Roles of Bone Morphogenetic Protein Signalling in the Bony Repair of Injured Growth Plate Cartilage in Young Rats

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DECLARATION

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ABBREVIATIONS

%	Percentage
ALP	Alkaline Phosphatase
BMP	Bone Morphogenetic Protein
BMPR1a	Bone Morphogenetic Protein Receptor-1a
BMPR2	Bone Morphogenetic Protein Receptor-2
BrdU	Bromodeoxyuridine
Cbf-α1	Core Binding Factor-α1
cDNA	complementary Deoxyribonucleic Acid
Col-2a	Collagen-2a
Col-10	Collagen-10
COX2	Cyclooxygenase 2
CT	Cycle Threshold
EDTA	Ethylenediaminetetraacetic acid
FGF-2	Fibroblast Growth Factor-2
GPC	Glypicans
H&E	Haematoxylin and Eosin
IGF-1	Insulin-like Growth Factor-1
Micro-CT	Micro tomography
ml, µl	Millilitre, Microlitre

mm, µm	Millimitre, Micrometre
mRNA	messenger Ribonucleic Acid
MSCs	Mesenchymal Stem Cells
OCN	Osteocalcin
OSX	Osterix
PBS	Phosphate Buffer Saline solution
PBS/BSA	Phosphate Buffer Saline solution/ Bovine Serum Albumin
PCR	Polymerase Chain Reaction
PDGF-BB	Platelet Derived Growth Factor-BB
RNA	Ribonucleic Acid
Rpm	Rate per minute
RT	Reverse transcriptase
SEM	Standard Error Mean
Sox-9	Sex reversal-Y-related high mobility group box proteins
TGF-β	Transforming Growth Factor-β
TNF-α	Tumour Necrosis Factor-α
VEGFα	Vascular Endothelial Growth Factor-α

THESIS ABSTRACT

The growth plate cartilage is solely responsible in the elongation of long bones. However, injuries upon the growth plate could result in orthopaedic complications such as limb length discrepancy and bone angulation deformity due to faulty bony repair. Although previous growth plate injury studies have uncovered the growth plate injury repair responses following an injury, the molecular events leading to the undesirable bony repair remain unknown. Using a well-established rat growth plate drill-hole injury model, this PhD study examined the roles of bone morphogenetic protein (BMP) signalling in the injury repair responses within the growth plate injury site, the adjacent growth plate cartilage and within the bone drill-track injury site. This study also investigated how the injury repair responses were regulated by a novel BMP regulator glypican-3 (GPC3).

Study on the remaining adjacent growth plate following the growth plate injury revealed significant upregulation of Col10 and downregulation of Sox9 gene expression level suggesting increment of hypertrophy and reduction in chondrogenesis. Subsequent gene expression study on the BMP genes showed significant upregulation of BMP7, BMPR1a and Dlx5 suggesting the presence of increased BMP activity at the remaining adjacent growth plate. Immunohistochemical and Western blot analyses also demonstrated BMP activation within the remaining adjacent growth plate. Further histological analyses through the systemic inhibition of BMP signalling showed an increase in height of the proliferative and hypertrophic zones while Col2a and Col10 were upregulated and downregulated, respectively. This part of the study suggests that the BMP signalling is involved in regulating the cartilage hypertrophy and that inhibiting BMP signalling could potentially prevent cartilage degeneration of the adjacent remaining growth plate.

While BMPs had been reportedly upregulated following growth plate injury, its role in the undesirable bony repair at the injury site remains poorly understood. Using a similar rat growth plate injury model, BMP signalling was systemically inhibited using the recombinant noggin protein. Quantitative gene expression analysis showed downregulation of BMP2, BMP4, Id1 and BMPR1a following the inhibition of BMP signalling. Histological studies showed that the inhibition of BMP signalling led to significant reductions of the percentage and total number of immunopositive pSmad1/5/8 cells during the growth plate injury repair. Micro-CT scans also revealed a significant reduction in bone volume and bone trabeculae formed within the growth plate injury site. Apart from the growth plate injury site, the bone drill-track injury site also showed a significantly reduced percentage of bone volume and repaired bone following BMP inhibition. This study suggests that the BMP signalling is indeed important for the bony repair at the growth plate injury site and the bone drill-track injury site.

While glypicans (GPCs, heparan sulphate proteoglycans) are known to regulate BMP signalling, their roles in bony repair at the growth plate injury site remain unknown. RT-PCR analyses showed GPC1 downregulation but GPC3 upregulation during the bony repair at the growth plate injury site. To investigate potential roles of GPC3 in regulating growth plate bony repair, rats with growth plate injury received rhGPC3 or anti-GPC3 neutralising antibody or vehicle delivered locally at the injury site via chitosan hydrogel. At the growth plate injury site, rhGPC3 treatment showed a significant reduction in phosphorylated Smad1/5/8 (pSmad1/5/8) immunopositive nuclear staining at day 15 when compared to the chitosan alone treated group. The presence of rhGPC3 also significantly reduced bone volume fraction within the growth plate injury site when compared with IgG treated groups. Consistently, bone-related genes and BMP downstream targeted gene, runx2, osteocalcin and Id1 were downregulated following rhGPC3 treatment. Analyses also showed a reduction in bone volume within the bone drill-

track injury site following rhGPC3 treatment. Anti-GPC3 appeared to have opposite trends in the above measurements when compared with the rhGPC3 treatment. This study suggests that locally expressed GPC3 is involved in suppressing BMP signalling and bony repair during the growth plate bony repair and bone drill track healing.

In conclusion, this study has identified critical roles of BMP signalling in the growth plate injury site bony repair, in the hypertrophic degeneration of the adjacent growth plate cartilage, and the bone repair of the bone drill-track. This study has found potential modulation of these injury repair responses by suppressing BMP signalling with inhibitor noggin and by modulating activities of locally produced GPC3.

CHAPTER 1

Review of BMP signalling in regulating growth plate injury and bony repair response

INTRODUCTION

At the end of all growing long bones lies the growth plate cartilage which is solely responsible for long bones lengthening. The growth plate is built-up of cartilaginous tissues. Therefore, being cartilaginous in nature, the growth plate is relatively the weakest region of the entire growing long bone. Hence, it is highly susceptible to a trauma injury. Even though some growth plate injuries are capable of self-healing, some are often repaired by undesirable bony tissue (bone bridge) causing bone growth defects. This phenomenon leads to various orthopaedic complications such as limb length discrepancy and bone angulation deformity. Unfortunately, current medical technology and advancements were not able to repair or prevent such undesirable bony repair from occurring. The sole method available is mainly surgical-based correction, which is highly invasive and often is ineffective and thus needs to be repeated. It also requires a series of follow-up treatments after surgery as the repaired bone is weaker and often suffers from infections.

Currently, biological treatments are not available to treat or to prevent such bony repair from occurring for an injured growth plate. Before a biological treatment can be derived, it is necessary to better understand the cellular and molecular pathways that lead to such undesirable bony repair at the injured growth plate. While bone morphogenetic protein (BMP) signalling has been well-known to be a potent regulator of osteoblast differentiation and osteogenesis during bone growth and bone fracture repair, there have not been so many publications on the involvement of BMP signalling following growth plate injury. However, since BMP (particularly BMP-2) has been known critical for the bone healing, it is very likely that BMP signalling plays an important role in the bony repair of injured growth plate.

Therefore, this review will discuss the major molecular and cellular events involved in the growth plate injury repair responses and bony repair at the growth plate injury site. Then, this review will also summarise the previous and current understanding of BMP signalling during the bone fracture repair, its signalling intervention (eg by glypicans), and its likely role in regulating growth plate bony repair.

1.1 The growth plate

The growth plate (**Fig 1.1**) which is also known as the epiphyseal plate resides at both ends of a growing long bone, specifically in adolescent and children (Ballock and O'Keefe, 2003). They are solely responsible for achieving longitudinal growth of the long bone by producing mineralised cartilaginous template for the bone formation to occur. This process is known as the endochondral ossification which is a two-step process involving the replacement of cartilaginous tissue by bone tissue (Kronenberg, 2003, Olsen et al., 2000). The growth plate cartilage decreases in size as a child grows and closes when the child's maximum growth has achieved. Histologically, the growth plate can be separated into three distinctively regions namely the resting zone (farthest to the diaphysis), proliferative zone (middle region) and hypertrophic zone (closest from the diaphysis) (Iannotti, 1990).

1.1.1 The resting zone

Initial finding claims that the resting zone does not play any specific roles in the longitudinal growth of the long bones because the cells within this zone proliferate at a very slow rate while at some point, it shows no sign of proliferation (Forriol and Shapiro, 2005). However, a study by Abad *et al.* (2002) shows that the removal of the proliferative and hypertrophic zone did not stop the bone elongation process. In fact, within a week, a complete proliferative and

hypertrophic zones were regenerated (Abad et al., 2002). This suggested that the resting zone makes an important contribution to the endochondral bone formation at the growth plate as it potentially contains stem-like cells that give rise to clones of proliferative chondrocytes (Abad et al., 2002). Histologically, the cells within the resting zone are sparsely distributed with either singular or coupled round cells. It contains abundant cytoplasmic vacuoles within the matrix which are postulated to play the role as nutrient storage for the cell proliferation later on (Forriol and Shapiro, 2005, Iannotti, 1990, Savendahl, 2005). The resting zone is also known to be responsible for protein synthesis and maintaining a germinal structure while it remains relatively inactive (Burdan et al., 2009). Apart from these, subsequent studies also suggested that the cells within the resting zone are cells that are being migrated to the proliferative zone for proliferation as they contain stem cell-like properties (Abad et al., 2002). This study was later supported by Savendahl *et al.* (2005) when they showed that the chondrocytes within the resting zone are being recruited to the proliferative and hypertrophic zones for proliferation and differentiation, respectively (Savendahl, 2005), which is followed by cell apoptosis and later mineralisation forming new a calcified template for bone formation (Savendahl, 2005).

1.1.2 The proliferative zone

The proliferative zone is seen as the site of bone length creation. They are mainly involved in matrix production and cellular division or replication leading to the bone elongation (Iannotti, 1990). Histologically, the proliferative zone lies in between the resting zone and hypertrophic zone. The appearance of the chondrocytes is slightly flattened whereby cells begin to divide and become more organised into columns. They are often shown as well-defined columns and are separated by surrounding cartilage matrix that is rich in collagen-2a (Col2a) (Forriol and Shapiro, 2005). The mitotic division is shown to be the highest within the proliferative zone compared to the resting zone and hypertrophic zone. Apart from that, the length of the bone

lengthening is solely dependent on the rate of mitotic cell division within the proliferative zone. The higher the rate of cell division, the longer the bone will achieve. Failure of mitotic division in this region can result in shorter bone length (Burdan et al., 2009, Forriol and Shapiro, 2005). The chondrocytes at the end of the proliferative zone that is nearest to the hypertrophic zone no longer undergo proliferation. Those chondrocytes were later shown to begin terminal differentiation (Hunziker et al., 1987, Iannotti, 1990).

1.1.3 The hypertrophic zone

As stated previously, the cell proliferation ceased within the hypertrophic zone. Histologically, the chondrocytes within the hypertrophic zone are greater in size and length compared to the chondrocytes in the proliferative zone. It seems like the chondrocytes were enlarged, swollen and vacuolated in the process of maturation. In fact, collagen-10 (Col10) and alkaline phosphatase (ALP) were found to be synthesised from the extracellular matrix which aid in cell hypertrophy and matrix mineralisation (Kronenberg, 2003). The chondrocytes nearest to the metaphyseal region begin to undergo changes which lead to the calcification process (Amizuka et al., 2012, Reddi, 1981). As ALP enzyme activity increases, the phosphate ions increase as well which is essential for the calcification process. The hypertrophic chondrocytes are no doubt the largest chondrocytes among the three regions in the growth plate cartilage. Therefore, the hypertrophic region is also the weakest part of the growth plate which is highly prone to growth plate injury. Apart from that, the chondrocytes nearest to the metaphysis are destined for apoptotic cell death which causes calcified tissue/bone absorbing cells (osteoclast) to disband the calcified cartilage region (Burdan et al., 2009). This is then followed by the influx of bone building cells (osteoblasts) which synthesise bone matrix to replace the absorbed tissue forming a new trabecular bone (Ballock and O'Keefe, 2003, Brighton, 1987, Brighton et al., 1973, Iannotti, 1990, Savendahl, 2005).

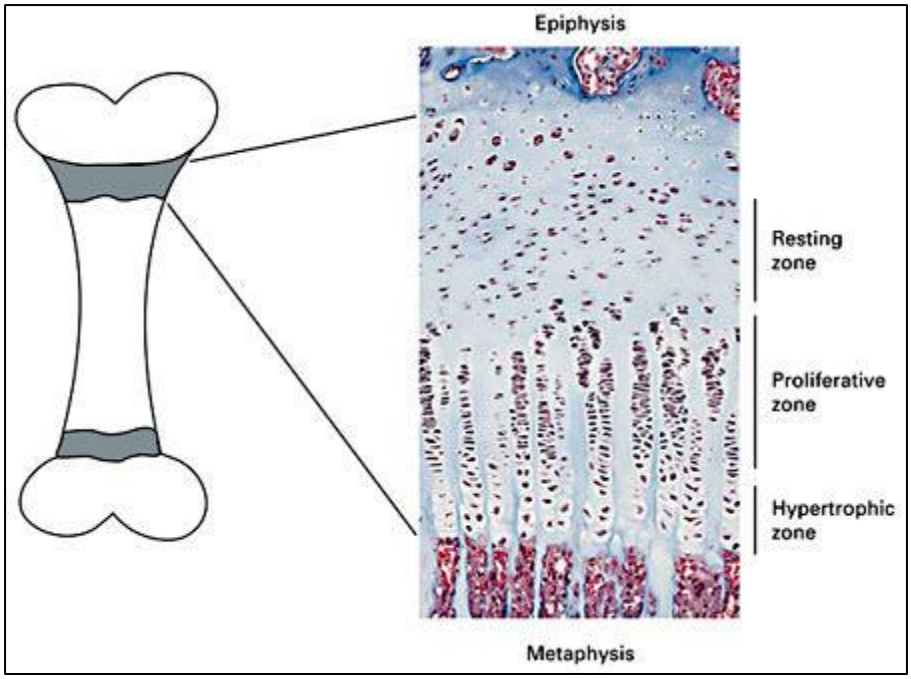

Figure 1.1. The structure of growth plate and metaphysis. The growth plate lies at the end of all long bones. It consists of cartilaginous tissue and contains three distinguished regions namely the resting zone, proliferative zone and hypertrophic zone (Nilsson et al., 2005).

1.2 Growth plate injuries and repair responses

1.2.1 Growth plate injuries and classifications

Bone fractures are common in sports and accidents (Caine et al., 2006). However, growth plate injuries are only common in adolescent or growing children. This is due to the larger growth plate region that they possess compared. The growth plate is deemed the weakest region of a growing long bone and it is highly susceptible to injuries. An approximate 20% of all bone fractures involve the growth plate (Mizuta et al., 1987).

To distinguish the types and patterns of growth plate injury, Salter-Harris classification system (**Fig 1.2**) has been introduced to characterise the degrees and types of growth plate injuries. The Salter-Harris classification system consists of five different classes (Type I-V) based on the severity and regional involvement of the growth plate (Mizuta et al., 1987, Perugia et al., 2014, Xian et al., 2004). Such classification system is important for prognoses and to determine the follow-up treatment for the growth plate injury. Previous statistical studies have indicated that the most common type of growth plate injuries in young children is the type I, governing 40% of total growth plate injuries (Perugia et al., 2014, Xian et al., 2004). Growth plate injuries that have been classified under type I injury often have a higher chance of self-healing and do not require any further surgical intervention as long as the growth plate blood supply remains undisturbed (Amizuka et al., 2012, Caine et al., 2006, DiFiori et al., 2006, Eastwood and De Gheldere, 2011, Mizuta et al., 1987, Xian et al., 2004). The type II injury also share a similar fate as the type I which also has a higher chance of recovery without any further orthopaedic complications in the future. However, type III, IV and V growth plate injuries could result in undesirable bony repair at the growth plate injury site. Statistical evidence shows that 30% of all growth plate injuries suffer from the undesirable bony repair at the injury sites leading to

orthopaedic problems later in life such as limb length discrepancy and bone angulation deformity (Eastwood and De Gheldere, 2011, Mizuta et al., 1987, Ogden, 1984, Perugia et al., 2014).

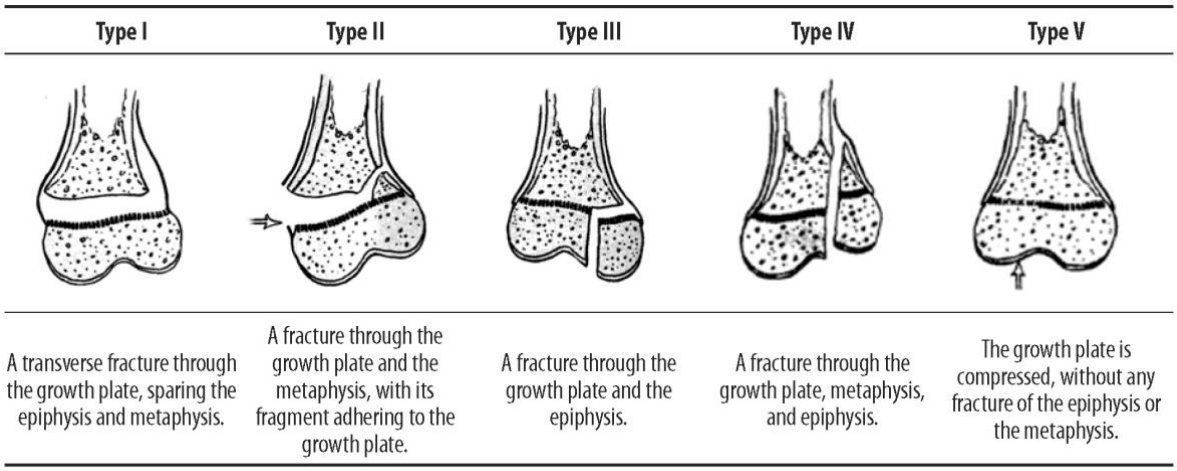

Figure 1.2. The Salter-Harris Classification system that determines the degree of growth plate injury based on the involvement of the growth plate. There are five categories in the Salter-Harris Classification, Types I, II, III, IV and V. (from Caffey's Pediatric Diagnostic Imaging, Mosby Elsvier, 2007).

1.2.2 Growth plate injury responses

Even though bone fracture repair has been studied extensively over the year, the studies on growth plate injuries have remained relatively scarce. The cellular and molecular mechanisms leading to the undesirable bone bridge formation (**Fig 1.3**) at the growth plate injury site remain largely unknown. Based on some earlier studies on the growth plate injury using a rat model, four different repair phases have been identified following a growth plate injury namely the inflammatory phase, fibrogenic phase, osteogenic phase and maturation and remodelling phase occurring during days 1-3, 3-7, 7-14 and 10-28, respectively (Xian et al., 2004). Consistently, such repair responses were also observed in murine, mice, rabbits and pigs growth plate injury models suggesting that these repair responses are common/similar in mammals (Jaramillo et al., 1990, Lee et al., 2000, Makela et al., 1988).

1.2.2.1 Inflammatory phase

Following growth plate injury, the initial injury repair response is the inflammatory phase (Xian et al., 2004). The inflammatory phase prepares the cartilage for repair by supplying the 'building blocks' necessary for repair and remodelling (McCullough, 1989). During the inflammatory phase, the infiltration of inflammatory cells was obvious at 8 hours after injury, reaches its peak on day 1 and subsides on day 3 (Chung et al., 2011). Similarly, such occurrence was also demonstrated by the bone fracture repair and soft tissue injury repair studies whereby an influx of inflammatory cells (neutrophils, macrophages and lymphocytes) was found predominantly at the injury site (Arasapam et al., 2006, Chung et al., 2006). The leukocyte infiltration plays an important role in the inflammation, and this is regulated by local factors, which mediate the influx of leukocyte infiltrates and cytokines such as the interleukin-1β (Il-1β), interleukin-8 (IL-8), cytokine-induced neutrophil chemoattractant-1 (CINC-1), cell-

surface adhesion molecules, chemotactic molecules or chemokines, and tumour necrosis factor-α (TNF-α).

Further immunohistochemical studies have demonstrated that the inflammatory cells at the growth plate injury site are responsible for the synthesising of both Il-1β and TNF-α (pro-inflammatory cytokines) which is involved in regulating inflammatory response to tissue injury and bone fracture (Barone and Feuerstein, 1999, Kon et al., 2001).

One previous study has shown that the bone morphogenetic proteins (BMPs) particularly the BMP2, 3, 4 and 7 were significantly expressed during the late inflammatory phase growth plate injury site, suggesting that BMPs could be involved in regulating the inflammatory response or downstream healing responses at the injury site (Ngo et al., 2006). Consistently, following a bone fracture, BMP4 mRNA expression was shown to be upregulated at the bone fracture site following injury (Nakase et al., 1994) while the influx inflammatory cells were also reportedly synthesising BMP4 at the bone fracture site (Scherbel et al., 2001). The BMPs were known to be important in regulating chondrogenic and osteogenic differentiation, and pre-natal and post-natal bone development (Anderson et al., 2000). Interestingly, a study suggested that the BMPs are able to induce chemotaxis of monocytes to the fracture site while stimulating osteogenesis-promoting growth factors which are known to enhance bone formation (Sykaras and Opperman, 2003). BMP4 was found to be significantly expressed in the regenerating tissue in a rabbit leg lengthening model while at the same time, the vascular endothelial growth factor (VEGF) that is known to stimulate the migration of endothelial cells was also upregulated during acute inflammation (Li et al., 1998, ten Dijke et al., 1994). Microarray analysis study of the growth plate injury repair had also demonstrated a significant upregulation of BMP signalling during the late inflammatory and early fibrogenic phase which further suggest that

the BMP signalling may play a vital role in the downstream healing of the growth plate injury site (Macsai et al., 2012). However, it is still not known if the inhibition of the BMPs could result in the cessation of bony repair at the growth plate injury site and bone trabecular fracture.

Apart from BMP4, BMP2 which is a potent inducer of osteoblast differentiation is upregulated during the inflammatory phase of the growth plate injury repair which is consistent with the gene expression study by Ngo *et al.* (2006) (Macsai et al., 2012, Ngo et al., 2006, Ogasawara et al., 2004). Similarly, during bone fracture repair, BMP2 expression was shown to be at its peak during the inflammatory phase of the bone fracture repair suggesting that the BMP2 is involved in regulating downstream bone healing of the fractured bone (Cho et al., 2002). Consistently, a study showing that mice lacking the ability to produce BMP2 in limb bones also lack the ability to heal bone fracture which suggests the crucial role of BMP2 in bone healing (Tsuji et al., 2006). This is further supported by a recent study which shows that the subsequent inhibition of BMP2 resulted in a delay in bone fracture healing (Sheu et al., 2014). Previous *in vitro* studies also revealed that the BMP2 has the ability to control the activity of other BMPs in an autogenously regulatory manner (Edgar et al., 2007). Due to the early detection of the BMP2 gene expression at the growth plate injury site, BMP2 could potentially be the initiator of the production of other BMPs involved in regulating the bony repair responses at the growth plate injury site. Even though BMP2 is found to peak during the inflammatory phase, its involvement in regulating the downstream bony repair response is yet to be studied.

1.2.2.2 Fibrogenic phase

The fibrogenic phase commences during days 3-7 following a growth plate injury (Xian et al., 2004). Histologically, the influx of vimentin-immunopositive mesenchymal cells into the growth plate injury site was observed during the fibrogenic phase (Xian et al., 2004). A similar injury response was observed in the bone fracture repair which is known as the soft callus formation stage (Claes et al., 2002). Consistently, the mice growth plate injury model also found the presence of undifferentiated cells predominantly at 7 days post-injury (Lee et al., 2000). Based on the histological observation, these cells do not resemble any of the bone cells or cartilaginous tissues; however, they can differentiate into either bone cells or cartilaginous tissues at the later stages following the growth plate injury (Chung et al., 2011, Xian et al., 2004). These studies suggest that these cells may be predetermined chondroprogenitor, osteoprogenitor or even multipotent mesenchymal stem cells. The presence of such cells at the growth plate injury site plays an important role in the cellular repair of the injured growth plate. However, more studies will be required to examine the potential of the fibrogenic cells within the growth plate injury site (Gerstenfeld et al., 2003, Macsai et al., 2008, Zhou et al., 2004).

Previous quantitative gene expression study with a rat growth plate injury model had also identified several growth factors being upregulated during the fibrogenic phase such as the fibroblast growth factor (FGF-2), platelet derived growth factor (PDGF-BB) and BMPs, indicating their possible roles in regulating bony tissue formation during the growth plate repair (Chung et al., 2009, Fiedler et al., 2002, Ngo et al., 2006, Zhou et al., 2004). The FGF-2 has been known to regulate cell proliferation, differentiation and migration (Farre et al., 2007). Consistently, FGF-2 was found to be upregulated at the bone fracture site during the bony repair at the injury site (Fujii et al., 1999). Apart from being involved in mesenchymal cell migration and proliferation, FGF-2 was also found to repress chondrocyte differentiation and

ALP activity suggesting a role in skeletal development and bony repair (Harada and Rodan, 2003, Kawaguchi et al., 2004, Rodan et al., 1989). An *in vitro* study using MC3T3E1 osteoblast-like cells also showed that FGF-2 had the greatest ability to induce proliferation while reducing gene expression associated with mineralisation such as Col1a1, fibronectin, osteocalcin and BMP2. In fact, BMP2 was found to stimulate the expression of the mineralisation-associated genes but had little effect on the gene expression associated with growth. The above studies suggest that the FGF-2 and BMP2 play a vital role in modulating early fracture repair (Hughes-Fulford and Li, 2011).

BMPs are potent regulators in determining cell fate specifically during embryogenesis and tissue homeostasis (Boergermann et al., 2010). It is a multi-functional growth factor that belongs to the transforming growth factors (TGF-β) superfamily (Cao and Chen, 2005). During the fibrogenic phase, BMPs were found significantly upregulated, indicating involvement in regulating the mesenchymal recruitment during the growth plate injury repair (Ngo et al., 2006). Consistently, bone fracture repair also demonstrated the importance of BMPs in the recruitment of mesenchymal-like cells as well as the initiation of osteoblast differentiation (Bragdon et al., 2012). To be more precise, a microarray analysis with the rat growth plate injury model detected increased involvement of four major bone-related gene expression pathways/groups, namely the BMP signalling, Wnt signalling, osteoblast differentiation and skeletal development groups (Macsai et al., 2012). This study observed upregulation of the BMP signalling during the late inflammatory and early fibrogenic phase which is an important time-point for the cellular differentiation process (Macsai et al., 2012). At this time-point, the undifferentiated fibrogenic cells will differentiate to either osteoblast or chondrocytes depending on the growth factors and cytokines that are being upregulated. An *in vitro* study on BMP2 have been shown to modulate osteogenic activity (Hiraki et al., 1991, Jiao et al., 2007).

Apart from promoting osteogenic differentiation, BMP signalling has also been found to promote chondrogenesis by inducing chondrocyte maturation. These studies suggested that the BMP could play an important role in regulating the endochondral ossification process (Kakoi et al., 2014). Even though previous studies have shown upregulation of BMPs following growth plate injury, their functions during the fibrogenic phase are still unknown.

1.2.2.3 Osteogenic and bone remodelling phase

This growth plate injury repair phase involves a series of osteogenic responses including cell differentiation of the mesenchymal infiltrate into the bone trabeculae tissue and/or cartilaginous tissue, which may be dependent on the cytokines and growth factors that are being expressed at that particular time-point (Xian et al., 2004). Quantitative real-time PCR analyses during the osteogenic phase have shown significant upregulation of the Runx2 and osteocalcin gene expression, which suggests potential osteogenic differentiation at the growth plate injury site (Chung et al., 2011). Consistently, Runx2 and ALP were immunopositively stained at the growth plate injury site which signifies osteoblast differentiation and maturation of the bone cells (Arasapam et al., 2006, Xian et al., 2004). On the contrary, cartilaginous genes such as Sox9 and Col2a were found to be significantly downregulated, suggesting a reduction of chondrogenesis at the growth plate injury site during the osteogenic and remodelling phase (Chung et al., 2006, Chung et al., 2009). Consistently, histological staining of the growth plate injury site at day 60 (at the remodelled phased) found a significantly large proportion of mature bone trabeculae and bone marrow cells when compared to the presence of cartilaginous and mesenchymal infiltrate tissues during the osteogenic phase (Xian et al., 2004). Apart from the bone trabeculae and osteoblast cells that were visible within the growth plate injury site, a small amount of osteoclast cells (bone resorption cells) were also found to be present which indicates that the bone maturation and remodelling processes happen during this repair phase (Chung et

al., 2011, Chung et al., 2009). Similarly, this osteogenic and remodelling phase was found to be present in the bone growth and bone fracture repair (Marsell and Einhorn, 2009). In addition, BMPs were also significantly expressed at this repair phase (Marsell and Einhorn, 2009). A recent study has shown that the BMP7 (Osteogenic Protein-1) has the potential in modulating the vascular endothelial growth factor (VEGF) which is known to be essential for angiogenesis and bony repair response at the growth plate injury site (Chung et al., 2014, Ngo et al., 2006). Angiogenesis plays a critical role in physiological bone growth and remodelling as well as during the growth plate injury and bone fracture repair. This is because vascularization is required for bone formation and remodelling (Chim et al., 2013). Although BMP7 is known to be involved in regulating downstream bone osteogenesis, its contribution to the growth plate injury repair remains largely unknown. Apart from BMP7, several other BMPs were found being expressed during the osteogenic and bone maturation phase as well (Chen et al., 2004b, Hahn et al., 1992). Therefore, further studies on the involvement of BMP signalling is essential to review the cellular and molecular mechanism as well as regulation of the bone bridge formation at the growth plate injury site.

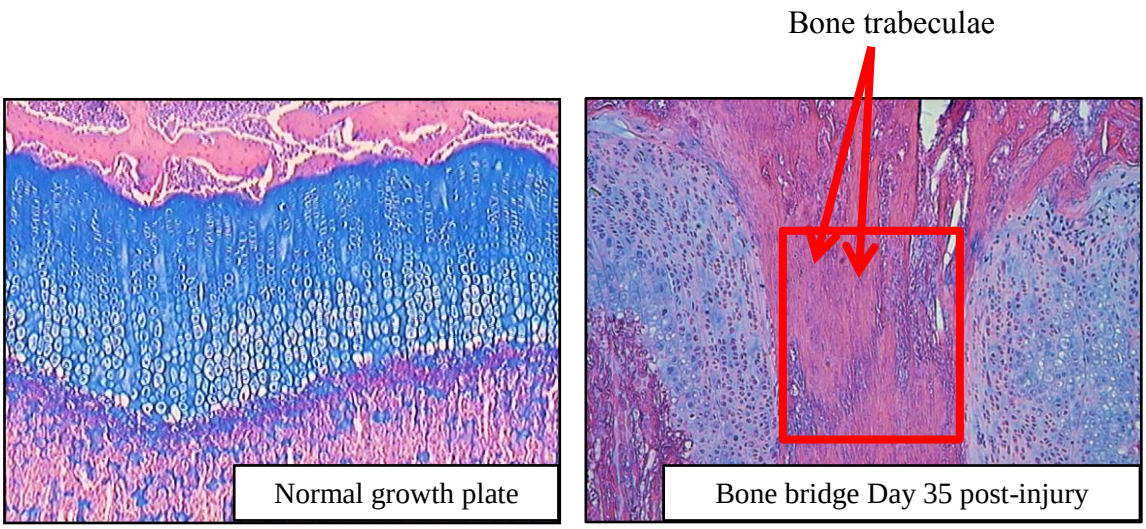

Figure 1.3. The bone bridge formation within the growth plate injury site due to the undesirable bony repair following growth plate injury. This often resulted in the cessation of the growth plate functions thus results in orthopaedic problems later in life such as limb length discrepancy and bone angulation deformity (Chung et al., 2011).

1.3 Key regulators of the growth plate injury repair response

1.3.1 Chondrogenic regulators

Previously, growth plate injury repair was initially thought to undergo intramembranous ossification which resulted in the bone bridge formation based on the murine and rat growth plate injury model (Lee et al., 2000, Xian et al., 2004). However, further studies have discovered that the functional chondrogenic genes such as Sox9, Col2a and Col10 were also being expressed during the growth plate injury repair, suggesting that the growth plate injury site also undergoes chondrogenesis apart from osteogenesis (Chung et al., 2013, Chung et al., 2009, Savendahl, 2005). Consistently, histological staining revealed the presence of cartilaginous tissues coupled with bone trabeculae tissue within the growth plate injury site, suggesting presence of both endochondral ossification and intramembranous ossification during the growth plate injury repair (Arasapam et al., 2006, Chung et al., 2006, Chung et al., 2011, Chung et al., 2009).

The Sox9 is an early transcription factor for chondrogenesis during the endochondral ossification in embryo development and post-natal skeletal growth (Akiyama et al., 2002, Bell et al., 1997, Bi et al., 1999). Sox9 gene expression was also being upregulated during chondrogenesis of the mesenchymal stem cells (MSCs) (Bell et al., 1997, Bi et al., 1999, Lefebvre et al., 1997). The absence of Sox9 in the *in vitro* MSCs also resulted in the total abolishment of the cartilage development (Henry et al., 2012), making Sox9 a vital transcription factor for the chondrocyte differentiation and cartilage formation (Bi et al., 1999). As the growth plate injury repair progresses through the inflammatory phase, the Col2a gene expression was significantly downregulated at the growth plate injury site, suggesting a reduction in chondrogenesis (Ngo et al., 2006). Col2a is a major cartilage matrix protein that

was found in the growth plate cartilage which involves the formation of fibrils that entrap the proteoglycan aggregate (Bell et al., 1997). A previous chondrogenesis study using a mouse model found that the Sox9 was co-expressed with Col2a (Bell et al., 1997). The author demontrated that the Sox9 protein binds specifically to the sequences in the first intron of human Col2a and that mutation of these sequences abolishes Sox9 binding along with the Col2a gene expression in transgenic mice model which concludes that the Col2a gene expression is directly regulated by the Sox9 protein (Bell et al., 1997). As the growth plate injury repair moves into the osteogenic and maturation phase, Col10 was found to be upregulated (Arasapam et al., 2006). Briefly, the Col10 is produced by the hypertrophic chondrocytes which is responsible for the matrix mineralisation. When both Col10 and VEGF are being upregulated, blood vessels will invade from the adjacent metaphysis region. This will allow migration of mineralised cartilage resorptive cells (called chondroclasts), osteoclasts and osteoblasts to remodel the mineralised cartilage scaffold into the trabecular bone at the metaphysis (Gerber and Ferrara, 2000, Shapiro, 1987).

1.3.2 Osteogenic regulators

During the bony repair at the injured growth plate cartilage, a series of osteogenic-related genes such as the Runt-related transcription-factor 2 (Runx2), Osteocalcin, Osterix and BMP2, BMP4, BMP7 were found to be upregulated (Chung et al., 2011, Chung et al., 2009, Macsai et al., 2012, Ngo et al., 2006, Xian et al., 2004). The Runx2 is better known as a master transcription factor that regulates the osteoblast differentiation and also a potent regulator for chondrocyte maturation (Ding et al., 2012). Earlier growth plate injury repair studies using a rat injury model had demonstrated an upregulation trend for Runx2 gene expression at Day 6 following growth plate injury (Chung et al., 2014). A study using MSC demonstrated that the overexpression of the Runx2 in the hypertrophic chondrocytes resulted in the Col10

upregulation which suggest that the Runx2 is also a regulator for chondrocyte hypertrophy, maturation and apoptosis (Ding et al., 2012). Additionally, Runx2 is also a downstream target gene of BMP signalling following the activation by BMP2, BMP4 and BMP7 (Chen et al., 2004b, Liu et al., 2007, Shimizu et al., 2012, Viereck et al., 2002, Wan and Cao, 2005, Wang et al., 1990, Yu et al., 2010). BMPs were initially discovered as proteins capable of inducing bone formation when implanted at the extraskeletal area of the bone (Tsumaki and Yoshikawa, 2005). They are expressed during the normal bone growth and bone fracture repair (Liu et al., 2007, Tsuji et al., 2006).

As growth plate repair progresses, osteoblasts at the growth plate injury site produce an increased amount of bone matrix protein, osteocalcin, at days 8-14 (osteogenic phase) (Xian et al., 2004). Osteocalcin plays a potent role in the body's metabolic regulation, bone formation and is often used as a marker for the bone formation process (Bharadwaj et al., 2009, Lee et al., 2007). The osteocalcin gene expression was found upregulated and Sox9 and Col2a were downregulated at day 14 onwards (Chung and Burdick, 2009, Zhou et al., 2004). Apart from the growth plate injury site, osteocalcin expression was also found to be prominent at the remaining adjacent growth plate at day 8 post-injury, which suggest that the remaining adjacent growth plate is also undergoing hypertrophic changes and/or bone formation. In fact, osteocalcin expression remains to be increasing on day 60 suggesting degeneration at the remaining adjacent growth plate cartilage (Macsai et al., 2011).

Another osteogenic gene is the Osterix (Osx), a zinc-finger containing transcription factor which is required for osteoblast differentiation and bone formation during embryonic development, postnatal growth and in bone homeostasis (Celil et al., 2005, Nakashima et al.,

2002, Sun et al., 2008). During embryonic development, Osx was found expressed in the osteoblast lineage cells, prehypertrophic chondrocytes, but not in the osteoclasts (Zhou et al., 2010). So far, Osx can be modulated by BMP2, insulin-like growth factor-1 (IGF-1) and the mitogen-activated protein kinase signalling (MAPK) (Celil et al., 2005, Harada and Rodan, 2003) with the ability to regulate osteoblastic gene expression such as osteocalcin, osteopontin and collagen-1 (Sun et al., 2008).

Following a recent microarray study, Wnt and BMP signalling were shown to be upregulated during the bony repair at the growth plate injury site (Macsai et al., 2012). Wnt signalling plays an important role in the development and maintenance of many organs and tissues, including bone where Wnt signalling is critical for bone formation (Cadigan and Nusse, 1997, Macsai et al., 2008). Although Wnt proteins can be found in several pathways, the canonical Wnt-β-catenin signalling pathway appears to be more important in bone growth and homeostasis (Tamura et al., 2010, Wang et al., 2014, Westendorf et al., 2004). In addition, Rodda *et al.* (2006) also observed an increase in chondrocyte differentiation when Wnt signalling was being silent (Rodda and McMahon, 2006). Furthermore, the activation of Osx transcription factor has been shown to stimulate Wnt signalling to upregulate osteoblast differentiation and maturation (Zaidi, 2007). Although Wnt signalling has been shown upregulated in the growth plate microarray study (Macsai et al., 2012) and while it is known to be upregulated during bone growth and bone fracture repair (Xu et al., 2014), its role in growth plate injury repair is still poorly understood.

As for BMPs, they are multifunctional growth factors that have the ability to initiate ectopic bone formation in adult animals (Urist, 1965). Due to their unique features in osteoblast differentiation and bone formation, BMPs have been used as a therapeutic agent for bone healing, by enhancing bone regeneration specifically for spine fusion surgeries and tibial fracture repair (Browne and Reddan, 2009, Ghodadra and Singh, 2008, Govender et al., 2002, Rosen, 2009). From the previous microarray analyses of rat growth plate injury sites, BMPs were found to be upregulated during the late inflammatory and early fibrogenic phase which is a crucial time-point that determines the direction of cellular differentiation to either osteoblast or chondrocyte (Chung et al., 2011, Macsai et al., 2012). During the inflammatory phase, BMP3, BMP4, BMPR1a and BMPR2 had increasing trends of mRNA expression following growth plate injury (Macsai et al., 2012). Consistently, quantitative gene expression of BMP2, BMP3, BMP4, BMP7 and BMPR1a were also found to be significantly upregulated during the fibrogenic phase following growth plate injury (Ngo et al., 2006). Due to the dynamic functions of the BMPs, the upregulation of BMP2 and BMP7 gene expression also resulted in the enhancement of osteogenic transcription factors such as the Runx2 and Osx in both mice and human MSCs (Celil et al., 2005). However, while there have been significant studies published to unveil the roles of BMPs in bone fracture repair, potential roles of BMPs in the growth plate bony repair following a growth plate injury remain unclear.

While most growth plate injury studies discussed above were focussed on the bony repair at the growth plate injury site, only a few studies investigated effects of growth plate injury on the remaining adjacent growth plate tissues, which also demonstrated progressive cellular and molecular changes which resemble cellular degeneration (Macsai et al., 2011). Macsai *et al.* (2011) showed that the bone bridge formation at the adjacent growth plate tissue was found in almost 60% of injured rats at day 60 post-growth plate injury, suggesting that the adjacent

growth plate is also affected by the growth plate injury (Macsai et al., 2011). Apart from that, the chondrocyte proliferation was significantly reduced while apoptosis was found to be increased at the adjacent growth plate, which further suggest a potential disruption to the normal bone growth process (Macsai et al., 2011). Furthermore, the expression of the osteocalcin and apoptosis-regulatory genes were found to be upregulated at the adjacent growth plate which was also shown on the growth plate injury site during the bony repair response (Macsai et al., 2011). Micro-CT scanning on the growth plate cartilage following injury showed a significant decrease in the overall growth plate thickness and volume at day 35 post-growth plate injury which further support the potential cartilage degeneration following injury (Coleman et al., 2010). Evidently, the bone bridge formation within the growth plate injury site is also affecting the uninjured adjacent growth plate cartilage area which plays important role endochondral ossification during long bone elongation. Unfortunately, the molecular and cellular changes associated with the degeneration of the remaining adjacent growth plate remain unclear.

1.4 Degeneration of the remaining adjacent growth plate

Following growth plate injury, osteocalcin (osteogenic gene) was upregulated along with the reduction in chondrocyte proliferation at the remaining adjacent growth plate area. Such progressive changes in the gene expression shown in the remaining adjacent growth plate strongly suggest increasing chondrocyte hypertrophy which could lead to the degeneration of the remaining adjacent growth plate (Macsai et al., 2011). Unfortunately, the molecular mechanisms involved has not been fully investigated in that study.

Cartilage degeneration is often seen as a clinical syndrome of osteoarthritis and is the most common cause of pain and disability in older people (Buckwalter and Mankin, 1998). This is due to the increase of the chondrocyte hypertrophy at the articular cartilage for patients suffering from osteoarthritis. Interestingly, using a guinea pig animal model to develop spontaneous osteoarthritis, a study demonstrated a progressive switch of the expression of phosphorylated Smad2/3 to Smad1/5/8 at the articular cartilage (Zhao et al., 2016), suggesting activation of the BMP signalling during the chondrocyte differentiation following osteoarthritis progression. Inhibition of BMP2/Smad1 activity using Matrilin-3 has shown to be able to inhibit premature chondrocyte hypertrophy by slowing down the progressive development of osteoarthritis (Yang et al., 2014). Furthermore, immunohistochemical analyses showed strong BMPR1a and BMPR2 being expressed at the osteoarthritic articular cartilage which further support that the BMP signalling is involved in the articular cartilage homeostasis (Bobinac et al., 2008). Similarly, the BMP2 expression has been reported in the chondrogenic and ossifying sites of calcified tendinopathy and traumatic tendon injury rat model whereby it is postulated that the ectopic BMP2 expression might potentially induce tissue transformation into ectopic bone/cartilage and promote structural degeneration in calcific tendinopathy (Lui et al., 2009).

1.5 BMP Signalling

1.5.1 Earlier studies on BMP signalling

Bone morphogenetic proteins (BMPs) (**Fig 1.4**) were originally founded by Dr Marshall Urist. They were identified as proteins because the demineralized bone segments and extracts of demineralized bone were found to have the ability to induce bone formation when implanted subcutaneously or intramuscularly in animals (Urist, 1965, Urist et al., 1997). Most of the BMPs play an important role in the embryonic development and postnatal development such as cellular growth, cell differentiation, apoptosis of certain cell types, and regulation of osteoblasts, chondroblasts, neural crest formation and epithelial cells (Kingsley, 1994). Further studies show that BMPs and BMPs receptors are important for the osteoblast formation, skeletal tissue development and bone maturation while the absence of BMPs can result in the disruption of a normal bone development (Anderson et al., 2000, Bragdon et al., 2012, Celil et al., 2005).

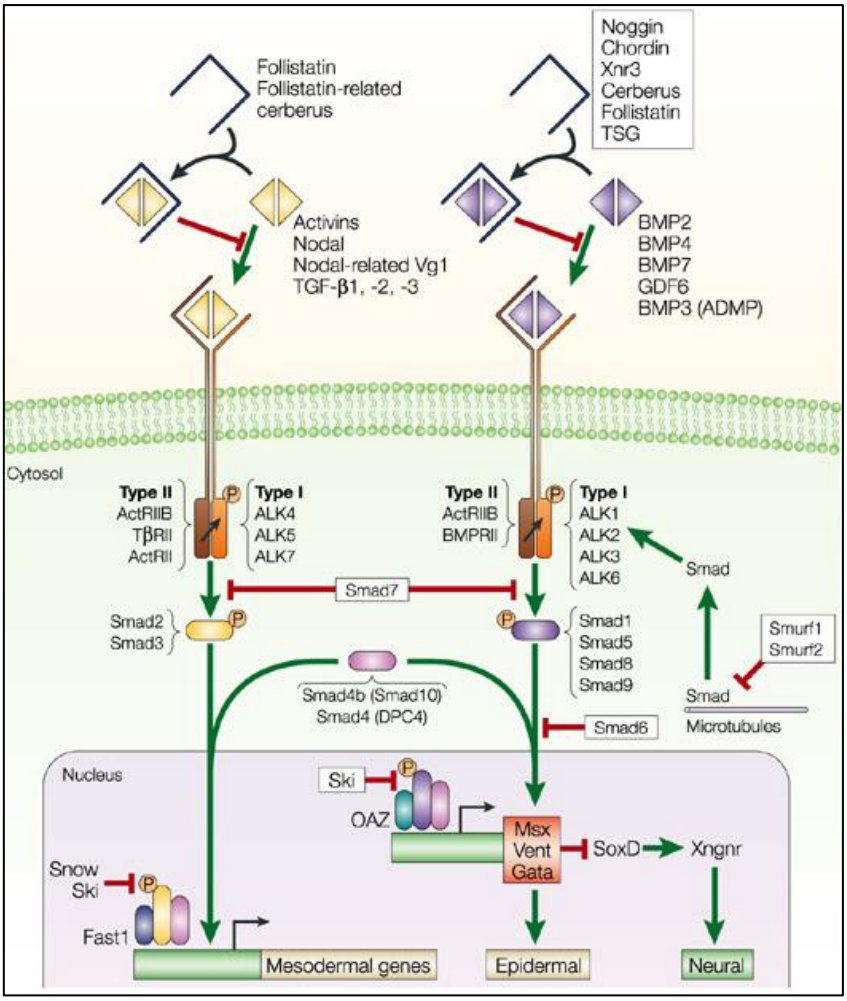

Figure 1.4. BMP signalling pathway consists of Smad-dependent and Smad-independent signal transduction. BMP is activated when BMP ligand binds to the type-2 receptors which in turn phosphorylates type-1 receptors. This will activate the Smad signalling pathway through the phosphorylation of Smad1, Smad 5 and Smad8. These associate with Smad4 to form a heteromeric complex that translocates to the nucleus to stimulate the expression of the targeted genes. Inhibitors such as Noggin and Chordin act upstream in modulating BMP signalling activity (Brivanlou, 2002).

1.5.2 Members of BMP signalling pathway

As mentioned earlier, BMP is one of the three subfamily in the TGF-β superfamily. BMPs encode-polypeptides share a common structural feature and configuration (Kingsley, 1994). Currently, approximate 40 members of BMPs proteins have been isolated and identified. The most common BMPs are BMP2, BMP4, BMP5, BMP6, BMP7 (osteogenic protein-1, OP-1), BMP8 (osteogenic protein-2, OP-2) and growth differentiation factor-5 (GDF-5), GDF-6, GDF-7 (Celeste et al., 1990, Chang et al., 1994, Ozkaynak et al., 1990, Ozkaynak et al., 1992, Wozney et al., 1988). These individual BMPs are different from one another due to their significant amino acid sequence at the carboxyl-terminal. BMPs are synthesized within the cell as precursor forms, and then proteolytically processed at the carboxyl-terminal regions into mature proteins with approximately 130 amino acids. Interestingly, most of the BMPs share a conserved of seventh cysteine residues in the mature domain that can covalently bind to an extracellular domain at their cell surface receptors which is similar with several members of the TGF-β superfamily (Kirsch et al., 2000, Thompson et al., 2003).

1.5.3 BMP receptors

All BMPs (ligands) bind to two key types of membrane-bound serine/threonine kinase receptors namely the type-1 (BMPR1) and type-2 receptors (BMPR2) (Massague, 1998, Zwijsen et al., 2003). These BMP receptors have a close resemblance in structure and functions to TGF-β receptors and activin receptors (ActRs) (ten Dijke et al., 1994). The BMPR1 consist of several subunits which are highly similar in their molecular structure, BMPR1a (ALK-3), BMPR1b (ALK-6) and ActR1A (ALK-2) (Natsume et al., 1997, Nishitoh et al., 1996, Yamashita et al., 1995). Due to the different molecular structure between the receptors and BMP ligands, the BMP ligand-receptor binding are more specific as the BMPs binding site

have to be complement to the BMP receptor binding site. This can be seen in the case of BMP2, BMP4, BMP6, BMP7, GDF5 and GDF9 which bind exclusively to BMPR2 (Liu et al., 1995, Nohno et al., 1995, Rosenzweig et al., 1995, Vitt et al., 2002). In addition, mice lacking BMPR1b had shown to be viable but exhibited defects in the appendicular skeleton. This was increasingly evident when a marked reduction in the chondrocyte proliferation and differentiation in the phalangeal region. As for the adult mutant mice, the proximal interphalangeal joint was absent while the size of the metacarpal and metatarsal bones were significantly reduced (Yi et al., 2000). Suggesting that the BMPR1a and BMPR2 are both indispensable in the activation of BMP signalling. Interestingly, both BMP receptors were found to be upregulated during the bony repair response suggesting activation of BMP signalling pathway at the growth plate injury site. However, the information on the expression of BMPs and other BMP signalling key components in the growth plate injury site and the remaining adjacent growth plate is still not known.

1.5.4 Mechanism of BMP signalling activation

The BMP signalling activation mechanism (**Fig 1.4**) is highly similar to the activation of TGF-β signalling (Sakou, 1998). Both type-1 and type-2 receptors are essential for the BMP signal transduction to occur. Upon the binding of the BMP ligand to the BMP receptors, they form a heterotetrameric-activated receptor complex consisting of two pairs of type-1 and type-2 receptor complex (Moustakas et al., 2001). This will then trigger the activation of the Smad pathway in the cell cytoplasmic region (Wan and Cao, 2005). Smad proteins play an important role in the intracellular signal transduction which includes the activation of downstream targeted genes such as Runx2 that regulates osteoblast differentiation (Bais et al., 2009, Heldin et al., 1997). The binding between the BMPR1 and BMPR2 resulted in the phosphorylation of the intracellular targets, the receptor-regulated (R-Smad) Smad proteins 1, 5 and 8. Those

phosphorylated Smads will form heteromeric complexes with the “common” (Co-Smad) Co-Smad4 and translocate to the nucleus where they regulate expression on their target genes, synergistically with other transcription factors (Nohe et al., 2004, Shi and Massague, 2003, Sountoulidis et al., 2012).

1.5.5 BMP intracellular signal transduction

A previous study on the activation of canonical BMP signalling shows the importance of Smad proteins in regulating the intracellular signal transduction which leads to the expression of targeted transcription factors such as Runx2 for bone endochondral ossification and bone fracture repair (Sountoulidis et al., 2012). Smad proteins are a vital signal transducer in the intracellular signalling following the binding of BMP ligands with the BMP receptors. Basically, Smad1, 2, 3, and 5 act in a ligand-specific manner. Smad1 and Smad5 are activated following the BMP ligand binding (Liu and Niswander, 2005, Liu et al., 1995). In contrast, Smad2 and Smad3 have similar structural configuration and they only mediate TGF-β/activin signals transduction. The phosphorylation of Smad2 and Smad3 are through the TGF-β signalling pathway which is not related to the BMP signalling activation. The R-Smads are consistently being phosphorylated by type-1 receptors. In particular, the phosphorylation of Smad1 is induced by BMP2, or through the expression of active type-1 receptors (Hoodless et al., 1996, Liu et al., 1996). In fact, Smad1 can only be induced though the BMP ligand binding activation as neither TGF-β nor activin are able to do so, and therefore, making the phosphorylated Smad1 a potential indicator for the activation of BMP signalling (Kretzschmar et al., 1997). The Smad4 is the common partner for the Smad1, Smad5 and Smad8 following the BMP ligand binding. In contrast, Smad 6 and Smad 7 (I-Smad) act as regulator or inhibitor for the ligand-specific Smads such as Smad1 and Smad2 (Hayashi et al., 1997, Imamura et al., 1997).

The Smad signalling pathway has been well-characterized over the past few years specifically during the activation of the BMP signalling. However, the initiation of the BMP signalling does not solely rely on the activation of the Smad pathway alone as the BMP signalling can also be initiated through non-Smad intercellular signalling pathway (Guzman et al., 2012). For example, previous studies demonstrated the activation of the MAPK family signalling molecules such as ERK1/2, p38 and stress-activated protein kinase/Jun N-terminal kinase following BMP activation (Hassel et al., 2003, Nakamura et al., 1999, Nohe et al., 2004). Another study by Shibuya *et al.* (1998) and Kimura *et al.* (2000) also observed that the activation of TAK1/TAB1 is the initiator for the activation of p38 pathway by BMP2 and BMP4 (Kimura et al., 2000, Shibuya et al., 1998). Even though the activation of the BMP signalling can happen through Smad or non-Smad signalling pathway, the non-Smad signalling pathway has not been studied extensively. In fact, most of the current BMP signalling studies solely focus on the Smad signalling pathway alone. Therefore, this PhD study has decided to concentrate on the BMP-Smad signalling pathway to elucidate the role of BMP signalling at the growth plate injury site following injury.

1.5.6 Modulation of BMP signalling

The modulation of BMP signalling occurs in three individual sites: intracellular region, plasma membrane and extracellular matrix (Wang et al., 2008). Currently, seven antagonists have been discovered in vertebrates, namely the Noggin, Chordin, Chordin-like, Follistatin, FSRP, the DAN/Cerberus protein family and Sclerostin (Balemans and Van Hul, 2002). All BMP antagonists play an important in maintaining the cellular and molecular homeostasis of the BMP signalling specifically for the organogenesis and postnatal development (Liu and Niswander, 2005, Tsumaki and Yoshikawa, 2005, Zhao, 2003). A previous mouse vertebral development model study has illustrated the BMP signalling is regulated by the BMP

antagonists, Chordin and Crossveinless 2 (Ambrosio et al., 2008, Zakin et al., 2010). Chordin acts as a BMP regulator which binds to the BMP ligands competitively preventing BMP ligand interaction with the BMP receptors at the cell surface thus inhibiting BMP signalling activation. Chordin and Crossveinless 2 are known to express at a different parts of the body. Chordin is found in the vertebral body whereas the Crossveinless 2 in the intervertebral disk. Chordin knockout studies observed an increase in BMP signalling; however, studies have also indicated the essential role of Crossveinless 2 for the re-localization and accumulation of Chordin in the vertebral body for BMP signalling activation (Zakin et al., 2010).

As another common BMP extracellular antagonist, Noggin binds competitively to the BMP ligands preventing interaction with cell surface receptors like BMPR1a and BMPR2. Noggin has been shown to be downregulated during the bone fracture repair (Devlin et al., 2003). In fact, immunohistochemical staining on a bone fracture shows higher expression of Noggin in areas of cartilage formation and noggin staining was predominantly in active osteoblasts in area of bone formation. This study highlighted the important role of the BMP antagonist in maintaining the balance of cartilage and bone development in the fracture site (Kwong et al., 2009). The extensive roles played by Noggin in the regulation of BMP signalling were shown in a previous transgenic mouse studies, whereby transgenic mice with overexpressed Noggin in the bone microenvironment displayed higher susceptibilities to osteopenia, bone fractures, decreased trabecular bone volume and impaired osteoblastic functions (Devlin et al., 2003, Wu et al., 2003). Although Noggin is known to be a major player in the bone fracture repair, its role in the growth plate injury repair is not known. Therefore, this study will investigate the potential role of BMP signalling during the growth plate injury repair through its controlled delivery (Wu et al., 2003) following growth plate injury in a rat model.

1.5.7 *In vivo* functions of BMP signalling

1.5.7.1 Roles in embryonic development

BMPs had been used to stimulate ectopic or orthotopic bone-induction in vertebrates (Urist, 1965). Apart from being regulating the bone osteogenesis, BMPs such as recombinant human proteins rhBMP2, rhBMP4, rhBMP5 and rhBMP7 also have the ability in regulating chondrogenesis, suggesting that the activation of different BMPs can result in the differentiation of osteoblast or chondrocytes (Wozney et al., 1988). Previous immunolocalization studies of BMPs during the limb bud development discovered significant localization and expression of the BMP2 and BMP4 in the epithelium of the ventral surface of limb buds which suggest potential proliferation or differentiation of the mesodermal cells in the underlying zone (Wozney, 1989). In addition, BMP2 and BMP4 were also found in the interdigital mesenchyme where the cartilaginous tissues form within the limb bud (Wozney et al., 1988). Besides that, during the chondrocyte hypertrophy, BMP6 was upregulated which indicates that the BMP signalling is also crucial for chondrocyte hypertrophy (Hahn et al., 1992). Consistently, immunohistochemistry analyses also discovered immunopositive localization of BMP6 at the hypertrophic chondrocyte region, which further affirm that the BMP6 plays a regulatory role during the endochondral ossification such as the terminal differentiation of chondrocytes and the growth plate hypertrophy (Carey and Liu, 1995, Gitelman et al., 1994).

1.5.7.2 Activities of BMP signalling during bone fracture repair

The bone has the capability to initiate self-healing specifically during bone fracture repair and such intrinsic activity was coined as the bone morphogenetic protein (BMPs) to characterise the molecules in respond to the bone fracture repair (Urist, 1965). The bone fracture repair

involves an orchestrated regulation of growth factors, cytokines, extracellular matrix and differentiation factors systemically and locally (Chhabra et al., 2005, Devlin et al., 2003, Gazzerro et al., 2005). Such synergic combination and interaction of all factors resulted in the migration of primitive, undifferentiated mesenchymal cells, proliferation and differentiation to the bone fracture site (Bolander, 1992, Simmons, 1985). A recent molecular study on the cellular proliferation, chondrogenesis and osteogenesis during the bone fracture healing has discovered upregulation of several growth factors including BMPs during the bone fracture repair (Ghodadra and Singh, 2008). In fact, rhBMP2 have been used clinically on human since 2004 which sees a significantly higher union rate, reduced time to union, improved wound healing, reduced infection rate and fewer secondary invasive interventions in the group treated with rhBMP2 (Govender et al., 2002). Another study using rhBMP7 has also shown to enhance the repair of scaphoid non-unions and fibular defects. The administration of rhBMP7 resulted in better formation of bone and bone bridge of the segmental defects compared to controls (Chim et al., 2013). Consistently, patients with distal tibial fractures that has been stabilised with external fixation with rhBMP7 showed significant increase in their bone healing rate when compared to patients who received the same treatment without rhBMP7, suggesting that BMP7 enhances the union of distal tibial fracture (Bharadwaj et al., 2009). However, the role of BMP2 or BMP7 in the bony repair at the growth plate injury site remain unknown.

In the early stage of a mouse bone fracture repair model, BMP4 was significantly upregulated in a less differentiated osteoprogenitor cells while another study discovered that the BMP4 immunopositive cells fail to express GLA protein (essential marker for mature osteogenic cells), suggesting that BMP4 could regulate the cell proliferation and initiate osteoblast differentiation (Li et al., 1998, Nakase et al., 1994). Consistently, BMP2 and BMP4 were also shown to be localized in the cambium cell layer of the periosteum which is adjacent to the

fracture region during the early stage of fracture repair and subsequently decreases across time (Nakase et al., 2003). At the endochondral ossification site adjacent to the bone fracture site, the expression of BMP2 and 4 were downregulated following bone fracture. However, the BMP2 and BMP4 expression levels increased significantly in the infiltrated mesenchymal cells at the fracture site and during their proliferation when hematoma starts to organise.

Chondrogenesis was fully developed at days 7-4 post bone fracture, when BMP2 and BMP4 were shown to be upregulated in chondrocyte precursor cells. BMP2 and BMP4 were immunopositively stained in the mature and hypertrophoid chondrocytes along with the newly differentiated osteoblasts (Nakase et al., 2003, Nakase et al., 1994, Wozney, 1989). Consistently, an established chronology study of the BMP in fracture repair model showed that the bone fracture site had an increase in the BMP immunopositive staining following bone fracture (Chhabra et al., 2005). Initially, only a small number of primitive cells were immunopositively stained by the BMP2 and BMP4 antibody. However, as the endochondral ossification progress, the presence of BMP2 and BMP4 was increasingly evident in the primitive mesenchymal and chondrocytic cells (Bostrom et al., 1995).

A critical discovery by Tsuji *et al.* (2006) in a bone fracture repair study showed the upregulation of BMP2 expression during the bone fracture repair which suggest the importance of BMP2 in regulating the bone fracture repair (Tsuji et al., 2006). Interestingly, the absence of BMP2 in newborn mice did not have any significant changes in the skeletal development. Therefore, it was postulated that the absence of BMP2 can be compensated by other BMPs present in the developing limb (Kawaguchi et al., 2004, Lyons et al., 1995). However, mice lacking limb-specific BMP2 soon showed significant disruption in the skeletal functions. Most

1-week old mice experienced a delay in formation of secondary ossification in all endochondral bones of limb followed by a significant downregulation of the Col2a at the articular cartilage surface (Rountree et al., 2004). A spontaneous bone fracture injury study in mice lacking limb-specific BMP2 model did not show any significant bone healing response at the injury site. At 20-weeks post-fracture, there were no signs of bone healing at the fracture site, while a large discontinuity remains. This study suggests the critical role of BMP2 for the initiation of bone fracture repair (Tsuji et al., 2006). Again, the role of BMP2 in the regulation of the growth plate injury repair and growth plate degeneration have yet to be examined.

Additional studies have also shown increased BMPR1 gene expression during the bony repair at the bone fracture site (Nakase et al., 1994). In particular, BMPR1a was found in expressed in the osteoblasts within the periosteum. BMPR1a and BMPR1b were later found to be upregulated in the cells within the proliferating osteogenic layer of the periosteum at day 3 post-bone fracture. The authors also showed expression of BMPR1a and BMPR1b in the fibrogenic-like cells, chondrocytes in the endochondral ossification site and osteoblasts in the newly formed trabecular bone (Ishidou et al., 1995).

The distinctive ability of the BMP signalling in the initiation of osteoblast differentiation leads to the development of recombinant BMP proteins that could potentially be a biological treatment for skeletal injuries and to speed-up bone grafting (Govender et al., 2002). However, the results of the recombinant protein treatment remain inconsistent between the animal and human trials. Even though the exact same delivery method and vehicle being used in the preclinical trials and in animal models, the effects on the bone healing were drastically different (Dimitriou et al., 2005, Govender et al., 2002). Furthermore, the use of the recombinant

proteins can also lead to numerous side effects (Armstrong and Burton, 2008). The drastic differences in the recombinant protein treatment outcomes between animal and human are still unclear. Therefore, further studies on the effects of BMP recombinant proteins on the bone fracture repair and growth plate injury repair is needed.

1.5.7.3 Activities of BMP signalling during growth plate injury repair

Since BMP signalling is important for bone fracture repair, it may also be a potential regulator in the bony repair during the growth plate injury repair. The previous study by Ngo *et al.* (2006) examined the gene expression of BMPs and BMP receptors during the course of growth plate injury repair (Ngo et al., 2006). As expected, at day 1 post-injury, BMP3, BMP4, BMPR1a and BMPR2 were upregulated at the growth plate injury site. Upregulation of BMP2, BMP3, BMP4, BMP7 and BMPR1a was also seen during the fibrogenic phase at days 3-14. These findings suggest the potential involvement of BMP3 and BMP4 during the early inflammatory phase, which could potentially lead to the following downstream events such as the upregulation of BMP2, BMP3, BMP4 and BMP7 during the fibrogenic and osteogenic responses during the growth plate injury repair (Ngo et al., 2006).

A recent microarray analysis of the growth plate injury site in a rat model successfully revealed four major functional groupings of differentially expressed genes (known for skeletal development) that were upregulated during the bony repair of the injured growth plate (Macsai et al., 2012). Those functional groups are Wnt signalling, BMP signalling, osteoblast differentiation and skeletal development. In particular, several components of the BMP signalling was found upregulated during the early fibrogenic phase of the growth plate injury, namely the BMP2, BMP6, BMP7, Chordin, Chordin *like*-2 and Id1 (Macsai et al., 2012). The

early fibrogenic phase is a critical time-point that determines the cellular differentiation of the potential mesenchymal infiltrates at the growth plate injury site. The result from this study strongly indicate the potential role of BMP signalling in the bone bridge formation during the growth plate injury repair. However, while studies on the BMP signalling during the growth plate injury repair were limited, the cellular and molecular mechanism of BMP signalling in bony repair of growth plate remains unknown. Knowing the importance and action mechanisms of BMP signalling in regulating bony repair in the bone fracture, BMP could be a potential therapeutic target to limit the bone formation within the growth plate injury site.

1.6 Glypican

1.6.1 Glypican gene organisation

Glypicans have been shown to regulate the Hh, Wnt, BMP and FGF growth factor signalling pathway in specific tissues. However, depending on the growth factor signalling and the tissue of interest, glypicans can either act as a stimulator or inhibitor to that particular signalling pathway (Filmus and Capurro, 2008). A previous study by Song *et al.* (2006) has suggested that the glypican has the potential in regulating the bone formation during bone fracture repair (Song et al., 2006). Furthermore, a recent study by Dwivedi *et al.* (2013) has shown that glypicans can potentially regulate BMP signalling in skeletal formation(Dwivedi et al., 2013a). Glypicans are proteoglycans that are able to become co-regulators in growth factor signalling. They are found on the plasma membrane, tethered by a glycosylphosphatidylinositol (GPI) anchor, giving them the opportunity to bind with extracellular signalling molecules with their receptors (Campos-Xavier et al., 2009, Litwack et al., 1998). There are six glypicans in human genome (GPC1 to GPC6). In fact, the mouse genome also has six glypicans while smaller amount of glypicans are found in invertebrates eg. 2 proteoglycans in the fruit fly *Drosophila*

melanogaster (Dally and Dally-like protein [Dlp]) and two in the worm, *Caenorhabditis elegans* (Lon2 and Gpn1) (Belenkaya et al., 2004, Fico et al., 2011, Grisaru and Rosenblum, 2001, Taneja-Bageshwar and Gumienny, 2013, Veugelers et al., 1999, Veugelers et al., 1998). Glypicans can be divided into two subfamilies: GPC1, 2, 4, 6 and GPC3, 5 with approximately 25% amino-acid identity between groups. GPC3 and GPC5 have almost 43% similarity in their primary structure compared to the GPC1, 2, 4 and 6 (De Cat and David, 2001). Vertebrate glypicans are smaller in size which comprises of 555-580 amino acids compared to the *D. melanogaster* glypicans, Dally and Dlp (2 isoforms) which contain 626 and 765 and 939 amino acids, respectively (Wu et al., 2010). The size of these genes can extend from 7.7 kb for human GPC2 to 1.5 Mb for human GPC5 (Fico et al., 2011). Most glypicans have a conserved arrangement of 14 cysteine residues that probably contains the core protein with a tertiary structure through a disulphide linkage. Another distinctive feature of the glypican is the GPI anchor that anchors the glypicans to the outer surface of the plasma membrane (Dwivedi et al., 2013a, Haupt et al., 2009, Viviano et al., 2005).

1.6.2 Glypican localization and functions

As mentioned earlier, the glypican are proteins that carry GPI anchors, therefore, they are mostly found in the cell membrane. GPI-anchored proteins are usually located at the apical membrane in polarised cells (Mayor and Riezman, 2004). *In vivo* studies have shown that the main functions of membrane-attached glypicans are able to regulate Wnt, Hedgehog (Hh), FGF and BMP signalling pathways (De Cat and David, 2001, Dwivedi et al., 2013a, Dwivedi et al., 2013b, Midorikawa et al., 2003, Song et al., 2005). For example, the inhibition of endogenous GPC1 and GPC3 have been shown to upregulate BMP2 responsiveness in the human suture mesenchymal cells (Dwivedi et al., 2013a). Furthermore, *Drosophila* with mutated glypican also showed defective Hh, Wnt, BMP and FGF signalling in specific tissues (Dwivedi et al.,

2013a, Lin and Perrimon, 1999, Ohkawara et al., 2003). More importantly, as these growth factors (Hh, Wnt, BMP and FGF) are critical for the bone growth and bone fracture repair, the above studies suggest that the glypicans could play a crucial role in regulating bony repair at the growth plate injury site. Although most of the glypicans have been shown to regulate BMP signalling, role of glypicans in the growth plate injury repair and bone trabecular tissue formation is still unknown.

1.6.3 Functional roles of glypicans in bone growth and development

Previous studies have found that GPC1, 2, 3, 4 and 6 were expressed in the growth plate cartilage region at the late-stage of limb development (Ahrens and Schlosser, 2005, Campos-Xavier et al., 2009, Veugelers et al., 1998). Expression of the glypicans is different from one another based on the specific site of expression or tissue. Studies have showed that GPC1 is mostly expressed during the late chondrogenic stage in the prehypertrophic and hypertrophic chondrocytes; GPC2 is expressed in the resting zone of the growth plate; GPC3 is expressed in proliferative, prehypertrophic zone and perichondrial cells; GPC4 is expressed solely in the prehypertrophic zone and GPC6 is expressed in resting and proliferative zones (Ahrens and Schlosser, 2005). Furthermore, RT-PCR quantification have also shown significant upregulation of the glypican gene expression (GPC6) in the proliferative, prehypertrophic and hypertrophic zones during bone formation and bone growth (Campos-Xavier et al., 2009). This study also suggest that the difference in glypicans expression in the growth plate structure could indicate that the glypicans have selective roles during the bone growth and development.

1.6.4 Functional roles of glypicans during bone fracture repair

Apart from being downregulated during the bone growth and development, a current bone tibia fracture rat model have also shown significant downregulation of the GPC3 following bone fracture which indicates the activation of the BMP signalling while the GPC1 gene were only upregulated after 1-2 weeks of the bone fracture signifying a decrease in the BMP signalling (Song et al., 2006). This study suggest that the glypican has the potential in regulating the bone formation during bone fracture repair. Consistently, the GPC3 knockout mice study by Viviano *et al.* (2005) also suggested possible regulation by the GPC3 in the differentiation of bone marrow macrophage precursor cells into osteoclasts (Viviano et al., 2005). Similarly, other glypican studies also implicated the significant downregulation of the GPC3 during the chondrogenesis and osteogenesis of long bones specifically through either endochondral ossification or intramembranous ossification (Djouad et al., 2007, Dwivedi et al., 2013a, Teplyuk et al., 2009, Viviano et al., 2005). During the skeletal and craniofacial development, GPC3 was found expressed in both limb bud mesenchyme and cartilage-associated with limb and vertebrate (Haupt et al., 2009, Pellegrini et al., 1998).

1.6.5 Effects of GPC3-knockout in bone biology

The deletion of GPC3 gene in mouse model has shown a range of features such as the Simpson-Golabi-Behmel syndrome (SGBS), perinatal death, developmental overgrowth, cystic and dysplastic kidneys and abnormal lung development (Cano-Gauci et al., 1999, Chiao et al., 2002), suggesting important roles of GPC3 in various tissues. GPC3 knockout mice showed delayed ossification in the trabecular bones and caused abnormal hypertrophic chondrocyte differentiation (Viviano et al., 2005). Furthermore, GPC3 knockout mice had a lower count of osteoclast-like cells within the hypertrophic chondrocytes compared to the control mice,

suggesting that the GPC3 could also be involved in osteoclastogenesis (Viviano et al., 2005). Besides that, Capurro *et al.* (2009) also discovered a potential cross-talk between GPC3 and Indian hedgehog (Ihh), a regulator of endochondral bone growth (Capurro et al., 2009). During the absence of the GPC3, Ihh protein levels as well as target genes such as Gli1 and Ptch1 were upregulated. This study suggested that GPC3 can mediate Ihh degradation during the bone development (Capurro et al., 2009).

1.6.6 Relationship between glypicans and BMP signalling pathway

Several *in vivo* and *in vitro* studies had demonstrated the functional role of the glypicans in regulating the BMP signalling pathway that led to osteoblast differentiation during the bone fracture repair and cranial suture repair (Dwivedi et al., 2013a, Teplyuk et al., 2009). Evidently, glypicans, which are a major type of membrane proteins that supply heparan sulfate at the cell surface, have emerged to play a pivotal role in the reception and dissemination of BMP signal transduction (Paine-Saunders et al., 2000). Furthermore, an *in vitro* analysis also demonstrated that the overexpression of GPC3 in hepatocellular carcinoma cells are able to modulate the FGF2 and BMP7 signalling responses suggesting the critical link between glypicans and BMP signalling pathway (Midorikawa et al., 2003). In fact, BMPs are heparin-binding proteins, and BMP-mediated developmental process involves the interaction between the heparan sulfate and BMPs (Ruppert et al., 1996). A previous *Xenopus* embryonic study demonstrated that the glypicans-BMP4 interaction resulted in the restriction of BMP4 expression pattern in the *Xenopus* embryos (Ohkawara et al., 2002). Moreover, *Drosophila* mutants of Dally, a gene (encoding the homolog of GPC3), displayed abnormalities in regulating *Decapentaplegic* (Dpp), the homolog of BMP2/BMP4 (Jackson et al., 1997). A genetic study also demonstrated that the combined genetic deficiency of both GPC3 and BMP4 leads to abnormal skeletal development in vertebrates (Grisaru and Rosenblum, 2001). Despite significant *in vitro* and *in*

vivo evidence, the direct role of GPC3 and BMP signalling in the growth plate injury repair in young rats remain poorly understood. Since GPC3 has been shown to modulate the expression of the BMP2 during the cranial suture study, this PhD will investigate the involvement of the GPC3 in modulation bony repair at the growth plate injury site in young rats.

CONCLUSION

As most of the previous growth plate injury repair studies suggested, the growth plate is highly prone to physical injury and can often result in the undesirable bony repair and bone bridge formation at the injury site. As a result, orthopaedic problems such as limb length discrepancy and bone angulation deformity often befall the patient following growth plate injury. To date, the only treatment is through invasive surgical correction of an established defect which is highly prone to infection and not always successful. Thus, a search for a biological treatment is required to prevent the bone bridge formation and at the same time enabling the injured growth plate to regenerate. During the previous growth plate injury repair studies, BMP signalling, which is a well-known to a play crucial role in bone fracture repair, has been identified to have an increased activation at the growth plate injury site. However, its role and its potential action mechanisms in the modulation of growth plate injury repair is yet to be unveiled. In addition, while the proteoglycans glypicans have been recognised to be able to regulate BMP signalling, whether it can do so in the growth plate repair setting is unknown. Therefore, this PhD study aim to investigate the potential involvement of BMP signalling and glypicans in modulating BMP signalling during the growth plate injury repair. New knowledge from this project will hopefully provide information in identifying new therapeutic targets and/or in the development of a biological treatment to prevent the orthopaedic problems resulting from growth plate injuries and to promote growth plate regeneration.

RATIONALE and AIMS

Chapter 2: Roles of bone morphogenetic protein signalling in the remaining adjacent growth plate post injury

The rationale for a focus on the bone morphogenetic protein signalling in the remaining adjacent growth plate is based on the previous microarray discovery demonstrating the upregulation of BMPs at the growth plate injury site following injury and yet it is unknown whether BMP signalling activation is also enhanced at the adjacent growth plate. At the same time, a structural study of the remaining adjacent growth plate also reported significant bone formation and showed signs of degeneration at the remaining adjacent growth plate following growth plate injury. As BMP signalling is known to be involved in growth plate chondrocyte maturation and hypertrophic as well as bone formation, osteoblast differentiation and bone fracture repair, this study will examine the roles of BMP signalling at the remaining adjacent growth plate following growth plate injury.

Chapter 3: Roles of bone morphogenetic protein signalling in the bony repair of injured growth plate cartilage in young rats

The rationale for the focus on BMP signalling in the growth plate injury site following injury is due to the discovery of enhanced expression BMPs and receptors and signalling activation in real-time quantitative PCR BMP gene expression and microarray studies at the growth plate injury site following growth plate injury. While BMP signalling is known to be critical in bone formation during bone development and bone healing, the roles of BMP signalling in regulating the growth plate injury bony repair have yet to be confirmed. Therefore, to reveal roles of the BMP signalling and its mechanisms of action during the growth plate bony repair, this study

utilises a common BMP antagonist, Noggin, to examine the functional roles of BMP signalling during the bony repair response at the growth plate injury site.

Chapter 4: Roles of glypican-3 (GPC3) in regulating BMP signalling and bony repair at the injured growth plate in young rats

The rationale to investigate the roles of GPC3 in regulating the BMP signalling at the growth plate injury site is due to the discovery that the GPC3 are able to regulate bone healing and BMP signalling and yet it is unknown whether GPC3 can also regulate growth plate bony repair and the BMP signalling in the growth plate injury repair setting. Therefore, using a recombinant human GPC3 (rhGPC3) protein and anti-GPC3 protein, this study aim to investigate the potential roles of GPC3 in the bony repair response and BMP signalling at the growth plate injury site in rats.

HYPOTHESES

1. The BMP signalling is involved in regulating the bone formation and degeneration of the remaining adjacent growth plate following injury.
2. BMP signalling is involved in regulating bone bridge formation following growth plate injury and the systemic inhibition of the BMP signalling using the BMP antagonist, Noggin, can inhibit the undesirable bony repair response at the growth plate injury site.
3. Glypican-3 (GPC3) can modulate the BMP signalling and thus the bony repair at the growth injury site.

CHAPTER 2

Roles of bone morphogenetic protein signalling in the degeneration of the uninjured adjacent growth plate post injury

Roles of bone morphogenetic protein signalling in the degeneration of the uninjured adjacent growth plate post injury

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HIGHLIGHTS

- Injury upon the growth plate often leads to bony repair response at the growth plate injury site.
- Cellular and molecular signalling pathways leading to the bony repair is still unknown.
- Injury upon the growth plate also affected the normal growth of the remaining adjacent growth plate cartilage
- Growth plate injury resulted in an increased growth plate cartilage hypertrophy resulting in the cartilage degeneration.
- BMP signalling pathway was found to be activated at the remaining adjacent growth plate following growth plate injury
- Rat treated with BMP antagonist, noggin resulted in the reduction of growth plate degeneration at the remaining adjacent growth plate cartilage.

ABSTRACT

Injured growth plate is often repaired by undesirable bony tissue which leads to orthopaedic complications such as limb length discrepancy and bone angulation deformity. Previous investigations using a rat tibial growth plate injury model demonstrated potential degeneration of the uninjured remaining adjacent growth plate area following a growth plate injury. However, the underlying mechanisms are unclear. This study examined the effect of growth plate injury on the structural and molecular changes at the remaining adjacent growth plate and potential roles of BMP signalling in the growth plate degeneration. Histological analyses showed no significant changes in the zonal height of the growth plate between the control and uninjured adjacent area of injured growth plate; however, the hypertrophic zone/proliferative zone ratio was significantly increased at day 35 (D35) injured growth plate. Consistently, gene expression studies showed that Col10 was upregulated, Runx2 had a trend of upregulation, while Sox9 was downregulated and Col2a showed a trend of downregulation at day 14, suggesting enhanced growth plate hypertrophy and reduced chondrogenesis of the adjacent area. Immunohistochemical and Western blot analyses revealed increased expression of BMP signalling molecule pSmad1/5/8 at the adjacent growth plate. Consistently, mRNA expression of BMP signalling pathway molecules such as BMP7, BMPR1a and Dlx5 in the adjacent cartilage was upregulated at D14 and D35 following growth plate injury, suggesting increased activation of BMP signalling at the adjacent growth plate. In the injured rats treated with BMP inhibitor Noggin, the hypertrophic zone/proliferative zone ratio was significantly reduced compared to the vehicle control, suggesting potential rescue of the growth plate structure. Noggin treatment also saw an increase in Col2a and a reduction in Col10 gene expression, suggesting a potential rescue of chondrogenesis and attenuation of hypertrophy in the adjacent growth plate. Overall, this study demonstrated that growth plate injury causes hypertrophic degeneration of the adjacent region involving increased activation of the BMP signalling.

INTRODUCTION

The growth plate cartilage is solely responsible in regulating elongation of all growing long bones through endochondral ossification (Olsen et al., 2000). However, being cartilaginous in nature, the growth plate is in fact the weakest part of the growing long bone making it prone to injuries (Chung et al., 2006). Injuries of the growth plate cartilage can be classified using the Salter-Harris classification (I-V) based on the involvement of the growth plate and severity of the injury (Perugia et al., 2014). Growth plate injuries classified under Salter-Harris I & II are less severe and have higher chances of being restored (Perugia et al., 2014). However, a statistical study shows that 20% of the growth plate injuries are more likely to undergo undesirable bony repair at the injury site (Mizuta et al., 1987). This undesirable bony repair often lead to orthopaedic complications during the child's growth causing limb length discrepancy and bone angulation deformity (Basener et al., 2009, Caine et al., 2006, Chung et al., 2011). Treatment available is solely surgical correction which is highly invasive and with a low success rate (Chung et al., 2006). Unfortunately, biological treatments are not available to treat or prevent such biological defects from occurring.

Besides affecting the growth plate injury site following injury, a previous investigation using a rat tibial growth plate injury model demonstrated potential degeneration or premature thinning of the uninjured remaining adjacent growth plate area (Macsai et al., 2011). However, the underlying cellular or molecular changes in the uninjured adjacent growth plate area are still unclear. Cartilage degeneration is often seen as a clinical syndrome of osteoarthritis and it is the most common cause of pain and disability in older people (Buckwalter and Mankin, 1998). This is generally due to an increase of chondrocyte hypertrophy at the articular cartilage for osteoarthritis patients. Interestingly, using guinea pig as an animal model to develop spontaneous osteoarthritis, a study demonstrated a progressive switch of the expression of

phosphorylated Smad2/3 to Smad1/5/8 at the articular cartilage (Zhao et al., 2016), suggesting upregulation of the bone morphogenetic protein signalling (BMP) during the chondrocyte differentiation following osteoarthritis progression. Subsequent inhibition of BMP2/Smad1 activity using Matrilin-3 have also shown to inhibit premature chondrocyte hypertrophy by slowing the progressive development of osteoarthritis (Yang et al., 2014). Furthermore, immunohistochemical analyses showed strong BMPR1a and BMPR2 being expressed at the osteoarthritic articular cartilage implying potential involvement of BMP signalling in the articular cartilage homeostasis (Bobinac et al., 2008). Similarly, BMP2 expression has been reported in the chondrogenic and ossifying sites of calcified tendinopathy and traumatic tendon injury rat model whereby it is postulated that the ectopic BMP2 expression might potentially induce tissue transformation into ectopic bone/cartilage and promote structural degeneration in calcific tendinopathy (Lui et al., 2009).

Interestingly, apart from potential roles in cartilage degeneration, some members of BMPs such as BMP7 is known to be reduced during the progression of cartilage degeneration and thus have been used for cartilage repair following cartilage degeneration (Chubinskaya et al., 2007). Therefore, BMPs plays an important role in bone/cartilage homeostasis by regulating chondrogenesis/hypertrophy and osteoblast differentiation during the postnatal bone development and during cartilage degeneration (Cao and Chen, 2005, Kim et al., 1998, Rountree et al., 2004). Therefore, this study examined the effect of growth plate injury on the structural, cellular and molecular changes at the remaining adjacent growth plate and to establish the potential roles of BMP signalling in the growth plate degeneration following a growth plate injury.

METHODS AND MATERIALS

2.1.1 Growth plate injury, treatment trial and specimen processing

A total of thirty five 5-weeks old male *Sprague Dawley* rats were randomly distributed into three treatment groups of noggin treated, injured and uninjured control groups. Growth plate injuries were inflicted at the proximal tibial of both hind legs under anaesthetic condition. The rat growth plate injury model was adopted from an established rat growth plate injury model by Xian *et al.* (2004). All injuries were inflicted using a drill-hole method using a 2-mm dental drill which disrupts the central part of the proximal tibia growth plate structure. All injured drill-holes were irrigated with 0.9% saline solution to remove any loose organic debris before wound closure with sterile surgical clips. At day 2 post-injury, noggin-treated and vehicle rats were anaesthetized while a small incision was made at the rats' dorsal. An osmotic pump, a controlled and continuous drug release minipump (model 2002, Alzet, Cupertino, CA) filled with 90 ng/ml rhNoggin (R&D Systems, Minneapolis, MN) or 0.9% saline solution was subcutaneously implanted into the incision of the noggin-treated and vehicle rats, respectively. All wounds were closed with surgical clips and all animals were allowed to recover prior to being placed back to general rat housing. Osmotic pumps were left within the rats until the day of cull. This protocol was performed under the approval of the Animal Ethics Committee of the Institute of Medical and Veterinary Services (IMVS), South Australia.

Rats (n=8/group) were humanely sacrificed through carbon dioxide (CO_2) overdose. Specimen collection was carried out at day 14 and day 35 post-injury. After all soft tissues on the tibia were removed, the proximal left tibia was fixed with 10% formalin solution for 24 hours at 4°C before subjecting decalcification in 14% EDTA solution (Sigma-Aldrich, NSW, Australia) for 4 weeks at 4°C. Following decalcification, tibia were halved longitudinally through the growth plate injury site prior to being processed for paraffin embedding and sectioning at 4µm

thickness using the microtome machine (Microm H<325, Charleston, SC) for histology staining and immunohistochemistry studies.

As for the right proximal tibia, the injury site was exposed by snapping the metaphysis away from the growth plate at the border, and the growth plate injury site tissue as well as the remaining adjacent growth plate tissue were collected respectively by scrapping using sterile surgical blades. All collected tissues were snapped-frozen using liquid nitrogen and kept at -80°C until being used for quantitative real-time PCR and Western blot detection analyses.

2.1.2 H&E and alcian blue staining and image analysis of remaining adjacent growth plate

To measure changes of the height of the remaining adjacent growth after the growth plate injury, paraffin tissue sections were deparaffinized using Solv21c (United Biosciences, Queensland, Australia), and stained with alcian blue, haematoxylin and eosin (H&E alcian blue). Light microscope examination of the stained sections will allow the visualization and measurements of the various zones in the adjacent growth plate, namely the resting, proliferative and hypertrophic zones. Height measurements of the whole thickness and the separate zones of the remaining growth plate were taken using the Image-Pro Plus (Media Cybernetics, Silver Spring, MD). These measurements were averaged from a total of four rats per group (n=4) from the growth plate injury and normal uninjured growth plate.

2.1.3 Real-time quantitative RT-PCR expression of cartilage-, bone-related and BMPs, BMP receptors and BMP downstream target genes

To examine the effect of growth plate injury on the expression of cartilage- and bone-related genes at the remaining adjacent growth plate, the remaining adjacent growth plate tissues collected from the right proximal tibia were used. For the control groups, uninjured growth plate tissues from rats aged 7 weeks (day 14) and 10 weeks (day 35) were used. All RNA samples were extracted using the Aurum™ Total RNA Mini Kit (BIO-RAD, NSW, Australia) according to the manufacturer's instructions. Following RNA extraction, all RNA were treated with DNase from the Ambion DNA-free kit (Ambion, NY, USA) according to the manufacturer's instructions. All cDNAs were later synthesized from the purified RNA using random decamers (Geneworks, SA, Australia) and Superscript-II RNase H Reverse Transcriptase (Stratagene, La Jolla, CA). Using the synthesized cDNA, the expression of cartilage- and bone-related genes was analysed through the SYBR green real-time PCR assay, Applied Biosystems 7500 RT-PCR system (Warrington, UK), and using specific primers (**Table 2.1**). Gene analyses was carried out as described in Chung *et al.* (2006). Using cyclophilin-A (Cyc-A) as an internal control, all results of targeted genes were expressed in relative to the Cyc-A gene. Results from the real-time quantitative RT-PCR gene expression were grouped as growth plate injury against the uninjured normal control samples.

Table 2.1. Primer sequences used for real-time quantitative RT-PCR analysis.

Gene	Forward Primer (5' - 3')	Reverse Primer (3' - 5')
Runx2	CAGTAAGAAGAGCCAGGCAG	GCATTCGTGGGTTGGAGAAG
Sox9	AGTACCCGCATCTGCACAAC	ACTTGTAATCGGGGTGGTCT
Col2a	CTCATCCAGGGCTCCAATGAT	TCTGTGATCGGTACTCGATGA
Col10	GAGGCAGGTCCAAGAGGTGA	CCTGGCTTATTGAGTGAGTGTCT
BMP2	GGAAAACTTCCCGACGCTTCT	CCTGCATTTGTTCCCGAAAA
BMP4	TTATGAGGTTATGAAGCCCAC	GCTCACATCGAAAGTTTCCCAC
BMP7	AACCGTCCAAGACTCCAAAG	CCTCTGGTCACTGCTGCTGTT
BMPR1a	GGTTCAGCGAACTATTGCCAAA	TCACCACGCCATTTACCA
BMPR2	CAGAACGAACGCAACCTATCAC	GCTGGACATTGAATGCTCAGA
Id1	CATGAAGGTCGCCAGTAGGA	AGCCGTTCATGTCGTAGAGC
Dlx5	CACCACCCGTCTGAGGAATC	TGGTATGGGTTGAGCGCTTT
Cyclophilin A	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG

2.1.4 Immunohistochemical analysis

To examine growth plate injury and treatment on BMP signalling activation, phosphorylated-Smad1/5/8 (pSmad1/5/8) was immunolocalized using the immunohistochemistry analysis method on the injured and uninjured growth plate injury sections. Briefly, deparaffinized tissue sections on the superfrost glass slides were rehydrated through sequential ethanol washes from 100% to 70% and quenched in 3% H_2O_2. All slides were treated with 0.01M citrate buffer (pH 6.0) for antigen retrieval and blocked with 5% normal swine serum in phosphate-buffered saline/bovine serum albumin (PBS/BSA, pH 7.4). After the overnight incubation at 4°C with the primary rabbit anti-pSmad1/5/8 antibody at 1:200 (Santa Cruz, Texas, USA). Sections were then labelled with biotinylated polyclonal swine anti-rabbit IgG at 1:500 (Dako, NSW, Australia) and liquid DAB Plus substrate (Dako, NSW, Australia). The primary antibody for the negative control sections were replaced with normal rabbit IgG (PeproTech, Rocky Hill, NJ) at 1:200. Following secondary antibody incubation and DAB colour development, all

sections were washed and counter-stained with haematoxylin and mounted with cover slip for further image analysis (ImageJ, NIH).

2.1.5 Western blot detection of pSmad1/5/8 at the remaining adjacent growth plate

To analyse the activation of BMP signalling at the adjacent growth plate, a Western blot analysis of expression of the BMP downstream target protein, pSmad1/5/8, was performed on the injured growth plate tissues collected from 7 and 10 weeks old rats. The uninjured normal growth plate tissues from 7 and 10 weeks old rats were used as the negative controls. All Western blot blots were analysed and quantified using the Image Lab 5.0 (Bio-Rad Laboratories).

2.1.6 Statistical analysis

All data were presented as means ± SEM comparing between the growth plate injury and uninjured control groups between different time-points. All data collected from the *in* vivo study were analysed using of two-way ANOVA. Significance levels ($P<0.05$) were determined and post hoc analysis of groups were done using the Tukey's test. In the figures, *, ** and *** represent $P<0.05$, $P<0.01$ and $P<0.00$, respectively. While ^ represents $P<0.05$.

RESULTS

2.2.1 Effects of growth plate injury on the zonal height measurements at the remaining adjacent growth plate cartilage

To examine the effect of growth plate injury on the remaining adjacent growth plate zonal heights (resting, proliferative and hypertrophic zone), histology staining and examination were performed (**Fig 2.1**). Zonal height measurements taken at the remaining adjacent growth plate (n=4) did not reveal any significant differences between the uninjured control and injured growth plate at day 14 post-injury for the resting, proliferative and hypertrophic zones respectively. At day 35 post-injury, the proliferative zone for the injured growth plate was shown to have a non-significant reduction when compared to the control growth plate. Apart from that, there were no significant changes observed between the control and remaining adjacent growth plate at day 14 and day 35 post-injury. The quantitative analyses of the hypertrophic/proliferative zonal height ratio showed a significant increase in the remaining adjacent growth plate at day 35 post-injury, suggesting a possible increase in the hypertrophic and/or a reduction in proliferative zone, respectively.

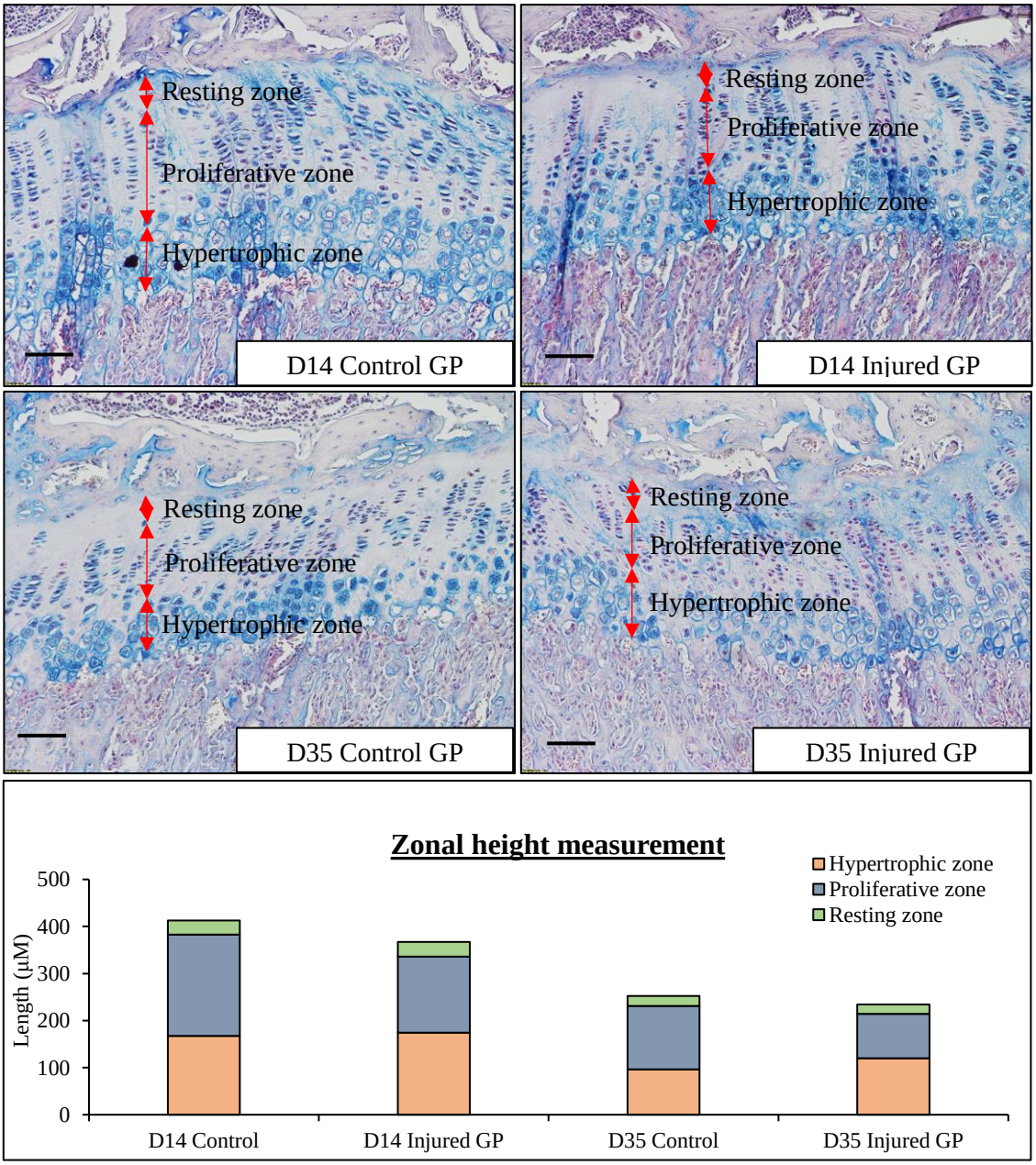

Figure 2.1. H&E and alcian blue histology staining on the uninjured adjacent growth plate to measure the individual resting zone, proliferative zone and hypertrophic zone at day 14 and 35 post-injury. Quantitative analyses on the zonal height measurements and the hypertrophic zone/ proliferative zone height ratio of the adjacent growth plate at day 14 and day 35 post-injury followed between the control and injured growth plate. Scale bar = 100µm.

2.2.2 Effects of growth plate injury on the bone-related gene at the remaining adjacent growth plate cartilage

Quantitative RT-PCR was performed on the RNA samples of remaining adjacent growth plate tissues to reveal potential changes in the expression of bone-related genes post-injury (**Fig 2.2**). Quantitative RT-PCR gene expression of the Runx2 (an early osteoblastic marker commonly used to mark the early osteoblastic differentiation and a marker of chondrocyte hypertrophy) at the remaining adjacent growth plate did not reveal a significant difference between the uninjured and injured rats at day 14 and day 35 post-injury. However, Runx2 demonstrated a trend of upregulated gene expression ($P<0.06$, non-significant), which further suggests a slight increase in the hypertrophy at the remaining adjacent growth plate following growth plate injury.

2.2.3 Effects of growth plate injury on the cartilage-related genes at the remaining adjacent growth plate cartilage

Quantitative RT-PCR examination of the expression levels of cartilage-related genes (Sox9, Col2a and Col10) at the remaining adjacent growth plate showed a significant reduction ($P<0.05$) in the Sox9 gene expression at day 14 post-injury (**Fig 2.2**). However, day 35 Sox9 expression did not show any significant differences between the uninjured and injured growth plate. Col2a gene expression was shown to have a trend of reduction at 14 and day 35 post-injury although the results were non-significant statistically. At day 14 post-injury, there was a significant increase ($P<0.05$) in Col10 gene expression level. However, day 35 adjacent growth plate did not show any significant changes in the Col10 gene expression. Results from day 14 Sox9 and Col10 could indicate presence of reduced chondrogenesis and enhanced

hypertrophy activity at the remaining adjacent growth plate during the growth plate injury repair.

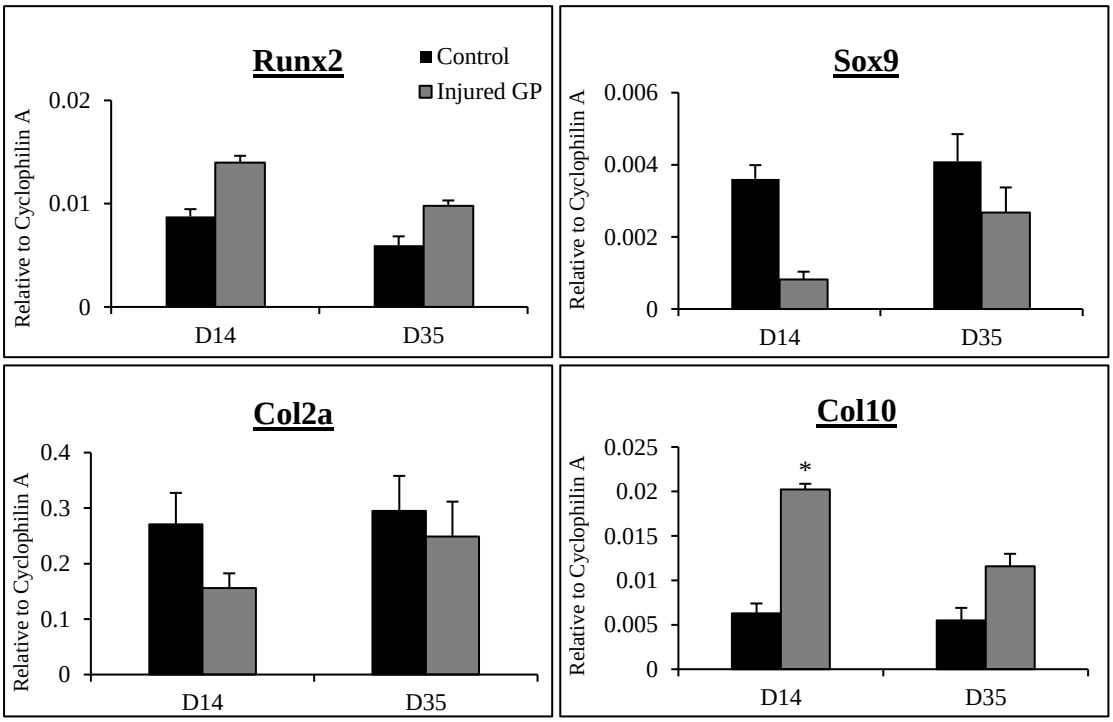

Figure 2.2. Quantitative RT-PCR analyses of bone-related gene (Runx2), cartilage-related genes (Sox9, Col2a and Col10) at the remaining adjacent growth plate at day 14 and day 35 following growth plate injury. * $P<0.05$ vs control group.

2.2.4 Immunohistochemical detection of pSmad1/5/8 at the remaining adjacent growth plate

Immunohistochemical detection of pSmad1/5/8 was used to detect the BMP activation at the remaining adjacent growth plate (**Fig 2.3**). Using day 14 injured growth plate rats, pSmad1/5/8 immunopositively stained cells were present at the remaining adjacent growth plate specifically at the pre-hypertrophic and hypertrophic region. Immunopositively stained cells were distinguished by the dark brown staining within the nuclei region as BMP signalling is only activated following the translocation of pSmad1/5/8 into the nuclei resulting in BMP downstream target gene transcription. Quantitative analyses on the percentage of pSmad1/5/8 immunopositively stained cells showed a significant higher ($P<0.05$) percentage of pSmad1/5/8 immunopositively stained chondrocytes following growth plate injury at the remaining adjacent growth plate compared to the uninjured control.

2.2.5 Western blot detection of pSmad1/5/8 levels at the remaining adjacent growth plate

Western blot analysis was performed to examine levels of pSmad1/5/8 protein at the remaining adjacent growth plate tissues (**Fig 2.3**). Results from the Western blot analyses revealed significant presence of pSmad1/5/8 protein at day 14 injured growth plate but a lesser amount was found in the uninjured growth plate. Further quantitative analyses were performed using the Image Lab software from Bio-Rad Laboratories, which showed a significant upregulation ($P<0.001$) of pSmad1/5/8 at the remaining adjacent growth plate compared to the uninjured growth plate. Consistent with the immunohistochemical data above, Western blot data again suggest enhanced BMP activation (higher levels of pSmad1/5/8) at the remaining adjacent growth plate.

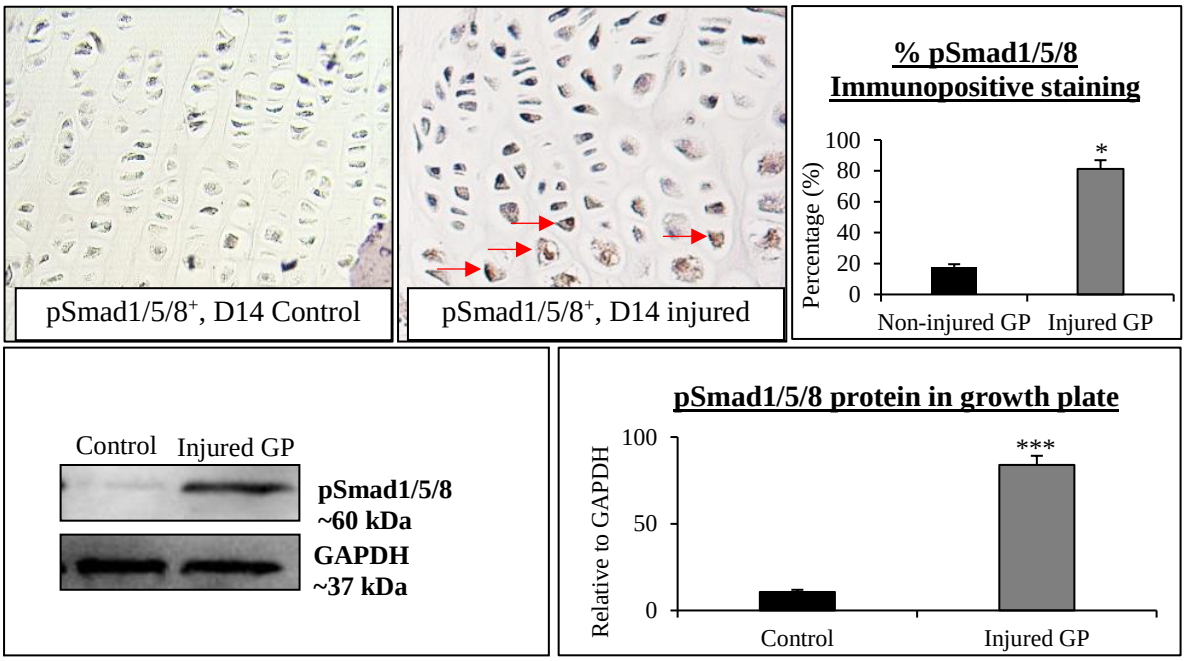

Figure 2.3. Detection of pSmad1/5/8 immunopositive cells within the remaining adjacent growth plate at day 14 post-injury in comparison to non-injury control. Immunopositively stained cells were detected in the remaining adjacent growth plate at day 14. Quantitative analyses on the percentage of immunopositive cells within an area of 500μm^2. Western blot detection of pSmad1/5/8 protein within the remaining adjacent growth plate through nucleic extraction at day 14 post-injury shows significant upregulation of pSmad1/5/8 protein in the nucleic extraction in the remaining adjacent growth plate compared to the uninjured control. Scale bar = 20μm. * P<0.05 and *** P<0.001 vs control group.

2.2.6 Effects of growth plate injury on the BMP signalling genes at the remaining adjacent growth plate

Quantitative RT-PCR gene expression analyses were performed to determine the effects of growth plate injury on expression of BMP signalling pathway genes at the remaining adjacent growth plate (**Fig 2.4**). There was a significant increase ($P<0.05$) in the BMP7 plate gene expression at day 35 post-injury for the injured growth plate compared to the uninjured growth. However, BMP2 and BMP4 did not show any significant difference in the gene expression level on day 14 and day 35 between the uninjured and injured rats.

Quantitative RT-PCR were performed on both BMP receptor genes (BMPR1a and BMPR2) following growth plate injury at day 14 and day 35 (**Fig 2.4**). Interestingly, BMPR1a gene expression was significantly increased ($P<0.05$) in the remaining adjacent growth plate at day 14 post-injury. However, there were no significance differences between the uninjured and injured rats in the BMPR2 gene expression at day 14 and day 35 post-injury.

Quantitative RT-PCR on the BMP targeted genes (Id1 and Dlx5) was also been performed at the remaining adjacent growth plate samples (**Fig 2.4**). Id1 did not reveal any significant differences between the uninjured and injured rats at day 14 and day 35 time-points. However, Dlx5 showed a significant increase ($P<0.05$) in gene expression following growth plate injury at day 14. Consistent with the upregulation of pSmad1/5/8 protein level as shown above, this finding further suggests an increase in BMP signalling activity within the adjacent growth plate following growth plate injury.

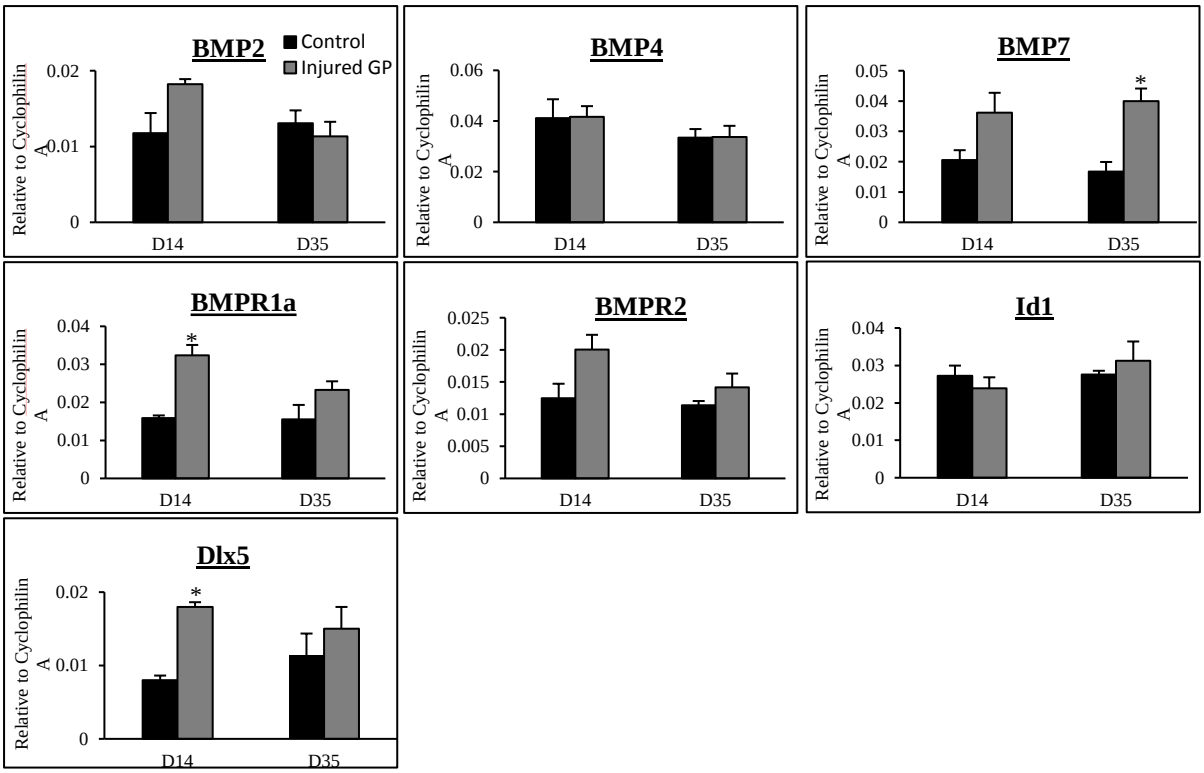

Figure 2.4. Quantitative RT-PCR analyses of gene expression of BMP signalling pathway component genes including BMPs (BMP2, BMP4 and BMP7), BMP receptor genes (BMPR1a and BMPR2) and BMP downstream targeted genes (Id1 and Dlx5) at the remaining adjacent growth plate at day 14 and day 35 post-injury. * $P<0.05$ vs control group.

2.2.7 Effect of BMP signalling inhibition on the height of remaining adjacent growth plate

To examine the effect of BMP inhibition on the height of remaining adjacent growth plate, histology staining and analyses were performed on the noggin-treated growth plate injury group against the vehicle-treated growth plate injury group (**Fig 2.5**). While the histology analyses did not show any significant changes between the heights of the noggin-treated and vehicle-treated injured groups, it was noticed that the overall remaining adjacent growth plate height for the noggin-treated group was slightly larger ($P<0.06$, non-significant) when compared to the vehicle-treated injured group at day 14. In particular, the proliferative zone of

rats treated with noggin was slightly thicker ($P<0.07$, non-significant) when compared to vehicle-treated group, indicating a possible prevention of growth plate degeneration following the suppression of BMP signalling. At day 35, there were almost no changes observed in the heights of the growth plate between the vehicle- and noggin-treated groups.

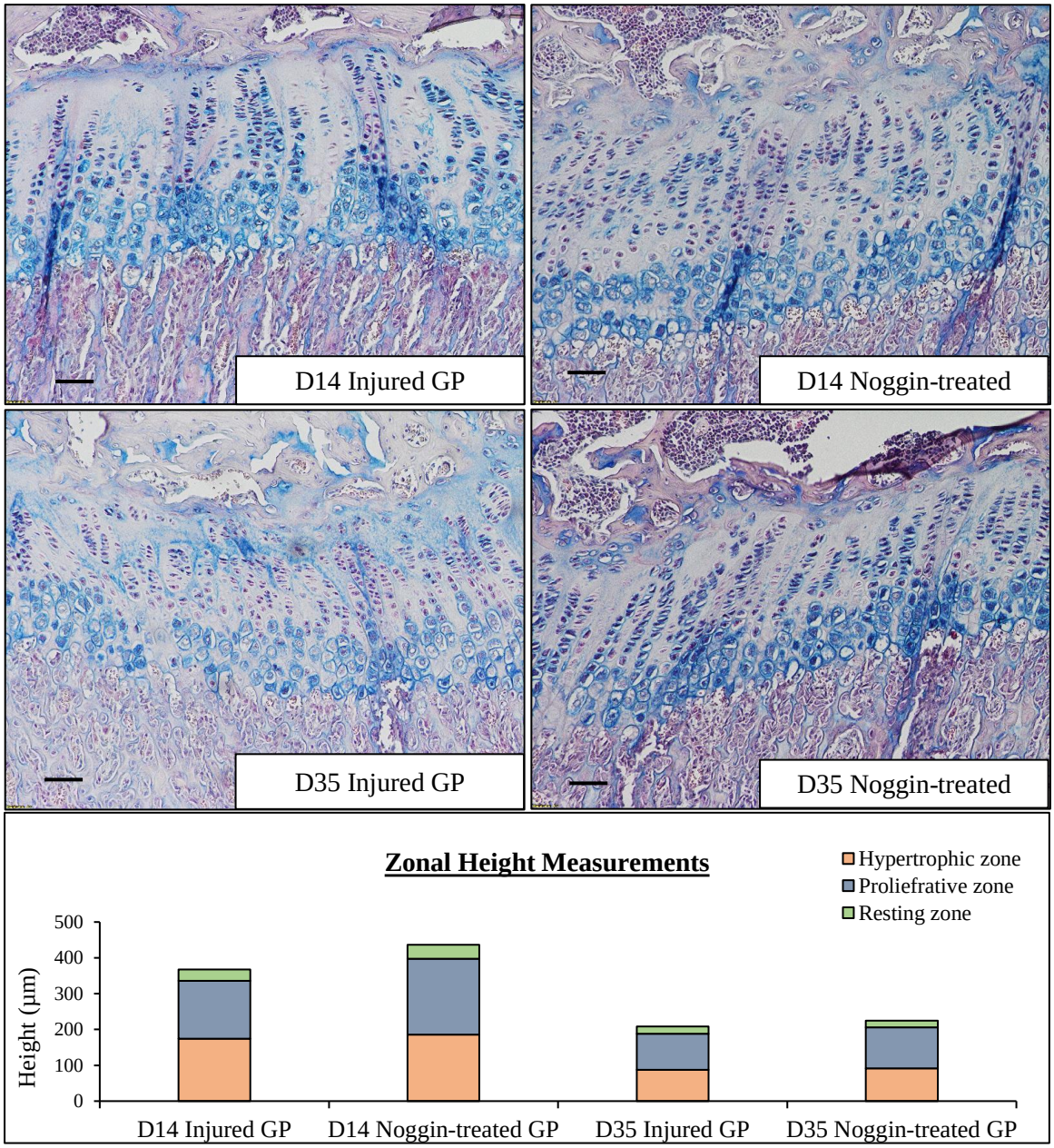

Figure 2.5. Histology staining to measure the effect of noggin treatment on zonal height differences (resting zone, proliferative zone and hypertrophic zone) following growth plate injury at day 14 and 35. Quantitative analyses on the hypertrophic zone/proliferative zone height ratio were graphed following noggin treatment. Scale bar = 100µm.

2.2.8 Effect of BMP signalling inhibition on the bone- and cartilage-related genes following growth plate injury at the adjacent growth plate

Quantitative RT-PCR were performed to examine the effects of noggin treatment (to inhibit BMP signalling) on the early osteogenic and chondrogenic genes at the adjacent growth plate following growth plate injury (**Fig 2.6**). At day 14 post-injury, the gene expression level of the Runx2 gene was slightly lower ($P<0.07$, non-significant) in the noggin-treated rats compared to the vehicle treated group showing an almost similar level as with the uninjured control group. While Sox9 gene expression was significantly ($P<0.05$) downregulated in the vehicle treated group compared to the control group at day 14, noggin-treated group did not show any significant differences although the gene expression level was shown to be slightly higher ($P<0.06$, non-significant) compared to the vehicle treated group. Interestingly, Col2a gene expression level was significantly upregulated ($P<0.05$) in the noggin-treatment group when compared to the vehicle group at day 14. The expression level of the Col2a was almost similar to the control group at day 14. However, no significant differences were recorded for the Col2a at day 35. While Col10, which is expressed by the hypertrophic chondrocytes, was significantly ($P<0.05$) upregulated in the vehicle treated group at day 14, it was significantly suppressed ($P<0.05$) by noggin treatment when compared to the vehicle group and to the similar level as with the control group. In fact, similar trends were also shown at day 35 ($P<0.06$) although results were insignificant.

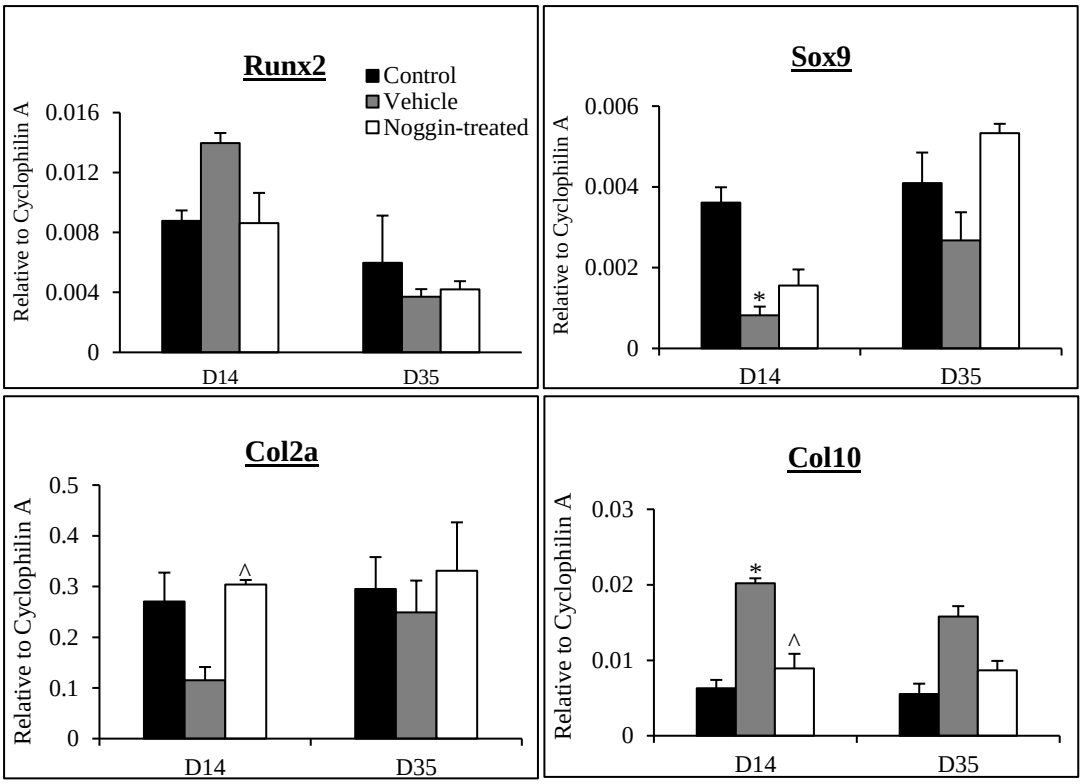

Figure 2.6. Real-time quantitative RT-PCR analyses on the bone or cartilage hypertropy-related (Runx2) and cartilage-related genes (Sox9, Col2a and Col10) following introduction of noggin at the adjacent growth plate. Col2a gene expression level was upregulated while Col10 was downregulated at day 14 following BMP inhibition. * $P<0.05$ vs uninjured control and ^ $P<0.05$ vs vehicle group.

DISCUSSION

Undesirable bony tissue repair is commonly seen following injury to the growth plate which leads to bone growth defects particularly in children with significant growth remaining. A previous rat growth plate injury model has shown potential degeneration of the uninjured remaining adjacent growth plate area following growth plate injury. Furthermore, longitudinal examination through µ-CT analyses also demonstrated significant time-dependant increase of bony repair at both growth plate injury site and bone formation at the adjacent uninjured growth plate area (Macsai et al., 2011). Previous studies have indicated that the bone bridge formation within the growth plate injury site may not be the major role player in bone growth defects. In fact, more studies are beginning to emerge studying the role of uninjured adjacent growth plate in regards to the bone growth discrepancies (Janezic et al., 2010, Macsai et al., 2011). The current study examined potential mechanisms for the degeneration changes at the adjacent growth plate cartilage. Structural and molecular analyses of this study showed significant increases in the hypertrophic zone/proliferative ratio, expression of bone-related and BMP signalling activation-related genes at the remaining adjacent growth plate area following growth plate injury. This study also demonstrated an important role of BMP signalling for this degeneration change.

2.3.1 Growth plate injury affects structure of the adjacent growth plate

Study by Macsai *et al.* (2011) has demonstrated progressive degeneration of the uninjured remaining growth plate in response to growth plate injury and the undesirable bony repair at the injury site. Cartilage degeneration is commonly diagnosed in patient with Osteoarthritis (OA) which is a chronic disease of articular joints that often leads to pain and disability and would be one of the leading cause of disability in the near future (Bedson et al., 2005, Kour et

al., 2016). Histological analyses of the articular cartilage lesion found uneven and disorganized chondrocytes in the superficial, mid and deep zones while proliferative chondrocytes were randomly clustered in the superficial and mid zone (Bai and Li, 2016). Furthermore, a recent mice medial meniscus injury model study also found progressive articular cartilage degeneration following surgery which suggest the onset of cartilage thinning (Chen et al., 2015). Similarly, a recent growth plate injury study using magnetic resonance imaging (MRI) also revealed a significant reduction ($P<0.05$) in the medial and lateral growth plates in the remaining adjacent growth plate following injury (Nakase et al., 2015). While the current study did not show any significant changes in the height of the remaining adjacent growth plate, it showed a decreasing (non-significant) trend of reduced growth plate thickness following the injury. In addition, the current study found a significant increase ($P<0.05$) in the hypertrophic/proliferative zone ratio at day 35, suggesting a potential decrease or increase in the proliferative or hypertrophic zone, respectively. Our findings suggest that the injury on the growth plate indeed affects the structure of the uninjured remaining adjacent growth plate and may cause its degeneration.

2.3.2 Growth plate injury downregulated the cartilage-related and upregulated hypertrophic gene expression at the remaining adjacent growth plate

The significant increase ($P<0.05$) in the hypertrophic/proliferative zone ratio could signify the suppression of chondrocyte proliferation and increase in chondrocyte hypertrophy following growth plate injury (Zhang et al., 2015). Consistent with the structural changes in the adjacent uninjured growth plate region after an injury, molecular analyses of the remaining adjacent growth plate also demonstrated a significant decrease in chondrogenesis-related genes (eg Sox9) and an increase in cartilage hypertrophy-related genes (Col10) at day 14 following the growth plate injury. Sox9 is one of the cartilage-related gene that is expressed by proliferating

chondrocytes in the growth plate and plays a critical role in regulating chondrocyte proliferation (Bell et al., 1997, Bi et al., 1999, Henry et al., 2012), while Col10 is expressed by hypertrophic chondrocytes involved in controlling the rate of chondrocyte hypertrophy (Ding et al., 2012). Thus, it is highly possible that the injury at the growth plate could potentially affect the molecular activity within the remaining adjacent growth plate structure by reducing the chondrocyte proliferation and increasing the chondrocyte hypertrophy. Consistent with the growth plate degeneration findings of the current study, work by Macsai *et al.* (2011) also demonstrated a decreased Sox9 gene expression at day 7 and 14 post-injury (Macsai et al., 2011). The increase in Col10 gene expression was also seen in patients with OA whereby significant expression of Col10 was detected through immunohistochemical analyses suggesting a similar phenomenon in both remaining adjacent growth plate and cartilage degeneration in OA patients (Aigner et al., 1993).

Furthermore, this current study showed an increasing trend of Runx2 (non-significant) gene expression at day 14 and 35 post-injury. Runx2 is a transcription factor involved in osteoblast differentiation and chondrocyte hypertrophy. A recent human OA study suggested that Runx2 expression is important in inducing osteoarthritis though the increase in cartilage degeneration activity, suggesting that the OA cartilage degeneration is also Runx2 gene expression-dependent (Cao et al., 2014). These studies suggest that injuries to the growth plate and articular cartilage may induce differential chondrocyte proliferative and hypertrophy responses in the uninjured adjacent cartilage area. However, further studies are required to investigate the underlying mechanisms leading to hypertrophic and other injury responses at the uninjured growth plate.

2.3.3 Upregulated BMP signalling activation at the remaining adjacent growth plate after an injury

As a step to investigate potential mechanisms for the hypertrophic changes in the adjacent region after an injury in the growth plate, potential roles of BMP signalling were investigated. BMP signalling is known for its potential in regulating osteoblast differentiation, bone formation, growth plate chondrogenesis and hypertrophy as well as hyaline cartilage maintenance and hypertrophic degeneration (Cao and Chen, 2005, Rountree et al., 2004). Consistently, our immunohistochemical and Western blot analyses of pSmad1/5/8 (marker of BMP signalling activation) showed its significant increase at the remaining adjacent growth plate, suggesting increased activation of BMP signalling at the hypertrophic change of adjacent area following the growth plate injury. Similarly, a previous study also demonstrated increasing BMP activity was involved in the premature chondrocyte hypertrophy in Matrilin-3 knockout mice (Yang et al., 2014). These results demonstrated increased activation of BMP signalling at the remaining adjacent growth plate following growth plate injury.

To dissect out mechanisms for the increased BMP signalling activation, the current study examined expression of the BMPs, BMP receptors and BMP downstream target genes at the remaining adjacent growth plate. The BMP7, BMPR1a and Dlx5 were significantly upregulated at day 35 ($P<0.05$), day 14 ($P<0.05$) and day 14 ($P<0.05$), respectively. An increase in BMP7 gene expression at the remaining adjacent growth plate following injury was an unexpected finding. BMP7 is known to possess an inhibitory effect on cartilage degeneration (Sekiya et al., 2009). Therefore, the upregulation of BMP7 could indicate a potential repair response initiated by the remaining adjacent growth plate to delay the progression of cartilage degeneration induced by the injury to the growth plate. However, further analyses and investigation are warranted to define the role of BMP7 at the remaining adjacent growth plate

following injury. On the contrary, BMPR1a and Dlx5 gene expression levels were significantly upregulated ($P<0.05$) at day 14 post-injury, which further suggest the potential increase in chondrocyte hypertrophy at the remaining adjacent growth plate. Studies such as the bony repair at the growth plate injury site, bone fracture repair and articular cartilage degeneration have also demonstrated the presence of BMP signalling in regulating bony repair responses (Chhabra et al., 2005, Ngo et al., 2006). Consistently, the upregulation of BMP signalling at the remaining adjacent growth plate could potentially be involved in the bone bridge formation in the uninjured adjacent growth plate area as reported by Macsai *et al.* (2011) (Macsai et al., 2011).

2.3.4 Inhibition of BMP signalling prevents the hypertrophic degeneration of the remaining adjacent growth plate following growth plate injury

As a means to investigate roles of BMP signalling at the hypertrophic change of the adjacent growth plate cartilage, the effect of inhibiting BMP signalling by noggin treatment on the remaining adjacent growth plate was also examined in rats with growth plate injury. Noggin is a competitive BMP inhibitor binding to BMPs which prevents the formation of BMPs-BMP receptor complex, and presence of noggin could potentially reduce the mRNA expression of the osteoblastic differentiation markers, including collagen type 1a, osteocalcin, osteoprotegerin, BMP2 and Runx2 (Liou et al., 2015). Histologically, noggin treatment was found to slightly increase the height of the adjacent growth plate and particularly the height of the proliferative zone (non-significant) at day 14 post-injury. Interestingly, noggin treatment was found to significantly suppress the injury-induced increase in the hypertrophic/proliferative zone ratio, suggesting a potential rescue/prevention in the growth plate degeneration of the adjacent growth plate. Consistently, a murine model study through the delivery of noggin also demonstrated enhanced recruitment of cells into cartilage causing

growth plate overgrowth (Brunet et al., 1998). Our study suggests that the suppression of BMP signalling reduces adjacent growth plate degeneration following the growth plate injury.

Consistent with our observation of suppression of BMP signalling inhibits injury-induced degeneration of the adjacent growth plate, the current study also found that noggin treatment upregulated chondrogenesis-related (Col2) gene expression and downregulated hypertrophic (Col10 and Runx2) gene expression at day 14 following injury. These findings further suggest that inhibition of the BMP signalling following growth plate injury could potentially induce chondrogenesis and reduce injury-induced hypertrophic degeneration at the adjacent growth plate. Similarly, Caron *et al.* (2013) demonstrated an increase in the chondrocyte hypertrophy during chondrogenic differentiation when BMP2 dose was increased (Caron et al., 2013).

The major limitation of this study was the sample size. Due to the small tissue sample that could be extracted from the remaining adjacent growth plate of each animal plus the number of PCR analyses that were performed, only a small group of 8 individual rats were used in each comparison, which potentially caused some sample variations in the comparisons.

In summary, data from this study suggest that growth plate injury causes hypertrophic degeneration of the adjacent growth plate cartilage, and that an increase in BMP signalling activation at the adjacent cartilage plays a role in the hypertrophic change. Consistently, after growth plate injury, growth plate chondrogenesis could be rescued and hypertrophy could be reduced at the adjacent growth plate following the inhibition of BMP signalling using inhibitor noggin treatment. Further studies will be required to confirm the role and action mechanism of the BMP signalling in the remaining adjacent growth plate following injury as it could be a

useful target for exploring a potential treatment for protecting remaining adjacent growth plate following growth plate injuries.

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CHAPTER 3

Roles of bone morphogenetic protein signalling in the bony repair of injured growth plate cartilage in young rats

Title: Roles of bone morphogenetic protein signalling in the bony repair of injured growth plate cartilage in young rats

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HIGHLIGHTS

- Growth plate injury often leads to undesirable bony repair at the growth plate injury site.
- Cellular and molecular pathways leading to the bony repair remain unclear.
- BMP signalling was shown to be expressed during the bony repair at the growth plate injury site, but its potential function is unclear.
- Rat growth plate injury sites were treated locally with Noggin (BMP inhibitor) to inhibit BMP signalling.
- Noggin treatment has been shown to suppress BMP signalling activation and bony repair at the growth plate injury site and bone drill-track injury site.

ABSTRACT

Growth plate cartilage is solely responsible for the longitudinal bone growth in children. An injury at the growth plate often undergoes bony tissue repair instead of cartilage repair leading to long term orthopaedic problems such as limb length discrepancies and bone angulation deformities. While earlier investigations in a rat growth plate injury model have identified various repair events/phases during injury, namely inflammatory, fibrogenic, osteogenesis and remodelling repair phases, molecular mechanisms underlying the bony repair remain unclear. A recent study has identified that BMP signalling was up-regulated during the growth plate injury repair. Using a rat tibial growth plate injury repair model and with or without systemic osmotic pump infusion of BMP signalling antagonist noggin at 90ng/ml commencing at day 2 post injury, the current study investigated the potential roles of BMP signalling pathway during the growth plate injury repair. Micro-CT scanning showed a significant reduction in the bony repair at the growth plate injury site at both days 8 and 14 with the administration of noggin. Consistently, histological image analyses also showed a reduction in the amounts of bone trabeculae and RT-PCR analyses exhibited a reduction in Runx2 and Osteocalcin gene expression following Noggin treatment. Similarly, micro-CT and histological examinations of the tibial bone drill-track injury site also revealed a significant reduction in the bone volume following noggin treatment. These data suggest that the BMP signalling is critically important in the bony repair at both the growth plate injury site and the bone drill-track injury site.

INTRODUCTION

The growth plate cartilage plays an important role in maintaining longitudinal bone growth during bone development. Being cartilaginous in nature, the growth plate is fragile and highly susceptible to injuries (Chung et al., 2006, Chung et al., 2009, Macsai et al., 2011, Xian et al., 2004). Furthermore, with a poor cartilage regeneration potential, bony repair (with newly formed bone trabeculae) is often seen (instead of cartilaginous tissue regeneration) within the growth plate injury site leading to various bone growth defects such as limb length discrepancy and bone angulation deformity (Basener et al., 2009, Chung et al., 2011). Currently, treatments available are solely surgical-based that are invasive and riddled with issues such as the needed repeated procedures and pin site infections (Chung et al., 2009). Unfortunately, there are no known biological treatments that can successfully treat or better yet, prevent this undesirable bony repair from occurring. To identify potential targets for developing biological therapies, further studies are required to gain a better understanding of the mechanisms underlying the growth plate bony repair.

Previous studies using a rat growth plate injury model have so far identified four major repair response phases, namely the inflammatory, fibrogenic, osteogenic and remodelling phases during days 1-4, 5-8, 8-14 and 14 onwards post-injury, respectively (Xian et al., 2004). During the inflammatory phase, inflammatory cells were shown infiltrating the growth plate injury site while fibrogenic phase involves the fibrogenic-like cell recruitment to the injury site (Chung et al., 2011). Differentiation of fibrogenic cells to tissue-specific cells such bone trabeculae tissue, cartilaginous tissue and fat tissue as well as the formation of bone marrow tissue took place during the osteogenic and remodelling phases. Furthermore, previous studies have also suggested that these specific cell differentiation events may be regulated by locally expressed cytokines and growth factors such as TNF-α, BMPs, Wnts, VEGF and PDGF-BB during the

growth plate injury repair (Chung et al., 2009, Lee et al., 2000, Macsai et al., 2008, Rodda and McMahon, 2006, Sakata et al., 2012, Tatsuyama et al., 2000, Zhou et al., 2006).

In particular, bone morphogenetic proteins (BMPs) are known to play critical roles such as chondrogenesis and osteoblast differentiation during the postnatal bone development (Leboy, 2006, Reddi, 1992) and bone repair (Li et al., 1998, Nakase et al., 1994, Tatsuyama et al., 2000, Tsuji et al., 2006). BMPs were first discovered as components of demineralized bone extract with bone growth properties (Urist, 1965). BMPs are cytokines that are part of the transforming growth factor-β superfamily (TGF-β) (Cao and Chen, 2005). The biological signals of BMPs are transduced through the serine/threonine kinase receptor namely the BMP receptors type I (BMPR1a) and type II (BMPR2). BMPs ligate to the BMPR1a and BMPR2 forming heteromers (Cao and Chen, 2005, Chen et al., 2004b). This event triggers BMPR2 to phosphorylate the intracellular R-Smad proteins, Smad1, Smad5 and Smad8 which interact with the co-Smad protein, Smad4 and translocate into the nucleus regulating gene transcription (Chen et al., 2004a, Khanal et al., 2008, Yamaguchi et al., 2000). During bone fracture repair, gene expression levels of BMPs were found up-regulated (Chen et al., 2004b, Chhabra et al., 2005, Diwan et al., 2013, Ngo et al., 2006, Yu et al., 2002), which suggest the involvement of BMP signalling during the of bone fracture repair (Chhabra et al., 2005, Rosen, 2006, Tang et al., 2009, Wan and Cao, 2005, Yoon and Lyons, 2004). *In vitro* BMP signalling studies using BMP2 have shown to induce alkaline phosphatase expression and osteoblast mineralization activity which is critical for bone development (Rawadi et al., 2003). BMP2 is currently being used clinically to treat bone fractures for human by inducing bony repair at the bone fracture site (Garrison et al., 2010, Ghodadra and Singh, 2008). Moreover, BMP signalling inhibition using the BMP inhibitor, Noggin, during the bone fracture repair was found to significantly reduce the repair rate and affect the bony repair responses such as osteoblast differentiation and bone formation at the bone fracture site (Brunet et al., 1998, Canalis et al., 2003, Epperlein

et al., 2007, Wu et al., 2003). Consistently, overexpression of Noggin in mice was found to impair osteoblastic function and decrease bone trabecular volume, leading to orthopaedic problems such as bone osteopenia and bone fractures (Devlin et al., 2003).

While the importance and roles of BMPs during bone fracture repair have been well established as discussed above, there is still a lack of knowledge on the role of BMPs during the bony repair at the growth plate injury site. A recent microarray gene expression study in a rat growth plate injury model found upregulation of several bone-related genes and signalling pathways such as BMP and Wnt pathways throughout the course of growth plate injury repair (Macsai et al., 2012). Macsai *et al.* (2012) found that during the growth plate injury repair, several BMPs were found to be up-regulated at the early fibrogenic phase and osteogenic phase suggesting the potential importance of the BMP signalling in the undesirable bony repair at the growth plate injury site (Macsai et al., 2012). To get a better understanding of the potential roles of the BMP signalling during the growth plate injury repair, this study examined the effects of treatment with BMP inhibitor, Noggin, on the bony repair outcomes in a rat growth plate injury model (Rawadi et al., 2003).

3.1.1 Growth plate injury and treatment trial

A total of sixty male *Sprague Dawley* rats (5-weeks old) were randomly stratified into three treatment groups; Noggin treatment, vehicle control and non-treated uninjured control groups. Both Noggin and vehicle groups were subjected to experimental growth plate injuries at the proximal tibiae of both hind legs. Injuries were inflicted using a drill-hole method which disrupts the central part of the proximal tibiae growth plate under the anaesthetic condition as described in Xian *et al.* (2004) (Zhou et al., 2004). Following injury, the drilled-hole track was irrigated with 0.9% saline solution prior to wound closure with sterile surgical clips. At day 2 (D2) post-growth plate injury, rats were anaesthetized while a small incision was made at the dorsal end of both Noggin and vehicle groups. An osmotic pump, a controlled and continuous drug release minipump (model 2002, Alzet, Cupertino, CA) filled with 90 ng/ml Noggin (R&D Systems, Minneapolis, MN) or 0.9% saline solution was subcutaneously implanted into the incision of both Noggin and vehicle rats, respectively. All wounds were closed with surgical clips and all animals were allowed to recover prior to being placed back to general rat housing. All osmotic pumps for D8 and D14 time-points were left within the rats until the day of cull. However, osmotic pumps for D35 rats were surgically removed after 14 days of the implant. This protocol was performed under the approval from the Animal Ethics Committee of SA Pathology, South Australia.

Rats (n=8) were sacrificed humanely through carbon dioxide (CO_2) overdose for tissue specimen collection at D8, D14 and D35 post injury. Briefly, tibiae were dissected and all soft tissues were carefully removed. The proximal left tibia was fixed in 10% formalin for 24 hours at 4°C and wrapped in PBS-soaked gauze for micro-CT scans (SkyScan 1076, Kontich, Belgium). Following micro-CT, all bones were decalcified for 4 weeks with 14% EDTA solution (Sigma-Aldrich, NSW, Australia). Once decalcified, all bones were halved longitudinally through the growth plate injury site and processed overnight for paraffin embedding. All paraffin-embedded tibiae were sliced using microtome (Microm HM325, Charleston, SC) at 4μm thickness for future histological and immunohistochemical analyses. The right proximal tibia of the rat was snap-opened at the metaphyseal/growth plate border exposing the growth plate. The bony repair at the growth plate injury site as well as the remaining adjacent growth plate cartilage was collected respectively using a sterile surgical blade and placed in two separate Eppendorf tubes. All collected samples were immediately snap-frozen using liquid nitrogen and stored at -80°C until future use.

3.1.2 Real-time quantitative RT-PCR expression analysis of cartilage- and bone-related genes as well as BMPs, BMP receptors and BMP downstream targeted genes

To examine the effect of Noggin treatment on the cartilage-, bone-related, BMPs, BMP receptors, and BMP downstream target genes during the growth plate injury repair, the growth plates injury site tissues harvested from the right proximal tibiae were used. Normal growth plate tissues were collected from rats aged 7 weeks old as controls for D8 and D14 groups while 10 weeks old for the D35 group. All RNA samples were extracted from the growth plate injury site tissue samples using the Aurum™ Total RNA Mini Kit (BIO-RAD, NSW, Australia). Extracted RNA was treated with DNase using the Ambion DNA-free kit (Ambion, NY, USA) according to manufacturer's instructions to prevent genomic DNA contamination.

Due to limited sample size, purified RNA samples from two growth plate injury sites of the same group were pooled together to increase the total concentration of RNA. Furthermore, cDNAs were synthesized from the pooled RNA samples using random decamers (Geneworks, SA, Australia) and Superscript-II RNase H Reverse Transcriptase (Stratagene, La Jolla, CA) according to manufacturer's instructions. To analyse the expression of cartilage-related, bone-related, BMPs, BMP receptors and BMP downstream target genes, PCR was conducted with specific primers (shown in **Table 3.1**), a SYBR Green Real-Time PCR assay employing the Applied Biosystems 7500 RT-PCR system (Warrington, UK). Gene analyses were performed as described in Chung *et al.* (2006). Cyclophilin-A (Cyc-A) was used as internal control whereby all results of other genes are expressed relative to Cyc-A gene. Levels of gene expression between Noggin and vehicle groups were compared against the uninjured normal control samples.

Table 3.1. Primer sequences used for real-time quantitative RT-PCR analyses

Gene	Forward Primer (5' - 3')	Reverse Primer (3' - 5')
Sox9	AGTACCCGCATCTGCACAAC	ACTTGTAATCGGGGTGGTCT
Runx2	CAGTAAGAAGAGCCAGGCAG	GCATTCGTGGGTTGGAGAAG
Col2a	CTCATCCAGGGCTCCAATGAT	TCTGTGATCGGTACTCGATGA
Osteocalcin	GCTGGCCCTGACTGCATTCTG	ATTCACCACCTTACTGCCCTCCTG
BMP2	GGAAAACTTCCCGACGCTTCT	CCTGCATTTGTTCCCGAAAA
BMP4	TTATGAGGTTATGAAGCCCAC	GCTCACATCGAAAGTTTCCCAC
BMPR1a	GGTTCAGCGAACTATTGCCAAA	TCACCACGCCATTTACCA
BMPR2	CAGAACGAACGCAACCTATCAC	GCTGGACATTGAATGCTCAGA
Dlx5	CACCACCCGTCTCAGGAATC	TGGTATGGGTTGAGCGCTTT
Id1	CATGAAGGTCGCCAGTAGCA	AGCCGTTCATGTCGTAGAGC
Cyclophilin A	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG

3.1.3 Immunohistochemical analysis

To localize the BMP signalling-related protein expression, phosphorylated-Smad1/5/8 (pSmad1/5/8) immunohistochemical analysis was performed on the growth plate injury sections. Briefly, deparaffinized tissue sections were rehydrated through sequential ethanol washes and quenched in 3% H_2O_2. All sections were subjected to antigen retrieval in 0.01M citrate buffer (pH 6.0) and blocked with 5% normal swine serum in phosphate-buffered saline/bovine serum albumin (PBS/BSA, pH 7.4). All sections were incubated overnight at 4°C with primary antibody pSmad1/5/8 at 1:200 (Santa Cruz, Texas, USA) and labelled with biotinylated polyclonal swine anti-rabbit IgG at 1:500 (Dako, NSW, Australia) and liquid DAB Plus substrate (Dako, NSW, Australia). As for a negative control, the primary antibody was replaced with normal IgG (PeproTech, Rocky Hill, NJ) at the equivalent concentration. All sections were counter-stained with haematoxylin and mounted with a cover slip for image analysis.

3.1.4 Micro-CT analyses of bony repair at growth plate injury site and bone drill-track injury site

To examine the effect of Noggin treatment on overall bony repair at D14 and D35 post injury rats, tibiae were subjected to micro-CT scans. All scans were performed using a high resolution micro-CT system on the proximal left tibiae (n=5). Following scanning, reconstruction of images was carried out by realigning and saving images as transaxial dataset using DataViewer (SkyScan 1076, Kontich, Belgium) for further analyses with CTan software (SkyScan 1076, Kontich, Belgium). In order to minimalize variations among the growth plate injury sites, a total of twenty scan images per injury site were selected for analyses using the CTan software. The region of interest (ROI) governing the entire growth plate was selected to measure the

amount of bone volume within the growth plate injury site. Following that, 3-dimensional analyses were conducted to quantify the amount of bone volume and tissue volume within the growth plate injury site. Results from these measurements were used to calculate the bone volume fraction (BV/TV%) within the growth plate injury site that will reflect the amount of bony repair within the growth plate injury site.

Additionally, to examine the effect of Noggin treatment on the bone repair within the tibial bone drill-hole injury track that connects with the growth plate injury site examined above, micro-CT scans were also performed on D14 and D35 tibiae. The whole tibiae (n=5) were scanned for 45min and images were similarly saved as transaxial dataset using DataViewer. The bone volume fractions (BV/TV%) were measured similarly as the total bone volume against the total tissue volume within the bone drill-track injury sites. In addition, micro-CT analyses at the uninjured metaphysis region immediately after the growth plate of proximal tibia were also conducted to quantify the trabecular bone volume fractions following Noggin treatment.

3.1.5 H&E and alcian blue staining and image analysis of tissue repair at growth plate injury site and bone drill-hole injury tracks

Deparaffinized tissue sections were stained with alcian blue, haematoxylin and eosin (H&E) to visualize the types of repair tissues at the growth plate injury site. Histological analyses revealed the presence of mesenchymal infiltrate, cartilaginous, bone marrow and bony repair tissues within the growth plate injury site. The area of each repaired tissue type within the growth plate injury site was measured using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) and quantified against the total growth plate injury site area to calculate the proportion of

respective repaired tissues. These measurements were taken from four rats (n=4) from each treatment groups and then averaged and expressed as a percentage of the total growth plate injury site.

Alcian blue and H&E staining was also performed on the bone tibiae tissue sections to reveal the types of repair tissues at the bone drill-track injury site. The areas of the different repair tissues within the drill-track were similarly measured using the Image-Pro Plus and their proportions were calculated. These measurements were taken randomly from four rats (n=4) for each treatment group and then averaged and expressed as a percentage of the total drill-track injury site area.

3.1.6 Statistical analysis

Data were presented as mean ± SEM. Comparisons between Noggin and vehicle treated groups and between different time-points were conducted using two-way ANOVA. When significance levels ($P<0.05$) were achieved, a post hoc analysis of groups was done using the Tukey's test. In the figures, *, ** and *** represent $P<0.05$ $P<0.01$ and $P<0.001$, respectively. While ^ represent $P<0.05$.

RESULTS

3.2.1 Expression analysis of BMPs, BMP receptors, and downstream target genes

As a first step to examine potential roles of BMP signalling at the growth plate injury site bony repair, quantitative RT-PCR gene expression study was performed to analyse changes in expression of major osteogenic BMPs, BMP receptors (BMPRs) and BMP downstream target genes (**Fig 3.1**). Upon growth plate injury, among BMPs, BMP2 showed significant upregulation at D14 ($P<0.05$) and at D35 ($P<0.01$) in the vehicle treated groups compared to the control groups. However, the Noggin-treated group showed significantly suppressed BMP2 gene expression at D14 ($P<0.05$) and D35 ($P<0.05$) when compared to the vehicle group. Consistently, gene expression of BMP4 at the growth plate injury site were significantly upregulated ($P<0.05$) at D14 and D35 vehicle groups while D35 Noggin-treated group remained suppressed compared to the vehicle group.

BMP receptor genes BMPR1a and BMPR2 also showed a trend of changes in gene expression consistent with BMPs above. Vehicle groups showed significant upregulation of the BMPR1a gene expression at D14 ($P<0.01$) and D35 ($P<0.01$), while the Noggin-treated group at D35 was significantly suppressed when compared to the vehicle group. Similarly, BMPR2 gene expression was upregulated at D14 ($P<0.05$) and D35 ($P<0.01$) in the vehicle treated group when compared to the control group, while D35 Noggin-treated group showed significantly downregulated BMPR2 gene expression ($P<0.05$) when compared to the vehicle group.

BMP downstream target gene, Id1, did not show significant difference at D14 post-injury. However, Id1 was significantly upregulated ($P<0.05$) in the vehicle group at D35 when compared to the control. On the other hand, Dlx5 gene expression did not show a significant

difference between the treatment group and control group. These results indicate a potential increase in BMP activity within the growth plate injury site during the course of growth plate bony repair.

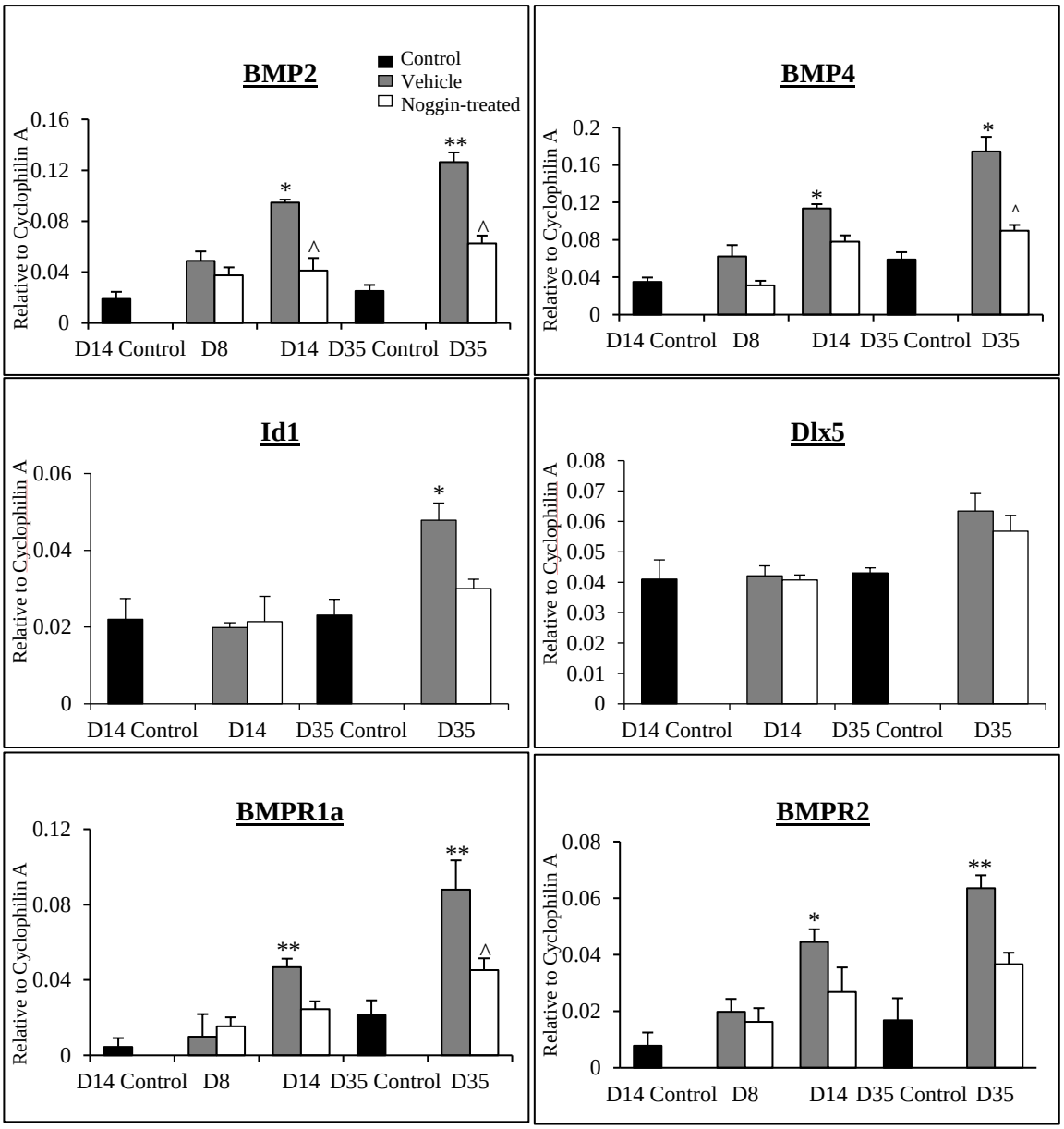

Figure 3.1. Quantitative RT-PCR analysis on the BMPs, BMP receptors and BMP downstream target genes at D8, D14 and D35. BMP2, BMP4, BMPR1a and BMPR2 were significantly up-regulated at D14 following growth plate injury. BMP2 gene expression was significantly suppressed at D14 following noggin treatment. However, BMP2, BMP4 and BMPR1a gene expression were consistently lower in the noggin treatment groups. * $P<0.05$ and ** $P<0.01$ vs normal control; and ^ $P<0.05$ vs vehicle group.

3.2.2 BMP signalling activation within the growth plate injury site

To further establish the presence and changes in BMP activity within the growth plate injury site, an immunohistochemical study was performed on the vehicle and Noggin-treated groups at D14 and D35 using the anti-phosphorylated Smad1/5/8 antibody which detects pSmad1/5/8 that can only be found following BMP signalling activation. Results showed that some stromal cells within the growth plate injury site of both vehicles and Noggin-treated rats were immunopositively stained indicating the presence of BMP activity at D14 and D35 following growth plate injury (**Fig 3.2**). Initial qualitative analyses of all immunohistochemical images showed more pSmad1/5/8-immunopositively stained cell nuclei in the vehicle rats at D14 and D35 when compared to the Noggin-treated rats. Further quantitative analyses on the percentage of pSmad1/5/8 immunopositive cells showed a significant reduction ($P<0.05$) of the immunopositive nuclei in the Noggin-treated groups compared to vehicle groups at D14 and D35 post-injury. Consistently, the quantitative analyses of the number of pSmad1/5/8 immunopositive cells per unit area of the growth plate injury site ($500\mu m^2$ area) also indicated significant reductions ($P<0.05$) in the number of immunopositive cells at the growth plate injury site for the Noggin-treated rats at D14 and D35. These results indicate the successful systemic suppression of BMP signalling activity via systemic treatment with rhNoggin which binds competitively to the BMPs.

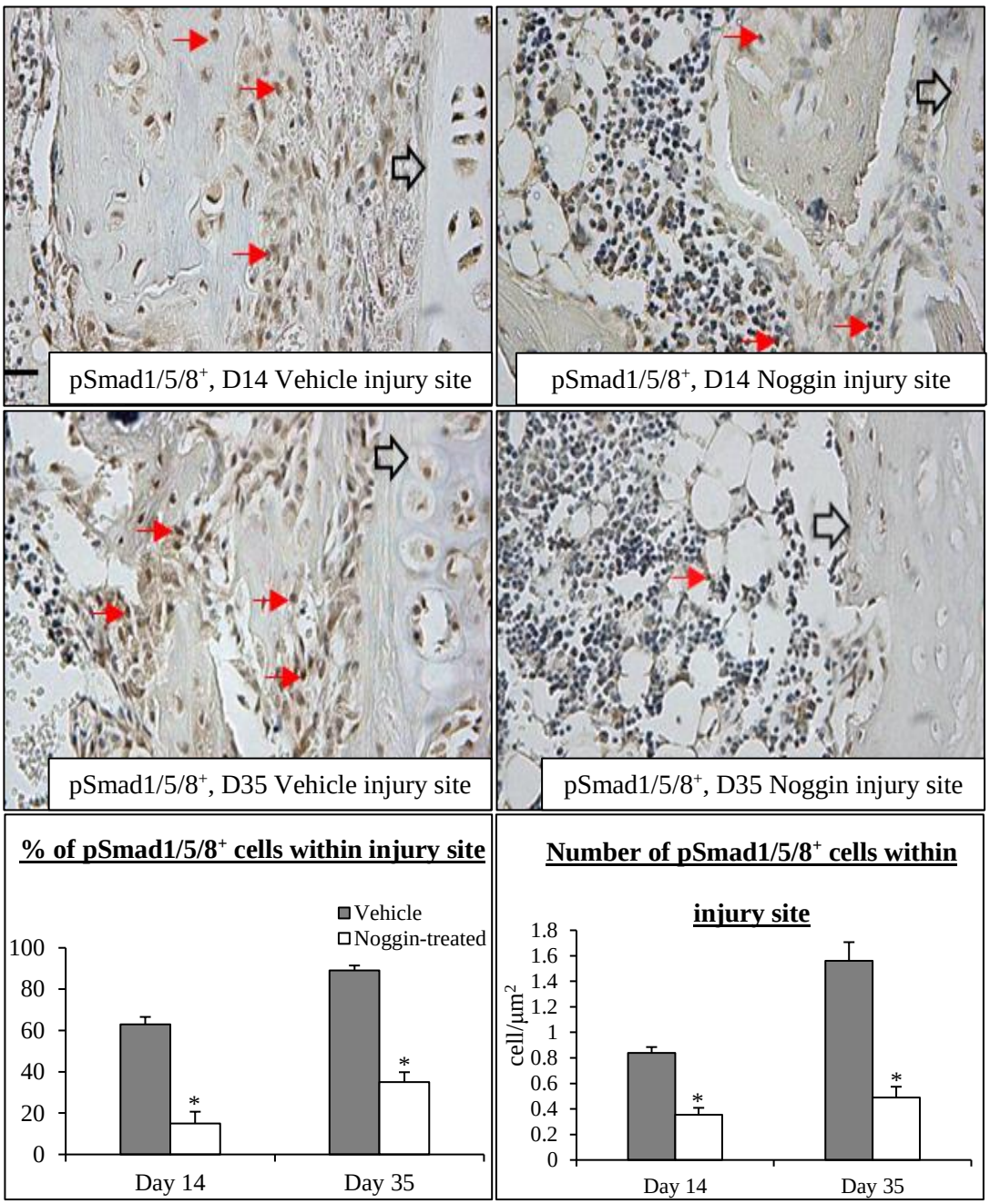

Figure 3.2. Immunohistochemical staining of pSmad1/5/8 at D14 and D35 growth plate injury sites following vehicle or noggin treatment. Immunopositive nuclei were indicated with a red arrow and remaining adjacent growth plate was indicated as an open arrow. Original scale bar is 20µm. Quantitative analyses on the percentages and numbers of pSmad1/5/8 immunopositively stained cells showed significant a lower percentage of pSmad1/5/8-stained

cells following Noggin treatments at both D14 and D35 post-growth plate injury. * $P<0.05$ vs vehicle group.

3.2.3 Effects of the Noggin treatment on bony repair at the growth plate injury site

To examine the relationship between the BMP activation and growth plate injury bony repair, micro-CT scans were performed on the vehicle and Noggin-treated groups at D14 and D35 time-points (**Fig 3.3**). Quantitative analyses of all micro-CT images revealed lesser bony repair (bone volume fractions, BV/TV%) at the growth plate injury site Noggin-treated rats when compared to the vehicle treated rats at both D14 ($P<0.05$) and D35 ($P<0.05$). These data suggest that the suppression of BMP activation could potentially lead to the lesser bone formation and bony repair at the growth plate injury site. In addition, micro-CT analyses at the uninjured metaphysis region of proximal tibia did not show a significant difference in the trabecular bone volume fractions between the vehicle and Noggin-treated groups (**Fig 3.3**).

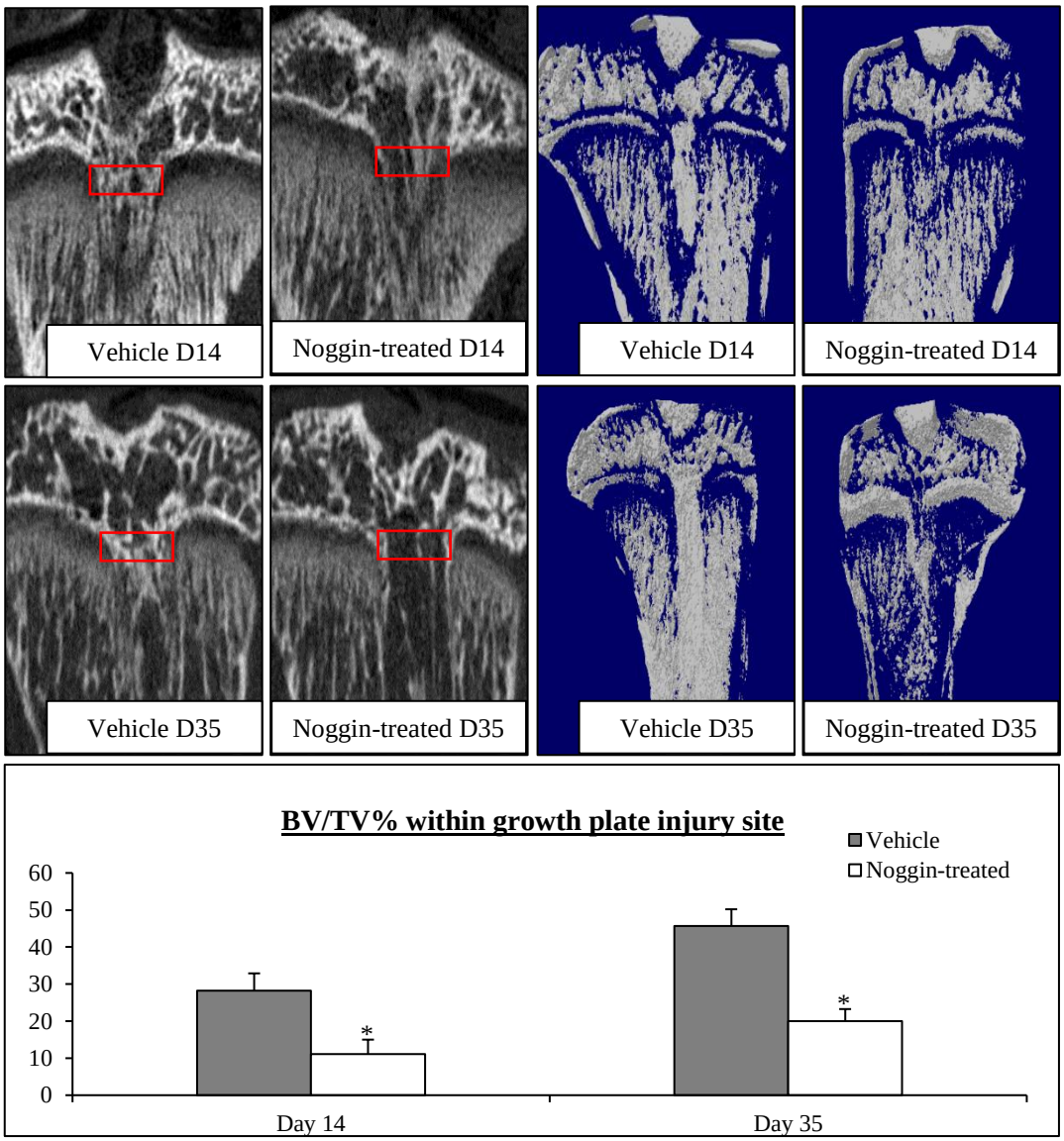

Figure 3.3. Effects of noggin treatment on the bone repair within the growth plate injury site following growth plate injury at D14 and D35. The graph indicates the percentage of bone volume over the total tissue volume within the growth plate injury site. Significant suppression of bone repair within the growth plate injury site as evident in both D14 and D35 noggin-treated rats. * $P<0.05$ vs vehicle group.

3.2.4 Effects of Noggin treatment on types of repair tissues at the growth plate injury site

Histological analyses showed that, at the growth plate injury site, four types of repair tissues were identified, namely the mesenchymal-like cells, cartilaginous tissue, bone trabeculae and bone marrow tissue (**Fig 3.4**). A significant amount of mesenchymal-like infiltrates were found within the growth plate injury site for both D8 vehicle and Noggin-treated group. However, little amount of bone trabeculae tissues was noticeable within the vehicle treated group. At D14, tissues within the growth plate injury site started to differentiate to bone trabeculae and bone marrow tissues with little remnants of mesenchymal-like infiltrates. Bone trabeculae tissues were evident in the vehicle treated group at D14 while the Noggin-treated group had a large amount of mesenchymal infiltrates within the growth plate injury site. Both growth plate injuries within the D35 vehicle and Noggin-treated rats consist high amount of bone trabeculae tissues, especially with the vehicle treated rats.

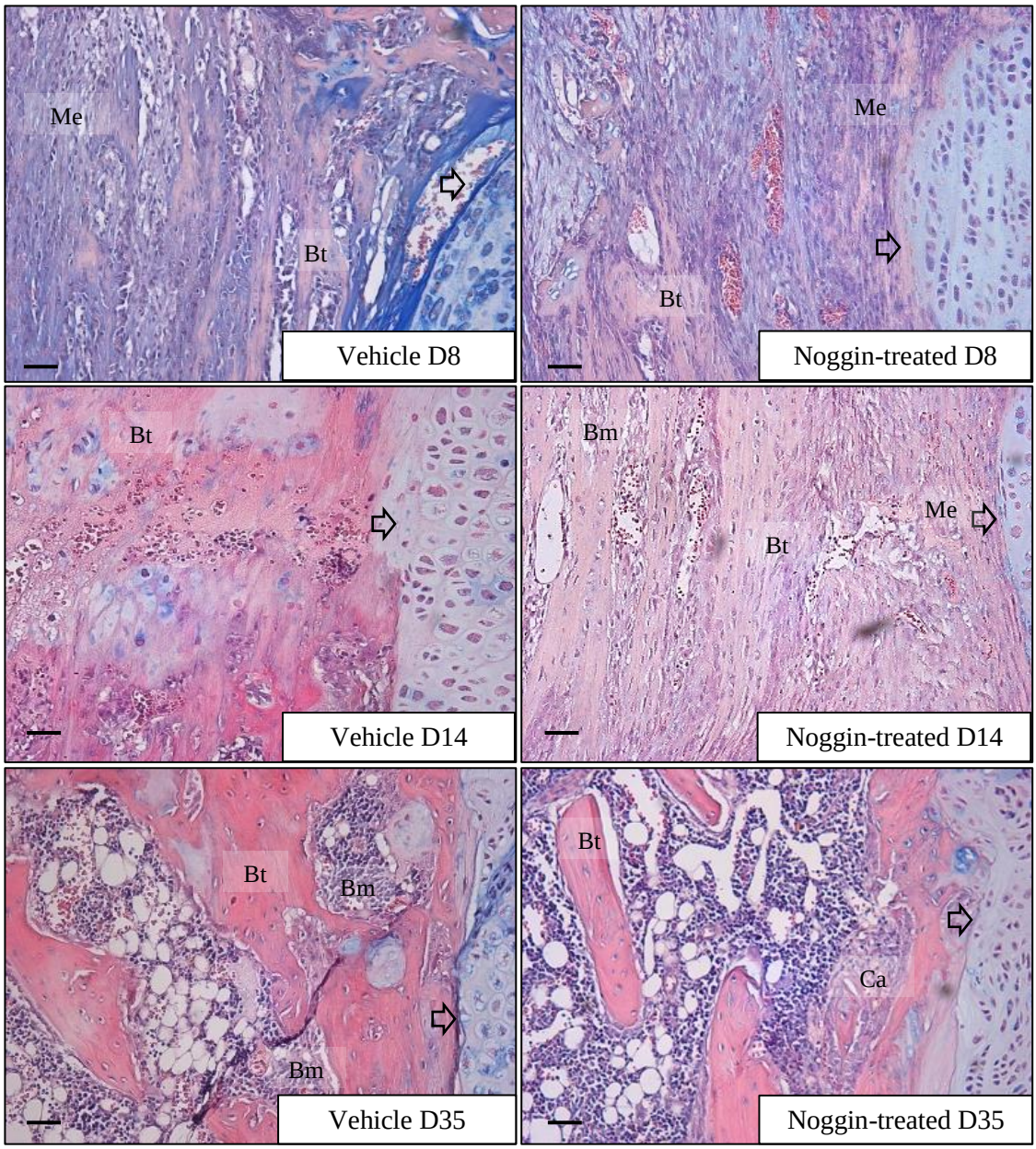

Figure 3.4. Histological images of injury repair tissues at the growth plate injury sites at D8, D14 and D35 post-injury and following noggin treatment. Injury site from D8 vehicle- and noggin-treated rats showed a larger amount of mesenchymal infiltrates (Me). Injury site at the D14 vehicle- and noggin-treated showed smaller amounts of bone trabeculae (Bt) and bone marrow (Bm). A larger amount of bone trabeculae was later shown at D35 vehicle treated rats while noggin-treated rat at D35 had a smaller amount of cartilaginous tissue found present at

the injury site. The remaining adjacent growth plate is indicated by the open arrow. Original scale bar is 50μm.

3.2.5 Effects of Noggin treatment on the percentage of different repaired tissue within the growth plate injury site

At D14 and D35, significant decreases in proportions of bone trabeculae ($P<0.05$) were observed following Noggin treatment compared to the vehicle treated group (**Fig 3.5**). While mesenchymal-like cells did not show any significant differences between the Noggin and vehicle treated groups for D8, D14 and D35 groups, the percentage of mesenchymal-like cells tended to be higher in the Noggin-treated group. The percentage of cartilaginous tissue also did not show any significant differences between the Noggin and vehicle treated groups, although there was a trend that there may have been a slight increase (non-significant) in the cartilaginous repair for the Noggin-treated group. There were no significant differences in the area of bone marrow tissue between the Noggin and vehicle treated groups at all time-points.

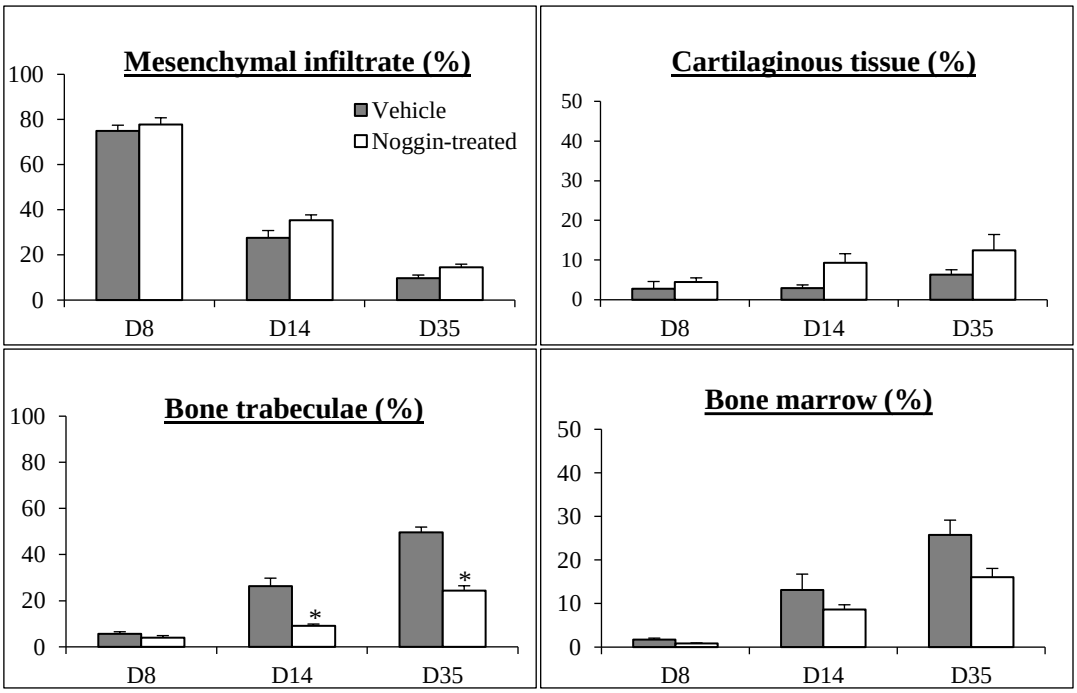

Figure 3.5. Effects of noggin treatment on the proportions of different repair tissues present at the growth plate injury sites at D14 and D35 post-injury. Quantitative histology image analysis measurements on the repair tissues present at the growth plate injury sites (over the total injury areas, measured in n = 4 samples). Mesenchymal infiltrate, cartilaginous tissue, bone trabeculae and bone marrow. * $P<0.05$ vs vehicle group.

3.2.6 Effects of Noggin treatment on the bone-formation and cartilage-related gene expression

Quantitative RT-PCR gene expression analysis for cartilage formation-related genes (Sox9 and Col2a) did not show any significant differences between the Noggin and vehicle treated rats at D8, D14 and D35 time-points (**Fig 3.6**). However, Noggin and vehicle treated rats showed significant reductions ($P<0.01$) of Col2a gene expression at D8 and D14 following growth plate injury compared to control group. For bone formation-related genes, Runx2 was significantly upregulated in all treatment groups at D8 and D14 post-injury. Osteocalcin gene expression

showed significant upregulation ($P<0.05$) in the vehicle treated group at D14. However, it was significantly suppressed ($P<0.05$) for the Noggin-treated group at D14 when compared to the vehicle group. At D35, the osteocalcin gene expression level was shown increased significantly ($P<0.05$) for the vehicle treated group compared to the normal control group. However, there were no significant differences found between the Noggin treatment and vehicle treated group.

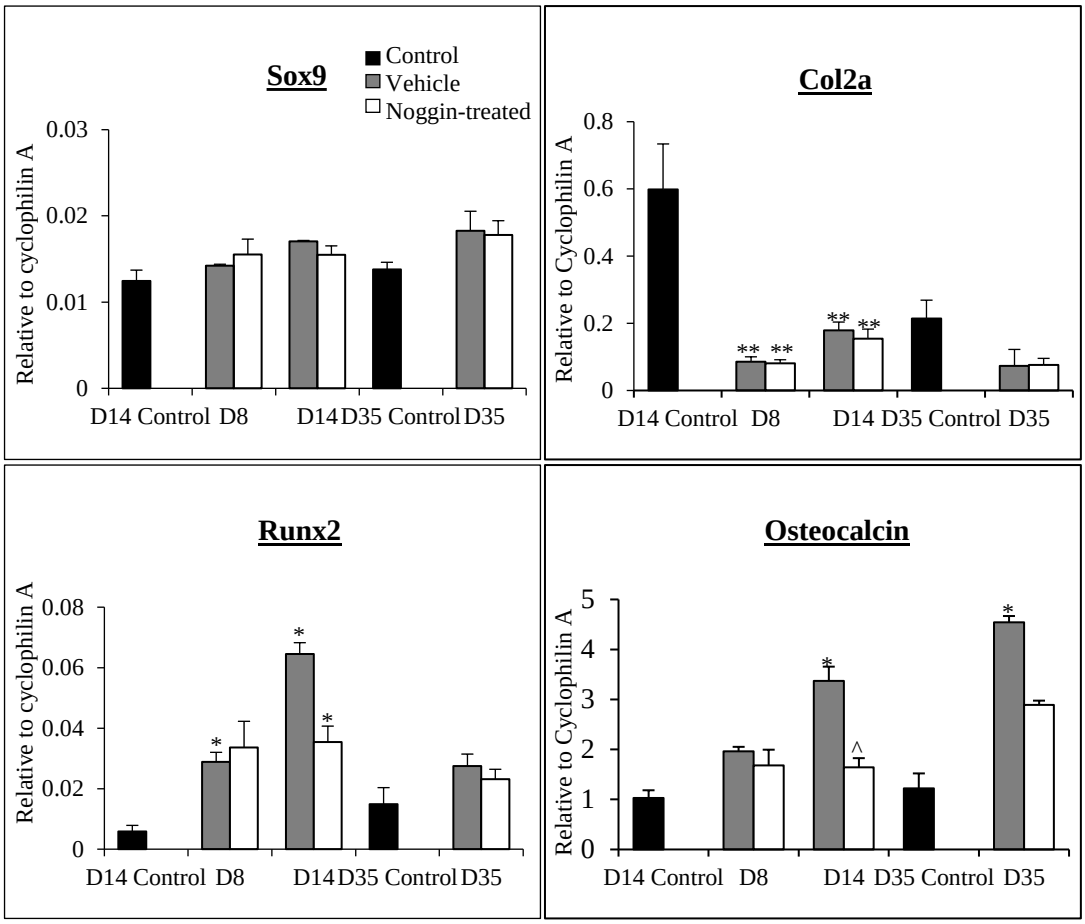

Figure 3.6. Effects of noggin treatment on mRNA expression of bone-related genes Runx2 and Osteocalcin and cartilage-related genes, Sox9 and Col2a, at the growth plate injury site on D8, D14 and D35 following injury. Runx2 and Osteocalcin gene expression levels were significantly upregulated following growth plate injury at D8 and D14. Treatment with Noggin significantly suppressed Runx2 gene expression at D14 following growth plate injury. Cyclophilin-A was used as an internal control in the quantitative RT-PCR assays. * $P<0.05$, ** $P<0.01$ vs normal control; and ^ $P<0.05$ vs vehicle group.

3.2.7 Effects of the Noggin treatment on bone repair at the bone drill-track injury site

To inflict injury on the growth plate cartilage, a 2mm burr-drill was used to penetrate from a window at the diaphysis through to the growth plate, which generated a bone drill-track injury site. At D14 and D35 following injury, it was noticed that the bone drill track contained mostly mature bone trabeculae in the vehicle-treated groups and yet there were delays in the bony repair in the drill-track injury site in the Noggin-treated rats (**Fig 3.7**). Micro-CT scans were performed to quantify the bone volume fractions within the drill tracks at D14 and D35 vehicle and Noggin-treated rats. Based on the micro-CT scanned images, Noggin-treated rats at D14 and D35 had a lesser bony repair at the drill-track injury site when compared to the D14 and D35 vehicle treated rats. Further quantification on the bone volume fractions (BV/TV%) within the drill-track of the bone tibia (n=5) showed that, consistent with the growth plate injury site, the drill-track injury site also revealed significant reductions ($P<0.05$) in the trabecular bone volume fractions at D14 and D35 Noggin-treated rats when compared to the vehicle treated rats. These suggest that the suppression of BMP signalling was able to reduce the bony repair at the bone drill-track injury site.

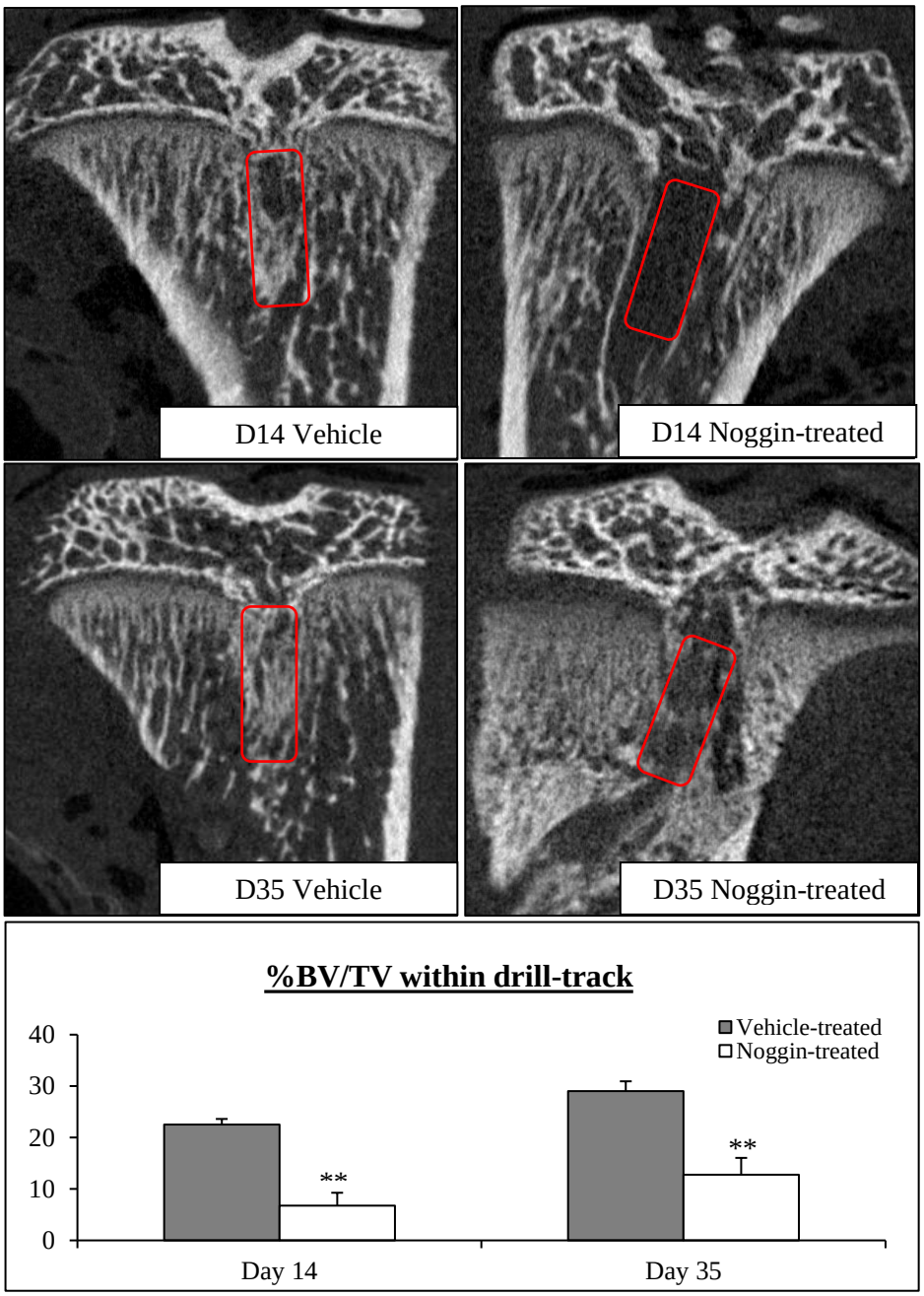

Figure 3.7. Micro-CT analyses of D14 and D35 bone drill-track injury sites showing the total bone formed in the vehicle and noggin-treatment groups. The amounts of bone formed within the noggin-treatment groups at D14 and D35 were significantly lesser compared to the vehicle treated groups. Analyses of bone volume fractions (% BV/TV) showed a significant reduction in the bone repair in the drill-track injury site following noggin treatment on D14 and D35. * $P<0.05$ vs vehicle group.

3.2.8 Effects of the Noggin treatment on the types of repair tissues at the bone drill-track injury site

Bone sections from D14 and D35 time point Noggin and vehicle treated rats were subjected to histological staining (H&E and alcian blue). From the images, it is apparent that the bone trabeculae were present in the bone drill-track injury site at D14 vehicle treated rat (**Fig 3.8**). However, bone trabeculae tissue was less apparent in the D14 Noggin-treated rat. At D14 Noggin-treated rats, the drill-track injury site consisted mainly of fat tissue and a small amount of bone trabecular tissue scattered within the drill-track injury site. At D35 time-point, bone trabeculae are also more apparent in the vehicle treated rats compared to the Noggin-treated rats. Quantification of proportion percentages of various repair tissues at injury track showed that bone trabeculae at D14 ($P<0.05$) and D35 ($P<0.05$) were significantly reduced in the Noggin-treated groups when compared to the vehicle treated groups. These again suggest that the inhibition of BMP signalling through the systemic delivery of recombinant human Noggin not only suppressed the bony repair at the growth plate injury site but also within the bone drill-track injury site.

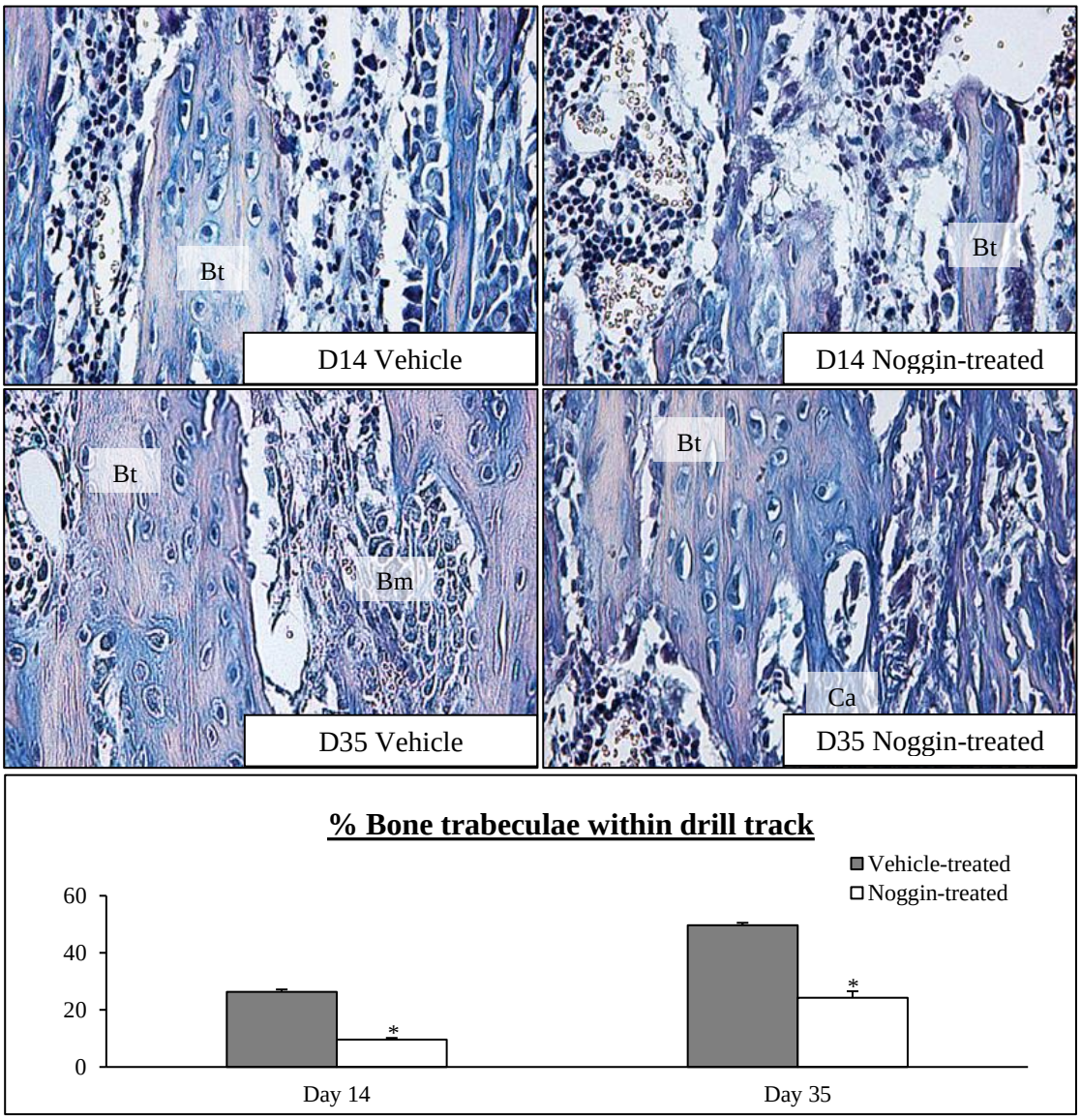

Figure 3.8. Effects of noggin treatment on the drill-track injury repair tissues at D14 and D35. At D14 and D35 vehicle treated rats, a significant amount of bone trabeculae (Bt) was formed within the drill-track injury site with the small amount of mesenchymal infiltrate (Me) at D14. At the D35 Noggin-treated group, mesenchymal infiltrate tissue was minimal and injury site was mainly comprised of fat tissues in the bone drill-track injury site. Measurements of the total bone present showed a significant reduction ($P<0.01$) of bone trabeculae formed within the drill-track injury site for both the D14 and D35 Noggin-treatment. * $P<0.05$ vs vehicle group.

DISCUSSION

While previous studies on tissue injury repair responses had revealed some cellular and molecular mechanisms during the growth plate injury repair, the reason behind the bony repair at the growth plate injury site is still unclear (Chung et al., 2011, Xian et al., 2004). To date, established roles of BMP signalling have been shown in many previous studies on bone fracture repair and bone development. However, little work was reported on the potential role of BMP signalling during the growth plate bony repair. Recent data collected from a microarray study revealed that the BMP signalling pathway was upregulated during the bony repair at the growth plate injury site (Macsai et al., 2012). This recent study investigated the potential role of BMP signalling in the growth plate bony repair, and the results suggested that BMP signalling is indeed involved in regulating bone formation at the growth plate injury site and bone repair at the bone drill-hole injury track. In the current study, BMP inhibitor, recombinant human Noggin protein was used to study the effect of suppressing BMP signalling pathway during the growth plate injury repair. Results from this study revealed that the suppression of the BMP signalling could subdue bony repair at the growth plate injury site as well as in the bone drill-hole injury track.

3.3.1 Noggin treatment downregulated BMP signalling during the osteogenic repair phase at the growth plate injury site

Through the recent microarray gene expression analyses, it is known that BMP signalling was upregulated during the osteogenic phase. To further determine the BMP signalling gene expression during the growth plate injury repair, this study examined the gene expression of BMP signalling pathway during the late fibrogenic (D8), osteogenic (D14) and maturation/ remodelling phases (D35). Gene expression of the BMP2 ($P<0.05$) and BMP4 ($P<0.05$) (two

major osteogenic BMP members) were significantly upregulated at D14 and continued to upregulate at D35 for BMP2 ($P<0.01$) and BMP4 ($P<0.05$). Interestingly, following Noggin treatment, BMPs such as BMP2 and BMP4 were significantly downregulated in both D14 and D35 post injury. Consistently, a study by Pathi *et al.* (1999) demonstrated the critical role of Noggin in the reduction of BMP2 gene expression in the perichondrium (Pathi et al., 1999). Similar results were also shown by Trzeciakiewicz *et al.* (2010) whereby the introduction of higher Noggin protein led to the suppression of BMP2 and BMP4 in the rat primary osteoblast culture (Trzeciakiewicz et al., 2010).

Apart from the BMPs, BMP receptors, BMPR1a and BMPR2 also showed a similar trend of gene expression whereby both receptor genes were significantly upregulated following growth plate injury at D14 and D35. Consistently, a study by Fischerauer *et al.* (2012) also demonstrated the upregulation of BMPR1a gene expression in the growth plate following tibia fracture (Fischerauer et al., 2013). Similar to the BMP gene expression, BMPR1a gene expression was also significantly downregulated ($P<0.05$) at D35 following Noggin treatment. Noggin is described as a competitive antagonist that inhibits BMP signalling by preventing the interactions of BMPs with the BMP receptors (Zehentner et al., 2002). These results suggest that the presence of the recombinant Noggin protein may reduce the need for BMP receptors at the growth plate injury site due to the limited BMPs available at the growth plate injury site.

BMP downstream target gene, Id1 gene expression was significantly up-regulated ($P<0.05$) at D35 for the following growth plate injury; however, Dlx5 did not reveal any significant following growth plate injury and Noggin treatment. This is consistent with the report by Ruzinova *et al.* (2003) and Valdimarsdottir *et al.* (2002) showing the upregulation of Id1

expression following activation of BMP signalling (Ruzinova and Benezra, 2003, Valdimarsdottir et al., 2002). These gene expression results of BMPs, BMP receptor and BMP downstream target genes further suggest a potentially important role of BMP signalling and addition of Noggin may be able to suppress BMP signalling during the process of growth plate injury bony repair.

The activation of the BMP signalling pathway leads to phosphorylation of the effector protein Smad1/5/8 to phosphorylated Smad1/5/8 (pSmad1/5/8) protein followed by upregulation of downstream target gene such as Runx2 (Leboy, 2006, Retting et al., 2009, Ueki and Reh, 2012). Vehicle treated rats at D14 and D35 showed more intense pSmad1/5/8 immunopositive nuclear staining when compared to the Noggin-treated rats at the growth plate injury site. Quantification of the immunopositive nuclei within the injury site also showed a significant suppression ($P<0.05$) of pSmad1/5/8 immunostaining following Noggin treatment. Consistently, a significant increase in the pSmad1/5/8 immunolocalization was shown in the cancer cells, kidney cells and bone cells following activation of the BMP signalling (Kakoi et al., 2014, Perugia et al., 2014, Rawadi et al., 2003). Our Noggin treatment results were consistent with the findings by Dummula *et al.* (2011) and Dudley *et al.* (2007) demonstrating the reduction of pSmad1/5/8 immunopositive staining following Noggin treatment (Dudley et al., 2007, Dummula et al., 2011). This immunohistochemical study clearly indicates the activation of BMP signalling following growth plate injury and treatment with the recombinant Noggin protein suppresses the BMP signalling during the growth plate injury repair.

3.3.2 Suppression of bony repair at the growth plate injury site following Noggin treatment

Apart from the suppression of BMP signalling activation following Noggin treatment, the systemic delivery of BMP antagonist Noggin also revealed successful suppression of bony repair at the growth plate injury site. Micro-CT scans and analyses clearly displayed a much less bony tissue at the growth plate injury site in both D14 and D35 Noggin-treated groups when compared to the vehicle treated groups. Quantitative analysis on the percentage of bone (BV/TV%) at the growth plate injury site demonstrated a significant reduction ($P<0.05$) of bone tissue within the growth plate injury site at D14 and D35 following Noggin treatment. This micro-CT analysis finding is consistent with a mouse study showing that the overexpression of Noggin impaired osteoblastic differentiation thus reduced the bone formation resulting in severe osteoporosis (Wu et al., 2003), and with previous findings demonstrating the skeletal overexpression of Noggin led to osteopenia and reduction in bone formation (Devlin et al., 2003).

Furthermore, to get a better understanding of the types of repaired tissue at the growth plate injury site following treatments, histochemical of the growth plate injury sites revealed a significant decrease ($P<0.05$) in the proportion of bone trabeculae at D14 and D35 following Noggin treatment when compared to the vehicle treated groups, which are consistent with the micro-CT results described above. Thus, our findings on injury site bony repair further confirm that the BMP signalling is indeed involved in the bony repair response following growth plate injury.

3.3.3 Noggin treatment downregulated bone-related gene expression at the growth plate injury site

Consistent with results from above, the gene expression analyses found significantly upregulated expression of the early osteoblast transcription factor Runx2 and bone protein osteocalcin following growth plate injury at D8 and/or D14, and noggin treatment significantly downregulated Runx2 and osteocalcin expression at D14 when compared to the vehicle control. These results suggest that the presence of Noggin not only reduces BMP signalling but also reduces Runx2 and osteocalcin gene expression. Consistently, an *in vitro* study by Phimphilai *et al.* (2006) demonstrated that blocking of BMP signalling is able to inhibit Runx2 gene expression which later leads to the disruption of osteoblast differentiation (Luppen et al., 2003). Jang *et al.* (2014) reported that the inhibition of BMP2 results in the impairment of the osteocalcin expression (Ueki and Reh, 2012). Similarly, Matsumoto *et al.* (2013) also revealed an increase in osteocalcin gene expression in a time-dependent manner following BMP2 treatment on osteogenic cells (Matsumoto et al., 2013). Our results suggest that noggin treatment suppressed bony repair at the growth plate injury site by inhibiting BMP signalling and osteogenic differentiation. Similarly, the critical role of BMP signalling has been reported during bone fracture repair and bone development by ensuring osteogenic differentiation of mesenchymal progenitor cells to mature bone (Tang et al., 2009).

On the other hand, noggin treatment did not appear to affect chondrogenesis-related genes including chondrogenic transcription factor Sox9 and cartilage protein collagen-2 (Col2a). However, Col2a gene was found to be significantly downregulated at D8 ($P<0.01$) and D14 ($P<0.01$) for both vehicle and Noggin-treated groups at the growth plate injury site when compared to the normal growth plate control group.

3.3.4 Noggin treatment suppressed bony repair at the bone drill-track injury site

Apart from the effect seen in the growth plate injury site, the suppression of BMP signalling following Noggin treatment was also shown to affect the bone drill-track injury site as well. Quantitative micro-CT and histological analyses data exhibited significant lesser bone within the bone drill track in Noggin-treated rats when compared to the vehicle treated rats. These results were consistent with studies by Wu *et al.* (2003), Rawadi *et al.* (2003) and Aspenberg *et al.* (2001) showing overexpression of noggin inhibits osteoblast differentiation, bone mineralization and bone regeneration at the bone fracture site following injury, respectively (Matsumoto et al., 2013, Rawadi et al., 2003, Wu et al., 2003). The outcome of the drill-track injury repair further suggests that the systemic inhibition of BMP signalling not only affect the bony repair at the growth plate injury site but also the repair of trabecular bone.

In summary, the current study has revealed the role of the BMP signalling pathway during the bony repair of injured growth plate cartilage and the bone drill-track in young rats. It is shown that BMP signalling pathway was activated at the growth plate injury site and that the suppression of BMP signalling through Noggin inhibitor was able to suppress bony repair at the growth plate injury site and the bone drill track. However, while it has been shown that BMP2 is critical for bone healing and is important in mediating the promoting effect of neurotrophic factor-3 in the growth plate bony repair (Rosen, 2006, Su et al., 2016, Tsuji et al., 2006), further studies will be required to pinpoint the exact BMP members and other specific BMP signalling components that are involved in regulating the bony repair and also action mechanisms of BMP signalling in growth plate repair.

CHAPTER 4

Roles of glypican-3 (GPC3) in regulating BMP signalling and bony repair at the injured growth plate in young rats

Roles of glypican-3 (GPC3) in regulating BMP signalling and bony repair at the injured growth plate in young rats

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HIGHLIGHTS

- Growth plate injury often leads to undesirable bony repair at the growth plate injury site.
- Cellular and molecular pathways leading to the bony repair remain unclear.
- GPC3 was recently shown to suppress BMP2 activity, but its potential function in growth plate repair is unclear.
- Rat growth plate injury sites were treated locally with anti-GPC3 or rhGPC3.
- GPC3 has been shown to suppress BMP signalling activation and bony repair at the growth plate injury site.

ABSTRACT

The growth plate cartilage injury is often repaired by undesirable bony tissue in young children. Although a previous study has identified Wnt signalling and the previous chapter has identified BMP signalling to be critical in mediating the bony repair at the growth plate injury site, the underlying molecular mechanism is unclear. While the previous chapter has identified that BMP signalling was important in promoting growth plate and bone drill track bony repair and a recent study has demonstrated that BMP signal activation can be suppressed by glypican-3 (GPC3), potential roles of GPC3 at the growth plate bony repair and the bone healing at the bone drill track are unknown. To address this question, firstly, mRNA expression of GPC1 and GPC3 at the growth plate injury site was investigated at different time points following growth plate injury in rat tibia. Interestingly, during the bony repair phase (days 14 and 28), while GPC1 was downregulated, GPC3 was induced. To investigate potential roles of GPC3 in regulating growth plate bony repair, rats with growth plate injury received 2.5 mg/kg rhGPC3 or 2.5 mg/kg anti-GPC3 neutralising antibody or vehicle delivered locally at the injury site via chitosan hydrogel. At the growth plate injury site, rhGPC3 treatment showed a significant reduction in phosphorylated Smad1/5/8 (pSmad1/5/8) immunopositive nuclear staining at day 15 when compared to the chitosan alone treated group. Presence of rhGPC3 also significantly reduced bone volume fraction within the growth plate injury site at both days 15 ($P<0.05$) and 35 ($P<0.05$) when compared with IgG treated groups. Consistently, bone-related genes and BMP downstream targeted gene, runx2, osteocalcin and Id1 were downregulated following rhGPC3 treatment. Analyses also showed reduction in bone volume within the bone drill-track injury site following rhGPC3 treatment. Anti-GPC3 appeared to have opposite trends in the above measurements when compared with the rhGPC3 treatment. This study suggests that locally expressed GPC3 is involved in suppressing BMP signalling and bony repair during the growth plate bony repair and bone drill track healing.

INTRODUCTION

The elongation of a growing long bone is achieved at the growth plate cartilage (Ballock and O'Keefe, 2003, Olsen et al., 2000). Due to its cartilaginous-nature, the growth plate is the weakest region of a growing long bone, and thus it is prone to injuries (Tatsuyama et al., 2000). Furthermore, with low cartilage regeneration ability, injury upon the growth plate is often repaired by the undesirable bony tissue which results in orthopaedic complications such as limb length discrepancy and bone angulation deformity (Chung et al., 2006, Chung et al., 2014, Xian et al., 2004). A previous statistical analyses shown an approximate of 20% of all childhood bone fractures involve growth plate injuries, which are often repaired by the bone tissue (Mizuta et al., 1987). Unfortunately, the cellular and molecular mechanisms leading to this undesirable bony repair remain largely unknown.

Earlier studies using a rat tibial growth plate injury model have identified four different repair response phases following growth plate injury, namely the inflammatory, fibrogenic, osteogenic and bone bridge maturation remodelling phases occurring during days 1-3, 3-7, 7-14 and 14 onwards, respectively (Xian et al., 2004). Consistently, these growth plate injury repair phases resemble the bone fracture repair phases (Bolander, 1992, Fujii et al., 1999, Tatsuyama et al., 2000). Histologically, the inflammatory phase involves rapid influx of inflammatory cells and neutrophils into the growth plate injury site which produce pro-inflammatory cytokines and growth factors (Chung et al., 2006, Kim et al., 2008, Wright et al., 2010). Studies had also observed the presence of BMP3 and BMP4 following the growth plate injury (Ngo et al., 2006), with BMP4 shown to be produced by the inflammatory cells, suggesting its potential roles in regulation of the inflammatory event and the subsequent mesenchymal cell migration and osteogenic differentiation (Ngo et al., 2006).

Following the inflammatory phase is the fibrogenic phase involving the infiltration of fibrous mesenchymal cells which were postulated to contain pre-determined chondroprogenitor and osteoprogenitor cells (Hadjidakis and Androulakis, 2006, Jaramillo et al., 1990). As stated previously, the fibrogenic phase is an important phase in regards to cell migration, differentiation and the formation of new repair tissues (Chung et al., 2011, Zhou et al., 2006). During the course of fibrogenic phase, a series of cytokines and growth factors such as the TNF-α, TGF-β, BMPs, VEGF, PDGF-BB and WNTs were reportedly expressed which could be involved in the undesirable bone tissue formation within the growth plate injury site (Chung et al., 2014, Chung et al., 2009, Macsai et al., 2012, Ngo et al., 2006, Zhou et al., 2004, Zhou et al., 2006). In particular, BMP signalling was found to be upregulated during the late inflammatory and early fibrogenic phase of growth plate injury repair (Macsai et al., 2012, Ngo et al., 2006). Similarly, BMP signalling was also localized and upregulated at the bone fracture site during the bone fracture repair (Diwan et al., 2013, Ghodadra and Singh, 2008, Nakase et al., 1994, Tsuji et al., 2006, Yu et al., 2002). During bone fracture repair, upregulation of the BMP signalling has been shown to initiate osteoblast differentiation and bone formation at the injury site. Furthermore, a previous study has shown that the lack of endogenous BMP2 in a mouse bone fracture model resulted in the blockage of the early bony repair response, suggesting that the BMP2 is critical for the bone fracture repair (Bragdon et al., 2012, Leboy, 2006, Matsumoto et al., 2013, Tsuji et al., 2006, Yamaguchi et al., 2000). Moreover, our earlier rat growth plate injury model study using a BMP inhibitor, Noggin, has demonstrated reduced bone formation at the growth plate injury site following injury which suggests the importance of BMP signalling during the growth plate bony repair.

The BMP signalling system is known to be regulated by several regulatory molecules, including noggin and heparan sulphate proteoglycans such as glypicans (Dwivedi et al., 2013a, Hartwig et al., 2005, Haupt et al., 2009, Iglesias et al., 2008, Paine-Saunders et al., 2000). One cranial osteogenesis study by Dwivedi *et al.* (2013) with administration of glypican-1 (GPC1) or glypican-3 (GPC3) recombinant protein had demonstrated a reduction in the premature bone suture fusion accompanied by reduced the BMP2 activity (Dwivedi et al., 2013a), suggesting GPCs can modulate BMP signalling and thus bone formation. Glypicans are found abundant on cell surface or extracellular matrix. The glypicans are known to regulate cell signalling (including BMP signalling) depending on the extracellular growth factors and the presence of glypicans on the cell surface (Campos-Xavier et al., 2009, Hartwig et al., 2005, Haupt et al., 2009, Iglesias et al., 2008, Litwack et al., 1998, Paine-Saunders et al., 2000, Viviano et al., 2005). The study by Jiao *et al.* (2006) also had shown that the glypicans were able to modulate BMP2 osteogenic activity through the sequestering of BMP2 at the cell surface hence disrupting the BMP signalling pathway (Jiao et al., 2007). Additionally, the loss of GPC3 function has also shown to be associated with the delay in endochondral ossification and abnormal persistence of hypertrophic chondrocytes of growth plate at embryonic day 16.5 (Viviano et al., 2005).

However, it is unknown whether GPCs are involved in regulating BMP signalling during growth plate bony repair and bone injury repair. To investigate the potential roles of the GPCs in modulating BMP signalling and bony repair at the injured growth plate and bone drill track, this study examined the GPC gene expression and effects of rhGPC3 or anti-GPC3 antibody treatment on the repair outcomes in a rat growth plate injury and tibial bone drill-hole model.

METHODS AND MATERIALS

4.1.1 Growth plate injury and treatment trial

A total of seventy male *Sprague Dawley* rats (5-weeks old) were randomly stratified into five treatment groups (n=8), namely the Chitosan gel alone-treated, normal IgG-treated, Anti-GPC3-treated, recombinant human GPC3 (rhGPC3)-treated and non-injured normal control. All treatment groups except non-injured control were subjected to experimental growth plate injury in the proximal tibia of both hind legs. The surgical injuries were performed using the drill-hole method by disrupting the central part of the proximal tibial growth plate followed by drill-hole irrigation with 0.9% saline solution under anaesthetic condition as described in Xian *et al.* (2004). Localized treatments injected into the injury sites were achieved by using the thermosensitive chitosan gel that solidifies when the chilled gel solution is exposed to body temperature, 37 °C. Chitosan gel made was a mixture of 2% (v/v) acetic acid, 10% (w/v) β-glycerol and 10% (w/v) chitosan (Ahmadi and de Bruijn, 2008). The gel was kept on ice to maintain in liquid state prior to use. All Chitosan gel alone-treated rats were injected with 0.5 ml of thermosensitive chitosan solution into the growth plate injury site and bone drill track and allowed to solidify prior to wound closure. For other drug-treated groups, drugs were contained in the injected Chitosan gel solution: normal mouse IgG (Peprotech, Victoria, Australia) at the dosage of 2.5 mg/kg, Anti-GPC3 IgG (Cell Marque, CA, USA) at the dosage of 2.5 mg/kg (Zhu et al., 2013), or recombinant human GPC3 (rhGPC3) (R&D Systems, Victoria, Australia) at the dosage of 2.5 mg/kg (Feng and Ho, 2014). This protocol was performed under the approval from the Animal Ethics Committee of the SA Pathology, South Australia.

All treated rats (n=8) were humanely sacrificed through carbon dioxide (CO_2) overdose for tissue specimen collection at day 15 (D15) and D35 post-growth plate injury. Both tibia were dissected and soft tissues were removed. All proximal left tibia was fixed in 10% formalin for 24 hours at 4°C, washed in water and then stored at 70% ethanol prior to micro-CT scanning followed by histological studies (see below). All right proximal tibia was exposed by snapping the metaphysis away from the growth plate at the border, thus exposing the growth plate and its injury site. The growth plate injury site and the remaining adjacent growth plate were collected separately using a sterile surgical blade and frozen using liquid nitrogen and stored at -80°C until being used for quantitative real-time RT-PCR analysis.

4.1.2 Micro-CT analyses of bony repair at the growth plate and bone drill-track injury sites

All fixed left tibia (n=5) were *ex vivo* scanned with a high resolution micro-CT scanner (SkyScan 1174, Kontich, Belgium) to examine the overall bony repair within the growth plate injury site following Chitosan, IgG, Anti-GPC3 or rhGPC3 treatment at D15 and D35 post-injury rats. After scanning, image reconstructions were carried out by realigning and saving images as transaxial dataset using the DataViewer (SkyScan 1076, Kontich, Belgium). An average of twenty scanned images per injury site was selected for the overall bony repair analyses using the CTan software to minimalize variations among samples. To measure the total bone volume within the growth plate injury site, the region of interest (ROI) of the entire growth plate were selected (Coleman et al., 2010). All selected ROIs were subjected to 3D analyses to quantify the total bone volume and tissue volume (BV/TV%) within the growth plate injury site. These results will reflect the degree of bony repair within the growth plate injury site following injury and treatment. Apart from measurements of the bone repair within the growth plate injury site, the bone volume fractions (BV/TV%) within the bone drill-hole

track that connects with the growth plate injury site (n=8) were also measured and quantified against the total tissue volume within the bone drill-track injury site as described above.

4.1.3 H&E and alcian blue staining and image analyses of tissue repair at growth plate injury site and bone drill-hole injury tracks

Following micro-CT scan and decalcification in 14% EDTA solution (Sigma-Aldrich, NSW, Australia), all tibia were halved longitudinally through the growth plate injury site and processed for paraffin embedding and sectioning at 4μm thick using the Microtome (Microm HM325, Charleston, SC) for histology and immunohistochemical analyses. To measure the percentage of various types of tissue repair at the growth plate injury site and bone drill-hole injury site, all paraffin sections were deparaffinized using Solvc21 solution (United Biosciences, Queensland, Australia) and stained with 1% alcian blue, haematoxylin and eosin (H&E). All stained tissue sections will reveal the types of repair tissues within the injury sites under light microscopy: mesenchymal infiltrate (fibrous tissue), cartilaginous tissue (stained blue), bone marrow tissue, and bony repair tissue (stained pink and appeared small surface). Using Image-Pro Plus (Media Cybernetics, Silver Spring, MD), the area of each type of repair tissue was measured and quantified against the total growth plate injury site area to calculate the proportion of respective repair tissue present. Measurements taken (n=8) were averaged and expressed as percentage over the total growth plate injury site for all treatment groups.

Apart from the growth plate injury site measurements, this project also quantified the different repair tissues within the bone drill-hole injury track similarly (n=8). The total drill-hole injury track area quantified was limited to 500µm^2 for each section of all treatment groups to reduce variations between drill-tracks. These measurements were averaged and expressed as percentage of total drill-track injury site area.

4.1.4 Immunohistochemical analyses

To examine the activation of BMP signalling at the growth plate injury site, immunohistochemical analyses on the phosphorylated-Smad1/5/8 (pSmad1/5/8) were performed on growth plate injury sections of all treatment groups. Firstly, deparaffinized tissue sections were rehydrated through a sequential ethanol solutions from 100% to 70% and quenched with 3% hydrogen peroxide before being subjected to antigen retrieval treatment using 0.01M citrate buffer (pH 6.0, 65°C, 90 min). All sections were then blocked with 5% normal swine serum in phosphate-buffered saline/bovine serum albumin (PBS/1%BSA, pH 7.4) and then incubated overnight with a primary rabbit anti-pSmad1/5/8 IgG antibody at 1:200 (Santa Cruz, Texas, USA) at 4°C. After washes, all sections were labelled with biotinylated swine polyclonal anti-rabbit IgG at 1:500 (Dako, NSW, Australia) and DAB Plus substrate (Dako, NSW, Australia). The primary antibody for the negative control sections was replaced with a normal rabbit IgG (PeproTech, Rocky Hill, NJ) at 1:200. All processed sections were washed and counter-stained with haematoxylin and mounted with cover slip for light microscopy and image analyses (ImageJ, NIH).

4.1.5 Real-time quantitative RT-PCR gene expression analyses of GPCs, cartilage- and bone-related genes, BMPs and BMP downstream target molecules

Initial quantitative gene expression analyses on the major GPCs (GPC1 and GPC3) were performed using the non-treated growth plate injury site tissue samples (n=8) (from Chapter one study) to determine changes of GPC expression following growth plate injury. To examine effects of Chitosan alone, normal IgG, Anti-GPC3 and rhGPC3 treatments on tissue repair and BMP signalling, gene expression analyses were performed on the cartilage- and bone-related genes, BMPs and BMP downstream target molecules at the growth plate injury sites at different time points (non-injured control, D15 and D35) (n=8). RNA samples were extracted from the normal growth plate control or injury sites using the Aurum™ Total RNA Mini Kit (BIO-RAD, NSW, Australia) according to the manufacturer's directions. All extracted RNA was treated with DNase using the Ambion DNA-free kit (Ambion, NY, USA) as per manufacturer's instructions to prevent genomic DNA contamination. Due to the limited RNA that can be obtained from the small injury site and the many genes to be analysed, RNA extracted from the growth plate injury site of the same treatment group were pooled together (2 rats per pool, 4 pools per treatment) for the RT-PCR analyses. cDNA synthesis with 1 µg total RNA was carried out using random decamers (Geneworks, SA, Australia) and Superscript-II RNase H Reverse Transcriptase (Stratagene, La Jolla, CA) according to manufacturer's instructions. Using the synthesized cDNA, the expression of GPCs, cartilage-, bone-related, BMPs and BMP downstream target genes were analysed using SYBR Green Real-Time PCR assays (in duplicate), specific primers (shown in **Table 4.1**) and an Applied Biosystems 7500 RT-PCR system (Warrington, UK). Cyclophilin-A (Cyc-A) was used as an internal control whereby all gene expression levels were expressed in relation to Cyc-A gene expression. The respective gene expression for all treatment groups were compared against the non-injured control group.

Table 4.1. Primer sequences used for real-time quantitative RT-PCR analysis

Gene	Forward Primer (5' - 3')	Reverse Primer (3' - 5')
Runx2	CAGTAAGAAGAGCCAGGCAG	GCATTCGTGGGTTGGAGAAG
Sox9	AGTACCCGCATCTGCACAAC	ACTTGTAATCGGGGTGGTCT
Col2a	CTCATCCAGGGCTCCAATGAT	TCTGTGATCGGTACTCGATGA
Osteocalcin	GCTGGCCCTGACTGCATTCTG	ATTCACCACCTTACTGCCCTCCTG
GPC1	TCCCGACTATTGCCGAAATGT	GTCAGTGATGAGCACCATGGAG
GPC3	CCCTGTGCCAGGATCAGATTT	ATGTTCAGGCGCGCTGTTAGT
Id1	CATGAAGGTCGCCAGTAGGA	AGCCGTTCATGTCGTAGAGC
BMP2	GGAAAACTTCCCGACGCTTCT	CCTGCATTTGTTCCCGAAAA
BMP4	TTATGAGGTTATGAAGCCCAC	GCTCACATCGAAAGTTTCCCAC
Dlx5	CACCACCCGTCTGAGGAATC	TGGTATGGGTTGAGCGCTTT
Cyc-A	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG

4.1.6 Statistical analyses

All data were presented as mean ± SEM. Comparisons were made between the treatment groups (GPC3 intervention study) and/or between different time-points compared to non-injured control group using the two-way ANOVA. Significance level was determined and post hoc analyses of groups were done using the Tukey's test. In the figures, *, ** and *** represent $P<0.05$ $P<0.01$ and $P<0.001$, respectively and ^, ^^, ^^^ represent $P<0.05$, $P<0.01$ and $P<0.001$, respectively and #, ## represent $P<0.05$ and $P<0.01$, respectively and @ represents $P<0.05$.

RESULTS

4.2.1 Increased GPC3 gene expression during bony repair at the growth plate injury site

Firstly, to determine the potential roles of glypicans at the growth plate injury site during the bony repair, quantitative gene expression study was performed to analyse changes in mRNA expression of major glypicans GPC1 and GPC3 (**Fig 4.1**). Following growth plate injury, GPC1 was shown to significantly downregulated at all time points (D6, D10, D14, D28) when compared to the non-injured rats. GPC3 gene expression levels, at D6 and D10 were also significantly downregulated ($P<0.001$) following growth plate injury. At D14 and D28, the gene expression levels remained significantly downregulated ($P<0.01$) when compared to the control group. However, the GPC3 gene expression was shown to increase ($P<0.01$ when compared to D6 and D10) at D14 onwards. While the reduced expression of GPC3 compared to the normal control was due to physical removal of growth plate tissue at the injury site, the increased expression of GPC3 during the bony repair phase (when compared to D6 and D10) suggests potential involvement and importance of GPC3 in regulating the growth plate bony repair.

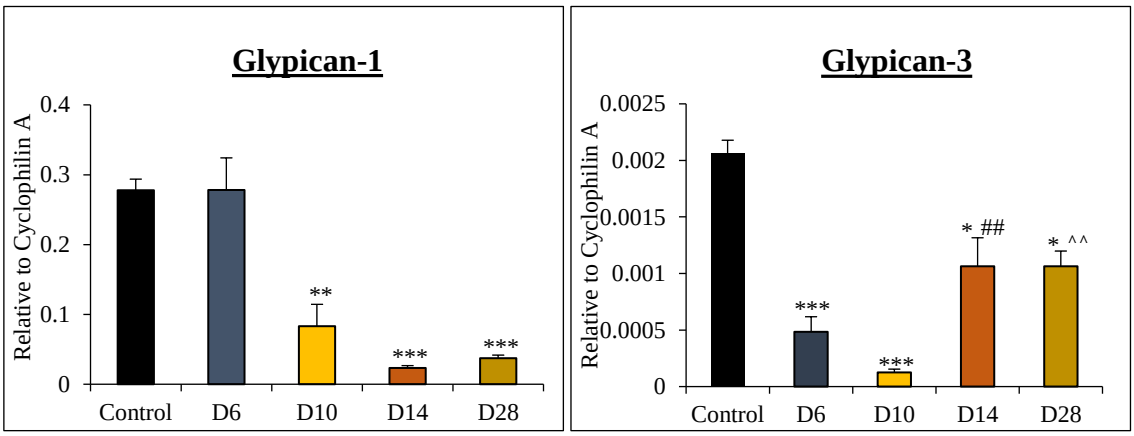

Figure 4.1. Quantitative RT-PCR detection of Glypican 1 and Glypican 3 gene expression level following growth plate injury at the remaining adjacent growth plate displayed significant changes in the GPC1 and GPC3 gene expression during the osteogenesis phase. **P<0.01 vs control, ***P<0.001 vs control, ##P<0.01 D10 vs D14 and ^^P<0.01 D10 vs D28.

4.2.2 Effects of GPC3 intervention on the bony repair within the growth plate injury site

To examine the potential role of GPC3 in the bony repair within the growth plate injury site, micro-CT analysis was performed to quantify the bony repair within the growth plate injury site by measuring the bone volume fraction (BV/TV%) at the growth plate injury site for the Chitosan alone, normal IgG, Anti-GPC3 or rhGPC3 treated rats (**Fig 4.2**). From the scanned-images, bony repair was obvious within the growth plate injury site for the Chitosan, IgG and Anti-GPC3-treated rats at D15 and D35 post-growth plate injury. However, bony repair was less obvious in the GPC3-treated rats. Quantitative analyses also revealed significantly lesser (P<0.05) bony repair at D15 and D35 post-injury for the GPC3-treated rats when compared to the IgG and Anti-GPC3-treated rats, respectively. These results suggest that the presence of GPC3 reduces bony repair at the growth plate injury site.

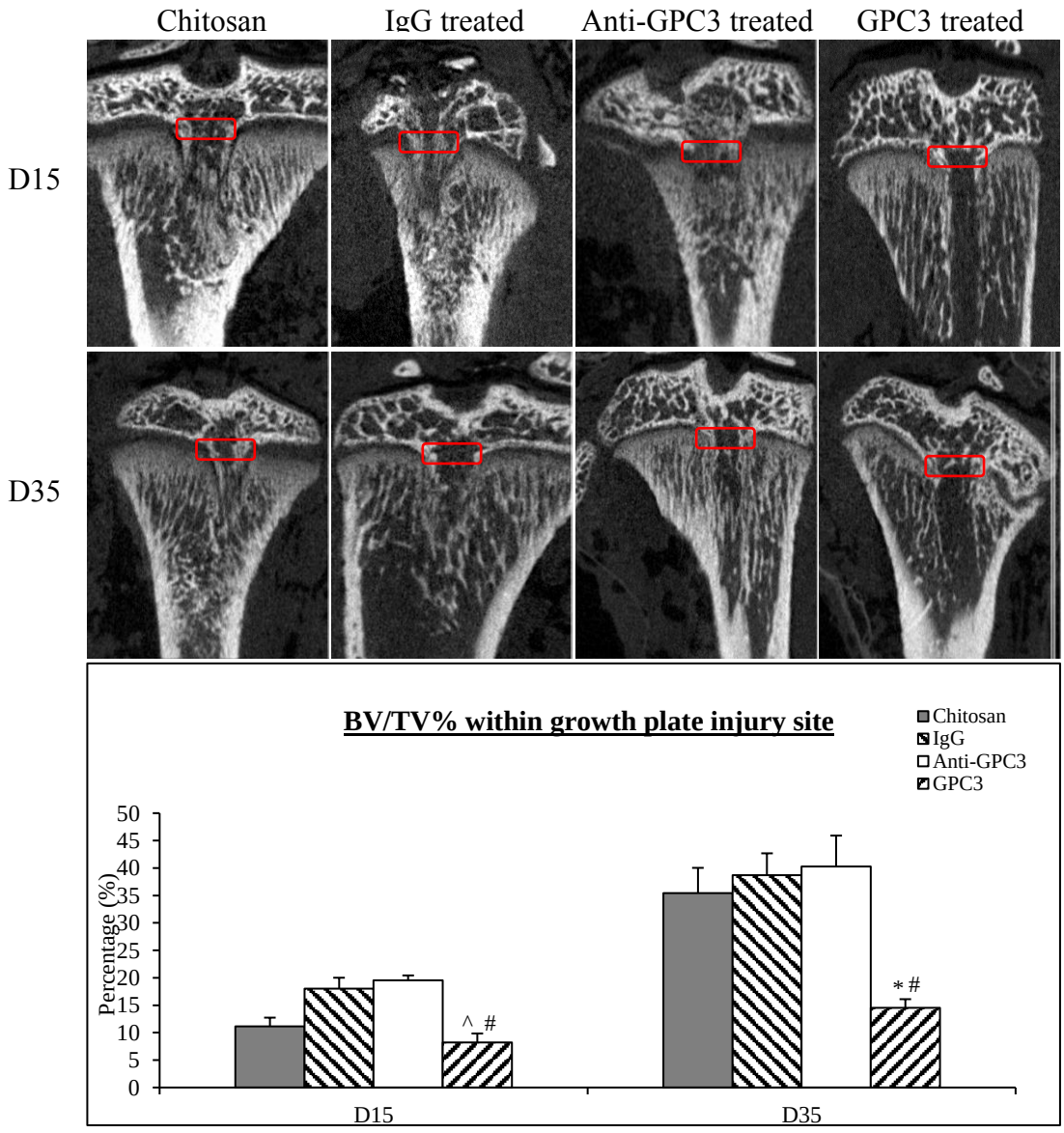

Figure 4.2. The effect of GPC3 treatment on the bony repair following growth plate injury at D15 and D15. Qualitatively, the Chitosan, IgG, Anti-GPC3 and GPC3 treated groups showed presence of bony repair at the growth plate injury site at D15 and D35 following growth plate injury. Quantitative analysis (BV/TV%) showed significant suppression of bone formation at GPC3 D15 following growth plate injury compared to the IgG and Anti-GPC3 groups. While D35 showed significant suppression of bone formation in the GPC3 treated group compared to IgG and Anti-GPC3 treated groups. *P<0.05 chitosan vs GPC3, ^P<0.05 Anti-GPC3 vs GPC3, #P<0.05 IgG vs GPC3.

4.2.3 Effects of GPC3 intervention on the repaired tissues at the growth plate injury site

Histological H&E and alcian blue staining revealed changes in the repair tissues at the growth plate injury sites following Chitosan, normal IgG, Anti-GPC3 or GPC3 treatment (**Fig 4.3**). Images from the Chitosan alone treatment at D15 showed a smaller proportion of bone trabeculae and but a larger proportion of mesenchymal infiltrate; however, D35 rats had a higher proportion of bone trabeculae but a reduced amount in mesenchymal infiltrate at the injury site. Normal IgG-treated rats at D15 showed approximately equal proportions of bone trabeculae and mesenchymal infiltrate tissues; however, D35 had a higher proportion of bone trabeculae at the injury site compared to mesenchymal infiltrate. At D15, anti-GPC3-treated rats had a significant deposition of bone trabeculae tissue and a small proportion of mesenchymal infiltrate at the injury site. By D35, the repair tissue of the growth plate injury site in the anti-GPC3 treatment rats was mainly bone trabeculae with a small proportion of bone marrow tissue. In contrast, GPC3-treated rats at D15 showed a higher proportion of mesenchymal infiltrate and little bone trabeculae and cartilaginous tissue present. At D35, the proportion of bone trabeculae was significantly smaller when compared to other treatment groups. However, there was a significant amount of fat tissue formed in the growth plate injury site following GPC3 treatment.

Quantitative analyses on repair tissues showed a significant increase ($P<0.05$) in the percentage of mesenchymal infiltrate in the rhGPC3-treated group against the Anti-GPC3-treated group at D15 (**Fig 4.3**). However, there were no significant changes found when compared to the Chitosan alone and normal IgG-treated groups. The percentages of mesenchymal infiltrate were shown to be reduced for all groups at D35 compared to D15 treatment groups. However, there were no significant differences observed among D35 treatment groups. The percentages of cartilaginous tissue within the growth plate injury site also did not reveal any significant

differences among all treatment groups at D15 and D35. While the percentages of bone trabeculae did not show any significant differences at D15 among treatment groups, at D35 there was a significant reduction ($P<0.05$) of bone trabeculae in rhGPC3-treated rats compared to the anti-GPC3 treatment. No significant changes were observed for the bone marrow tissue proportion at D15 and D35 treatment groups although there seemed to be a slight decrease (non-significant) in the GPC3 treatment group when compared to the Anti-GPC3 treatment at D35.

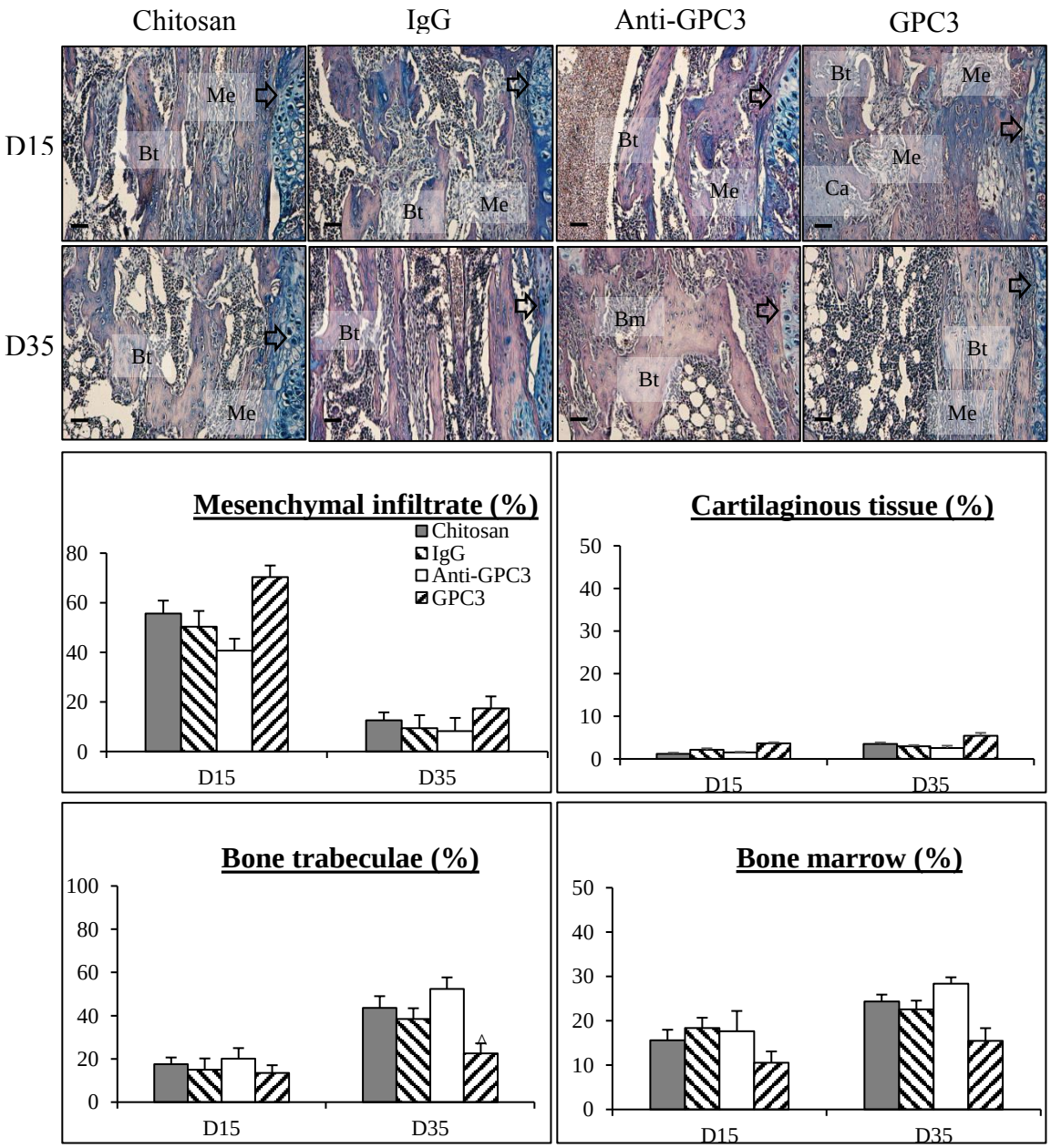

Figure 4.3. Effects of GPC3 treatment on the repair tissue at the growth plate injury site at D15 and D35 post-growth plate injury. Injury site from D15 Chitosan, IgG and GPC3 rats showed larger amount of mesenchymal infiltrate (Me) at the growth plate injury site compared to the Anti-GPC3 rats. While GPC3 treated rats showed negligible amount of cartilaginous tissues (Ca). Anti-GPC3 rats had larger amount of bone trabeculae (Bt) and bone marrow (Bm) at D35 compared to the other treatment groups. Quantitative histology image analysis measurements of area present (over total growth plate injury site areas, measured n=4 samples) of each type of repair tissue. The proportion of bone trabeculae formation were significantly suppressed in

the GPC3 treated rats compared to the Anti-GPC3 rats at D35. The remaining adjacent growth plates were indicated by closed arrows. Original scale bar is 50μm. ^P<0.05 Anti-GPC3 vs GPC3.

4.2.4 Effects of GPC3 intervention on expression of cartilage- and bone formation- related genes

To examine the effect of GPC3 intervention on the cartilage-related gene expression (such as Sox9 and Col2a) (**Fig 4.4**), quantitative real-time RT-qPCR was performed on all treatment groups. Firstly, the early chondrogenic gene, Sox9, did not show any significant changes between the rhGPC3-treated group and non-injured control group at D15. However, the Sox9 gene expression level was significantly upregulated (P<0.05) in the rhGPC3-treated group when compared to the Anti-GPC3 group. In contrast, Sox9 gene expression was downregulated in Chitosan alone-treated, normal IgG treated and Anti-GPC3 treated groups following growth plate injury at D15 compared to the control group. At D35 post injury, there were no significant changes recorded in the Sox9 gene expression level for all treatment groups. Col2a gene expression level for the rhGPC3-treated group at D15 showed no significant differences when compared to the non-injured control group. However, rhGPC3-treated group showed a significant increase (P<0.001) in Col2a gene expression when compared to the Anti-GPC3-treated group at D15. While the levels of Col2a gene expression for the Chitosan, IgG and Anti-GPC3-treated groups were shown to be reduced following growth plate injury, GPC3-treated group is the only group that showed no significant reduction in Col2a gene expression at D15. At D35, there were no significant differences among all treatment groups for Col2a expression level.

Levels of mRNA expression of Runx2 (an early osteogenic transcription factor and early marker commonly used to mark osteoblastic differentiation) and osteocalcin (a protein component of bone extracellular matrix synthesized by mature osteoblasts) (**Fig 4.4**) were examined at D15 and D35 to reveal the potential changes in the bone formation-related genes during the growth plate injury repair and following GPC3 intervention. Runx2 did not show any significant changes in the rhGPC3-treated group when compared to the Control, Chitosan, IgG and Anti-GPC3-treated groups at D15. However, Runx2 expression was significantly lower ($P<0.05$) in the rhGPC3-treated group when compared to the Anti-GPC3 group at D35. As for the osteocalcin gene expression, there were no significant changes at D15 post-growth plate injury among all treated groups. However, at D35, osteocalcin expression in the rhGPC3 -treated rats was significantly lower ($P<0.01$) when compared to the Anti-GPC3 treated rats. These results suggest that the growth plate injury site is favouring towards bony repair and osteoblast differentiation at D35 rather than cartilaginous tissue repair. However, treatment with rhGPC3 saw a significant reduction ($P<0.05$) on the bony repair response at the growth plate injury site at both D15 and D35 treatment groups, which was also supported by trends of changes in trabecular bone proportion as examined by histological analyses and in Runx2/osteocalcin gene expression. These data suggest that the GPC3 intervention could suppress bony repair following growth plate injury.

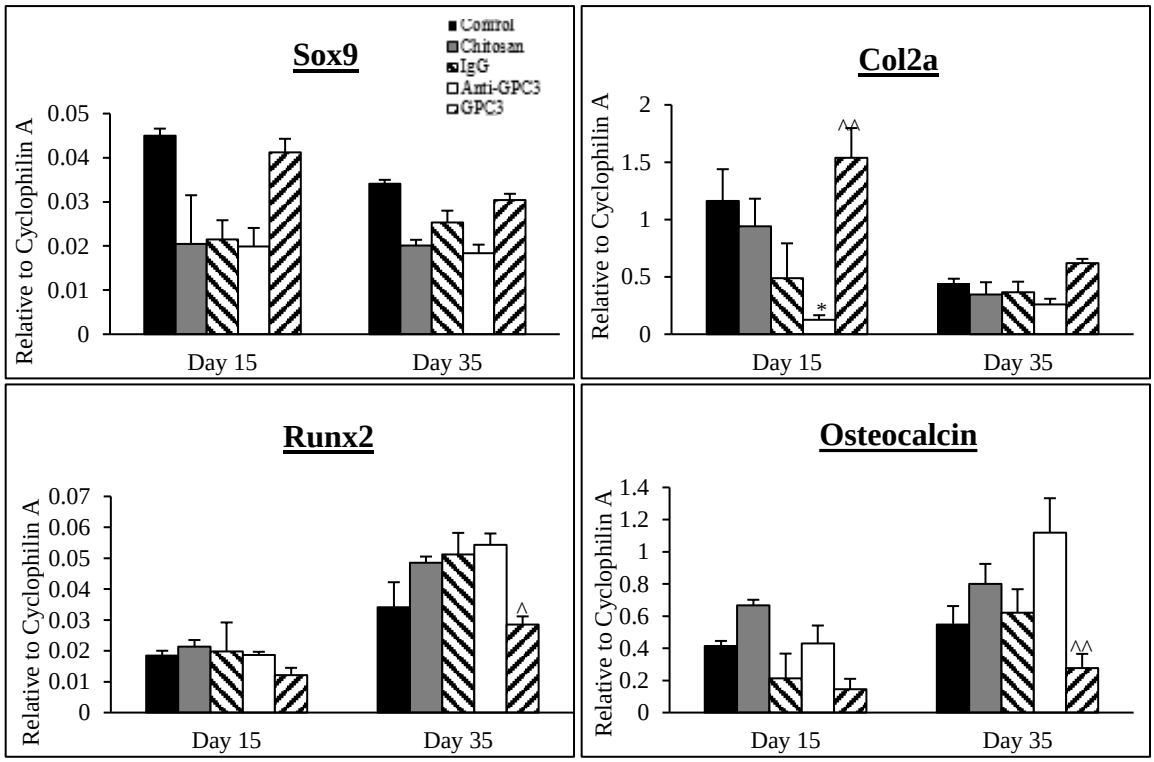

Figure 4.4. The effect of GPC3 treatment on the cartilage-related (Sox9, Col2a) and bone-related (Runx2, Osteocalcin) following growth plate injury at D15 and D35. There were no significant differences in Sox9 and Col2a between the GPC3 treated groups and the non-injured control group at D15 and D35. However, Col2a gene expression level is significantly higher in the GPC3 group compared to the Anti-GPC3 treated group. ^P<0.05 Anti-GPC3 vs GPC3, ^^P<0.01 Anti-GPC3 vs GPC3 and *P<0.05 Control vs Anti-GPC3.

4.2.5 Effects of GPC3 intervention on bone repair at the bone drill-track injury site

Furthermore, to examine treatment effects on the bony repair at the drill-hole injury track, micro-CT scans were also performed on the whole tibial bones comparing the Chitosan, normal IgG, Anti-GPC3 and GPC3 treatment groups (**Fig 4.5**). Preliminary examination revealed distinctive formation of bony tissue within the drill-hole tracks for Chitosan, IgG and Anti-GPC3 groups at D15. However, there were no obvious bone tissue found within the injury tracks of the rhGPC3-treated rats when compared to the other treatment groups. At D35, the total bone formation became more obvious in Chitosan, IgG and Anti-GPC3 group. While rhGPC3-treated rats at D35 began to show bone formation within the injury track, it was less obvious compared to the other treatment groups at D35. Quantitative analyses indicated significant suppression ($P<0.05$) of bone formation (BV/TV%) within the drill-track injury site for the rhGPC3-treated group compared to the Chitosan and Anti-GPC3 groups at D15. In addition, similar trend was shown at D35 whereby the total percentage of bone formation was significantly downregulated ($P<0.001$) compared to the Anti-GPC3 rats. These data suggest that the suppression of GPC3 signalling also affect the bony repair in the drill-track injury site.

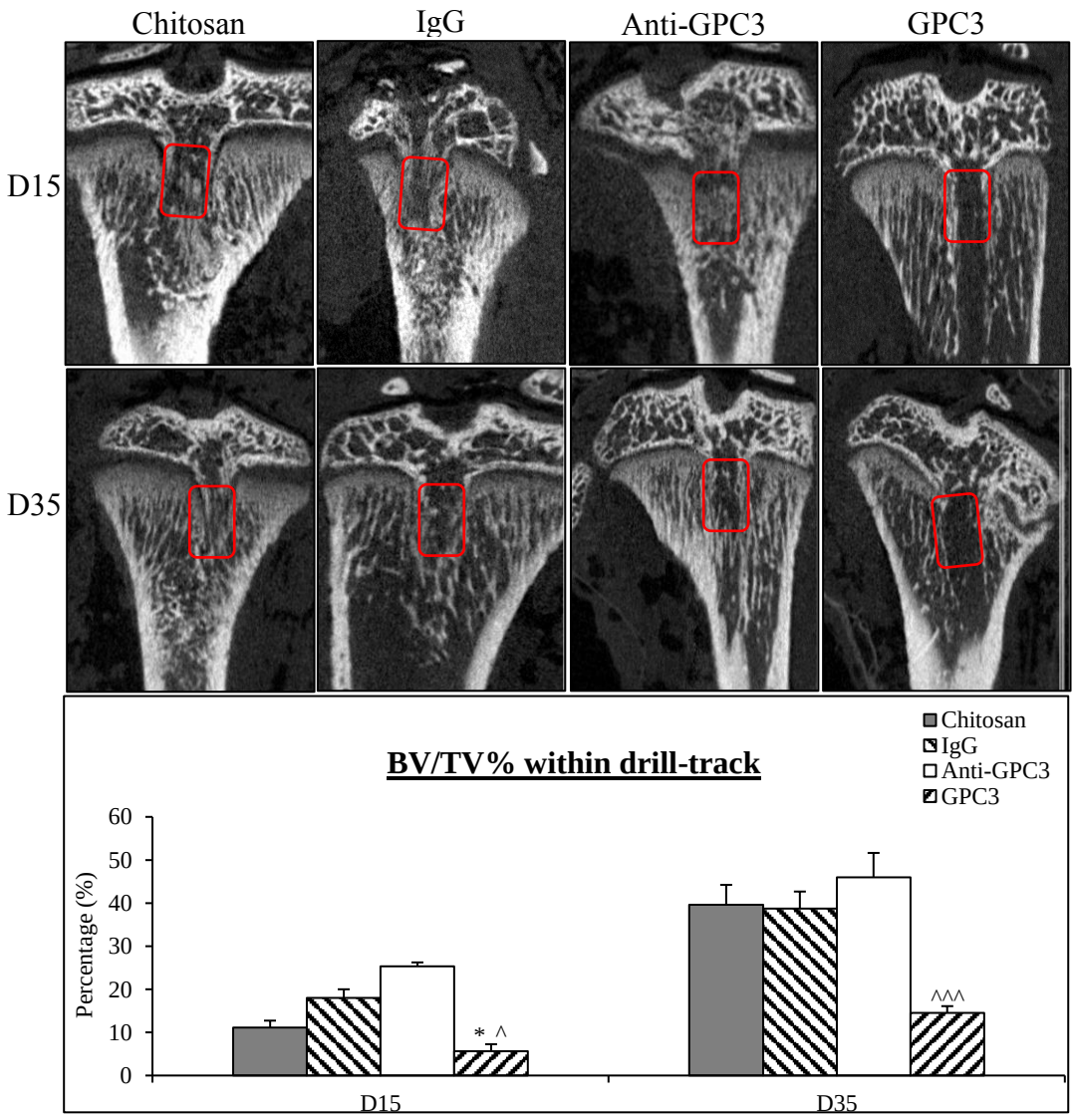

Figure 4.5. The effect of GPC3 treatment on the bone formation within the drill-track injury site at D15 and D35 post-growth plate injury. At D15 post-growth plate injury, Chitosan, IgG and Anti-GPC3 groups showed presence of bone formation within the drill-track injury site. Quantitative micro-CT analysis on the percentage of BV/TV showed significant reduction in the bone volume for GPC3 treated group compared to the Anti-GPC3 treated group at D15. ^P<0.05 Anti-GPC3 vs GPC3, ^^^P<0.001 Anti-GPC3 vs GPC3 and *P<0.05 chitosan vs GPC3.

4.2.6 Effects of GPC3 intervention on the bone trabecular content within the bone drill-track injury site

To determine the treatment effects on proportions of repair tissues at the drill-track injury sites, histological staining was performed on tibial sections collected from all Chitosan, IgG, Anti-GPC3 and rhGPC3 treatment groups (**Fig 4.6**). Bone trabeculae were found to be present in all treatment groups at D15. However, trabeculae were more obvious in the Chitosan, IgG and Anti-GPC3 treated groups when compared to rhGPC3-treated group at D15. While mesenchymal infiltrate was evident in the drill-track injury sites for all treatment groups, it was more obvious in the rhGPC3-treated group at D15, suggesting a delay of its conversion to bony tissue. At D35, the bone trabecular tissue was distinctively present in the Chitosan, IgG and Anti-GPC3 groups. However, little was found in the rhGPC3 group. Interestingly, the rhGPC3-treated rats had higher amount of fat tissues within the bone drill-track injury site which was also observed at the growth plate injury site. Quantitative analyses on the percentages of bone trabeculae within the drill-track injury sites showed a significant lower ($P<0.05$) amount of bone trabeculae in the rhGPC3-treated group compared to the IgG and Anti-GPC3 groups at D15. At D35, the percentage of bone trabeculae was also significantly lower ($P<0.05$) in the rhGPC3-treated group compared to the Chitosan and Anti-GPC3 groups. The lower bone trabecular tissue content following rhGPC3 treatment at the bone drill-track was also consistent with above results of a lower BV/TV% at the growth plate injury site following rhGPC3 treatment.

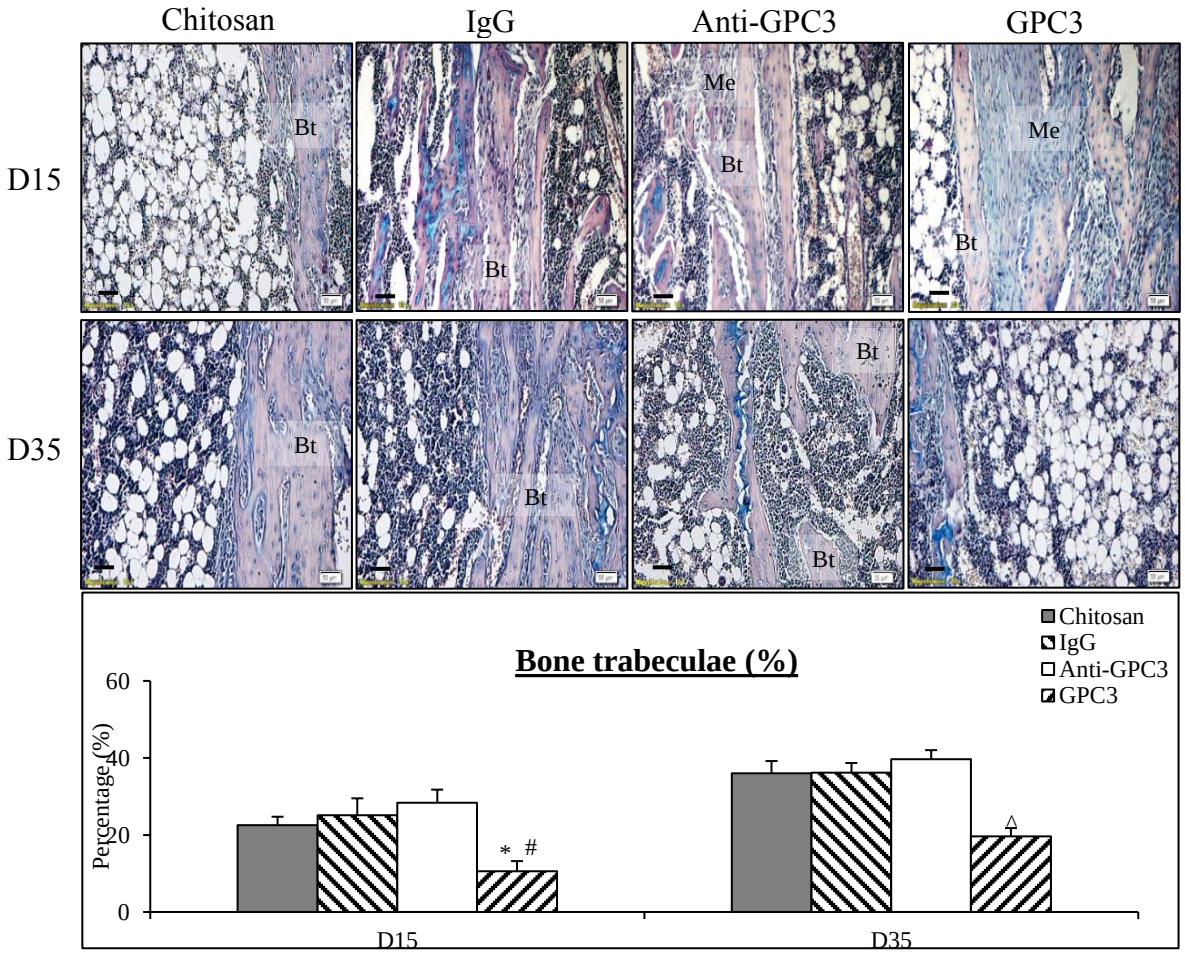

Figure 4.6. Effects of Chitosan, IgG, Anti-GPC3 and GPC3 treatments on the types of repaired tissue formed at the drill-track injury site at D15 and D35 post-growth plate injury. GPC3 treated group showed distinctive presence of mesenchymal infiltrate. At D35, the area repaired by bone trabeculae is more significant in the Chitosan and Anti-GPC3 treated groups. Quantitative histology image analysis measurement on the bone trabeculae tissue showed significant reduction in the GPC3 treated group compared at both D15 and D35 post-growth plate injury. Original scale bar is 50µm. *P<0.05 chitosan vs GPC3, ^P<0.05 Anti-GPC3 vs GPC3 and #P<0.05 IgG vs GPC3.

4.2.7 Effect of GPC3 intervention on the BMP signalling activation and expression of BMPs and BMP downstream target genes at the growth plate injury site

Immunohistochemical staining was performed using the anti-phosphorylated pSmad1/5/8 antibody to detect BMP signalling activation. Results showed obvious immunolocalization of pSmad1/5/8 within the stromal cells at D15 and D35 in the Chitosan-treated group (**Fig 4.7**). Normal IgG-treated group also showed significant immunolocalization of pSmad1/5/8 nuclear staining in the growth plate injury site at both D15 and D35, although the intensity of immunopositive staining appeared lesser at D15 than D35 at the growth plate injury site. pSmad1/5/8 immunopositively stained cell nuclei were also found in both D15 and D35 growth plate injury sites for the GPC3-treated group. However, the intensity of the immunopositive nuclear staining appeared lower at D15 compared to D35 group. In fact, the rhGPC3-treated group showed the least pSmad1/5/8 immunopositive staining compared to the other treatments at D15. Further quantitative analyses on total immunopositively stained cell nuclei against the total tissue area within the growth plate injury site showed significantly lower numbers of immunopositive cells in the growth plate injury site for the GPC3 treated group when compared to the Chitosan and Anti-GPC3 treated groups ($P<0.05$ and $P<0.01$), respectively.

To further examine treatment effects on activation of BMP signalling pathway, quantitative real-time RT-PCR gene expression analyses were performed on the BMPs and BMP target genes, namely BMP2, BMP4 and Id1, Dlx5, respectively. Gene expression trend for BMP2 and BMP4 were highly similar for all treatment groups. BMP2 and BMP4 were significantly suppressed ($P<0.05$) in the rhGPC3-treated rats at D15 and D35 when compared to the Anti-GPC3 rats. The BMP downstream target genes, Id1 and Dlx5 was significantly upregulated ($P<0.05$) in the Anti-GPC3 treated group when compared to the control and Chitosan-treated rats following growth plate injury at D35 post-injury. However, Id1 and Dlx5 genes were

significantly downregulated ($P<0.05$) in the rhGPC3 treated group compared to the Anti-GPC3 group at D35. Similarly, Id1 was also significantly downregulated ($P<0.05$) in the rhGPC3 treated rats compared to the Anti-GPC3 group at D15.

While the above pSmad1/5/8 immunohistochemical analyses and gene expression analyses of BMPs and BMP downstream target genes further suggest upregulation of BMP signalling at the growth plate injury site following growth plate injury, particularly following anti-GPC3 treatment. However, the treatment with rhGPC3 during the growth plate injury repair was able to downregulate the BMP signalling activity at the growth plate injury site.

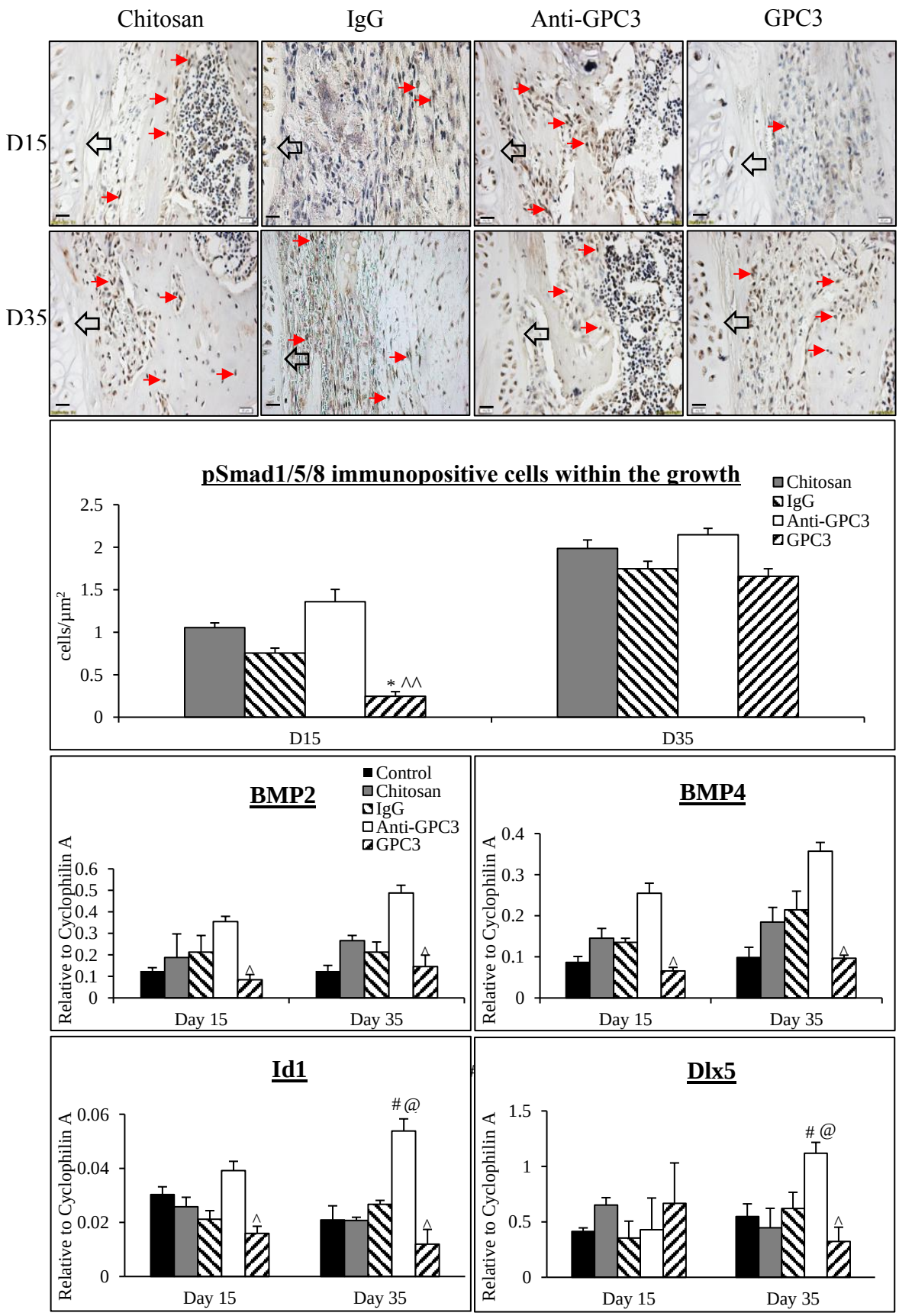
Chitosan
IgG
Anti-GPC3
GPC3
D15
D35
pSmad1/5/8 immunopositive cells within the growth
cells/µm²
2.5
2
1.5
1
0.5
0
* ^^
D15
D35
Chitosan
IgG
Anti-GPC3
GPC3
BMP2
Control
Chitosan
IgG
Anti-GPC3
GPC3
Relative to Cyclophilin A
0.6
0.5
0.4
0.3
0.2
0.1
0
Day 15
Day 35
BMP4
0.4
0.3
0.2
0.1
0
Id1
0.06
0.04
0.02
#@
Dlx5
1.5
1
0.5
@

Figure 4.7. Detection of pSmad1/5/8 immunopositive cells nuclei within the growth plate injury site at D15 and D35 post-growth plate injury for Chitosan, IgG, Anti-GPC3 and GPC3 treated groups. Using the total number of immunopositively stained cell nuclei within the area of 500μm^2, quantitative analysis was performed and graphed showing significant (P<0.05) and (P<0.01) reduction of pSmad1/5/8 immunopositive cells when compared to the Chitosan and Anti-GPC3, respectively. Quantitative gene expression analysis on the BMP signalling genes were performed on the growth plate injury site showing significant changes in the BMP component and downstream targeted genes at both D15 and D35 post-growth plate injury. Scale bar is 20μm. ^P<0.05 Anti-GPC3 vs GPC3, ^^P<0.01 Anti-GPC3 vs GPC3, @ P<0.05 chitosan vs Anti-GPC3, #P<0.05 Control vs Anti-GPC3.

DISCUSSION

Injury upon the growth plate often leads to the formation of undesirable bony repair at the injury site resulting in orthopaedic problems such as limb length discrepancy and bone angulation deformity. Even though tissue repair responses at the growth plate injury site have been studied extensively, the cellular and molecular mechanisms are still unclear. Following a recent microarray study on the growth plate injury, BMP signalling was found to be one of the four major signalling pathways upregulated during the growth plate injury repair (Macsai et al., 2012), and our recent study (Chapter 3) has confirmed the important role of BMP signalling in the bony repair of the injured growth plate. Until lately, while many BMP signalling studies were conducted on the bone fracture, little work has been reported on the roles of BMP signalling pathway and its regulation during the growth plate bony repair. While it has been previously identified that BMP signalling can be regulated by several antagonists such as Noggin, Chordin, and Gremlin (Rosen, 2006), a recent paper by Dwivedi *et al.* (2013) has revealed that the glypicans, particularly glypican-3 (GPC3), has the potential to downregulate BMP signalling activity during the cranial suture formation (Dwivedi et al., 2013a). Since the potential role of GPCs in regulating BMP signalling and growth plate bony repair or bone healing is not clear, this current study firstly examined gene expression of GPC1 and GPC3 during the bony repair of injured growth plate and found a substantial increase in the gene expression of GPC3. Then, this study administered a GPC3 neutralising antibody or recombinant human (rh) GPC3 protein at the injury site locally in a well-established rat growth plate and bone drill-hole injury model and found that GPC3 is involved in suppressing BMP signalling and bony repair during growth plate bony repair and bone drill track healing.

4.3.1 Suppressed bony repair response at the growth plate injury site following rhGPC3 intervention

Previously, genetic and biochemical analyses have demonstrated the potential role of GPC3 in regulating several bone-related signalling pathways such as Wnts (Song et al., 2005, Tsuda et al., 1999), BMPs (Belenkaya et al., 2004, Dwivedi et al., 2013a), Hedgehogs (Hh) (Capurro et al., 2014, Gutierrez and Brandan, 2010) and Fibroblast growth factors (FGFs) (Gutierrez and Brandan, 2010). Although glypicans are known to regulate BMP signalling, the mechanistic link between glypicans and bony repair response at the growth plate injury site is still not known. The results of this study demonstrated the potential role of glypicans in regulating bony repair response at the growth plate injury site as well at the bone trabeculae within the bone drill track. At the growth plate injury site, bone trabecular formation was suppressed following rhGPC3 intervention. Interestingly, apart from the reduction of bone trabeculae within the growth plate injury site, the mesenchymal infiltrate tissue was shown to be increased following rhGPC3 intervention, suggesting a potential delay in bony repair from progenitor cells among these mesenchymal infiltrated cells. While further cellular and mechanistic investigations are needed on the role of GPC3 in regulating bony repair, as of current, it is only understood that the GPC3 expression increases as cells developmentally mature from resting to proliferating chondrocytes (Grisaru and Rosenblum, 2001). Consistently, the reduction of bone formation following rhGPC3 intervention in our study is consistent with a genetic study using GPC3 knockout mice which showed a marked reduction in calcified trabecular bone formation while hypertrophic chondrocytes showed an abnormal persistence during the embryonic development at day 16.5 (Viviano et al., 2005).

Apart from the changes in the bone structure, this study also showed significant downregulation of the bone-related gene expression (Runx2 and osteocalcin) during the growth plate injury

repair following rhGPC3 intervention. Based on previous studies with this rat growth plate injury model, growth plate injury often resulted in an increase of bone-related genes such as Runx2 and osteocalcin at the injury site (Chung et al., 2011, Chung et al., 2014, Chung et al., 2009). Similarly, this study also showed increased levels of bone-related genes in the chitosan, IgG and Anti-GPC3 treated rats. However, these genes were significantly down-regulated following rhGPC3 treatment. Consistently, the Simpson-Golabi-Behmel syndrome (SGBS) due to the mutation of GPC3 in human has been known to exhibit skeletal defects such as fusion of the vertebra and ribs (Lindsay et al., 1997). Indicating that the absence of GPC3 can potentially increase bone-forming activity such as osteogenesis leading to cranial suture fusion as shown by Dwivedi *et al.* (2013). This is consistent with our study showing a clear reduction in Runx2 gene expression following the rhGPC3 intervention.

On the other hand, glypicans were also known for their role in regulating BMP signalling during the limb chondrogenesis (Gomes et al., 2002). BMP signalling, specifically BMP2 is known to stimulate chondrocyte hypertrophy (Horiki et al., 2004). Indicating that the glypicans have the potential to regulate chondrogenesis and chondrocyte hypertrophy through BMP signalling. Sox9 is a gene known to be expressed by the proliferating chondrocytes in the growth plate cartilage or cartilage tissue during bony repair which is involved in stimulating chondrocyte proliferation as well as delaying chondrocyte hypertrophy (Akiyama et al., 2002). Thus, upregulation of the Sox9 gene expression seen following rhGPC3 intervention could explain the potential delay in cartilage hypertrophy as found in this study. However, further investigation is still required to investigate the role of GPC3 in the cartilaginous formation and catabolism at the growth plate injury site or during bony repair.

4.3.2 Anti-GPC3 intervention enhances bony repair at the growth plate injury site

On the contrary, the anti-GPC3 intervention significantly increased the bony repair response at the growth plate injury site when compared to other treatment groups. As mentioned previously, rhGPC3 is fundamental for the regulation of osteogenesis through BMP signalling regulation (Dwivedi et al., 2013a, Horiki et al., 2004). Studies have also demonstrated that the release of GPC3 by the human primary suture mesenchymal cells into the media results in the inhibition of the BMP2, BMP4 and BMP7 gene expression with plays a pivotal role in bone osteogenesis (Dwivedi et al., 2013a, Dwivedi et al., 2013b, Grisaru and Rosenblum, 2001, Hartwig et al., 2005, Midorikawa et al., 2003). In fact, the inhibition of GPC3 resulted in the potentiation of the BMP2 responsiveness in human suture mesenchymal cells (Dwivedi et al., 2013a). Consistently, our study also showed a higher percentage of bone formation and bone trabecular repair at the growth plate injury site following the the anti-GPC3 intervention. Thus from our in vivo and these previous in vitro studies, it is proposed that the inhibition of GPC3 could result in the increase of BMP2-mediated osteogenesis, hence the increase in bony repair at the growth plate injury site. However, further investigations are still needed to establish this potential link.

4.3.3 Changes in bony repair response in bone drill-hole injury site

In addition to the effects on growth plate injury repair, we discovered that the rhGPC3 intervention also affected bone formation in the bone drill-hole injury site. Similar to the growth plate injury site, the drill-hole injury site also showed a significant reduction of bone formation following rhGPC3 intervention. A previous study using GPC3 knockout mice has shown that the absence of GPC3 resulted in a decreased osteoclast differentiation but more importantly, a delay in endochondral ossification (Viviano et al., 2005). As mentioned

previously, the increase of GPC1 and GPC3 gene expression can inhibit the BMP2 signalling and BMP2-mediated mineralization, therefore reduces the bone osteogenesis (Dwivedi et al., 2013a). On the contrary, the loss-of-function mutations to the GPC3 gene showed increased skeletal growth which leads to a disease known as the Simpson-Golabi-Behmel syndrome (DeBaun et al., 2001).

4.3.4 Effects of GPC3/anti-GPC3 intervention on the BMP signalling pathway activation at the growth plate injury site

As mentioned previously, GPC3 was found to be expressed in cranial sutures and appears to regulate BMP2-mediated osteogenesis (Cohen, 2000, Wilkie, 1997). Furthermore, the inhibition of GPC3 has resulted in an increase in the BMP2 responses by the human mesenchymal cells, indicating that the downregulation of glypican expression could be a potential factor for the bony suture fusion specifically craniosynostosis (Dwivedi et al., 2013a). As the GPC3 is able to affect BMP2 responsiveness, we proposed that it is possible that the localized rhGPC3 delivery suppresses BMP2 signalling pathway involved in regulating bony repair at the growth plate injury site.

Consistent with our Chapter 3 and previous microarray analyses, this study also demonstrated activation of the BMP signalling at the growth plate injury site during the bony repair response in all treatments except for the rhGPC3 treated groups (Macsai et al., 2012). pSmad1/5/8 immunostainings were significantly higher in all treatment groups specifically in the anti-GPC3 treated groups. In contrast, the rhGPC3 treatment significantly reduces pSmad1/5/8 immunostainings. Suggesting that the glypicans are involved in regulating the BMP signalling at the growth plate injury site during the bony repair response. Consistently, previous studies

had also proposed that the GPC3 can regulate BMP signalling pathway specifically the BMP2 and BMP4-mediated embryogenesis and osteogenesis (Balemans and Van Hul, 2002, Dwivedi et al., 2013a, Paine-Saunders et al., 2000). BMP2 is essential for osteoblast differentiation and bone formation. Study have shown that the inhibition of BMP2 expression prevented both mineralize nodule formation in vitro and bone formation in vivo whilst blocking Runx2 and Osterix (transcriptional determinants of terminal osteogenic differentiation) (Bais et al., 2009). Additionally, it was also demonstrated that the in vivo BMP2 is a central morphogenetic regulator of postnatal osteoprogenitor differentiation (Bais et al., 2009). From the Dwivedi *et al.* (2013) study, it was understood that the BMP2-mediated osteogenesis can be inhibited by GPC1 and GPC3 while the inhibition of the GPC1 and GPC3 potentiates BMP2 responsiveness. Additionally, the study also demonstrated that the presence of GPC3 can also inhibit BMP2-mediated osteogenesis (Dwivedi et al., 2013a). Based on our Chapter 3 results, it was evident that the BMP signalling plays a major role in regulating bony repair at the growth plate injury site as well as in the bone drill track. In fact, BMP signalling plays a vital role in regulating osteoblast differentiation, chondrocyte hypertrophy and bone fracture repair (Bragdon et al., 2012, Sheu et al., 2014, Tsuji et al., 2006).

On the contrary, rats receiving the anti-GPC3 showed opposing results to the rhGPC3 treated rats. Anti-GPC3 treatment showed increasing bony repair response structurally and molecularly. The presence of anti-GPC3 heightens the bone-related gene expression, bone formation and BMP signalling following growth plate injury. These results proposed that the absence of GPC3 can potentiate BMP signalling causing the increase in bone formation. Demonstrating the important role of the GPC3 in regulating the bony repair at the growth plate injury site as well as bone drill track.

In summary, the results of this study demonstrated that the GPC3 plays an indispensable role in regulating BMP signalling during the bony repair at the growth plate injury site as well as in the drill track injury site. GPC3 intervention inhibits BMP signalling activation at the growth plate injury site which resulted in the decrease of the undesirable bony repair at the growth plate injury site and drill track injury site. However, further mechanistic studies are still required to get a better understanding of the potential roles and action mechanisms of glypicans in modulating bony repair following growth plate and bone injury.

Chapter 5

General Conclusion and Discussion

THESIS DISCUSSION

The growth plate cartilage is situated at both ends of a long bone. It is solely responsible for the long bone elongation during childhood and adolescence through a process known as the endochondral ossification (Ballock and O'Keefe, 2003, Brighton, 1987). Unfortunately, being cartilaginous-nature the growth plate is the weakest region of a growing long bone, making it highly prone to injury (Tatsuyama et al., 2000, Williams et al., 2001). Statistical studies revealed that approximate 15-30% of all childhood skeletal injuries are growth plate-related, while 30% those injuries can result in the undesirable bony repair within the growth plate injury site (Mizuta et al., 1987), causing long-term orthopaedic problems such as bone angulation deformity and limb length discrepancy (Basener et al., 2009, Coleman et al., 2010). At present, only surgical-based corrective treatments are available which are highly invasive, not always effective, and often riddled with ongoing follow-up procedures due to growing long bones and pin-site infections (Kim et al., 2008). Regrettably, there are no known biological treatments or preventative therapies to prevent the undesirable bony repair from occurring. To enable developments of biological treatments, it is required to elucidate the key cellular and molecular mechanisms involved in the regulation of bony repair response at the growth plate injury site and degeneration of the remaining adjacent growth plate following growth plate injury. Therefore, using a rat drill-hole growth plate and tibial bone injury model, this PhD project has carried out *in vivo* and *in vitro* studies to unveil the underlying mechanism particularly the role of BMP signalling pathway behind bony repair/bone bridge formation at the growth plate injury site and bone formation degeneration of the adjacent growth plate following an injury, and to investigate the potential role of the glypican (heparan sulfate proteoglycan, GPC3) in regulating the BMP signalling in the bony repair response at the growth plate injury site as well as the bone drill track.

5.1 Growth plate injury resulted in degeneration of the remaining adjacent growth plate involving BMP signalling

As mentioned previously, the chondrocytes within the growth plate/articular cartilage responds to cytokines, growth factors and physical stimuli (Guerne et al., 1995), and any disruptions upon those factors can lead to cartilage matrix degradation. Degeneration of the articular cartilage is generally associated with osteoarthritis (OA) which is the most common for arthritis. It is a degenerative joint disease that involves thinning or destruction of the smooth cartilage that surrounds the ends of bones (Westendorf et al., 2004). In fact, the homeostasis of joints greatly relies on the balance of the cartilage matrix degradation and repair by the chondrocytes as in the growth plate (Wang et al., 2014). In a recent rat drill-hole growth plate injury model, it was discovered that the growth plate injury site is not the only region that underwent undesirable bony repair (Macsai et al., 2011). In fact, the growth plate injury also led to cartilage degeneration and the bone bridge formation in the remaining adjacent growth plate in 60% of injured rats at day 60 post-injury while others exhibited progressive changes in the cellular and molecular composition that strongly suggest degeneration in the remaining adjacent growth plate (Macsai et al., 2011).

In our current study, injury upon the growth plate in rats revealed an increase in the hypertrophic zone/proliferative zone ratio at the remaining adjacent growth plate over a time-course of 35 days. This increase in the hypertrophic/proliferative zone ratio is consistent with the real-time RT-PCR analyses which revealed significant upregulation of the Col10 and Runx2 gene expression levels, while Sox9 and Col2a were downregulated at day 14 post-injury. Furthermore, immunohistochemical staining analyses and western blot protein quantification have also demonstrated increased BMP signalling activation at the remaining adjacent growth plate compared to the non-injured growth plate with increased pSmad1/5/8

localization and protein expression in the remaining adjacent growth plate tissue, respectively. Evidently, BMP7, BMPR1a and Dlx5 were also found to be upregulated in the remaining adjacent growth plate at day 14 and 35 post-injury. Interestingly, a previous gene expression analysis at the growth plate injury site also revealed upregulation of the BMP signalling following growth plate injury (Ngo et al., 2006). BMP signalling is known to be involved in all phases of chondrogenesis such as mesenchymal condensation, chondrocyte proliferation, extracellular matrix deposition and terminal differentiation (Kon et al., 2001). In addition, the BMP signalling also plays an important role in regulating chondrocyte hypertrophy and cartilage matrix degradation (Nakase et al., 2003). Although extensive studies had been performed to reveal the cellular and molecular mechanisms leading to the bony repair response at the growth plate injury site, the association between this bone formation/cartilage degeneration and BMP signalling pathway has not been investigated. Our current study has demonstrated that growth plate injury activates BMP signalling pathway at the remaining adjacent growth plate which may be involved in mediating the bony degeneration of the adjacent uninjured cartilage.

To further determine the role of BMP signalling in regulating the cartilage degeneration at the remaining adjacent growth plate following injury, this study also examined the effect of inhibiting BMP signalling pathway through the systemic delivery of Noggin protein. Interestingly, the hypertrophic/proliferative zone height ratio was significantly reduced compared to the vehicle control, and gene expression analyses found increased Col2a and reduced Col10 gene expression levels, suggesting a potential rescue of chondrogenesis and attenuation of chondrocyte hypertrophy in the remaining adjacent growth plate after BMP signalling blockade following injury. These results illustrated that the BMP signalling pathway is involved in regulating the bone bridge formation/cartilage degeneration at the remaining

adjacent growth plate following growth plate injury. Despite these findings, the mechanisms underlying the activation of the BMP signalling at the remaining adjacent growth plate requires further examinations.

5.2 Involvement of BMP signalling pathway in the bony repair at growth plate injury site

Previous microarray analyses in a rat drill-hole growth plate injury model had discovered upregulation of several bone-related genes and signalling pathways such as BMP and Wnt signalling over a time-course of 60 days following injury (Macsai et al., 2012). Evidently, real-time RT-PCR quantification at the growth plate injury site found upregulation of BMPs following growth plate injury (Ngo et al., 2006). Therefore, in order to confirm the role of the BMP signalling (a key osteogenic signalling pathway known critical for bone healing) being also important for the bony repair at the growth plate injury site, the BMP signalling pathway was further investigated in the current study

In our current study, levels of BMP2 and BMP4 mRNA expression were found to be upregulated following growth plate injury at day 14 and 35. In addition, BMP receptor genes, BMPR1a and BMPR2 were also upregulated at days 14 and 35 following growth plate injury, and the BMP downstream target gene, Id1, was significantly upregulated at day 35, suggesting upregulation of BMP signalling pathway during bone formation at the growth plate injury site. However, in rats treated with BMP signalling inhibitor noggin, Id1 expression was not upregulated. Furthermore, immunohistochemical analyses also showed a significant reduction in the percentage of pSmad1/5/8 immunopositive cells at the growth plate injury site following noggin treatment at day 14 and 35. These findings suggest that the BMP signalling pathway

was upregulated at the growth plate injury site following growth plate injury and systemic noggin treatment blocking BMP pathway was able to suppress the upregulation of the BMP signalling during the bony repair at the growth plate injury site.

The current study further demonstrated that inhibition of BMP signalling pathway through noggin treatment reduces bone formation and bony repair within the growth plate injury site. Micro-CT analyses revealed a significant reduction in the bone formed at the growth plate injury site in the noggin-treated group over the time-course compared to vehicle treated group suggesting that the suppression of the BMP signalling reduces bony repair at the growth plate injury site. Consistently, a recent mouse calvarial study discovered that the suppression of noggin not only enhances *in vitro* osteogenesis but can also accelerate *in vivo* bone formation (Wan et al., 2007). Additionally, in the current study, osteogenic gene expression was investigated to examine the role of BMP signalling in regulating osteogenesis at the growth plate injury site following injury. Expression of Runx2, which is an osteoblast-related transcription factor plays an important role in early stage of osteoblast differentiation (Liu et al., 2007), was found to be upregulated following growth plate injury but was suppressed following noggin treatment over the time-course. Evidently, osteocalcin, which is specifically expressed in osteoblasts (Viereck et al., 2002), was also found to be upregulated during the growth plate injury; however, its expression was reduced following noggin treatment over the time-course. This gene expression data suggests that the injury upon the growth plate increases osteoblast differentiation at the growth plate injury site and the inhibition of BMP signalling following injury reduces osteogenesis-related gene expression and bone formation over the time-course. Moreover, the current study suggests that the upregulation of the BMP signalling induces bony repair at the growth plate injury site and blocking BMP signalling causes a

significant reduction in the osteogenesis-related gene expression and bony repair at the growth plate injury site over the time-course.

5.3 Glypican 3 suppresses BMP signalling and thus regulates bony repair at the growth plate injury site

While earlier chapters of this PhD thesis have demonstrated important roles of BMP signalling in promoting bony repair at the injured growth plate, and some previous studies have illustrated functions of glypicans (GPCs, heparan sulfate proteoglycan), particularly GPC1 and GPC3, in regulating BMP-mediated osteogenesis of human suture mesenchymal cells (Dwivedi et al., 2013b), this PhD project carried out further studies to investigate potential roles of GPCs in regulating BMP signalling and bony repair at injured growth plate. A previous study has observed decreased expression levels of glypican-1 (GPC1) and glypican-3 (GPC3) in the cranial sutures and were decreased during premature suture fusion in children and their potential role in the regulation of the BMP signalling pathway, BMP-mediated osteogenesis and bone formation (Dwivedi et al., 2013a). Similarly, the downregulation of glypican expression was found in suture tissue which has been suggested to lead to enhanced BMP signalling and the bony suture fusion that characterises craniosynostosis (Dwivedi et al., 2013b). As of interest, the loss of function mutations in the GPC3 gene has been shown to lead to an X-linked disorder in humans known as Simpson–Golabi–Behmel Syndrome (SGBS) which affects both pre- and postnatal growth development particularly, childhood cancers, congenital defect and skeletal abnormalities (Viviano et al., 2005). During embryonic development, E16.5, the GPC3-deficient animal showed a marked delay in the trabecular bone deposition and abnormal hypertrophic chondrocytes in the developing tibia (Paine-Saunders et al., 2000), suggesting that the GPC3 is involved in the regulation of endochondral ossification. However, although glypicans had been shown to affect BMP-mediated osteogenesis in the

premature cranial fusion, their potential roles and action mechanisms have not been previously investigated at the growth plate injury repair setting.

In the current study, injury upon the growth plate was found to alter the GPC1 and GPC3 gene expression levels at the growth plate injury site with GPC1 being downregulated but GPC3 upregulated. In order to examine the potential role of GPC3 in the growth plate injury repair response, the recombinant human GPC3 (rhGPC3) or a neutralising anti-GPC3 antibody was delivered locally to the growth plate injury site of rats following growth plate injury. Localized rhGPC3 protein treatment to the growth plate injury site revealed a significant reduction in trabecular bone volume fraction at the growth plate injury site, suggesting a reduced bony repair. In contrast, rats treated with the anti-GPC3 neutralising antibody had a significantly increased trabecular bone volume within the growth plate injury site.

Consistent with the previous cranial suture studies (Dwivedi et al., 2013b), rats treated with rhGPC3 showed a significant reduction in the pSmad1/5/8 immunopositive nuclear staining at the growth plate injury site at day 15 post-injury, suggesting a reduced BMP signalling following the rhGPC3 treatment. Consistently, levels of expression of BMP target genes Id1 and Dlx5 were significantly downregulated following GPC3 treatment at day 15 and 35 post-injury. Further mRNA gene expression analyses had also shown significant downregulation of the osteogenesis-related genes, Runx2 and Osteocalcin over the time-course following GPC3 treatment. These data suggest that rhGPC3 treatment can suppress BMP signalling and consequently inhibit the bony repair at the growth plate injury site.

The rhGPC3 treatment did not only alter the osteogenesis-related gene expression, it also change the cartilage-related gene expression as levels of Sox9 and Collagen-2a (Col2a) were significantly upregulated at day 15 post-injury at the injury site. Although this mRNA gene expression data suggests rhGPC3 treatment may favour cartilaginous repair at the growth plate injury site, volumes of the overall cartilaginous tissues within the growth plate injury site did not increase nor decrease following GPC3 treatment over the time-course.

Overall, data from this study suggest the GPC3 can suppress BMP signalling and thus modulate the bony repair at the growth plate injury site. Further studies are required to investigate potentials and action mechanisms of modulating growth plate repair by GPC3 functional interventions.

5.4 Inhibition of BMP signalling pathway suppressed the bone tibia drill-track injury repair

In addition, this PhD project also examined the effects of treatments with noggin, rhGPC3 and Anti-GPC3 neutralising antibody on healing of the tibia bone drill-track injury site. Bone drill-hole models are often used to study bone injury/fracture repair, and BMP signalling has been shown to be upregulated at the bone fracture site along with increasing BMP2 gene expression level (Sheu et al., 2014, Tsuji et al., 2006, Yu et al., 2010). BMP2 plays an important role in regulating bony repair response following a bone fracture, and a previous mouse genetic study identified BMP2 as a fundamental component of the inherent regenerative capacity of bone (Rosen, 2009). Confirming critical function BMP signalling in bone healing, in our study following noggin intervention (blocking BMP signalling), the percentage of pSmad1/5/8 immunostainings was significantly downregulated at the drill-track injury site suggesting the

inhibition of the BMP signalling activation following drill-hole injury, and a reduced bone volume fraction and reduced trabecular bone formation were observed at the bone drill-hole injury site. This data reaffirms that the BMP signalling is indispensable for the tibia drill-hole injury repair.

5.5 rhGPC3 intervention at the bone tibia drill-track injury site suppressed bone formation

Similarly, the tibia bone drill-track injury site was also being examined following local delivery of the rhGPC3 using the thermosensitive chitosan hydrogel. Although the *in vivo* function of the glypicans is still not known, spontaneous mutation and induced deletion of the GPC3 gene had led to malformation and postnatal overgrowth (Paine-Saunders et al., 2000). Furthermore, a previous distraction osteogenesis study found that the exogenous application of glypican in the distracted gap of the murine model did not increase bone healing, but had a negative impact on bone and wound healing (Gdalevitch et al., 2013). Also suggesting potential roles of GPCs in regulating bone healing, Song *et al.* (2006) found that the mRNA expression level for GPC1 was being upregulated post-bone fracture, reaching a peak at day 7. In contrast, expression of the GPC3 was decreased post-surgery (Song et al., 2006). Interestingly, a recent mandibular study using glypican knockout technology found that mandibles of the GPC3-knockout mice were larger than wild-type mandibles suggesting that the GPC3 expression is likely to mediate changes to mandibular size in craniosynostosis (premature fusion of the cranial sutures) (Mian et al., 2017).

While GPC3 expression is known to affect the bone formation and craniosynostosis, the link between the GPC3 protein and bone fracture repair has yet to be explored. Therefore, this part of the study examined the potential role of the glypicans (GPC3) in regulating trabecular bone repair at the drill-track injury site treated locally with the rhGPC3 and a GPC3 neutralising antibody. Our micro-CT analyses found that the localized delivery of rhGPC3 reduced bone repair at the drill-track injury site while the anti-GPC3 treatment enhanced bone repair.

In addition, a genetic study demonstrated that glypican (GPC3) have a significant relationship with the BMP signalling pathway when GPC3-deficient mice were mated with mice haploinsufficient for BMP4, their offspring displayed a high penetrance of polydactyly and rib malformation even though such phenotypes were not found present in their parents (Paine-Saunders et al., 2000). Consistently, an earlier study by Dwivedi *et al.* (2013) shows the importance of GPC3 in regulating BMP2 responsiveness during osteogenesis (Dwivedi et al., 2013a). In the current study, suggesting potential regulation of BMP signalling by GPC3 during bone healing, immunohistochemical analyses found reduced pSmad1/5/8 immunopositive stainings at the drill-track injury site compared to the vehicle treated rats. However, further studies are required to investigate whether and how GPC3 function regulates BMP signalling and/or other molecular mechanisms in influencing bone healing.

CONCLUSION

Data from this PhD project suggest that, following growth plate injury, the BMP signalling is activated at the remaining adjacent growth plate cartilage which resulted in the degeneration of the remaining adjacent growth plate, and systemic inhibition of BMP signalling could prevent the degeneration of the remaining adjacent growth plate cartilage. In addition, the current project clearly demonstrated that the BMP signalling plays an important role in the bony repair response in both growth plate injury site and bone drill-track injury site as BMP signalling blockade can suppress bony repair at both injury sites. Furthermore, data from this project also suggests that locally expressed GPC3 is involved in suppressing BMP signalling pathway during the growth plate and bone drill-track injury repair resulting in the reduction of bony repair. Therefore, the data from this PhD project strongly suggests that BMP signalling pathway is involved in the hypertrophic degeneration of the remaining adjacent growth plate cartilage and the bony repair at the growth plate injury site and bone drill-track injury site.

FUTURE DIRECTIONS

While knowledge from this PhD project has at least shown the important roles of BMP signalling in regulating bony repair at the growth plate injury site and bone drill track, to be able to develop a successful biological therapy for growth plate injuries in order to prevent the invasive surgery and to promote cartilage regeneration following growth plate injury, further studies will be required to unravel the cellular and molecular responses that happen during the growth plate injury repair. In fact, a few improvements from this study can be implemented to further affirm the role of the BMP signalling in the bony repair of the injured growth plate.

Following this PhD project, future investigations for modulating BMP signalling should be considered, which may lead to strategies for preserving the remaining growth plate cartilage adjacent the injury site, limiting bony repair at the growth plate injury site, and promoting bone healing at the bone injury site.

In the aspect of measuring the BMP signalling activation within the growth plate injury site, this study used the immunohistochemical analysis method. While this method can only prove the presence of pSmad1/5/8 protein, it is not quantitative. In order to quantify the pSmad1/5/8 protein at the growth plate injury site, Western blot analyses will be needed. This Western blotting method was not adopted due to the small amount of growth plate injury tissues that could be harvested from each rat. One possibility to overcome this limitation would be to pool protein proportionally from 3 rats of the same group to generate several pools of samples for the group, which would be sufficient for Western blotting analyses.

Furthermore, the inhibition of the BMP signalling either through noggin or rhGPC3 treatment saw a significant reduction in the bone trabeculae formation at the growth plate injury site and at the same time, a significant amount of fat tissue deposition. BMP signalling is known for regulating chondrogenic and osteoblastic differentiation. Therefore, it would be beneficial to examine the other signalling pathway and fat-related genes that were being upregulated following growth plate injury. Therefore, it will be interesting to investigate if the suppression of the fat-related genes could result in more cartilage tissue formation at the growth plate injury site.

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