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Injury responses and repair mechanisms of the injured growth plate

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1. ABSTRACT

The growth plate is responsible for longitudinal growth of children's long bones. However, being a cartilaginous tissue, the growth plate has a limited ability for regeneration and thus injured growth plate is often repaired by bony tissue resulting in bone growth defects of the involved limb. Understanding the pathophysiology of growth plate bony repair and developing preventative treatments remain a challenge. This review discusses previous and recent studies investigating growth plate injury responses and repair mechanisms in a rat tibial growth plate injury model. Following an injury, inflammatory, fibrogenic, osteogenic and bone-bridge maturation repair phases have been observed on days 1-3, 3-7, 7-14 and 10 onwards, respectively. Important roles of several growth factors and cytokines (such as PDGF-BB, FGF-2, TNF-alpha and IL-1beta) have been highlighted, regulating different phases of growth plate injury repair. Studies have also shown that while intramembranous ossification is the major mechanism responsible for the bony repair, endochondral ossification, to a lesser extent, also plays a role. Further understanding of the growth plate injury responses and bony repair mechanisms is still required.

2. INTRODUCTION

The growth plate is solely responsible for the longitudinal growth of long bones via a tightly controlled and regulated process called endochondral ossification, whereby a calcified cartilaginous template is first made and then replaced by bone (1-3). However, being the weakest region of the long bone, it is susceptible to injuries, which, depending on the location and severity, can lead to "undesirable" bony repair and a myriad of orthopaedic problems. Currently, the pathophysiology for the bony repair and bone growth defects remains unclear, and there lacks a biological treatment for the injured growth plates to prevent the bony repair and the bone growth defects. This review discusses and summarises the major molecular and cellular events involved with the growth plate injury responses and bony repair which have been reported in recent studies using rodent models.

3. MECHANISMS OF BONE GROWTH AND MODELLING

The developing long bones in children, such as the femur (thigh bone) or humerus (arm bone), can be divided into five distinguishable regions, including articular

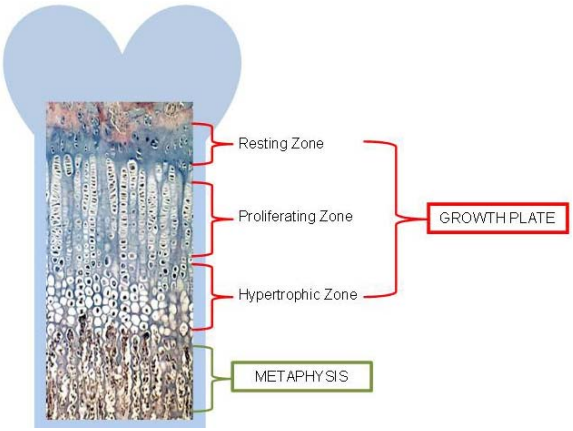

Figure 1. The structure of growth plate and metaphysis. The growth plate is a cartilaginous structure situated at the end of long bones and made up of three distinct zones, namely the resting, proliferative and hypertrophic zones. During the process of endochondral ossification, the hypertrophic cartilage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

cartilage, epiphysis, physis (referred as the growth plate), metaphysis and diaphysis (4, 5). The growth plate, a cartilage-like structure situated directly below the epiphysis and present only in developing long bones, is a layer of hyaline cartilage that allows the long bone to grow longitudinally but not in width. It functions to produce mineralised cartilaginous scaffold from which trabecular bone is formed through the endochondral ossification mechanism involving chondrogenesis and osteogenesis (3, 6, 7) (Figure 1).

There are three distinguishable zones within the growth plate: the resting (reserve) zone, the proliferative zone and the hypertrophic zone (4, 6, 8). Previously, the resting zone has been thought to play no direct roles in longitudinal growth of bones as the cells within the zone (pre-chondrocytes) proliferate very slowly or do not proliferate at all (5). Histologically, the resting zone is characterised by the sparse distribution of either singular or coupled round cells that are abundant in lipid and cytoplasmic vacuoles within the matrix, indicative of its proposed role as a storehouse for nutrients (5, 6, 9). Even though the resting zone possesses the ability to produce a cartilaginous matrix, it remains relatively inactive in both cell and matrix turnover (6), with very low rates of proteoglycan and collagen-2 production (10). On the other hand, research has suggested that the cells within the resting zone act as a pool of stem cell-like cells, producing proliferative chondrocytes for the proliferative zone (11, 12). In addition, Abad *et al* (2002) reported that, by producing an unknown growth plate orienting morphogen, the resting zone may be responsible for influencing the columnar directional arrangement of growth plate chondrocytes within the proliferative zone (12).

There are two main functions at the proliferative zone, matrix production and cellular division, which are vital contributions to the longitudinal growth of long bones (6). Histologically, the proliferative zone is characterised by longitudinal columns of slightly flattened chondrocytes. These columns are separated from each other via the surrounding cartilage matrix, which is enriched in collagen-II (5). The extent of total longitudinal growth can be determined by the thickness of the proliferative zone, with a greater number of cells present representing a greater potential of longitudinal growth (5). At the end of the proliferative zone, the chondrocytes no longer proliferate and instead begin to undergo hypertrophy as they enter into the hypertrophic zone. Histologically, the cells within the hypertrophic zone are 5 to 10 times greater in size than those in the proliferative zone. Producing collagen-X and alkaline phosphatase, the hypertrophic zone is involved with matrix mineralization. In addition, production of vascular endothelial growth factor (VEGF-a) as well as a low oxygen tension present attract vessel invasion from metaphysis, which will turn the calcified cartilage into trabecular bone in metaphysis. Since the hypertrophic chondrocytes are larger in size and this relatively thicker zone of calcified cartilage serves as a template for bone deposition, the hypertrophic zone is the principal engine of longitudinal bone growth, and thus the variation in the rate at which the hypertrophic zone increases in thickness has been regarded as the major reason behind the differences in growth rate in different parts of the body (5).

With the mineralisation and angiogenesis, chondrocytes within the lower hypertrophic zone particularly at the chondro-osseo junction are destined for apoptotic cell death, which causes calcified tissue/bone absorbing cells (osteoclasts or chondroclasts) to zone in and dissolve the calcified cartilage (9). The influx of bone building cells (osteoblasts) deposits bone matrix to replace the previously absorbed tissue to form trabecular bone (6, 8, 13, 14). Therefore, with vascularisation and coordinated action of osteoclasts/chondroclasts and osteoblasts, the calcified hypertrophic cartilage is modelled and remodelled into the metaphyseal trabecular bone, in which mineralised growth plate cartilage is first being replaced by primary woven bone (primary spongiosa) and then further modelled and remodelled into more mature laminar trabecular bone (secondary spongiosa) (8). In mature bone, the metaphysis is where the epiphysis and diaphysis meet.

4. GROWTH PLATE INJURIES, INJURY RESPONSES AND REPAIR MECHANISMS

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

Due to accidents in sports and play, skeletal fractures are common in children. Since the growth plate is the least rigid region of the long bone, its injuries are common, and it has been estimated that around 20% bone fractures involve growth plate (15). The Salter-Harris classification system has been used to distinguish the different types of growth plate injuries and relationship between the characteristics of the fractures and their prognoses (6, 16-18). Current literature indicates the most common types of fractures occurring in the distal tibias of younger children is type I (around 40%), which in most

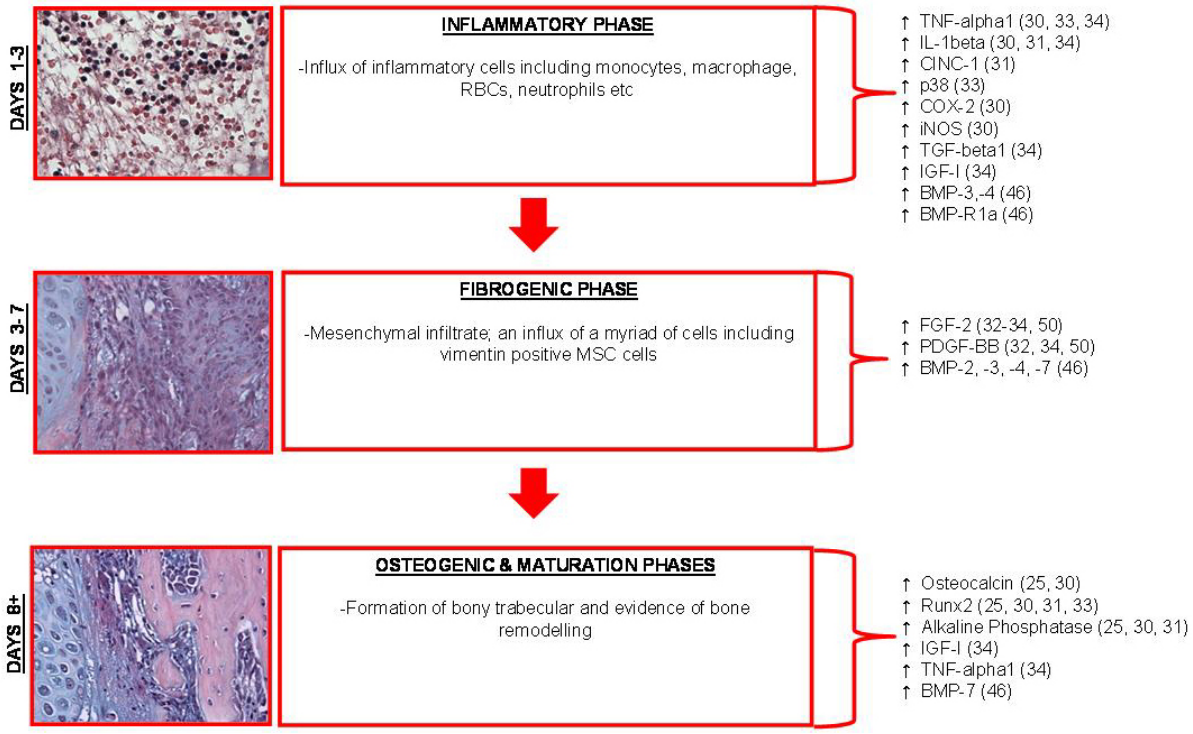

Figure 2. Growth plate injury repair responses and gene expression. In a rat growth plate injury model four distinct phases of injury repair were observed, namely the inflammatory, fibrogenic and osteogenic and maturation phases on days 1-3, 3-7 and 8 onwards, respectively, which are accompanied by elevated levels of mRNA expression of specific cytokines, inflammatory mediators, and growth factors during each phase.

cases has a reasonably good prognosis as the cells responsible for interstitial growth of the growth plate as well as the epiphyseal blood supply remain undisturbed (18-20). Similarly, the prognoses for future growth in type II fractures are also quite good. Other types of fractures, types III, IV, and V, however, may/will all result in bony formation at the injured site (21). It has been estimated that in up to 30% of all children with growth plate related injuries, undesirable formation of bony tissue and bone bridge at the injury site hinders normal growth of the developing long bone in the affected limb (22, 23), which results in significant orthopaedic problems such as limb length discrepancy and bone angulation deformity (22, 24).

4.2. Injury responses after a growth plate fracture

The cellular and molecular mechanisms for the bony repair of the injured growth plate remain largely unknown. An earlier study identified four different phases of injury responses in a rat growth plate injury model (25) - the inflammatory, fibrogenic, osteogenic and bone bridge maturation remodelling responses occurring during days 1-3, 3-7, 7-14, 10-25, respectively. Similarly, this pattern of growth plate injury repair was demonstrated in a murine growth plate injury model (26). In addition, similar injury responses were also observed in various growth plate injury models including mice, rabbits, pigs and sheep (26-29). Following from these studies, there have been some additional *in vivo* mechanistic studies using a rat tibial growth plate injury model (30-33).

4.2.1. Inflammatory phase

Common to bone fractures and soft tissue injuries, the first response after a growth plate injury is the inflammatory phase (25, 34, 35). During this initial phase there is an influx of inflammatory cells - predominately neutrophils together with macrophages/monocytes and lymphocytes entering into the growth plate injury site. This rapid influx of inflammatory cells has been shown to commence approximately 8 hours after the injury in a rat growth plate injury model, peaking at day 1 and gradually subsiding by day 3. Consistent with the abundant numbers of neutrophils seen within the infiltrate, the gene expression of rat neutrophil chemokine CINC-1 (similar to human interleukin-8) was shown to be significantly increased during the peak of the inflammatory phase (day 1) (31). By the end of the inflammatory phase (day 4) the levels of CINC-1 had decreased back to almost basal levels. Along with the influx of inflammatory cells entering the injury site, the infiltrate also secretes a myriad of growth factors and cytokines that are thought to regulate further downstream responses during growth plate injury repair. Pro-inflammatory cytokines tumour necrosis factor-alpha (TNF-alpha) and interleukin-1 (IL-1beta), which are known regulators of inflammation after tissue injury and bone fractures, were seen in their mRNA expression levels during the inflammatory phase - peaking between 8 hours to day 1 post injury (34) (Figure 2). Follow-up studies also showed a significant increase of these cytokines at day 1 post injury in a rat growth plate injury model (30, 31).

Growth factors insulin-like growth factor (IGF-I) and transforming growth factor (TGF-beta) were also found to be upregulated during this early phase of injury repair (34).

Previous studies have examined the potential role of the inflammatory phase in mediating the cascade of downstream events leading to the bony bridge formation after growth plate injury. As one of the key regulators of the inflammatory response, p38 mitogen activated protein kinase (MAPK) has been shown to be increased in activation at the injured growth plate (33) (Figure 2). Furthermore, Zhou *et al* (2006) found that TNF-alpha was needed for the activation of p38 at the injured growth plate as p38 activation was blocked in rats treated with a TNF-alpha antagonist (33). TNF-alpha inhibition also resulted in a reduced mesenchymal infiltration, proliferation as well as a reduced expression of FGF-2, indicating the potential role of TNF-alpha in mesenchymal infiltration and proliferation within the growth plate injury site (33). Similarly, Gerstenfeld *et al* (2001) found that in bone fractures, blocking TNF-alpha signalling resulted in a significant delay in bone callus formation (36). The role of TNF-alpha has also been studied in other types of tissue repair. Consistent with the finding of TNF-alpha role in mesenchymal cell infiltration into growth plate injury site (33), Fu *et al* (2009) reported that TNF-alpha had a strong chemotaxis role for mesenchymal stem cell migration during wound repair (37), and thus abrogation of TNF-alpha resulted in an obvious delay in MSC migration and wound healing. Overall, these studies highlight the importance of TNF-alpha during tissue repair.

Since the major inflammatory cells involved with the inflammatory phase are found to be neutrophils (25), a follow-up study examined the role of the neutrophil-mediated inflammatory response in growth plate injury repair by utilising an anti-serum to deplete the majority of neutrophils (31). As a result of the depletion, an increase in expression of osteogenesis genes such as osteocalcin and Runt- related transcription factor 2 (Runx2 or also commonly referred to as core binding factor alpha-1 or cbf alpha-1) was seen. In addition, neutrophil depletion also decreased the expression of chondrogenesis-related genes such as Sox-9 and collagen -2 (31). This study indicates that neutrophils play a role of initiating the growth plate injury response and consequently may enhance chondrogenic differentiation. During both soft tissue and bone healing repair, neutrophil recruitment has also been found to be vital, as they play an active role in the clearance of undesirable bacteria and microdebris within the injured zone (38, 39).

Furthermore, one previous study observed significant upregulated gene expression of injury-induced key inflammatory mediators cyclo-oxygenase-2 enzyme (COX-2) and inducible nitric oxide synthase (iNOS) during the inflammatory phase at the injured growth plate (Figure 2) and found that inhibition of COX-2 or iNOS by specific inhibitors caused an increased proportion of undifferentiated mesenchymal tissue but a decrease in chondrogenic differentiation within the injury site (30). This study confirms that the injury-induced inflammatory response in general at the growth plate injury site is necessary for enhancing the chondrogenic differentiation of mesenchymal cells. Overall, these studies suggest that the injury-induced inflammatory response has an important role early in regulating growth plate injury repair as it initiates and regulates a cascade of downstream events which lead to the bony repair at the growth plate injury site. Similarly during bone fracture healing, these two inflammatory mediators (COX-2 and iNOS) have been found to be important for triggering the cascade of events leading to tissue repair. More specifically, numerous bone fracture studies have demonstrated that inhibiting COX-2 resulted in a delay in bone formation and fracture healing (40-43), highlighting the importance of injury-induced inflammatory response and COX-2 enzyme during tissue repair.

Other studies have also shown that during the inflammatory phase there were increases in the levels of several members of the bone morphogenic protein (BMP) family. The BMPs have been known for having roles in chondrogenic and osteoblastogenic differentiation as well as encouraging mesenchymal cell proliferation and migration (44, 45). Ngo *et al* (2006) observed the presence and upregulation of BMP-3 and BMP-4 within the growth plate injury sites of young rats (46) (Figure 2). BMP-4 also appeared to be produced by inflammatory cells- indicating their role in mediating the initial inflammatory event in regulating mesenchymal cell migration and differentiation (46). BMP-4's proposed role in regulating mesenchymal cell migration and differentiation during skeletal repair was also echoed in another earlier study which examined BMP-4 potential role and level of expression in regenerating tissue of a rabbit leg-lengthening model (47).

4.2.2. Fibrogenic phase

Following the initial inflammatory phase in the rat growth plate injury model is the fibrogenic phase - occurring during days 3-7 post injury (25). The fibrogenic response involves the influx of fibrous vimentin-immunopositive mesenchymal cells into the injury site (25). This response was also observed in mice, whereby approximately 7 days post injury, there was presence of undifferentiated, spindle- shaped cells near the superior and inferior areas of the growth plate injury site (26). Although it is yet to be confirmed, previous findings of osteogenesis as well as chondrogenesis from these infiltrated cells (25, 30, 31, 48) suggest that these filtrating cells may contain pre-determined chondroprogenitor and osteoprogenitor cells as well as multipotent mesenchymal stem cells. The infiltration of such stromal progenitor cells (originating from periosteum, the circulation as well as from the bone marrow) following the inflammatory response has been confirmed in bone fractures, which is critical for the formation of the bridging "soft callus" as the next stage of the fracture repair process (27, 35, 49).

During the influx of fibrogenic cells in both injured growth plate and bone, mRNA levels of growth factors FGF-2 and PDGF-BB have been found to be significantly upregulated, indicating the possible involvement of both growth factors in regulating this

mesenchymal reaction phase in both bone or growth plate injury repair (Figure 2) (34, 48, 50). FGF-2 has functions in various biological responses such as cell proliferation, differentiation and migration (51). During bone fracture healing, various cells such as monocytes, macrophages, mesenchymal cells, osteoblasts and chondrocytes produce FGF-2 (52). Along with its well known roles in mesenchymal cell migration and proliferation (53, 54), FGF-2 has been found to inhibit chondrocyte differentiation (55), alkaline phosphastase activity (56, 57) as well as stimulating bone resorption *in vitro* (58, 59), suggesting its role in suppressing skeletal cell differentiation during bone fracture repair. Interestingly, a more recent *in vitro* study has shown that FGF-2 was able to increase the osteogenic and chondrogenic differentiation potentials of mesenchymal cells via suppression of key signalling from TGF-beta (54, 60). However, although it has been suggested that FGF-2 may play a possible role in mesenchymal and osteoprogenitor cell proliferation, migration and differentiation, further studies are required to investigate the functions of the upregulated FGF-2 at the injured growth plate during the fibrogenic phase (33, 34).

PDGFs have been documented to have many different roles including cell migration, cell proliferation and angiogenesis in wound healing (61-64). In particular, it is also a potent chemotactant for fibroblasts and smooth muscle cells (65). During bone fracture repair, PDGFs have been found to be essential for triggering the initial events leading to the migration and proliferation of fibroblasts and osteoblasts (52). Similarly, Zhou *et al* (2004) found that gene expression levels of PDGF-BB were significantly upregulated following the inflammatory phase in a rat growth plate injury model (34). A recent study by Chung *et al* (2009) found that inhibition of PDGF-R signalling during the fibrogenic phase reduced proliferation and the level of infiltration of mesenchymal cells by day 4 after injury and the amounts of bony or cartilage tissues at the injury site by day 14, suggesting a critical role of PDGF in the fibrogenic phase of growth plate repair (32).

4.2.3. Osteogenic and maturation phases

Following the fibrogenic phase, the subsequent osteogenic response involves some bone cell differentiation among some of the infiltrated mesenchymal cells, as indicated by positive immunohistochemical staining of Runx-2 and alkaline phosphatase (markers of osteoblast differentiation and maturation, respectively) (25, 30, 31, 33). Furthermore, the presence of active bone deposition containing bone matrix protein osteocalcin on the new trabecular bone surface within the growth plate injury site is indicative of the bony tissue formation (25, 30). During the remodelling and maturation of the bony bridge, bone trabeculae are found to be separated by abundant marrow cells, and were surrounded by flattened spindle-like inactive osteoblasts in resting phase - producing little or no osteocalcin which is characteristic of inactive bone formation (25). In addition, resorptive cells osteoclasts are sometimes seen on some areas of newly formed trabeculae at the injury site (48), suggesting that osteoclastic bone resorption is involved in the bone bridge matuation phase at the injured growth plate. While the molecular mechanisms regulating this maturation phase remains unclear, upregulation of TNF-alpha, IGF-I and BMP-7 at the injured growth plate (Figure 2) suggest their involvement in the bony bridge remodelling (34, 46). Consistently, TNF-alpha upregulation has been observed during the remodelling phase in bone fracture repair (66), and TNF-alpha has been shown to be important in regulating bone remodelling by promoting differentiation of bone resorptive cells, osteoclasts (67). Similarly, BMP-7 upregulation is known to be important for bone formation and remodelling at the bone fracture sites (68). Further studies are required to characterise the molecular and cellular mechanisms regulating the bony bridge maturation/remodelling at the growth plate injured site.

4.3. Mechanisms of bony repair of injured growth plate cartilage

Studies in both murine and rat growth plate injury models by Lee *et al* (2000) and Xian *et al* (2004) respectively showed that the bony bridge formation occurring after injury was a result from direct bone formation mainly via intramembranous ossification (25, 26). In support, Lee *et al* (2000) saw no changes in the levels of endochondral ossification-related molecules including collagen-2, Indian hedgehog (Ihh) and vascular endothelial growth factor (VEGF) at the time points examined (26), and Xian *et al* revealed $Runx2^{+}$ osteoblastic differentiation and bony trabecular formation from infiltrated mesenchymal cells (25). Similarly, Zhou *et al* (2004) reported no up-regulation of chondrogenic transcription factor Sox-9 and cartilage matrix protein collagen-2 at the injured growth plate in this rat model (34). However, more recent studies in the rat growth plate injury model (some with different post-injury time points examined) have found that apart from direct bone formation being as the major bony repair mechanism present, endochondral ossification, despite to a lesser extent, also occurred as a potential mechanism underlying the bony repair. Arasapam *et al* (2006) found increased expression of some cartilage related genes including collagen-2, collagen-10 and Sox-9 together with increased levels of some bone related genes (30). This indicates the presence of the formation of both cartilage and bone within the growth plate injury site and hence involvement of both endochondral and intramembranous ossification mechanisms during the bony repair. Similarly, Chung *et al* also found endochondral ossification involvement during bone bridge formation showing presence of cartilage-related molecules, Sox-9 and collagen- 2 and -10 at the growth plate injury site and positive immunostaining of both collagen-2 and -10 in cartilage-like tissue derived from infiltrated mesenchymal cells (31, 32). Further mechanistic studies are required to understand the bone formation pathways underlying the bony repair of injured growth plate.

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

While most growth plate injury studies have focused and looked at the events occurring purely within the injury site, very few have investigated the potential effects of injuries on adjacent growth plate chondrocytes.

An earlier study looking at the effects of growth plate trauma observed the intrusion of growth plate cartilage tissue into the metaphyseal region, and found that these islands of trapped cartilage disrupt the continuing bone growth of the surrounding tissue (69). Consequently, there was abnormal widening and irregularities of the remaining growth plate and hence potentially resulting in the deformities and discrepancies seen in many patients as a result from their growth plate related injuries (69). More recently, Coleman *et al* (2010) utilised micro-CT imaging to characterise changes occurring within the injured growth plates of rats as well as the effect on the whole tibial bone itself (70). Interestingly, Coleman *et al* (2010) observed that bone volume present within the injury site did not directly correlate with overall reduced bone growth by 35 days after the injury. Furthermore, using micro-CT imaging, by the time a bone bridge has formed, significant damage could already be detected in the remaining non-injured growth plate, including cellular disorganisation as well as a significant decrease in overall growth plate thickness and volume. Interestingly, Coleman *et al* (2010) also observed that tethers, which usually form with age as the growth plate begins to close (71), were present earlier in the adjacent growth plate after injury (70). These studies highlight the potential involvement of the adjacent remaining growth plate during growth plate injury repair and its contribution to limb length discrepancies and bone angulation deformities that form after growth plate injuries (69, 70). Further mechanistic studies are required to gain a better understanding how the bone bridge formation within the injury site and changes in the adjacent non-injured growth plate tissue contribute to the final undesirable bony repair and bone growth defects after a growth plate injury.

5. PERSPECTIVE & CONCLUSION

Growth plate cartilage injuries in children are common and are a significant clinical problem since they can often lead to undesirable bony repair and bone growth defects. Currently, pathophysiological mechanisms for the undesirable bony repair at the injured growth plate still remain elusive and understanding the detailed events for growth plate injury responses and bony repair is imperative towards the development of an alternative and effective biological therapy for inducing growth plate cartilage regeneration. However, studies in the last 10 years using rodent growth plate injury models have observed sequential injury repair phases (inflammatory, fibrogenic, osteogenic and maturation) and differential gene expression (including various growth factors and cytokines and inflammatory mediators) occurring after growth plate injury. Interestingly, it has been shown that the initial inflammatory response appears to play an important role in regulating downstream healing events and bony repair at the injured growth plate. In addition, after the inflammatory response, infiltration of mesenchymal progenitor cells occurs, which contributes to both intramembranous and endochondral bone formation mechanisms for the bony repair at the injured growth plate. Therefore, growth plate injury responses and bony repair mechanisms appear to be similar to the events that happen during bone fracture healing.

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Abbreviations: TNF-alpha: Tumour necrosis factor- alpha; Runx2: Runx-related transcription factor 2; cbf alpha-1: core binding factor- alpha1; IL-1 beta: Interluekin-1beta; PDGF-BB: platelet derived growth factor-BB; FGF-2: basic fibrogenic growth factor; COX-2: Cyclo oxygenase-2; iNOS: nitric oxide synthases; CINC-1: cytokine induced neutrophil chemoattractant; BMP: Bone morphogenic protein; TGF-beta: Transforming growth factor-beta; VEGF: Vascular endothelial growth factor

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