo* expanded MSCs may have some potential for developing a biological therapy.

1.5. PROJECT RATIONALE, AIMS AND HYPOTHESES

Previous work in a rat growth plate injury model has established stages of repair responses after growth plate injury – the inflammatory, fibrogenic and osteogenic and remodelling phases. Furthermore, several studies examined the potential role of inflammation during growth plate injury repair and found that it was important for not only unwanted bony tissue formation but also for desirable cartilage formation within the growth plate injury site.

However, the molecular events which occur after this phase have not been closely looked at. Therefore this PhD project utilised a rat tibial growth plate injury model to further understand the molecular events occurring after growth plate injury repair with a particular focus on the fibrogenic phase and various growth factors and transcriptional factors which lead to the bony tissue repair. The initial study of this project used various histology and immunohistochemical techniques to identify the potential multipotent MSCs which might be present at the injury site. In the second and third studies, respectively, synthetic inhibitors were also used in the rat growth plate injury repair model to examine the importance and potential role of known key chemoattractant PDGF-BB and osteogenic transcriptional factor osterix during the growth plate bony tissue repair. The information generated from this PhD project will hopefully aid in increasing our understanding of the mechanisms of growth plate bony repair and in the development of a future biological therapy for the prevention of bony tissue formation and regeneration of cartilage within the growth plate injury site.

Using a rat growth plate injury model, the aims of this project were:

1) To examine and identify the presence of progenitor cell infiltration, differentiation and vascularisation during growth plate injury repair.

2) To investigate he potential role of key chemoattractant PDGF-BB during growth plate injury repair particularly during the infiltration of mesenchymal tissue of the fibrogenic phase.

3) To determine the effect of inhibiting the known osteogenic transcriptional factor, osterix, during growth plate injury repair on formation of repair tissues such as cartilage and bone and the final repair outcomes.

It is hypothesised that alongside other cells, multipotent MSCs do exist within the fibrogenic infiltrate following growth plate injury, chemoattractant PDGF-BB plays an important role in the fibrogenic mesenchymal cell infiltration, and the key osteogenic transcription factor osterix signaling is critical in the bony repair of the injured growth plate.

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CHAPTER 2

Mesenchymal progenitor cell infiltration, differentiation and vascularisation during growth plate cartilage repair

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Funding sources: Bone Growth Foundation, Channel-7 Children's Research Foundation of South Australia, Australian National Health and Medical Research Council (NHMRC)

Running Title: Mesenchymal progenitor cells at growth plate injury site

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Conflicts of Interest: All authors have no conflicts of interest.

ABSTRACT

Previous studies using rodent growth plate injury repair models have identified different phases of growth plate injury repair, namely the inflammatory, fibrogenic, osteogenic and remodelling responses. While the myriad of infiltrated mesenchymal cells are known to contribute to form bone and cartilage during the subsequent healing events, it remains to be established whether these cells contain multipotent mesenchymal stem cells (MSCs) and/or osteo- or chondro- progenitor cells. In an attempt to identify the presence of these progenitor cells at the growth plate injury site, immunohistochemical analysis was conducted with rat proximal tibiae at different time points after a drill hole injury. Analysis showed that a portion of cells during the fibrogenic response were immunopositive for known MSC markers - alpha smooth muscle actin (αSMA) or bone morphogenic protein receptor 1a (ALK-3). In addition, some cells present within the infiltrate at days 4 were also found to be positive for von Willebrand factor (vWF) a MSC-negative but endothelial cell-positive marker. Further analysis also showed that some of these cells at day 4 were positive for osteogenic transcription factor core binding factor- alpha 1 (cbf-α1) or chondrogenesis marker collagen-IIa (col-IIa), suggesting the presence of progenitor cells for osteogenesis and chondrogenesis respectively. Furthermore, immunohistochemical analysis confirmed the presence of angiogenic factor vascularendothelial growth factor-a (VEGF-A) as well as vWF later on in the repair process. Moreover, further analysis of vascularisation using endothelial cell marker, isolectin-B_4, showed that vascularisation and isolectin-B_4 positive cells were present as early as day 4 post-injury. Therefore, these findings suggest that within the infiltrating mesenchymal tissue, there may potentially be MSCs as well as other pre-committed osteo- and chondro- progenitor cells which, together with some vascular precursor cells, contribute to the bony repair of the injured growth plate.

INTRODUCTION

Due to the weak mechanical strength, the growth plate is often submitted to trauma injuries, which can often result in various orthopaedic problems such as limb length discrepancy and angulations during childhood and adolescence. Previous studies on growth plate cartilage injury repair have observed three specific phases of growth plate injury repair – the inflammatory phase, the fibrogenic phase and the osteogenic and maturation phase. While previous studies have identified some growth factors and cytokines such as fibrogenic growth factor (FGF-2) and platelet derived growth factor (PDGF-BB) produced during the inflammatory and early fibrogenic phases that might have contributed to the infiltration of mesenchymal cells, the composition and potential functions of these infiltrated cells remain to be characterised.

Previous studies have demonstrated that the infiltrated mesenchymal cells contribute to the bone and cartilage formation during the bony repair of injured growth plate and thus it has been postulated that undifferentiated mesenchymal stromal (stem) cells (MSCs) and/or some already committed skeletal progenitor cells may exist at this fibrogenic phase of growth plate repair. MSCs are multipotent cells with the capability of differentiating under certain conditions or signals into various types of mesenchymal cells including chondrocytes, osteoblasts and adipocytes [1-4]. Unfortunately, distinguishing a true MSC has been a challenge as currently no exclusive markers exist which clearly or definitively identify these cells. However, previous studies have pinpointed a myriad of various so-called positive and negative surface markers using cell flow cytometry to establish the existence of these multipotent cells. These reported positive surface markers for MSCs include STRO-1, CD105, CD90, CD73, CD29, CD44, CD166 and CD146 [5-10]. In addition to these positive indicators, MSCs are reported as lacking the expression of CD45 and CD31, known markers for haematopoietic and endothelial cells, respectively. Immunohistochemically, MSCs have been

shown to be immunopositive for various markers including alpha smooth muscle actin (αSMA) and bone morphogenic protein receptor 1a also known as activin A receptor type II-like kinase 3 (ALK-3) [6, 11, 12] and negative for the endothelial marker, von willibrand factor (vWF) [6]. However, whether MSC-like cells exist within the growth plate injury site during the early fibrogenic phase or whether the infiltrated cells already contain pre-committed or differentiated skeletal or endothelial cells remain to be investigated.

Angiogenesis or neovascularisation is an integral part of bone formation, fracture healing as well as growth plate bony repair – needed for the transformation of fibrous tissue into bony tissue [13]. Previous studies have already demonstrated that vascular endothelial growth factor (VEGF-A) is the key factor that promotes angiogenesis during bone formation and fracture healing [14]. Although the importance of VEGF's role in neovascularisation/angiogenesis has been well studied during development and wound healing including fracture healing, further studies are needed to characterise VEGF expression and potential roles during growth plate injury repair.

Using immunohistochemistry in a rat growth plate injury model, the current study aimed to identify potential MSCs as well as pre-committed osteoprogenitor and chondroprogenitor cells which enter the injury site or are differentiating within the injury site. In addition to examine the time course of angiogenesis during growth plate bony repair, expression of VEGF-A as well as endothelial markers vWF and isolectin-B_4 (I-B_4) were examined immunohistochemically.

MATERIALS and METHODS

Growth plate injury repair time course and tissue specimens

Eight-week-old male Sprague-Dawley young rats were subjected to experimental growth plate injury in the proximal tibia of both hind legs as described [15]. All protocols followed the Australian code of practice for the care and use of animals, and were approved by the Animal Ethics Committee of the Children, Youth and Women's Health Service, South Australia. Under halothane inhalation anaesthesia, an incision was made to expose the anterior-medial aspect of the proximal tibial bone and growth plate of both hind-limbs. A 2 mm surgical drill was then used to make a cortical window in the metaphyseal bone on the medial side. A central disruption of the growth plate was then induced by the drill through the cortical window and perpendicular to the growth plate cartilage. Saline was then irrigated through the drill track to rinse out debris before the wound was closed. A group of normal rats receiving no injury was used as the normal control group.

Groups of rats (n = 8 per time point) were euthanized by CO_2 overdose for specimen collection on day 1, 4, 5, 8 & 10 post-operation, time points that have been previously shown appropriate for observing injury-induced inflammation, fibrogenic and bone formation responses at the injured growth plate [15]. Briefly, the left proximal tibia containing the injury site was collected, fixed in 10% buffered formalin for 24 hours at 4°C and decalcified for 6 days at 4°C in formic acid-based bone decalcifier solution Immunocal (Decal Corporation, New York). The decalcified left proximal tibia was cut perpendicular to the growth plate cartilage, longitudinally bisecting the point of injury. One half of each specimen was taken through alcohol solutions of increasing concentrations and processed routinely for paraffin embedding. For the current study, 5 μm sections were cut from

paraffin tissue blocks and collected on SuperFrost Plus glass slides for H&E and Alcian blue as well as immunohistochemical staining.

Immunohistochemistry

To examine the potential presence of potential MSCs, expression of a couple of known positive (αSMA and ALK-3) and negative (vWF) markers was analysed immunohistochemically using sections taken on day 4-5 post-growth plate injury. In addition, to examine the presence of possible osteo- and chondro- progenitor cells, core binding factor- alpha1 (cbfα-1), alkaline phosphatise (ALP) and collagen-IIa (col-IIa) were used respectively. Briefly, sections were dewaxed, rehydrated and quenched in 3% H_2O_2 for 20 minutes to remove endogenous peroxidase activity and incubated in antigen retrieval solution (Proteinase K, 0.01M Citrate buffer pH 6.0, or DAKO pH 6.0 antigen retrieval solution) at 70°C for 90 minutes or 20 mins at room temperature for proteinase K (Table 1). Sections were blocked in 5% normal pig serum in 1% bovine serum albumin/phosphate buffered saline (1%BSA/PBS pH 7.4) for 90 minutes. The sections were then incubated with appropriate primary antibodies overnight at 4°C (mouse anti-αSMA 1:200 (R&D System); rabbit anti-ALK3 1:400 (Santa Cruz Biotechnology); rabbit anti-von Willebrand factor 1:100 (DAKO); rabbit anti-cbfα-1 1:400 (Santa Cruz Biotechnology); swine anti-alkaline phosphatase 1:400 (DAKO); swine anti-col-IIa 1:800 (Santa Cruz Biotechnology)). Incubated sections were washed with PBS and labelled with appropriate secondary antibodies (αSMA- rabbit anti-mouse IgG (DAKO); ALK3 and vWF- swine anti- rabbit IgG (DAKO)), ABC-complex reagents (1:500) and liquid DAB Plus substrate (DAKO) [15]. Replacement of the primary antibody with 1% BSA in PBS was used as a negative control.

Table 1: Primary monoclonal antibodies and retrieval solutions used in this study

Antibody	Isotype	Dilution	Retrieval
αSM actin (R&D Systems)	Mouse	1:200	DAKO pH6
cbf-α1 (Santa Cruz Biotechnology)	Rabbit	1:400	Citric buffer
BMP R1a (Santa Cruz Biotechnology)	Swine	1:400	Citric buffer
Alkaline phosphatase (DAKO)	Swine	1:400	Citric buffer
Von Willebrand Factor (DAKO)	Rabbit	1:100	Citric buffer
Col-2a (Santa Cruz Biotechnology)	Swine	1:800	Proteinase K
VEGF-A (Santa Cruz Biotechnology)	Rabbit	1:200	Citric buffer

Isolectin-B_4 labelling of endothelial cells

To investigate neovascularisation during growth plate injury repair, Isolectin-B_4 (IB_4) labelling – (a known marker for endothelial cells) was conducted [16]. Isolated from *Griffonia simplicifolia* (Bandeiraea), isolectin-B4 has previously been used to identify neovascular structures in tumor networks [17]. I-B_4 recognises α–galactosyl residues expressed on various cells including endothelial cells [18-20]. Briefly, sections from Day 5, 8 and 10 post-injury were deparaffinised and endogenous peroxidase quenched for 30 minutes using 0.3% H_2O_2 in Methanol. Sections were then rinsed with PBS and further blocked for 30 minutes with 1%BSA/PBS solution to decrease non-specific binding. Biotinylated Isolectin-B_4 (Vector Labs, United Kingdom) was diluted in PBS (1:100) and then added and incubated for 30 minutes at room temperature. After several PBS washes, sections were incubated with ABC reagent for 30 minutes, then liquid DAB Plus substrate was added and slides

were then counterstained and cover-slipped. Replacement of the primary antibody with BSA at 1% in PBS was used as a negative control.

RESULTS

Growth plate injury and phases of repair

Haematoxylin, eosin (H&E) and Alcian blue staining revealed, in comparison to the uninjured control, a clear structural disruption of the growth plate in rats which had been subjected to growth plate injury surgery in the proximal tibia (**Fig. 1A-1B**). On day 1 post-injury, the growth plate injury site is flooded with inflammatory cells (**Fig. 1B**). Starting from day 3-4, the fibrogenic phase commenced, with infiltration of mesenchymal cells and other cells (**Fig. 1C**). By day 10 post-injury, the cells within the injury site have undergone differentiation and tissues such as bone trabeculae and cartilaginous tissue were present (**Fig.1 D**).

Identification of potential MSCs, osteo- and chondro- progenitors and repair tissues within the injury site

To determine whether potential MSCs are present within the mesenchymal infiltrate, immunostaining of two known positive markers of MSCs as well as one negative marker was performed. Analysis of the injury sites during the fibrogenic phase found immunopositive cells for both known positive markers αSM-actin and ALK-3 as well as for the known endothelial marker and negative MSC marker, von Willebrand factor (vWF) (**Fig. 2A-F**). Furthermore, fibrogenic cells were also found to be immunopositive for cbf-α1 (**Fig. 3A**) and col-IIa (**Fig. 3B**) –indicating the potential presence and

involvement of both pre-differentiated MSCs and already committed osteo- and chondro- progenitor cells during the fibrogenic phase of growth plate injury repair. In addition, as a means to examine the potential multi-potent capabilities of some of these cells to differentiate into various repair tissues (namely bone trabeculae and cartilaginous tissues), immunohistochemistry of osteocalcin and col-IIa was performed on specimens collected on days 5 and 10 post-growth plate surgeries (**Fig. 3C-D**). Immunopositive detection of all three antibodies show that during the mid-to-late stages of growth plate injury repair, infiltrated fibrogenic cells were able to undergo differentiation and form bony tissue and cartilaginous-like tissue.

Identification of VEGF and angiogenesis during growth plate injury repair

To investigate angiogenesis/vascularisation during growth plate injury repair and bony repair tissue formation, expression of VEGFa was examined by immunostaining. Positive immunodetection of this growth factor was found within the injury site (day 8) as well as within the hypertrophic zone of the growth plate where VEGF is expressed normally [21] (**Fig. 4A-B**). In addition, endothelial marker, vWF was used to show blood vessel formation during the later stages of growth plate injury repair. Presence of blood vessels formed as identified by vWF immunopositive cells was shown mainly during days 8 & 10 post-surgery (**Fig.4C-D**). Isolectin-B_4 was also used to label endothelial cells to further analyse blood vessels and angiogenesis during growth plate injury repair. Whilst the negative control displayed an absence of positively labelled cells (**Fig. 4E**), the growth plate/metaphyseal transitional area revealed some isolectin-B_4 positive cells (**Fig.4F**). Within the growth plate injury site itself, further qualitative analysis of isolectin-B_4 positive cells showed that the positive cells were

present as early as day 5 post-injury with obvious presence of blood vessels formed occurring later during Day 8-10 post-injury (**Fig. 4G-H**).

DISCUSSION

In long bones, the cartilaginous and dynamic nature of the growth plate cartilage makes it the most probable location for injury, as well as almost impossible for it to regenerate and recover back to its original structure and function. Previous observations of a wide range of growth plate related injuries have established that in 20% of patients, injury will result in a “faulty” or “undesirable” bony repair at the injured growth plate [22]. Unfortunately this faulty bony repair at the injured cartilage hinders the long bone’s growth and function resulting in orthopedic problems such as limb length discrepancy and bone angulation deformity [22-24]. However, despite numerous clinical studies and some experimental work, not enough is known about the molecular mechanism involved in growth plate bony repair. Previous studies have established three very distinct phases during growth plate injury repair namely the inflammatory, fibrogenic and osteogenic and maturation phases occurring during days 1-3, 3-7, 7-onwards, respectively [15, 25]. In particular however, the late inflammatory to early fibrogenic phase is of great interest – as this is when an influx of fibrogenic cells with the capability to form bone and cartilage repair tissues enter into the injury site. To date however, it remains unknown what types of stromal progenitor cells are present in this influx of fibrogenic cells. In addition, neovascularisation is known to be needed for the growth plate bony repair – involved in the transformation of fibrous tissue into bony tissue [13]. However, currently there have been no studies which have examined the time course presence/involvement of the key angiogenic growth factor VEGF and endothelial cells during the different stages of growth plate injury repair.

Although the infiltrated mesenchymal cells have shown the ability *in vivo* to differentiate into various types of cells such as chondrocytes and osteoblasts [26-28], it is unclear whether the cells have differentiated on their own accord at the injury site or whether pre-committed osteoprogenitor or chondroprogenitor cells are already contained in the infiltrate prior to moving into the injury site. Thus, this current preliminary study has attempted to characterise the cell types within the infiltrate – with a focus on examining presence of potential MSCs as well as any pre-committed skeletal cells within the infiltrate. Identification of MSCs within the infiltrate has been deemed to have a certain degree of difficulty, as currently there are no known markers specific for MSCs. Rather, studies have utilised a combination of various positive and negative cell surface markers [5-9, 24], immunohistochemistry techniques [6, 11, 15] as well as certain observed cellular characteristics such as plastic adherence and ability to differentiate into chondrocytes, osteoblasts and adipocytes etc under conditioned media [3, 29, 30]. In the case of immunohistochemistry on paraffin embedded sections, the markers selected for identification of potential MSCs included alpha-smooth muscle actin and ALK-3 (also known as BMPR1a). Previously, alpha smooth muscle actin has been detected on MSCs – more specifically on human bone marrow MSCs (BM MSCs) [6, 31, 32]. Alpha smooth muscle actin is a contractile actin isoform enabling cells to contract and hence is vital for the migration of cells. Positive detection of alpha-SM actin has also been shown in other multipotent types of cells including dental pulp stem cells [33], osteosarcoma cells [34] as well as in *vitro* rat BM MSCs [35] [34]. In this study, a portion of the cells within the infiltrate at the rat growth plate injury site was found to be immunopositive for alpha-smooth muscle actin, therefore indicating the presence of potential MSCs within the infiltration.

To further characterise the potential presence of multipotent cells, another positive marker was analysed. ALK-3 or otherwise referred to as BMPR-1a has previously been detected on both

human and rat MSCs [11, 12]. Previous work on mouse mesenchymal stem cell line C3H10T1/2 found that ALK-3 was expressed at greater levels on MSCs than the other receptor of BMPs, ALK-6 (BMPR-2), and it has been suggested that ALK-3 is responsible for initiating both osteoblastic and chondrogenic differentiation in the mulitpotent cells [36]. In this current study, a portion of the cells within the infiltrate were immunopositive for ALK-3, therefore once again suggesting the existence of potential MSCs within the infiltrate at the injured growth plate during the fibrogenic healing phase. In addition, currently since positive markers for MSCs are usually used together with other known negative markers for MSCs, in this study von Willebrand Factor (vWF), a known marker for endothelial cells but a negative marker of MSCs, was also used [37, 38]. Interestingly, some of the cells during the fibrogenic phase were found to be immunopositive for vWF, indicating the presence of cells other than MSCs. Overall, the above various preliminary immunohistochemical analyses have shown that the observed mesenchymal infiltration during the early fibrogenic phase contains a myriad of cells possibly including MSCs. However, as this preliminary immunolabelling study was done individually, further analysis utilising multiple labelling techniques will need to be performed to allow confirmation of MSCs being present within the fibrogenic cell population during growth plate injury repair. In addition, as a potential future study, one potential way of confirming whether MSCs enter into the injury site during the fibrogenic phase of growth plate injury repair could involve injection into bone marrow prior to injury and tracing their potential migration into the injury site of fluorescently labelling FACS-sorted MSCs.

Additionally, alongside potential MSCs, the infiltrated mesenchymal cells might contain cells which could have already pre-committed prior to entering the injury site and/or committing cells once inside the injury site. In an attempt to pinpoint whether some of the cells within the infiltrate could potentially be already committed or committing into osteoprogenitor or chondroprogenitor cells,

immunostaining with cbf-α1 and col-II antibodies was used, respectively [39, 40]. As a result, some of the cells within the growth plate site were found to be immunopositive for cbf-α1 or col-II. This indicates that during the early stages of the fibrogenic phase some of the cells entering the injury site were already displaying early characteristics of differentiating into either osteoblasts or chondrocytes. Furthermore due to the early timing of this phase, this could potentially indicate that the infiltrate contained some osteoprogenitor and chondroprogenitor cells prior to entering the injury site. Alternatively, the presence of these cells during this early phase could also indicate that the infiltrated MSCs may have already started differentiating into osteo- or chondro- progenitor cells. Although this current study has revealed potential presence of MSCs and/or osteoprogenitors/chondroprogenitors at the growth plate injury site at the early stage of repair, further studies are required to characterise the types of cells which exist within the fibrogenic influx of cells entering into the growth plate injury site. Unfortunately due to the lack of specific markers for MSCs (particularly for rat MSCs) and the difficulty in isolating the cells contained within the growth plate injury site, this study was only able to perform some basic qualitative analyses of the cell populations within the growth plate injury site.

It is known that neovascularisation of the growth plate injury site after the fibrogenic phase is a prerequisite for the conversion of the mesenchymal tissue into bony tissue [13]. However, the molecular mechanism for this remains unknown. One of the most important angiogenic factors expressed during neovascularisation is VEGF-A (vascular endothelial growth factor-A)- a known mediator for physiological and pathophysiological angiogenesis [41, 42]. Out of the six known isoforms of VEGFs, VEGF-A is the only one highly expressed in chondrocytes [43]. Correspondingly to other studies, VEGF-A immunopositive cells were detected within the hypertrophic zone of the growth plate cartilage [21, 42]. The importance of VEGF-A during endochondral ossification and bone formation has been shown via loss-of–function experiments whereby absence of VEGF-A by

genetic means resulted in reduced blood vessel invasion as well as increased hypertrophic zonal height and reduced bone lengthening [44]. Consistent with its obvious importance during bone formation and with the evident bony repair tissue occurring after growth plate injury, VEGF-A expression was found present within the growth plate injury site in this study. VEGF-A immunopositive cells were more evident during Days 8 & 10 post-injury compared to Day 4 sections, consistent with previous studies which outline the majority of bony tissue formation occurring after the fibrogenic phase [15, 25]. In addition, positive immunostaining with vWF (a marker of endothelial cells) also showed the formation of blood vessels during the late fibrogenic phase onwards. Blood vessel formation was further analysed on specimens collected Day 5, 8 and10 post-surgery using isolectin-B_4. Interestingly, I-B_4 positive cells were found within the growth plate injury site of Day 5 post-injury samples – indicating that the start of neovascularisation may be occurring fairly early on the fibrogenic phase. Overall our analyses suggest that neovascularisation of the growth plate injury site, which is essential for the development of bony repair tissue within the injury site, starts fairly early on in the cascade of events leading to the undesirable bony tissue repair.

In summary, identifying the presence of potential MSCs which enter the growth plate injury site is a difficult task due to the difficulty of accessibility and lack of specific cell markers for rat tissues. In addition the methods used in this particular study could also be improved such as utilising a double/triple labelling technique in order to directly compare the positive and negative markers on one observed section. Another potential way of identifying potential MSC and their presence within the growth plate injury site may involve the use of green fluorescence labelling of isolated MSC before their reintroduction just prior injury. The labelling would enable easy detection of how the cells behave during the injury repair process. However overall, results from this study suggest that the fibrogenic infiltrate entering into the growth plate injury site may contain a mixture of potential MSCs

(with the ability to differentiate into bone and cartilage cells) and pre-committed or committing osteoprogenitor or chondroprogenitor cells. In addition, VEGFa expression is present and neovascularisation starts early following the fibrogenic phase and during the subsequent bony repair events. However further studies are required to characterise the potential MSCs or progenitor cells that contribute to the bony repair of injured growth plate and regulation of the angiogenesis process involved.

FIGURES:

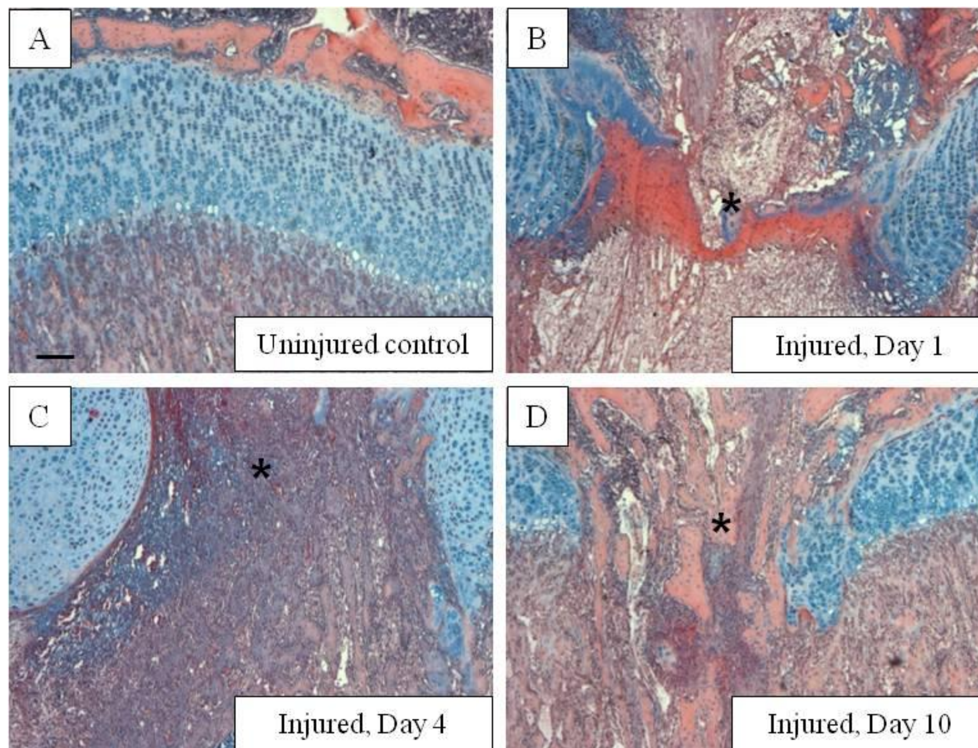

Figure 1: Growth plate injury and phases of repair in a rat growth plate injury model. Following injury, there is an initial influx of inflammatory cells on day 1 (**B**), followed by the fibrogenic phase with infiltration of mesenchymal cells during days 3-5 (**C**), and the bone formation and remodelling phase (day 7 onwards) (**D**). Bar in **A** (applies to **A-D**)= 50 µm

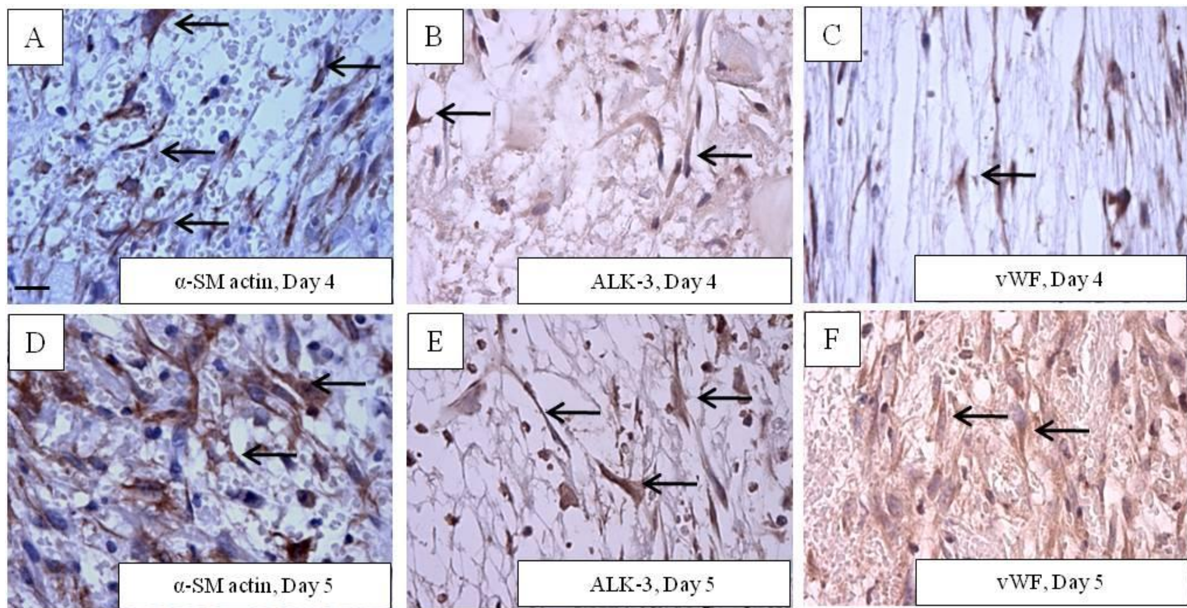

Figure 2: For identification of potential MSCs, Day 4 and 5 post-injury sections were stained with positive markers, α smooth muscle-actin (αSM-actin) and ALK-3 as well as negative MSC marker, von Willebrand factor (vWF). Immunopositive cells for αSM-actin (**A, D**) and ALK-3 (**B, E**) were detected on fibrogenic cells within the infiltrate entering the growth plate injury site at both days 4 and 5. Fibrous cells immunopositive for endothelial marker, vWF, were also observed within the infiltrate (**C, E**). Bar in **A** (applies to **A-F**)= 250 μm

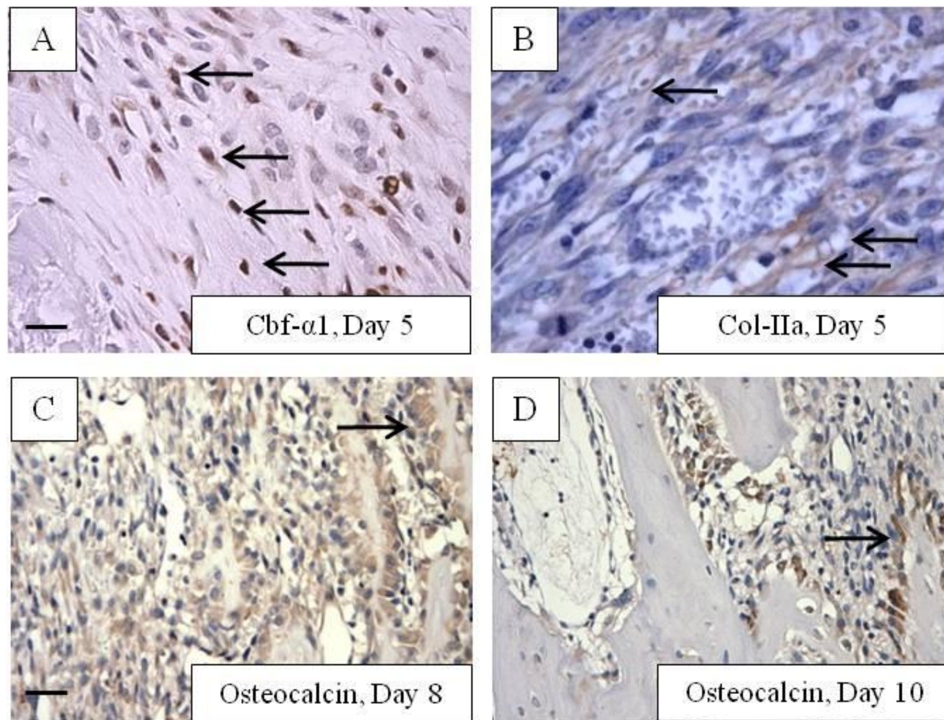

Figure 3: Presence of cells mmunopositive for osteoblastic differentiation factor cbf-α1 (**A**) and cartilage protein collagen-IIa (**B**) within the growth plate injury site during the fibrogenic phase (day 5), and cells positive for bone protein osteocalcin at Day 8 and 10 within the growth plate injury site (**C, D**). Bar in **A** (applies to **A,B**)= 250 μm. Bar in **C** (applies to **C,D**)= 125 μm.

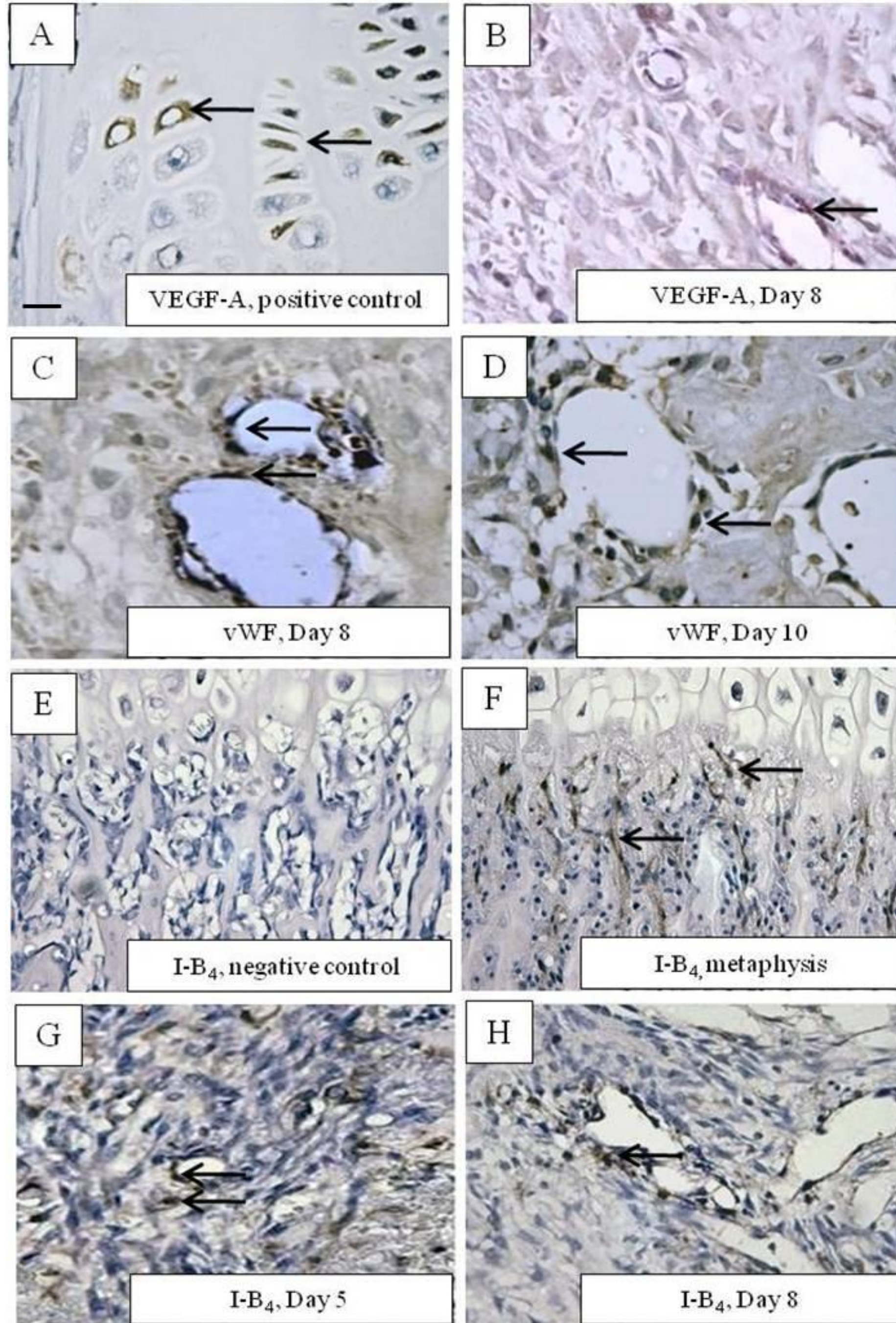
A
VEGF-A, positive control
B
VEGF-A, Day 8
C
vWF, Day 8
D
vWF, Day 10
E
I-B4, negative control
F
I-B4, metaphysis
G
I-B4, Day 5
H
I-B4, Day 8

Figure 4: Immunostaining of VEGF-A, vWF and I-B_4 for visualising presence of angiogenesis/vascularisation during growth plate injury repair and bony repair tissue formation. VEGF-A immunopositive cells were found within the growth plate hypertophic zone (normally the case) (**A**) as well as at the growth plate injury site (**B**). vWF- immunopositive cells were found during the later phases of growth plate repair (**C, D**). I-B_4 positive cells were shown within the metaphysis as vascularisation begins within the hypertrophic/metaphyseal transition zone (**F**). I-B_4 positive cells were also detected within the injury site days 4 and 8 post-injury (**G, H**). Bar in **A** (applies to **A-H**)= 250 μm

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CHAPTER 3

Potential roles of growth factor PDGF-BB in the bony repair of injured growth plate

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Published, Bone 2009

NOTE:
Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

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, v. 44(5), pp. 878-885

NOTE:
This publication is included on pages 84-91 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1016/j.bone.2009.01.377

CHAPTER 4

Inhibition of protein kinase-D promotes cartilage repair at injured growth plate in rats

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Bone, Submitted January 2011

NOTE:
Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

Inhibition of protein kinase-D promotes cartilage repair at injured growth plate in rats

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Funding sources: Bone Growth Foundation, Channel-7 Children's Research Foundation of South Australia,

Australian National Health and Medical Research Council (NHMRC)

Running Title: Osterix in growth plate repair

Key words:

- **Growth plate**
- **Osteoblasts**
- **Injury repair**
- **Stromal progenitor cells**
- **Differentiation**
- **PKD**
- **Osterix**

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Word/character counts: Abstract = 246/1472; Manuscript = 3789/21581 ; Figures = 5

Conflicts of interest: All authors have no conflicts of interest.

Abstract

Injured growth plate cartilage is often repaired by bony tissue, impairing bone growth and causing growth defects in children. Currently, molecular events leading up to the undesirable bony repair remain unclear. This study utilised a rat growth plate injury model to investigate the potential role during growth plate bony repair of protein kinase-D (PKD) which is known to regulate osteoblast differentiation transcription factor osterix. Immediately after infliction of surgical injury at the proximal tibial growth plate, young rats received four once-daily injections of vehicle or 2.35mg/kg gö6976 (a PKD inhibitor known for its inhibitory effects on osterix), and injured growth plate samples were collected at day 10. Micro-CT analysis revealed that bone volume at the injury site was significantly lower following gö6976 treatment ($P<0.05$). Histological analysis showed that, compared to the vehicle control, PKD inhibition resulted in an increase in percent of mesenchymal tissue ($P<0.001$), a decrease in bone trabeculae and bone marrow tissues, and more cartilaginous tissue within the injury site. Consistently, gö6976 treatment decreased mRNA expression at the injury site of bone related genes (osterix and osteocalcin) and increased levels of cartilage related genes (collagen-2a and Sox9). In support, in *vitro* experiments showed that addition of gö6976 during chondrogenic differentiation of rat primary bone marrow stromal progenitor cells resulted in a significant increase in collagen-2a 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 less bone formation and potentially more desirable cartilage repair.

Introduction

Being a large cartilaginous structure at the ends of all long bones in children, the growth plate is the most fragile region in the bone itself. Hence, growth plate related injuries are common in children, which are often (up to 30%, depending on the severity and location of the injuries) repaired by bony tissue that can result in orthopaedic problems such as limb length discrepancy and bone angulation deformity [1]. Out of the possible five clinically recognised Salter-Harris injury types, long term complications are thought to only arise from those with Type II-V growth plate injuries [2, 3].Currently, highly invasive and sometime ineffective surgical techniques are implemented as corrective procedures for growth plate injury-induced bone defects, and there are no known biological therapies for the prevention of these conditions [4, 5].

At present, detailed mechanisms for the undesirable bony repair of the injured growth plate remain unclear, and understanding the molecular events occurring during the bony repair would be invaluable towards the development of a potential biological therapy. Previous studies have outlined four distinct phases during the process of growth plate repair [6, 7], namely the initial inflammatory phase, the fibrogenic, the osteogenic, and the bone remodelling phases. After the inflammatory event and during the fibrogenic phase, there is an influx of mesenchymal stromal cells entering the injury site, which have been shown to be able to differentiate into both bone and/or cartilage cells [7-11].

During the process of osteoblastogenesis from mesenchymal stem cells (MSC) or stromal progenitor cells, transcriptional factors such as Runx2/cbfa-1 and Osterix are essential, as knockout of either factor results in a significant decrease or clear absence of bone formation [12-16]. Furthermore, studies have shown that Osterix acts downstream from Runx2 [16, 17] and is vital for preosteoblasts to differentiate into mature osteoblasts [18-20], with the inhibition of Osterix in mice resulting in a skeletal structure made entirely up of cartilage [21]. Osterix is a zinc-finger containing transcription factor that is reportedly regulated by similar Runx2-regulating anabolic signals like growth factors BMP-2 and IGF-I [21]. However, while it is the clear that osterix is critical in bone cell differentiation, promoting the expression of osteoblastic genes such as

osteocalcin, osteopontin, collagen-1 and bone sialoprotein [22], roles of osterix in growth plate bony repair have not yet been studied. In addition, whether inhibition of this vital osteoblast transcriptional factor could possibly promote cartilage repair at the injured growth plate remains to be investigated. Unfortunately, such potential studies have been limited by the lack of specific pharmacologic inhibitors for osterix.

Previous studies have shown that protein kinase-D (PKD) activation is required for osteoblast differentiation [23], and osterix up-regulation occurs via activation of PKD [24]. Inhibition of PKD with inhibitor gö6976 blocks BMP-2-induced osteoblast differentiation and mineralisation in *vitro* [24, 25] and FGF-2-induced increased bone formation in rodents [26]. In the current study using a rat growth plate injury model, inhibitor gö6976 was used to investigate whether inhibiting PDK (which potentially inhibits osterix in treated rats) could modulate growth plate repair.

Methods and Materials

Growth plate injury and treatment trial

Twenty two 7-week-old male Sprague Dawley rats were divided randomly into treatment and control groups and were stratified to have a similar starting average body weight between groups. These rats were subjected to an experimental growth plate injury in the proximal tibia of both hind legs as described [6]. All protocols followed the Australian code of practice for the care and use of animals, and were approved by the Animal Ethics Committee of the Children, Youth and Women's Health Service, South Australia. Under Halothane inhalation anaesthesia, an incision was made to expose the anterior- medial aspect of the proximal tibial bone and growth plate of both hind-limbs. A 2-mm surgical drill was then used to make a cortical window in the metaphyseal bone on the medial side. A central disruption of the growth plate was then induced by the drill through the cortical window and perpendicular to the growth plate cartilage, an injury model which resembles a Salter Harris Type IV injury of the growth plate. Care was taken not to disturb the articular cartilage. Saline irrigation through the drill track was carried out to rinse out debris before the wound was closed. Following five days post-surgery the rats were intraperitoneally administered daily injections of vehicle or PKD inhibitor, gö6976 (Sigma-Aldrich), at a dose of 2.35mg/kg for four consecutive days. Rats were treated during this time to coincide with the fibrogenic phase. A group of normal rats receiving no injury and no injections was used as the normal control group.

Groups of rats (n = 8 per treatment) were euthanized by CO_2 overdose for specimen collection on day 10 post-operation, a time point that has been previously shown appropriate for observing injury-induced fibrogenic and bone formation responses at the injured growth plate [6]. Briefly, both tibiae were dissected and cleared of soft tissues. To collect growth plate cartilage for RNA analysis, the metaphyseal bone from the left proximal tibia was carefully snapped apart from the epiphysis, clearly exposing the growth plate cartilage. Using a sterile, surgical scalpel blade, the tissue material present *within* the growth plate injury site was then carefully collected, immediately frozen in liquid nitrogen and stored at -80°C. The entire growth plate was

carefully collected from the non-injured controls. The right proximal tibia containing the injury site was collected, wrapped in saline-soaked gauze, snap frozen and stored at -80°C until needed for micro tomography (µCT) analysis.

H&E alcian blue staining and image analysis of tissue repair

To minimise damage to structural integrity, immediately after µCT scanning, the same proximal tibia was fixed in 10% buffered formalin for 24 hours at 4°C and decalcified for 4 days at 4 °C in decalcifier solution Immunocal (Decal Corporation, New York). The decalcified right proximal tibia was cut perpendicular to the growth plate cartilage, longitudinally bisecting the injury site. One half of each specimen was processed routinely for paraffin embedding. For the current study, 5µm paraffin sections were cut and collected on SuperFrost Plus glass slides for immunohistochemical and histology staining.

To assess the types of repair tissues at the growth plate injury site, haemotoxylin-eosin (H&E) and alcian blue staining was performed on paraffin sections from all animals. Briefly, de-waxed and rehydrated sections were first stained in 0.3% alcian blue in 3% acetic acid (pH 2.5) for 40 minutes and subsequently stained with H&E. The proportions of the different types of repair tissues (mesenchymal infiltrate, bony trabeculae, bone marrow and cartilaginous tissue) within the injury site were assessed by histological measurements based on cell morphology and staining (with fibrous cells being mesenchymal infiltrate, alcian blue colour-stained tissue being cartilage, and pink colour smooth solid structure being bony trabeculae) using an image analysis program (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Measurements were taken from 3 sections of 200µm interval for each sample. The area measurements of different kinds of tissues were then expressed as percentages of the total injury site area as previously described [11].

Real-time qualitative RT-PCR expression analysis of cartilage and bone related genes

Real-time quantitative RT-PCR assays were carried out to examine expression of cartilage and bone related molecules Coll-2a, Sox-9 and osterix and osteocalcin, respectively. In addition, expression of endochondral ossification related protein, collagen-10, was also examined. Total RNA from frozen samples was isolated using Qiagen MicroKit (Qiagen, Victoria, Australia). Samples were DNase-treated and total RNA quality and quantity determined by spectrophotometry. Due to a small yield of RNA from samples of the small injury site, purified samples were pooled proportionally (2 animals per pool of the same group) to yield 1μg of total RNA per pool needed for reverse transcription. Synthesis of cDNA was achieved using random decamers and Superscript III RNase RT (Invitrogen, NSW, Australia). Gene expression analysis of these five genes and Cyclophilin-A as the internal reference was performed using SYBR Green real-time PCR and the oligonucleotide primers as previously described [27]. Gene expression was calculated using the $2^{-\Delta\Delta}$ CT method, where threshold cycle (CT) values from triplicate runs were averaged and calibrated in relation to cyclophilin-A CT values. Levels of gene expression (fold changes) in injured growth plate samples were expressed in relation to normal growth plate control as described [11].

Immunohistochemical analysis

To confirm the presence of cartilage repair at the injury site, collagen-2 immunohistochemistry was carried out as described previously [6]. Furthermore, to examine presence of the process of endochondral ossification as part the potential bony repair mechanisms, collagen-10 immunostaining analysis was also carried out as previously described [6].

Effects of gö6976 on chondrogenic potential of bone marrow-derived stromal cells

To examine the effect of PKD inhibition on the chondrogenic potential of bone marrow derived stromal progenitor cells, in *vitro* chondrogenic assays were performed. Briefly, $1x10^6$ marrow stromal progenitor cells isolated from normal rats were added to 10mL sterile polypropylene tubes and aggregrated for 5 minutes at 600g. The supernatant was carefully removed and replaced with chondrogenic media containing 100μM ITS+ premix- containing insulin, human transferrin and selenous acid (BD Biosciences, Australia), 50U/mL Pen/ Strep (Invitrogen, Australia), 10^{-5} Dexamethasone (Sigma-Aldrich, Australia) and 0.125% BSA in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Australia) along with chondrogenic growth factor human TGF-β1 (10ng/ml) as described [28] and with or without PKD inhibitor, gö6976 (10μM) [24]. Cell aggregates were incubated at 37 ^{0}C/5% CO_2 for 4 weeks with media changed thrice weekly. For histological analysis, pellets were washed and immediately fixed in 10% buffered formalin for 24 hours at 4°C, left in 30% sucrose solution for 24 hours at 4 ^{0}C then embedded in OCT and snap frozen. Four μm OCT frozen sections were cut with a cryostat and stained with Alcian blue H&E for morphological analysis. In addition, immunohistochemical detection of cartilage matrix protein collagen-2a was also performed as described previously [6]. For gene expression analysis of collagen-2a, pellets were washed and digested with a collagenase/dispase solution. After a second wash, total RNA from pellets was isolated using the Qiagen MicroKit (Qiagen, Australia). Total RNA was also extracted from a control culture without the addition of any growth factor or inhibitor. Synthesis of cDNA and gene expression analysis of Collagen-2a (with Cyclophilin-A as the internal reference) were conducted as described above.

Statistical analysis

Data are presented as mean $\pm$ SEM. Comparisons between gö6976-treated and vehicle-treated groups were analysed using one-way ANOVA.

Results:

Effects on bone bridge formation and total bone volume within the growth plate injury site

To explore the role of osterix during the mesenchymal differentiation phase of growth plate injury repair, gö6976 was used to inhibit the activation of protein kinase D which has been shown to potentially suppress transcriptional factor, osterix. Following the administration of inhibitor during days 5-8 post injury, bony bridge formation within the growth plate injury site at day 10 was revealed via µCT analysis. Within the growth plate injury site, bone formation was observed in both injured groups, whilst non injured controls showed no signs of bone bridge formation within the growth plate area (Fig. 1A-C). Analysis of bone volume present within the growth plate injury site showed that rats treated with the inhibitor resulted in a significant ($P<0.05$) reduction in total bone volume in comparison with those treated with vehicle (Fig. 1D).

Effects on tissue repair at growth plate injury site

On day 10 post-injury, histological analysis of the growth plate injury site via H&E and alcian blue staining revealed a variety of different types of repair tissues including mesenchymal, cartilaginous, bone marrow tissues and bone trabeculae (Fig 2A-B). Rats treated with the inhibitor showed a significant increase in the proportion of mesenchymal tissue present within the injury site in comparison to rats of the vehicle group ($p<0.01$) (Fig 2C-D). Albeit statistically not significant, those rats treated with the inhibitor had greater proportions of cartilaginous tissue in comparison to vehicle rats (Fig 2C-D). Correspondingly, in the rats administered with the inhibitor lower proportions of both bony trabeculae and bone marrow repair tissues were noticed (Fig 2C-D).

Effects on expression of cartilage and bone related genes at the injury site

The effects of inhibiting PKD on growth plate injury repair and the formation of bone and cartilaginous repair tissues were analysed at the molecular level via mRNA expression of various cartilage and bone related genes. The change in the osterix expression was not significantly different between the inhibitor-treated injured group and the vehicle treated group although there was a slight decrease in the level of expression of osterix in the gö6976-treated group (Fig. 3A). Consistently osteocalcin gene expression levels also revealed a slight decrease after gö6976 administration in comparison to vehicle treated rats. Interestingly, both injured groups (vehicle and treated) expressed higher levels of osteocalcin in comparison to the normal control group (Fig. 3B). Chondrogenic transcription factor Sox-9 was found to be slightly up-regulated after gö6976 administration when compared to vehicle treated rats (Fig. 3C). Similarly, this effect was also shown with levels of collagen-2a mRNA (Fig. 3D). Interestingly, both chondrogenic related genes were slightly reduced in the vehicle group but returned to basal levels after treatment with the inhibitor gö6976 (Fig. 3C-D).

The presence of cartilage formation was confirmed by collagen-2a immunostaining in chondrocyte-like cells within the growth plate injury site (Fig. 4A). In addition, presence of collagen-10 immunopositive cells within the growth plate injury site suggest that the bony bridge repair involved the endochondral ossification mechanism of bone formation (Fig. 4B). Furthermore, gene expression analysis of collagen-10 also showed that this gene was up-regulated significantly after treatment with gö6976 in comparison to both normal control and vehicle-treatment groups (Fig. 4C).

Effects on chondrogenic differentiation of bone marrow derived stromal cells in vitro

To examine the potential role of PKD in chondrogenic differentiation of bone marrow derived stomal progenitor cells, an in *vitro* chondrogenesis assay was performed. After 28 days of culture, initial observation found cell pellets treated with TGF-β1+ gö6976 were of greater size in comparison to pellets only exposed to TGF-β1 (Fig. 5A). H&E (Fig. 5B) and alcian blue (Fig. 5C) staining of the pellets showed the presence of cartilage cells and glycoaminoglycans, in both treated and untreated pellets. Further immunohistochemical analysis also found that treatment with gö6976 resulted in a visually stronger staining of collagen-2a of the pellet (Fig. 5D). In support, RT-PCR gene expression analysis of the resulting pellets found that addition of inhibitor gö6976 resulted in a significant increase of expression of collagen-2a ($P<0.05$) in comparison to pellets treated with TGF-β1 alone as well as a control with neither growth factor nor inhibitor (Fig. 5E).

Discussion:

Our previous studies in a rat growth plate injury model have established four distinct phases of growth plate injury repair- the inflammatory, fibrogenic, osteogenic and the remodelling phases. In addition, it has been shown that this repair was more prone to result in bony tissue formation rather than back to its original cartilaginous form [6, 8-11]. Although the reason and mechanisms for this preference are currently unknown, investigation of potential roles of various key growth factors and transcriptional factors involved during growth plate injury repair has become of interest. Osterix is often referred to as the secondary master regulator of osteoblastogenesis. Various studies have highlighted the importance of osterix during osteogenesis as the partial or complete absence of osterix resulted in significant decreased bone formation or a skeletal structure made entirely up of cartilage [12-16, 21]. Although the importance of osterix during development has been well explored, its particular role during bony tissue formation during growth plate injury repair is unclear. However, lack of specific pharmacologic inhibitors for osterix has hampered investigations in this area. On the other hand, previous studies have shown that protein kinase-D (PKD) activation is required for osteoblast differentiation [23], and osterix up-regulation occurs via activation of PKD [24]. Therefore using a rat growth plate injury model, this study attempted to examine the potential role of PKD (which potentially suppresses osterix) during the bony repair at the injured growth plate and in addition to see whether PKD inhibition changes the final repair tissue outcome and potentially induces more chondrogenic healing.

In this current study, osterix transcription at the growth plate injury site was partially blocked by systemic administration of a known protein kinase D inhibitor, gö6976, which has previously been shown to successfully inhibit BMP-2 and IGF-I induced osterix expression [24, 25, 29, 30]. In our study, both histological and micro-CT analyses revealed that administration of gö6976 resulted in an apparently decreased amount of bony tissue within the growth plate injury site in comparison to the vehicle control. We showed that inhibition of PKD and partial suppression of osterix by gö6976 resulted in significantly more mesenchymal tissue and

cartilage repair tissues but significantly less bony tissue at the injury site in the treated rats, implying the possibility of PKD and potentially osterix in controlling the rate and fate of osteogenic vs chondrogenic differentiation from stromal progenitor cells with the growth plate injury site. Consistent with histological findings of less bony repair and an increase in cartilage formation at the growth plate injury site after treatment with PKD inhibitor, levels of bone and cartilage related genes were shown to decrease and increase, respectively. However, one noticeable shortfall of this study is the lack of significance for all gene expression data. Although why this has occurred is unknown, there are a few reasons which could have been involved. Animal variation could have potentially resulted in loss of significance of differences between treatments, and therefore the addition of more animals per treatment group may potentially rectify this situation. In addition, the drug dosing regimen chosen for this study may have also been a factor. Although rats were treated from days 5-9 post-injury to ensure the inhibitor was exerting its effect during the fibrogenic period of the growth plate injury repair process (where differentiation of fibrogenic cells into repair tissue should be at its peak), it is difficult to rule out that between the last dose to time of specimen collection (day 10 post-injury) any obvious treatment effects may have been lost. Furthermore, while our previous studies have shown that bone formation, within the growth plate injury site, occurs via endochondral ossification as well as intramembranous ossification [8, 9], the current study showed that gö6976 treatment can significantly enhance the endochondral ossification as indicated by the significant upregulation of collagen-10 at the injury site. Interestingly, Zhou et al (2010) found that complete postnatal osterix inactivation (by genetic means) in mice resulted in a massive accumulation of unresorbed calcified cartilage within the growth plate-metaphyseal border [31], which was due to lack of bone formation on the surface of the cartilage scaffold and hence a reduction in resorption of the cartilage [31]. Our findings suggest that PKD and perhaps osterix plays a role in promoting bony repair at the injured growth plate and PKD suppression can delay osteogenic differentiation of infiltrated mesenchymal cells and promote cartilaginous tissue repair and endochondral ossification.

Previous studies have shown that following different types of injury, MSCs from various locations migrate towards the injury site [32-34]. Shirley et al (2005) showed movement of MSCs into a bone fracture site from remote bone marrow cavities [35]. Hence, as the growth plate is located within the long bone itself, as it has been previously shown that mesenchymal cells from adjacent bone/bone marrow migrated into the growth plate injury site [6], it can be postulated that bone marrow/bone stromal cells would be involved in and contribute to the growth plate repair. Therefore, rat BMMSCs were used to examine the effect of PKD inhibition on chondrogenic potential *in vitro.* The decrease in osteogenesis but increase in chondrogenesis at the growth plate injury site after gö6976 treatment and in *vitro* chondrogenesis assay in the presence of gö6976 suggest the bi-potency or plasticity of the stromal cells. Nakashima et al (2002) found that in their osterix null mice, osteoprogenitor precursor cells still expressed cartilage related genes [16]. Similarly, Runx2-positive osteoprogenitor cells were still able to "switch" and differentiate into chondrocytes at a stage prior to the influence of osterix and osteoblast maturation [36]. More recently, Kaback et al (2008) showed that osterix actually possessed the ability to inhibit chondrocyte differentiation [37]. Similarly, a recent study also observed that the chondrogenic potential of mesenchymal cell line C3H/10T1/2 was abrogated by osterix expression [38]. Due to this plasticity and the ability of osteoprogenitor cells to become chondrocytes in the absence of osterix, modulating the osterix transcription and activity could be a potential approach to enhance cartilage repair. However, in the current study, our gö6976 approach of partially suppressing osterix was found not be able to completely block bony repair at the injured growth plate. A possible explanation may be due to gö6976 being shown to only partially inhibiting osterix by blocking only BMP-2 and/or IGF-I related osterix expression [24, 25]. Another potential reason for the partial switch from bony repair to cartilage formation could be due to the inability of this approach to induce a complete switch of already committed osteoprogenitor cells or pre-osteoblasts to chondrogenic differentiation at the growth plate injury site. Using a lentivirus-mediated shRNA transfection to silence the osterix gene, Tominaga et al (2009) found that chondrocyte differentiation was not significantly enhanced even though osteoblast differentiation was suppressed in human MSCs in *vitro* [38].

In summary, partial inhibition of osterix signalling using PKD inhibitor gö6976 has resulted in an apparent delay in the bony healing of the injured growth plate, increasing the amount of mesenchymal tissue and cartilage but decreasing the volume of bone formed present within the growth plate injury site. Overall, these results suggest that PKD may be an important factor for osteoblastogenesis and bony repair of injured growth plate and blocking PKD activity during growth plate injury repair may result in less bone formation and potentially more desirable cartilage repair. While PKD can affect many other molecules such as TrkA [39], further studies are required to investigate whether PKD can be a potential target for the development of a biological therapy to promote cartilage healing of injured growth plate.

Acknowledgments

The project was funded in parts by grants from Bone Growth foundation (BGF), Channel-7 Children's Research Foundation of South Australia (to CJX and BKF), and from the Australian National Health and Medical Research Council (NHMRC) (to CJX and BKF).

Figure Legends

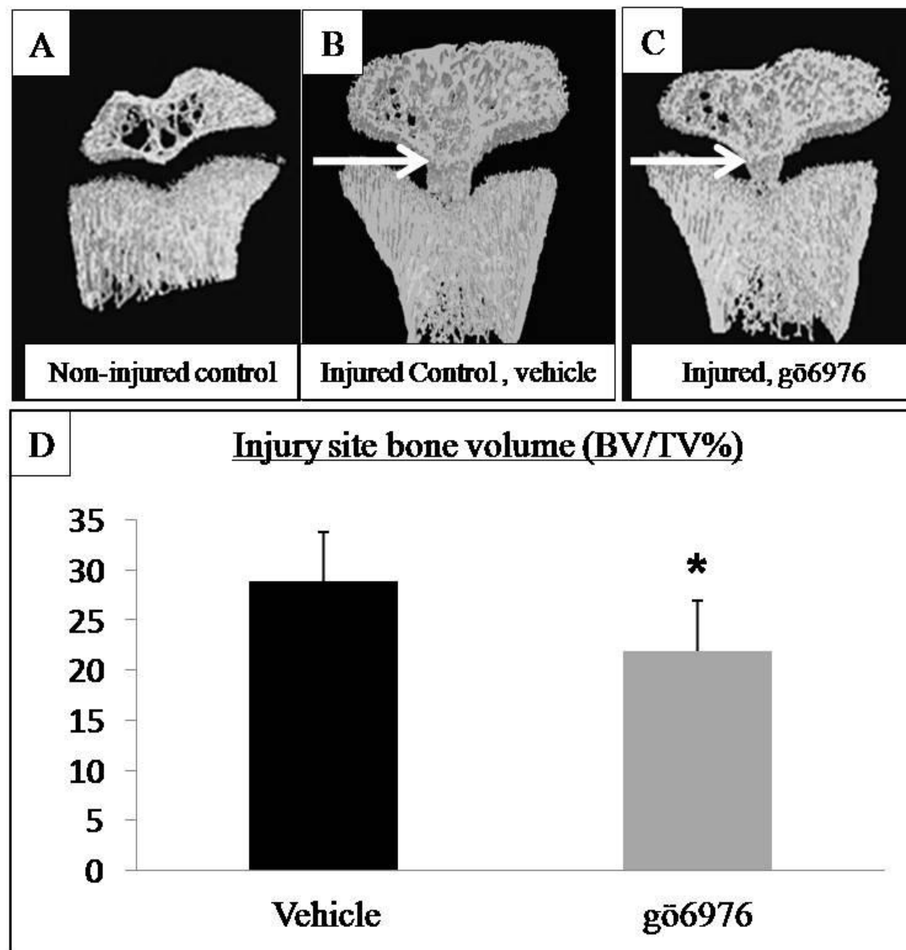


FIGURE 1: μCT analysis of treatment effects with protein kinase D inhibitor gö6976 on bone formation within growth plate injury site. A non-injured control (A); an injured saline-treated growth plate at day 10 (B) and an injured and gö6976-treated growth plate at day 10 (C) calculation of the total bone volume % per tissue volume (BV/TV, %) within the injury site (white arrow) (D), showing a significant reduction in the treated group compared to the non-treatment control ($P<0.05$).

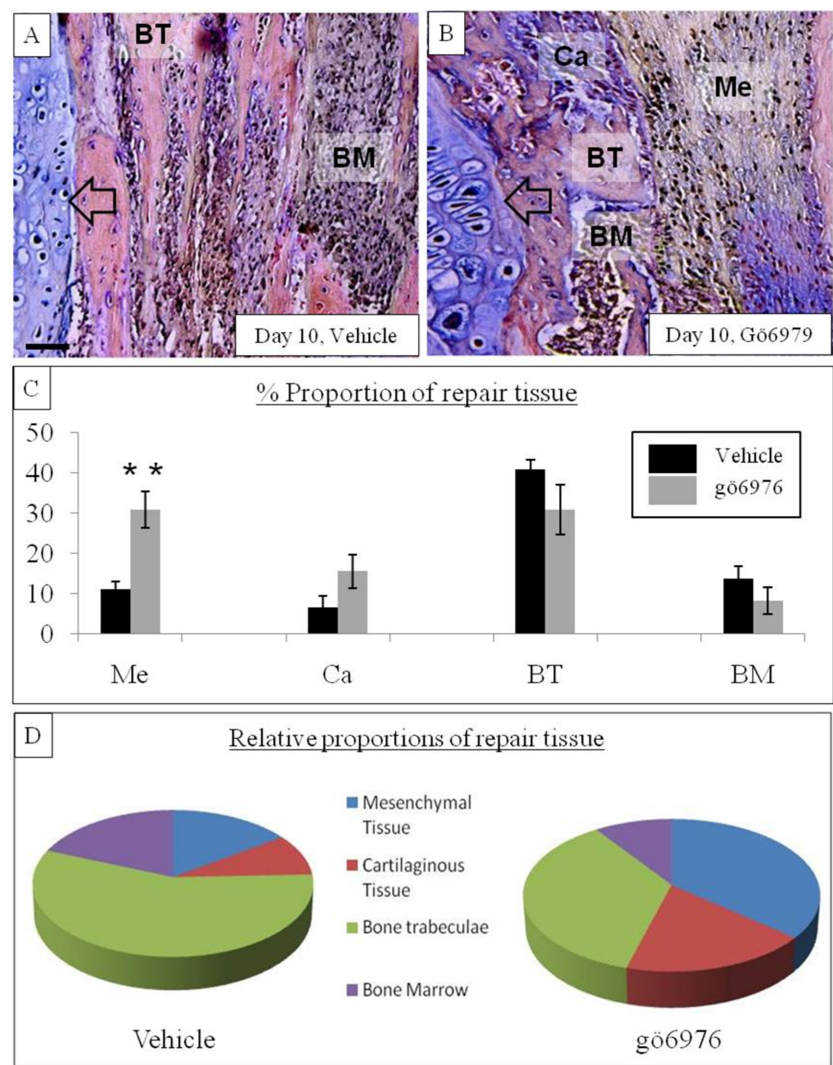


FIGURE 2: Effects of PKD inhibition using gö6976 on proportions of various repair tissues within the growth plate injury site. Injury sites from Day 10 vehicle-treated rats (A) and gö6976-treated rats (B) showed amounts of mesenchymal tissue (Me), bony trabeculae (BT), cartilaginous tissue (Ca) and bone marrow (BM). Treatment with the inhibitor resulted in a significant increase in mesenchymal tissue ($P<0.05$). The remaining adjacent growth plate cartilage is indicated by open arrows. Original scale bar =250 μm (applies to A and B). Quantitative histology image analysis measurements of area percent (over total growth plate injury site areas, n=8) (C) and proportion (D) of each type of repair tissue: mesenchymal tissue- Me, cartilaginous tissue- Ca, bone trabeculae- BT, and bone marrow- BM.

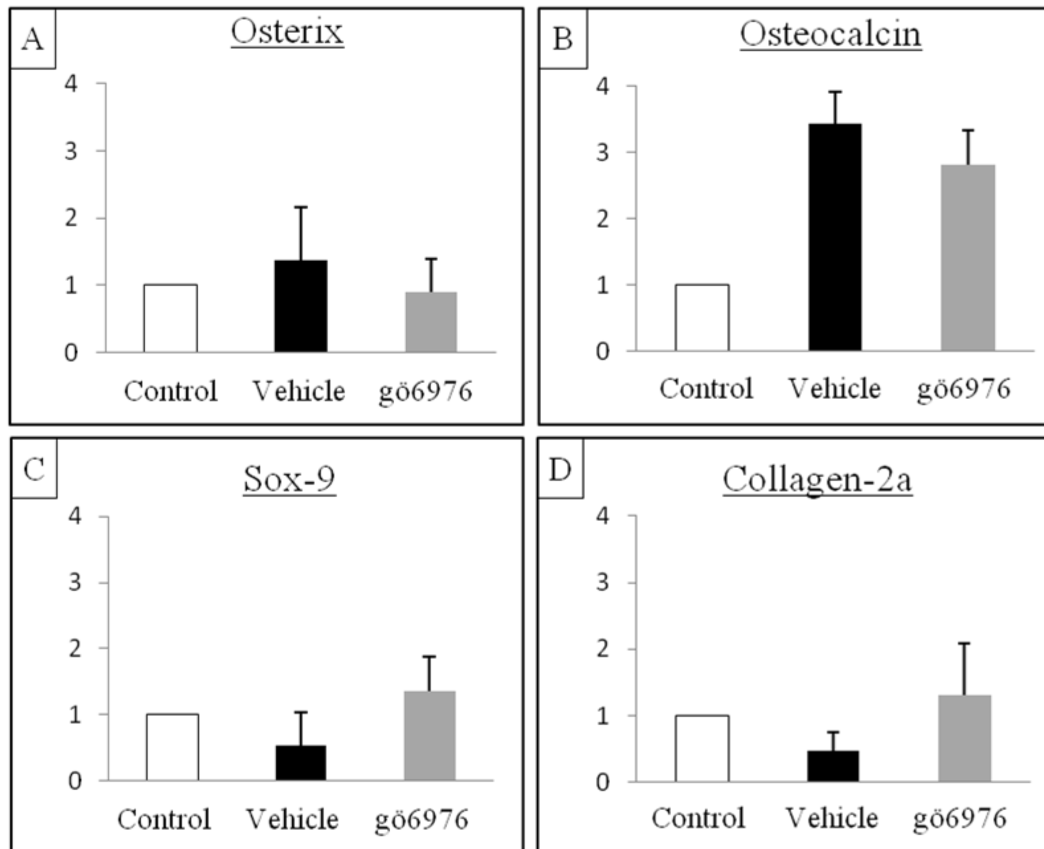
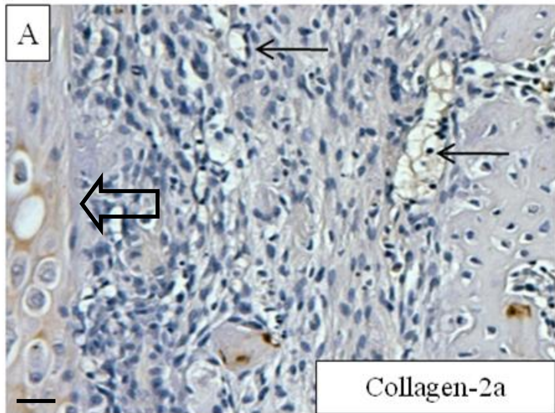
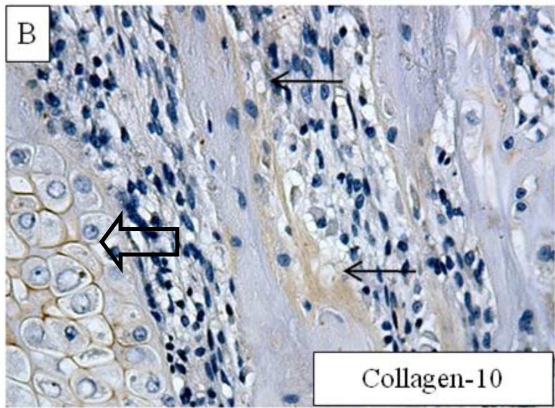


FIGURE 3: mRNA expression of genes involved with osteogenic and cartilaginous repair at the injured growth plate. Quantitative real-time RT-PCR expression data for osterix (A), Osteocalcin (B), Sox-9 (C) and Collagen-2a (D) are expressed as fold change in relation to noninjured, normal control after being normalized to the internal standard cyclophilin-A.


A
Collagen-2a


B
Collagen-10

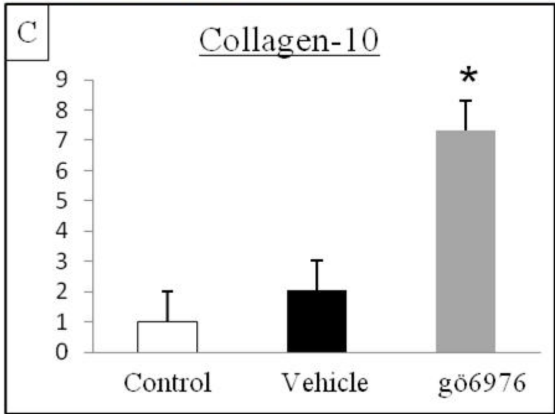

C
Collagen-10
9
8
7
6
5
4
3
2
1
0
*
Control
Vehicle
gö6976

FIGURE 4: Detection of cartilage formation and endochodral ossification at the injured growth plate. Immunohistochemical analysis of Collagen-2a (A) and Collagen-10 (B) were conducted. Immunopositive cells are indicated by arrows (←). The remaining adjacent growth plate cartilage is indicated by open arrows. Original scale bar =125 µm (applies to A,B). Collagen-10 mRNA expression of the growth plate injury site was also analyzed by quantitive RT-PCR showing a significant increased level of Collagen-10 in protein kinase D inhibitor-treated group compared to vehicle treated or non-injured control groups ($P<0.05$) (C).

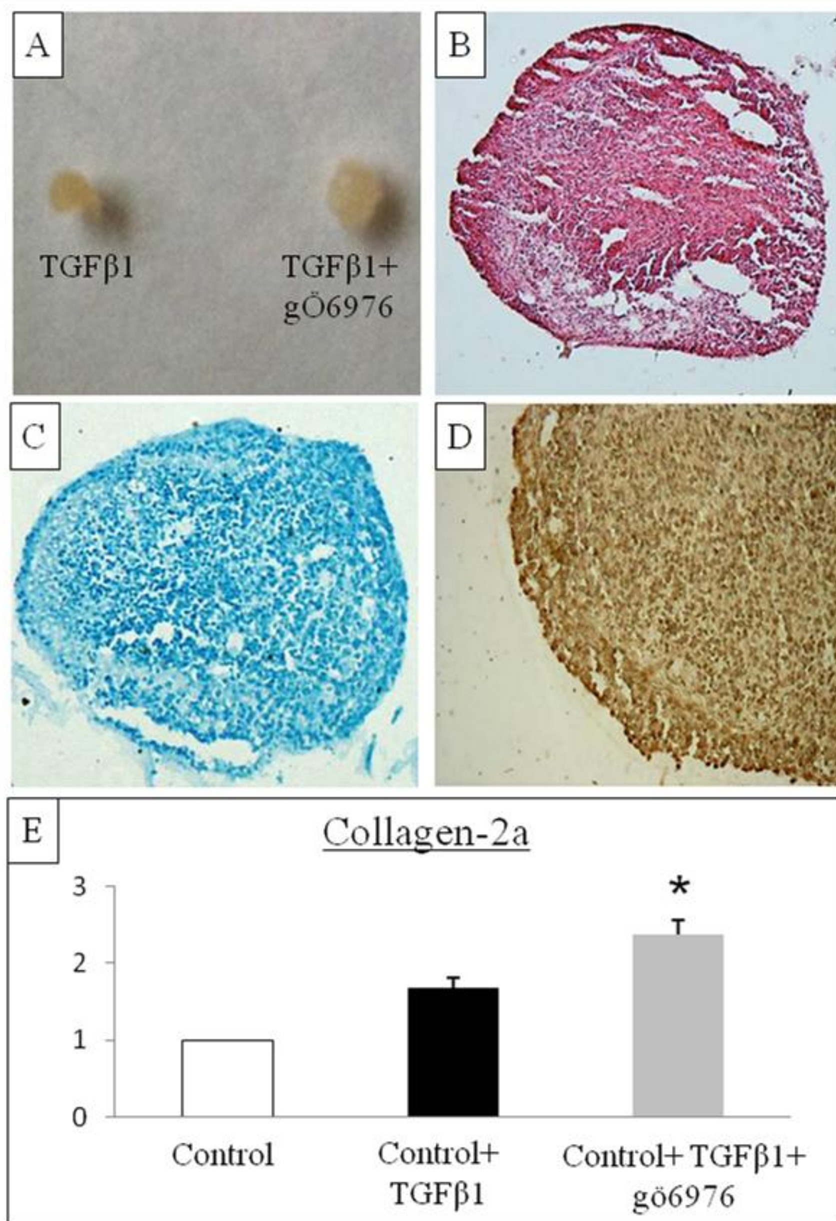


FIGURE 5: Histological analysis of *in vitro* chondrogenic differentiation of bone marrow derived MSC+ TGFβ1 with or without addition of inhibitor, gö6976 (A). Pellets were stained with Haematoxylin and Eosin (B) and with glycosaminoglycan indicator, Alcian Blue (C). Immunohistochemistry and gene expression analysis of cartilage extracellular matrix protein, Collagen-2a showed immunopositive detection of Collagen-2a (D) as well as significant increases in gene expression following exposure to TGF-β1 as well as PKD inhibitor, gö6976 in comparison to control ($p<0.05$) (E).

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CHAPTER 5

General discussion, conclusion and future directions

5.1. GENERAL DISCUSSION & CONCLUSION

5.1.1. Growth plate injury/repair responses and focuses of this PhD project

The growth plate is solely responsible for achieving all longitudinal bone growth during childhood and early adolescence. However, although being such an important part of the body, being of a cartilaginous tissue the growth plate is the weakest area and thus fairly prone to injuries. Unfortunately approximately 20% of the all growth plate related injuries will result in minor to major (depending on the location and severity of injury) orthopaedic-related problems such as bone angulations and limb length discrepancy due to the undesirable bony repair [1-3]. Currently, only surgical treatments are available to correct these orthopaedic problems, which are however not 100% effective, extremely painful and invasive, complicated and often needing to be repeated due to the dynamic and continuingly growing nature of growing bones in children and young adolescents. There are no existing biological or preventative therapies for growth plate injuries. Although previous studies have observed the cellular events and phases occurring at the injury site after growth plate injury – namely the inflammatory, fibrogenic, osteogenic and remodelling phases, respectively, not a lot is known about the molecular mechanisms which activate and control these cascades of events leading to growth plate tissue repair and more importantly the undesirable bone bridge formation [4]. Therefore, to gain a better understanding of this complex repair process, this PhD project has investigated various cellular and molecular aspects during growth plate injury repair using a well established rat growth plate injury model with a particular focus on the early fibrogenic phase and the multipotent cells and signals involved in regulating progenitor cell differentiation and repair tissue formation during the osteogenic and remodelling phases.

As briefly mentioned, many previous studies have outlined the major phases observed during the growth plate injury repair process leading up to undesirable bone bridge formation. Using a rat model, growth plate injury repair was observed to include the inflammatory, fibrogenic and osteogenic and remodelling phases [4]. Coincidently, very similar stages of repair have been found during bone fracture repair, with four recognised stages of fracture repair – the inflammatory, soft callus (fibrocartilage) formation, hard callus formation and finally bone remodelling phase [5]. However, although there have been many studies looking at fracture repair, not many have examined the molecular and cellular events during growth plate injury repair and bone bridge formation. Histologically, the initial inflammatory phase involves an influx of neutrophils and other inflammatory cells which are thought to release a variety of growth factors (PDGF-BB, FGF-2) and inflammatory cytokines (IL-1β, TNF-α) [6-9] potentially triggering a cascade of signals leading to the next healing phase, the fibrogenic response. Whilst the inflammatory phase is the first observed phase of growth plate injury, many previous studies have already examined various aspects of this phase, its potential role in regulating downstream events of growth plate injury responses and bony repair. Various published studies have shown that the inflammatory phase, involving a myriad of inflammatory cells and cytokines, is vital for proper repair tissue formation with the particular inhibition of the most dominant inflammatory cells present, neutrophils, as well as inflammatory cytokine, TNF-α, leading to delayed repair responses and more interestingly an increase in unwanted osteogenesis [7, 9].

Following the inflammatory event, the fibrogenic response (Day 3-7 post-injury) involves an observed influx of infiltrate containing various fibrogenic or mesenchymal cells [4]. Although not yet validated, it has been hypothesised that this infiltrate may contain MSCs – confirmed only by the cells' ability to differentiate into various types of repair tissues, namely chondrocytes and osteoblasts, later on the healing phases. The fibrogenic phase also involves the beginning of differentiation of fibrogenic cells

into various types of repair tissues (namely mesenchymal tissue, cartilage and bone); hence this particular phase is a very dynamic and important stage of growth plate repair. However, it is currently unknown what signals and transcriptional factors are causing the cells to be more in favour of differentiating into bone cells rather than the more desirable chondrocytes. In addition, it has remained unclear whether the absence of key transcriptional factors such as osterix (for osteogenesis) would alter the final differentiation outcome of repair tissues within the growth plate injury site.

The osteogenic and remodelling phases are the final two observed stages of growth plate injury repair. From day 8 post injury onwards, histologically these phases show late differentiation and formation of repair tissues with the obvious presence of bony tissue repair occurring within the growth plate injury site [4]. While bone formation involves bone remodelling and hence vascularisation of tissue, it is still unclear whether blood vessel invasion and the expression of pro-angiogenic factors such as VEGFa occur before or after bone bridge formation at the growth plate injury site.

Therefore, this PhD project has focused on the late inflammatory to early fibrogenic phase with an attempt to examine and identify potential mesenchymal progenitor cells within the infiltration and to investigate roles of chemotatic growth factor PDGF-BB and osteogenic differentiation factor osterix via protein kinase-D (PKD) in their migration, differentiation and bony repair of the injured growth plate. In addition, this project also examined whether VEGFa expression is associated with vascularisation of the injury site and bony repair.

5.1.2. Mesenchymal progenitor infiltration and vascularisation of injury site

The initial study of this project focused on the late inflammatory to early fibrogenic phase in an attempt to examine mesenchymal progenitor cell infiltration, differentiation and vascularisation of the growth plate injury site during the injured growth plate repair process. During bone fracture repair, an influx of multipotent mesenchymal stem cells and progenitor cells (originating from various locations including periosteum, bone marrow and endosteum) has been observed post- inflammatory phase [10-13]. Although the stages of repair are fairly similar, in regards to growth plate injury, the specific types of cells within the fibrogenic infiltration have not yet been characterised and it is unknown whether they contain MSCs-like cells and osteo- and chondro- progenitor cells. However, previous studies have shown the ability of infiltrated mesenchymal cells of injured growth plate to differentiate into various repair tissues [4, 6-8, 14], suggesting the presence of potential multipotent mesenchymal stromal cells within the fibrogenic infiltrate. Similarly in fracture repair, MSCs are shown to undergo proliferation and differentiate and generate repair tissue in a form of a callus [15]. Hence, for the first study of this PhD project, immunohistological technique was utilised in an attempt to identify types of mesenchymal stem cells and progenitor cells present during the fibrogenic phase. Keeping in mind the difficulties in being able to distinguish a true MSC from other mesenchymal cells due to the lack of specific markers, a few known positive (αSMA, ALK3) and negative (vWf) markers were used for this project due to cross-reactivity with the rat tissues [16-18]. Alpha smooth muscle actin (αSMA) is a contractile actin isoform enabling cells to contract and hence is vital for the migration of cells [19]. With the obvious morphological fibrogenic features as well as the ability to migrate into the injury site, a portion of cells within the infiltrate proved to be αSMA positive – indicating potential MSC characteristics. Similarly, previous studies have also found MSCs and other multipotent cells such as dental pulp stem cells [20], osteosarcoma cells [21] as well as rat bone marrow MSCs [21, 22] to be αSMA immunopositive.

However, this marker alone can not distinguish a MSC as other fibrogenic cells could also potentially express this marker. Therefore the second positive marker used for this study was BMP receptor-1a otherwise known as ALK-3. Similar to αSMA, some cells within the infiltrate presented ALK-3 immunopositivity – indicative of MSCs. Previous studies have proposed that ALK-3 was responsible for initiating both osteoblastic and chondrogenic differentiation in the mulitpotent cells [23], and in support, ALK-3 expression has been detected on both human and rat MSCs [17, 18]. Alongside positive markers, vWF was also used. As mentioned previously, there is no one specific marker for MSC identification; therefore identification is done via a combination of positive and negative markers. In this study, cells of the fibrogenic infiltrate showed signs of immunopositivity for known negative MSC and positive endothelial marker, vWF [24, 25]. However, as this is only a preliminary study and each marker was applied separately, further work such as double labelling techniques are required to confirm the potential presence of MSCs within the infiltrate.

In addition, antibodies for cartilage protein, col-IIa, and early bone transcription factor, cbf-α1, were also used to detect the presence of chondro- and osteo-progenitor cells, respectively, within the infiltrate. Detection of immunopositive cells for all of the above five markers within the infiltrate at the early stage of the fibrogenic phase indicated the potential presence of MSCs as well as other committed osteo- and chondro- progenitor cells within the infiltrate. However, although the current study was able to show that these were present within the infiltrate, it could not be confirmed what exactly these cells were – whether MSCs co-exist together with the skeletal progenitor cells within the infiltrate or the progenitor cells themselves were a result from the MSCs already undergoing differentiation just prior to entering into the injury site. Further studies will be needed to clarify this. However, overall this result

(presence of MSC-like and progenitor cells) suggests that it could be a possible therapy target for these undifferentiated cells to be encouraged to differentiate into more desirable types of repair tissue.

During normal endochondral ossification, angiogenesis is eminent for proper bone formation. Angiogenic factor VEGFa is important for vascularisation of the growth plate, as it is the only isoform which is highly expressed in chondrocytes [26]. The expression of angiogenic factor VEGF within the growth plate encourages vascularisation at the hypertrophic-metaphsyeal junction allowing bone cells to move and settle around the newly calcified cartilage scaffold [27]. Even more so, during fracture repair, without proper blood supply, formation of a hard callus can be compromised [28]. Interestingly, although much is known about VEGFa's role in angiogenesis during endochondral ossification as well as bone fracture repair, no previous work has been found that has examined VEGFa role and expression during the unwanted bony repair of the injured growth plate. Corresponding with the obvious bone formation occurring within the growth plate injury site, VEGFa immunopostive cells were detected during the later time points within the growth plate cartilage as well as within the injury site itself, indicating its possible role in the formation of bony trabeculae and bone bridge and it remodelling within the injury site. In addition, further analysis of vascularisation using endothelial cell marker, isolectin-B_4, showed that vascularisation and isolectin-B_4 positive cells were present as early as day 4 post-injury. Therefore, these findings suggest that within the infiltrating mesenchymal tissue, there may potentially be MSCs as well as other pre-committed osteo- and chondro- progenitor cells, which, together with some vascular precursor cells, contribute to the bony repair of the injured growth plate

5.1.3. Roles of PDGF signalling in the fibrogenic response and growth plate repair

As mentioned previously, the fibrogenic phase is a dynamic and important phase in regards to cell migration, differentiation and the repair tissue formation. Therefore the second study of this PhD project delved into gaining a better understanding about some of the molecular mechanisms behind possible triggers or factors which encourage the movement and differentiation of multipotent cells into the injury site. As mentioned earlier, previous work found that the inflammatory phase and cells involved were integral for the progression of repair of injured growth plate injury repair [6, 7, 9]. In particular, PDGF-BB is one of the growth factor released from platelets present during the inflammatory phase [8]. In addition to its significant upregulation during late inflammatory and early fibrogenic phases, PDGF-BB is also a well known potent chemotactic factor of MSCs during skeletal tissue repair [29-31]. During bone fracture healing, PDGF-BB, alongside other growth factors such as TGF-β2, IGF-I and FGF-1, has been shown to stimulate fibroblast proliferation, migration as well as differentiation [11, 32]. However, its particular significance and role during the fibrogenic phase as well as growth plate injury repair are unknown. Using a clinically available and known PDGF-BB receptor inhibitor, Imatinib, in our well established rat growth plate injury model, this part of the PhD project showed that PDGF signalling has potential roles in not only inducing the initial migration of fibrogenic cells into the growth plate injury site and their proliferation but also in promoting their chondrogenic and osteogenic responses and remodelling of the repair tissues. The significant delay in differentiation of fibrogenic cells into repair tissues, namely bone trabeculae and cartilaginous tissue, corresponded with other studies where inhibition of PDGF's receptor also resulted in delayed wound healing responses [33]. Therefore, this study demonstrates the importance of PDGF-BB as a powerful chemottactant and mitogen during the early fibrogenic phase of growth plate injury repair as well as having other roles during the later osteogenic and remodelling phases. The results gathered from this study potentially indicate that

PDGF-BB may be a potential target in the development of a future biological therapy (by modulating the level of infiltration of progenitor cells into the injury site).

5.1.4. Roles of PKD activation in growth plate bony repair

All other previous studies on growth plate injury have found that the cells within the fibrogenic mesenchymal infiltrate are more prone to differentiate into bone tissue rather than to its original cartilaginous tissue of the growth plate [4, 6-9, 14, 34]. Hence, gaining understanding not only about the initial influx of fibrogenic cells but also why the cells are more prone to undergo osteogenesis is of great interest. Thus, the final aim of this PhD project was to examine the potential role of Protein Kinase D (PKD)-induced osterix expression, during growth plate injury repair and bone bridge formation. Whilst the importance of the bone transcription factor, osterix in osteogenesis is well documented during bone development and bone formation [35-37], its role in bone bridge formation at injured growth plate has never been examined. Understanding its particular role during bone bridge formation would allow us to pinpoint the role of PKD induced osterix and whether its absence directly affects only osteogenesis or whether its absence affects other differentiation pathways such as chondrogenesis. Osterix (Osx) is a zinc-finger containing transcription factor that is reportedly regulated by similar anabolic signals regulating Runx2, like growth factors BMP-2 and IGF-I [38]. Previous studies have shown that protein kinase-D (PKD) activation is required for osteoblast differentiation [39], and one way of Osx up-regulation occurs via activation of PKD [40]. Inhibition of PKD with inhibitor gö6976 specifically blocks BMP-2-induced osteoblast differentiation and mineralisation *in vitro* [40, 41] and FGF-2-induced increased bone formation in rodents [42]. In this part of the PhD project using a rat growth plate injury model, inhibitor gö6976 was used to investigate whether the partial inhibition of

Osterix via PKD could modulate growth plate repair. As predicted, µCT scans revealed that inhibition of PKD using inhibitor, gö6976, found a significant decrease in bone volume within the injury site; however, more interestingly; it was also observed that there was a significant increase in undifferentiated mesenchymal as well as cartilaginous repair tissue. In support, *in vitro* work of the current project also found favourable chondrogenesis of bone marrow derived mesenchymal cells after blocking this osteogenic transcriptional factor (whereby 3D chondrogenic assays treated with the PKD inhibitor revealed a significantly greater gene expression of cartilage gene, col-IIa). Potentially the *in vitro* result of this study suggests a bi-potency or plasticity of the stromal cells – an ability to "switch" and differentiate into chondrocytes at a stage before osterix activation and osteoblast maturation. This potential switch at a later stage has also been previously observed in some other studies [37, 43, 44]. Overall, these results suggest that osterix via PKD is a critical factor for osteoblastogenesis and bony repair of injured growth plate and blocking osterix signalling during growth plate injury repair may result in less bone formation and potentially more desirable cartilage repair. Further studies are required to investigate whether PKD induced osterix expression can be a potential target for the development of a biological therapy.

5.2. CONCLUSIONS

In conclusion, the knowledge gained from this PhD project has allowed the author to grasp a better understanding of the complex nature of molecular and cellular events leading to the undesirable bone bridge formation following growth plate injury. It was demonstrated that multipotent MSCs may exist within the mesenchymal infiltrate alongside with osteo- and chondro- progenitor cells during the fibrogenic phase, and this represents a pool of cells with the potential to be stimulated or encouraged into a more desirable differentiation pathway. In addition, this PhD project has also shown the importance of key chemoattractant PDGF-BB not only with the migration of fibrogenic mesenchymal stromal cells but also as having a role in the differentiation of cells into cartilaginous or bony tissues. Furthermore, this PhD project has elucidated the importance of bone transcriptional factor, osterix, in bone bridge formation at injured growth plate. Therefore, even the partial inhibition of osterix expression via PKD inhibitor has resulted in less bony tissue formation and more desirable cartilage repair tissue. Overall, the knowledge and ideas that such key growth factors and transcriptional factors like PDGF-BB and osterix are able to regulate and control the differentiation of MSCs and hence the outcome of repair following growth plate injury, suggest their potential use in developing a future biological therapy for enhancing cartilage repair of injured growth plate.

5.3. FUTURE DIRECTIONS

Ideally, a successful biological therapy is to eliminate the need for invasive surgery and prevention of bone bridge formation in children and young adolescence after growth plate injuries. In order to prevent faulty repair tissue before it happens, more research into the molecular and cellular events occurring during growth plate injury repair is required. Stemming from the work in this thesis, there are a few aspects which can be further analysed, investigated, improved, or expanded.

In respect to identifying MSCs within the mesenchymal infiltrate, although the immunohistochemical analysis suggests the potential existence of multipotent MSCs this data is by no means concrete or definitive, partially due to the lack of mesenchymal markers available for the specific use against MSCs as well as the lack of markers specific for rats and more so for use against paraffin embedded sections. One potential way of validating the existence of MSCs at the growth plate injury repair site could involve the use of green flurorescence labelling of exogenous MSCs. By utilising the technique of fluorescence activated cell sorting (FACS), true MSCs could be isolated and then green fluorescent labelled before being injected into a rat just prior or immediately following growth plate injury. Analysis of collected specimens could be done to see whether the labelled MSCs actually move into the injury site and contributed to the bony repair, and hence their role in growth plate repair can be confirmed.

With the assumption that multipotent MSCs exist and the knowledge that PDGF-BB plays a critical role in migration of these cells, future work could also involve the local delivery of recombinant PDGF-BB into the injury site immediately or day 1-2 post surgery to see whether it can modulate

progenitor cell infiltration into the injury site and enhance repair. However, some of the challenges may involve the administration of this growth factor, keeping the growth factor within the injury site as well as having the desired activity at the specific time after growth plate injury. Although more research would be required for future tissue engineering based work, the addition of PDGF-BB or another more suitable bio-factor could potentially encourage the migration of endogenous MSCs into the injury site and hence increase the amount of cartilage formation with the right kind of signaling during its differentiation stage.

Work from the final study of this PhD project could aid in developing ways of encouraging chondrocyte differentiation whilst subduing bony repair. While limiting osterix signaling using PKD inhibitor during MSC differentiation could potentially encourage less bone and (together with pro-chondrocyte factors such as TGF-β2) more cartilaginous differentiation, more specific osterix inhibitors or other more suitable inhibitors for osteogenesis should be investigated. Some of the major shortfalls that should be addressed for this experimental chapter include lack of statistical significance of some treatment effects, which could potentially be related to not having enough animals per treatment group and/or inhibitor dosing regimen – where the rats could potentially show more obvious treatment effects if the inhibitor were given for a longer period.

Overall, more work is required on many molecular and cellular aspects of the growth plate injury repair process in order to develop that illusive biological therapy; however, knowledge from this thesis has at least in part highlighted some key points during growth plate injury repair where further research can be carried forth on.

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APPENDICES

Chung, R., Foster, B.K. & Xian, C.J. (2011) Injury responses and repair mechanisms of the injured growth plate
Frontiers in Bioscience (Scholar Edition), v. S3(1), pp. 117-125

NOTE:
This publication is included on pages 135-143 in the print copy of the thesis held in the University of Adelaide Library.

Elsevier Editorial System(tm) for Bone
Manuscript Draft

Manuscript Number: BONE-D-11-00093

Title: Inhibition of protein kinase-D promotes cartilage repair at injured growth plate in rats

Article Type: Original Full Length Article

Keywords: Growth plate; Injury repair; Stromal progenitor cells; Differentiation; PKD; Osterix
Corresponding Author: Dr Cory J. Xian, PhD
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First Author: Rosa Chung
Order of Authors: Rosa Chung; Bruce K Foster; Cory J. Xian, PhD

Abstract: Injured growth plate cartilage is often repaired by bony tissue, impairing bone growth and causing growth defects in children. Currently, molecular events leading up to the undesirable bony repair remain unclear. This study utilised a rat growth plate injury model to investigate the potential role during growth plate bony repair of protein kinase-D (PKD) which is known to regulate osteoblast differentiation transcription factor osterix. Immediately after infliction of surgical injury at the proximal tibial growth plate, young rats received four once-daily injections of vehicle or 2.35mg/kg gö6976 (a PKD inhibitor known for its inhibitory effects on osterix), and injured growth plate samples were collected at day 10. Micro-CT analysis revealed that bone volume at the injury site was significantly lower following gö6976 treatment ($P<0.05$). Histological analysis showed that, compared to the vehicle control, PKD inhibition resulted in an increase in % of mesenchymal tissue ($P<0.001$), a decrease in bone trabeculae and bone marrow tissues, and more cartilaginous tissue within the injury site. Consistently, gö6976 treatment decreased mRNA expression at the injury site of bone related genes (osterix and osteocalcin) and increased levels of cartilage related genes (collagen-2a and Sox9). In support, in vitro experiments showed that addition of gö6976 during chondrogenic differentiation of rat primary bone marrow stromal progenitor cells resulted in a significant increase in collagen-2a 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 less bone formation and potentially more desirable cartilage repair.

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