RECENT RESEARCH ON THE GROWTH PLATE

Mechanisms for growth plate injury repair and potential cell-based therapies for regeneration

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Abstract

Injuries to the growth plate cartilage often lead to bony repair, resulting in bone growth defects such as limb length discrepancy and angulation deformity in children. Currently utilised corrective surgeries are highly invasive and limited in their effectiveness, and there are no known biological therapies to induce cartilage regeneration and prevent the undesirable bony repair. In the last 2 decades, studies have investigated the cellular and molecular events that lead to bony repair at the injured growth plate including the identification of the four phases of injury repair responses (inflammatory, fibrogenic, osteogenic and remodelling), the important role of inflammatory cytokine tumour necrosis factor alpha in regulating downstream repair responses, the role of chemotactic and mitogenic platelet-derived growth factor in the fibrogenic response, the involvement and roles of bone morphogenic protein and Wnt/B-catenin signalling pathways, as well as vascular endothelial growth factor-based angiogenesis during the osteogenic response. These new findings could potentially lead to identification of new targets for developing a future biological therapy. In addition, recent advances in cartilage tissue engineering highlight the promising potential for utilising multipotent mesenchymal stem cells (MSCs) for inducing regeneration of injured growth plate cartilage. This review aims to summarise current understanding of the mechanisms for growth plate injury repair and discuss some progress, potential and challenges of MSC-based therapies to induce growth plate cartilage regeneration in combination with chemotactic and chondrogenic growth factors and supporting scaffolds.

Key Words

- ▶ growth plate injury
- ▶ cartilage tissue engineering
- ▶ chondrogenesis
- ▶ MSCs

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The growth plate cartilage and endochondral bone formation

Situated under the epiphysis at the ends of all long bones in an immature skeleton, the growth plate holds the sole responsibility in achieving longitudinal growth. It functions to produce a mineralised cartilaginous scaffold to which new trabecular bone is formed via a tightly

controlled two-step process (called endochondral ossification) involving chondrogenesis and osteogenesis (Iannotti 1990, Yang & Karsenty 2002).

The growth plate is made of three distinct zones, namely the resting zone, the proliferation zone and the hypertrophic zone (Iannotti 1990). The resting zone has previously been thought to play a very minimal role during

endochondral ossification as the pre-chondrocytes/cells within this zone proliferate minimally (Forriol & Shapiro 2005). However, studies have indicated the importance of the resting zone as it acts as a reservoir of stem cells/pre-chondrocytes for the chondrocytes in the adjacent proliferative zone (Abad *et al.* 2002). The proliferative zone is responsible for matrix production (including collagen-2 and aggrecan) and cellular division during endochondral ossification. The height of the proliferative zone directly correlates with the extent of longitudinal growth that can be achieved by the long bone (Iannotti 1990). As regulated by various signalling pathways including parathyroid hormone-related protein (Vortkamp *et al.* 1996), insulin-like growth factor (IGF1; Baker *et al.* 1993, Hunziker *et al.* 1994), bone morphogenic protein (BMP; Chen *et al.* 2004, Jing *et al.* 2013), Wnt/B-catenin (Macasai *et al.* 2008, Dao *et al.* 2012, Golovchenko *et al.* 2013, Lu *et al.* 2013), fibroblast growth factor (FGF; Mancilla *et al.* 1998, Du *et al.* 2012) and others (Minina *et al.* 2002, Xian 2007), chondrocytes cease to proliferate and become hypertrophic. The hypertrophic chondrocytes produce collagen-10 which is involved with matrix mineralisation. Together with the action of angiogenic factor vascular endothelial growth factor (VEGF) produced by hypertrophic chondrocytes and a low oxygen tension, the lower hypertrophic zone attracts blood vessel invasion from the adjacent metaphyseal bone (Gerber *et al.* 1999, Forriol & Shapiro 2005), which brings along mineralised cartilage-resorptive cells (chondroclasts), bone-forming cells (osteoblasts) and bone-resorptive cells (osteoclasts) to convert the mineralised cartilage scaffold into trabecular bone in metaphysis.

Growth plate injuries and their classification

Being cartilaginous in nature and the weakest region of a long bone, the growth plate is often an injury-prone area. Past and recent epidemiological data have shown that ~15–30% of all childhood skeletal injuries are growth plate related (Mizuta *et al.* 1987, Mann & Rajmaira 1990, Eid & Hafez 2002). While the prognoses in ~40% of these growth plate-related injuries are fairly good, depending on the degrees and locations of fractures, those of others are not (Leary *et al.* 2009, Mubarak *et al.* 2009). In about 30% cases, the injured growth plate cartilage is often repaired by an undesirable bony tissue (Ogden 1984, Barmada *et al.* 2003), which often results in substantial lifelong orthopaedic problems including discrepancies in limb length as well as bone angulation deformities (Ogden 1984, Wattenbarger *et al.* 2002).

The Salter–Harris classification system has been used to determine and predict the general outcome and severity of a growth plate injury (Brown & DeLuca 1992). The classification system grades a growth plate injury into five types. Briefly, type 1 and 2 injuries (with type 2 being the most common injury type) do not disturb the growth plate or the epiphyseal blood supply and thus will usually heal by itself with no overall growth problems (Kay & Matthys 2001, Leary *et al.* 2009). On the other hand, ~30% of growth plate-related injuries are of types 3, 4 or 5, which disturb not only the growth plate but also its blood supply and hence usually lead to the undesirable bony repair (Sailhan *et al.* 2004, Basener *et al.* 2009).

Growth plate injury repair responses

In the past 2 decades, a number of animal models have been developed and used as attempts were made to understand the underlying pathophysiology for the faulty bony repair and to develop potential biological treatments, including the mouse and rat central drill hole disruption models (Lee *et al.* 2000, Xian *et al.* 2004, Coleman *et al.* 2010), rabbit peripheral disruption model (Lee *et al.* 1993, 1998, Planka *et al.* 2008), miniature pig peripheral disruption model (Planka *et al.* 2012) and sheep peripheral disruption model (Foster *et al.* 1990) (Fig. 1). In all of these models, bony repair and/or bone growth defects that mimic the human clinical outcomes can be demonstrated. In particular, in the last decade more extensive work using a rat proximal tibial drill hole model has been performed (Xian *et al.* 2004, Zhou *et al.* 2004, 2006, Arasapam *et al.* 2006, Chung *et al.* 2006, 2009, 2013a,b, 2014, Ngo *et al.* 2006, McCarty *et al.* 2009, 2010, Macasai *et al.* 2011, 2012), which has demonstrated different phases of injury repair that lead to the undesirable bony repair, namely the inflammatory, the fibrogenic, and the osteogenic and remodelling phases, on days 1–3, 4–8 and 8 onwards respectively (Fig. 2).

The Inflammatory phase

As in cases of all bone fracture and soft tissue injuries, the first phase of growth plate injury repair is the inflammatory phase, involving an infiltration of inflammatory cells (including neutrophils, monocytes/macrophages and lymphocytes) into the growth plate injury site and up-regulation of inflammatory cytokines and mediators (Zhou *et al.* 2004, Arasapam *et al.* 2006, Chung *et al.*

Figure 1

A brief summary of the different animal models used to study growth plate injury repair responses, mechanisms and regeneration.

2006). Consistent with a predominance of neutrophils within the influx, an increase in mRNA expression of neutrophil chemokine, *Cinc1* (*Cxcl1*) (rat equivalent to human interleukin 8 (IL8)), was found on day 1, which declined on day 4 at the injured growth plate. Interestingly, neutrophil-mediated inflammatory response was found to modulate downstream injury repair events. Following the depletion of neutrophils with a neutralising antibody, Chung *et al.* (2006) observed an increase in the undesirable bony repair tissue with increased expression in bone-related genes such as *Runx2* and osteocalcin, but decreased expression in cartilage-related genes *Sox9* and collagen-2 (Chung *et al.* 2006).

In addition, up-regulation of some pro-inflammatory cytokines was also observed in this phase of injury response at the injured growth plate. Zhou *et al.* (2004) reported significant increases in the mRNA expression of tumour necrosis factor alpha (*Tnf*) and interleukin 1 beta (*Il1b*) (Zhou *et al.* 2004). Both of these cytokines are known to have roles in regulating inflammation after bone and soft

tissue injuries (Feghali & Wright 1997, Gerstenfeld *et al.* 2003). Moreover, at the injured growth plate, a higher level of activation of the major inflammatory mediator p38 kinase was observed, which was also found to be involved in TNF α and IL1B induction (Zhou *et al.* 2006). Utilising a TNF α antagonist in a rat growth plate injury model, Zhou *et al.* (2006) found that blocking TNF α resulted in a clear delay in the subsequent mesenchymal infiltration response and a reduction of the proliferation of these cells (Zhou *et al.* 2006). Similarly, separate studies looking at the role of TNF α during bone fracture repair and wound repair have also found that its inhibition leads to a significant delay in overall mesenchymal progenitor or stem cell (MSC) infiltration and subsequent healing (Gerstenfeld *et al.* 2001, Fu *et al.* 2009). More studies have reported the importance of TNF α in regulating recruitment of MSC, their proliferation and differentiation (Barnes *et al.* 1999, Martin *et al.* 2003, Dimitriou *et al.* 2005).

Furthermore, following growth plate injury, Arasapam *et al.* (2006) observed an increase in injury-induced

Figure 2

Injury repair response phases and their associated elevated cytokines, inflammatory mediators, growth factors, and molecules related to bone/cartilage formation and remodelling that lead to the undesirable bony repair at the injury site in a rat tibial growth plate drill-hole injury repair model. The initial inflammatory response involves an influx of key inflammatory cells into the growth plate injury site and up-regulation of inflammatory cytokines/mediators and some growth factors (A).

The fibrogenic phase involves an influx of fibrogenic and progenitor cells containing MSC-like cells (B). The osteogenic phase involves the osteogenic and chondrogenic differentiation, formation of bony trabeculae together with angiogenesis within the injury site (C). The remodelling phase involves the maturation and active remodelling of the newly formed bony trabeculae as well as disappearance of cartilaginous repair tissue (D).

inflammatory mediators, cyclooxygenase 2 enzyme, and inducible nitric oxide synthase. After blocking their activity pharmacologically, *Arasapam et al. (2006)* also found a decrease in chondrogenic differentiation of the infiltrated mesenchymal cells. Overall, the above studies indicate a potentially important role for the inflammatory phase, its cells, and released cytokines/mediators, not only in regulating the inflammatory phase itself but also in modulating downstream healing responses following growth plate injury.

The fibrogenic phase

Following the initial inflammatory phase, a fibrogenic phase is apparent at the growth plate injury site. The fibrogenic phase has been observed on days 3–7 post-injury

in injured growth plate of rats, involving an influx of vimentin (mesenchymal cell marker)-immunopositive mesenchymal cells (*Xian et al. 2004*), a response which is similar to the infiltration of mesenchymal cells following the inflammatory phase at bone fracture sites (*Jaramillo et al. 1990*, *Schindeler et al. 2008*). At the growth plate injury site, some of these cells were found to express growth factors including BMPs (*Ngo et al. 2006*), platelet-derived growth factor (PDGF) and FGF2 (*Zhou et al. 2004*, *Chung et al. 2009*) and receptors for BMPs and PDGF (*Ngo et al. 2006*, *Chung et al. 2009*). In addition, some of these cells were found to be MSC like as they expressed the stem cell marker alpha-smooth muscle actin (*Chung & Burdick 2009*). Furthermore, at the early time point day 4 post-injury, some of these cells already expressed chondrogenic marker collagen-2 and/or osteogenic marker

Runx2 (Xian *et al.* 2004, Chung *et al.* 2006), suggesting that this influx of mesenchymal cells may contain a myriad of cells including MSC-like cells, osteoprogenitor cells, pre-osteoblasts, and/or pre-chondroblasts (either pre-existing or newly derived from the infiltrated MSCs). Further indicative of potential multipotency for some of these cells, previous studies have shown that differentiation of these cells and growth plate repair outcomes could be influenced by some growth factors or other signals occurring during this phase (Arasapam *et al.* 2006, Chung *et al.* 2013a,b, 2014).

Alongside a myriad of signals at the growth plate injury or bone fracture site, the two more significantly up-regulated growth factors during this phase are PDGF and FGF2 (Zhou *et al.* 2004, Chung *et al.* 2009). In a rat growth plate injury model, Zhou *et al.* (2004) observed a significant peak in the mRNA expression of platelet-derived growth factor (*Pdgf*) and fibroblast growth factor 2 (*Fgf2*) following the initial inflammatory phase, suggesting a potential regulatory role for these two growth factors during this phase (Zhou *et al.* 2004). PDGFs in particular have been well documented to be involved in stimulating cell migration and proliferation as well as promoting angiogenesis during injury repair. In bone fracture repair, the chemotactic and mitogenic properties of PDGFs for mesenchymal cells and osteoblasts are essential for the initiation of correct bone fracture repair (Bordei 2011, Korsak *et al.* 2013, Elangovan *et al.* 2014). Similarly, in rats with growth plate injury, the inhibition of PDGF signalling caused a significant reduction in the amount of mesenchymal infiltrate, decreased amounts of bony and/or cartilage repair tissues, and thus an overall delay in bony repair 14 days post-injury (Chung *et al.* 2009), highlighting the importance of PDGF expression during growth plate injury repair in regulating the fibrogenic phase and downstream tissue repair processes. More studies are still needed to further elucidate the specific role of PDGF and other signal molecules occurring during this phase of growth plate injury repair.

The osteogenic and remodelling phases

At the injured growth plate, bone formation has been observed to commence around day 7 with the appearance of bony trabeculae, and bone remodelling has been observed by day 14 with the appearance of bone marrow cells in between bony trabeculae (Xian *et al.* 2004). A recent *in vivo* micro-computed tomography (micro-CT) imaging study has demonstrated that the bone volume fraction of the injury site was significantly higher on day 60 when

compared with that on day 14 (Macasai *et al.* 2011). During the osteogenic phase of the growth plate injury repair process, the cells within the fibrogenic infiltrate differentiate into Runx2 and alkaline phosphatase-immunopositive osteoblasts (Xian *et al.* 2004, Arasapam *et al.* 2006, Chung *et al.* 2006, 2009, Zhou *et al.* 2006) and produce increased levels of bone matrix protein osteocalcin (both mRNA and protein) during days 8–14 (Xian *et al.* 2004, Arasapam *et al.* 2006). Similarly, Fischerauer *et al.* (2011) also observed collagen-1-immunopositive bone tissue at the injury site ~14 days post-growth plate injury (Fischerauer *et al.* 2011). On day 35, histologic examination of the injury site has also revealed the presence of flattened inactive bone-lining osteoblasts that weakly expressed osteocalcin (Xian *et al.* 2004).

In addition, starting at day 14, there were also signs of bone remodelling, with the presence of multi-nucleated osteoclasts lining the newly formed bone trabeculae (Chung *et al.* 2009). During the bone remodelling phase, levels of osteocalcin mRNA expression were found increased, and expression of chondrogenesis-related genes such as *Sox9* and collagen-2a was lacking (Zhou *et al.* 2004). In addition, *Tnfa* (*Tnf*) mRNA expression was found increased during the remodelling phase (Zhou *et al.* 2004), which is similar to that seen during the bone maturation and remodelling phase of bony fracture repair (Gerstenfeld *et al.* 2001). Previously, TNF α has been associated with bone remodelling by promoting osteoclast differentiation (Horowitz *et al.* 2001). In addition, Fischerauer *et al.* (2011) have also observed an increase in expression of VEGF (VEGFA) (Fischerauer *et al.* 2011). VEGF has known roles not only in angiogenesis but also in osteoblast differentiation and osteoclast recruitment (Yang *et al.* 2012). The above studies indicate the active involvement of bone formation and remodelling within the growth plate injury site that lead to the formation of the undesirable bony repair tissue or bone bridge; however, more studies are required to characterise the specific molecular and cellular events that regulate these and bone bridge formation.

Potential changes at the adjacent non-injured region of the growth plate

Although a myriad of studies have investigated the phases of events that occur within the growth plate injury site, it is only recently that more interest has been shown on the potential effects of growth plate injury on the adjoining uninjured adjacent growth plate cartilage. Previous studies have suggested that injuries in the long bone can

inadvertently affect the tightly controlled process of endochondral ossification in the growth plate. These effects were found regardless whether the growth plate was injured or not. For example, [Fischerauer et al. \(2013\)](#) found that following a tibial fracture, there were up-regulated levels of BMP6 and BMP receptor BMPRI1A at the growth plate of the fractured or the contralateral intact bones on day 29 (when fractured bone was consolidated), suggesting possibly a late role of BMP6 and BMPRI1A in bone fracture-induced growth plate alterations and the potential existence of a regulatory 'cross-talk' mechanism between the different regions of the lower limbs after bone fractures ([Fischerauer et al. 2013](#)). A recent study by [Macasai et al. \(2011\)](#) evaluated injured growth plate of rats by micro-CT ([Macasai et al. 2011](#)) and found the presence of small bony 'tethers' at the uninjured adjacent growth plate on day 60 post-injury. These 'tethers' were not observed in age-matched non-injured animals, suggesting that the growth plate injury itself affected not only the injury site but also the surrounding areas.

In addition, further analysis of the adjacent growth plate also revealed that following growth plate insult, the uninjured adjacent cartilage had a significant reduction in chondrocyte proliferation but an increase in apoptosis ([Macasai et al. 2011](#)). [Macasai et al. \(2011\)](#) also observed that the adjacent growth plate showed decreases in expression of chondrogenic transcription factor *Sox9* and growth factors TGF β 1 and IGF1 in comparison to their non-injured controls ([Macasai et al. 2011](#)). Similarly, [Musumeci et al. \(2013\)](#) also reported an increase in expression of apoptotic marker, caspase-3 protein, over days 7–30 post-injury in the adjacent growth plate of their experimental rats ([Musumeci et al. 2013](#)), although this study did not use an antibody that distinguishes between the intact inactive and cleaved active forms of caspase-3. [Musumeci et al. \(2013\)](#) suggest that one potential mechanism for this histomorphological change at the adjacent growth plate may be related to the observed increase in levels of inflammatory cytokine TNF α during the bony repair tissue formation at the growth plate injury site ([Musumeci et al. 2013](#)). While the above studies have demonstrated that the growth plate injury also affects the uninjured adjacent areas of growth plate, the current evidence is quite limited. So far, there has been no evidence reported whether and how the alterations in the adjacent area of growth plate can affect bone growth. More studies are required to characterise cellular and molecular changes that lead to the bone formation at the

adjacent cartilage and whether and how these changes contribute to the final bone growth defect outcomes.

Vascularisation of the growth plate injury site in bone bridge formation

Angiogenesis is critical for endochondral bone growth, bone modelling, bone remodelling, and fracture repair ([Brandi & Collin-Osdoby 2006](#), [Chim et al. 2013](#)). It is known that, after a 'fibrous tissue' is formed from the infiltrated stromal cells at an injured growth plate, its invasion by new blood vessels is a prerequisite for its osseous transformation ([Odgen 2000](#)). While the process of vascularisation is vital for the formation of new bone tissue and bony repair following growth plate injuries, the molecular mechanism for the growth plate injury site angiogenesis remains unclear.

VEGF is a known key mediator of angiogenesis and has roles in both bone-forming processes – direct (intramembranous) and indirect (endochondral ossification) ([Emad et al. 2006](#), [Dai & Rabie 2007](#), [Li et al. 2009](#)). Highlighting VEGF's essential role during bony repair, [Street et al. \(2002\)](#) found that in a mouse bone fracture model, the absence of VEGF delayed bone formation by halting the initial soft callus from being converted into hard bony callus. Interestingly, in a rat growth plate injury study, VEGF was detected as early as days 1 and 3 post-injury, suggesting that VEGF may have roles in the repair process to form the bony repair tissue ([Fischerauer et al. 2011](#)). A recent study has examined the potential role of VEGF-induced angiogenesis on bony tissue formation at the injured growth plate. Using a rat growth plate injury model and applying an anti-VEGF antibody treatment immediately following surgery, [Chung et al. \(2014\)](#) found that the numbers of blood vessel-like structures were significantly decreased at the growth plate injury site. Importantly, anti-VEGF treatment was found to increase the amount of undifferentiated mesenchymal tissue infiltrate remaining and to decrease the bony tissue present at the growth plate injury site. Consistently, gene expression analysis revealed significant decreases in expression of bone-related genes such as osteocalcin at the growth plate injury site ([Chung et al. 2014](#)). Overall, this study suggests that VEGF is vital for not only angiogenesis but also the formation of bony repair tissue at the growth plate injury site. This finding may present a potential target of intervention for developing biological therapies to block bone bridge formation at the injured growth plate ([Chung et al. 2014](#)).

Mechanisms of bony repair tissue formation at the injured growth plate

Intramembranous and endochondral ossification

Earlier rodent growth plate injury repair studies have suggested the intramembranous bone formation mechanism at the injured growth plate as examined at some time points. Lee *et al.* (2000) in a mouse model observed no changes in expression of endochondral ossification-related genes such as collagen-2, *Vegf*, or Indian hedgehog (*Ihh*). Using a similar drill hole growth plate injury model in rats, Xian *et al.* (2004) and Zhou *et al.* (2004) observed the presence of *Runx2*-immunopositive osteoblasts during the formation of bony trabeculae within the growth plate injury site and no up-regulation in chondrogenesis-related genes such as *Sox9* and collagen-2 (Xian *et al.* 2004, Zhou *et al.* 2004). However, following on from these studies, others have shown that in addition to the direct bone formation mechanism, the endochondral ossification indirect bone formation was also in play in some time points examined. Several studies in the rat growth plate injury model found increases in cartilage-related genes such as *Sox9* and collagen-2 alongside the increased expression in bone-related genes such as *Runx2* and osteocalcin (Arasapam *et al.* 2006, Chung *et al.* 2006, 2009). More importantly, significant increases in expression of collagen-10 were observed (Arasapam *et al.* 2006, Chung *et al.* 2006, 2009). Collagen-10 is expressed by hypertrophic chondrocytes during endochondral ossification. These studies thus indicate that both mechanisms of bone formation may be involved during growth plate bony repair.

Osterix and the involvement of protein kinase D during bony repair

Osteogenic transcription factors, *Runx2* and osterix, are well known to be essential for osteoblast differentiation during embryonic development (Komori *et al.* 1997, Nakashima *et al.* 2002, Day *et al.* 2005) and during postnatal bone growth and homeostasis (Zhou *et al.* 2010). In addition, increased osterix expression has been observed during bone fracture healing, and osterix over-expression can induce bone healing (Tu *et al.* 2007, Xu *et al.* 2009). A recent study examined the potential role of osterix in mediating the bony repair tissue formation following growth plate injury. Chung *et al.* (2013a,b) utilised a synthetic inhibitor, gö6976, to block protein kinase D (PKD) during growth plate injury repair in a rat

model. PKD has previously been shown to up-regulate osterix and be important for osteoblast differentiation (Celil & Campbell 2005, Jensen *et al.* 2009). This study found that inhibition of PKD suppressed bony repair but induced more chondrogenic differentiation at the injury site (Chung *et al.* 2013a,b). Consistent with this finding, Kaback *et al.* (2008) found that the over-expression of osterix in osteochondroprogenitor cells resulted in a decrease in chondrogenic transcription factor *Sox9*. These studies suggest that osterix may have a role in inhibiting chondrogenic differentiation while encouraging osteoblast differentiation. However, more studies are required to elucidate the role and importance of PKD and osterix during growth plate bony repair, and whether PKD and osterix may potentially be a target for interventions to block bony repair but to increase cartilage healing following growth plate injury.

Involvement of BMP and Wnt signalling pathways for the growth plate bony repair

BMPs are known key regulators for skeletal repair (Sakou 1998). In particular, BMP2 is consistently up-regulated during the initial stages of bone fracture healing (Yaoita *et al.* 2000, Marsell & Einhorn 2009) and, as shown by mutant mouse studies, is critical for bone fracture healing (Tsuji *et al.* 2006). In addition to its role in the recruitment of osteoprogenitor cells into a bone fracture site, BMP signalling is ultimately required for their differentiation into osteoblasts (Ghodadra & Singh 2008). Therapeutically, BMP2 has been shown to induce the bone healing response in many animal models of critical-sized segmental non-union defects in both small and large animals (Kirker-head & Gerhart 1998, Murakami *et al.* 2002). However, roles of BMPs in growth plate repair remain largely unknown and have only started to be addressed recently. In a rat tibial drill hole growth plate injury model, levels of *Bmp2* mRNA expression were found notably increased in the early part of the fibrogenic phase and then again later during the osteogenic phase (Macasai *et al.* 2012). Similarly, Ngo *et al.* (2006) also detected BMP2 protein expression immunohistochemically within the injury site during the fibrogenic and osteogenic responses. Furthermore, a microarray study of a rat growth plate injury model has also identified BMP signalling pathway as one of the most active pathways during the osteogenic phase of the bony repair of the injured growth plate (Macasai *et al.* 2012). Overall, these gene expression studies suggest that BMP signalling may play an important role in regulating or mediating the

formation of the undesirable bony repair tissue at the injured growth plate, and further intervention studies are required to confirm whether indeed BMP signalling (particularly from BMP2) is required for the growth plate bony repair.

Another known integral signalling pathway during bone formation and fracture repair is the Wnt/B-catenin signalling pathway (Day *et al.* 2005, Chen *et al.* 2007, Macsai *et al.* 2008). Similarly, recent studies have demonstrated that Wnt/B-catenin signalling pathway is also important for growth plate bony repair (Macsai *et al.* 2012, Chung *et al.* 2013a,b). In a rat model study, B-catenin expression levels were found to be significantly up-regulated on day 4 in comparison to day 8 post-injury, and the Wnt/B-catenin signalling pathway was identified as one of the most active pathways involved in the growth plate bony repair (Macsai *et al.* 2012). More recently, when the Wnt/B-catenin signalling pathway was inhibited using a B-catenin inhibitor in rats within the growth plate injury, Chung *et al.* (2013a,b) observed a significant decrease in proportion of bony repair tissue and coincidentally an increase in chondrogenic differentiation within the injury site. Overall, with their notable essential roles in bone fracture healing, the above studies strongly suggest that both BMP and Wnt/B-catenin signalling pathways are important in regulating the growth plate bony repair. However, further studies are required to confirm this, to investigate their action mechanisms, and to explore whether they can be potential targets for interventions to block bony repair but to enhance cartilage regeneration.

Current corrective treatments for growth plate injuries

Currently, there are no known biological therapies for the prevention of bony repair following growth plate injuries. At this present time, dependent on the severity and types of the injury, bone growth defect outcomes, and the age of the patient, some bone growth defects resulting from growth plate injuries are managed by corrective surgeries, including limb lengthening and interpositional implantation of materials. Limb lengthening is used to correct limb length discrepancies using an external frame or scaffold such as the Ilizarov frame to stabilise and stretch the limb following surgical fracturing. However, requiring multiple pins and involving an invasive surgically created fracture, this procedure is riddled with issues such as pin site infections and lengthy lengthening time. More recently, another bone lengthening procedure using an

internally implanted nail called 'Fitbone' is being utilised (Baumgart 2009), but it can only be used in adults. Furthermore, bone angulation deformities are often treated via the insertion of various interpositional materials following the surgical removal of bony repair tissue at the growth plate injured site. Referred to as the Langenskiöld method (Langenskiöld 1981), this procedure has previously been done with various interpositional materials for implantation, including fat, muscle, bone wax, cement and polymeric silicone materials (Tobita *et al.* 2002). Currently, there have been no biological treatments that are clinically used to prevent the growth plate faulty repair and to prevent the associated bone growth defects. However, in the last 2 decades, various experimental studies have been carried out attempting to develop chondrocyte or MSC-based biological therapies for growth plate regeneration.

Previous studies and current advances in cartilage tissue engineering for growth plate regeneration

Advances in cartilage tissue engineering include developments in areas such as autologous cell transfers, direct chondrocyte transplantations, MSC transplantations, as well as advances in the way these are implanted into the desired sites using various carriers such as natural biomaterials, synthetic materials and hydrogel scaffolds in combination with a cocktail of chemotactic and cartilage-inducing growth factors. In addition, in more recent years the focus has also shifted in potentially utilising the patient's own endogenous MSCs to initiate a more desirable chondrogenic repair. The following sections have outlined some of these advances directly in relation to growth plate injury repair (Table 1).

Chondrocyte transplantation and the regeneration of injured growth plates

Owing to the difficulty in regenerating cartilage, earlier studies have attempted cartilage repair via allogeneic or autologous chondrocyte transplantations. The concept of this transplantation involves the collection of healthy chondrocytes from a donor or patient, the *ex vivo* expansion, followed by their re-implantation into the patient. As allogeneic chondrocyte transplantation involves the removal of chondrocytes from one host followed by the transplantation of chondrocytes into another host (Toolan *et al.* 1998), it has the obvious disadvantages of the high risk of disease transmission from one source to the other

Table 1 A summary of experimental studies of cell therapies for growth plate regeneration

Animal model	Cell type	Growth factor used	Scaffold used	Effect observed	Reference
Rabbit	Cultured chondrocytes	–	Agarose (0.3%)	Decreased bone angulation and length difference compared with agarose alone	Lee <i>et al.</i> (1998)
Rabbit	–	adIGF1 and adBMP2 vectors	Muscle interposition	adIGF1, decreased limb length discrepancy and adBMP2, bone bridge formed	Lee <i>et al.</i> (2002)
Rabbit	MSC from periosteum	–	Agarose	Limb angulation and length corrected	Chen <i>et al.</i> (2003)
Rabbit	BMMSCs	hTGFB3 (10 ng/ml)	Porcine skin gelatin	Degree of angulation deformity was reduced	Anh <i>et al.</i> (2004)
Rabbit	MSC from periosteum	–	Chitin	Reduced length discrepancy	Li <i>et al.</i> (2004)
Rabbit	BMMSCs	hTGFB1 (100 ng/ml)	Hyaluronan and collagen-1	Limb lengthening increased and angulation was decreased compared with scaffold alone	Planka <i>et al.</i> (2008)
Rabbit	–	hIGF1 (5.9 µg/scaffold)	PLGA scaffold	Histology revealed regeneration of cartilage (disorganised)	Sundararaj <i>et al.</i> (2012)
Miniature pig	BMMSCs	–	Chitosan and collagen-1	Limb length was greater compared with scaffold alone	Planka <i>et al.</i> (2012)
Miniature pig	Chondrocytes	–	Chitosan and collagen-1	Limb angulation was reduced compared with scaffold alone	Planka <i>et al.</i> (2012)
Ovine	BMMSCs	hTGFB1 (10 ng/ml)	Gelfoam sponge	Undesirable fibrous tissue formation at the injury site	McCarty <i>et al.</i> (2010)
<i>In vitro</i>	hBMMSCs	hTGFB1 and hBMP2	Agarose	Cells differentiated into growth plate-like structure	Schmitt <i>et al.</i> (2012)

(Detterline *et al.* 2005). Autologous chondrocyte transplantation, on the other hand, requires the removal of chondrocytes from one host followed by the re-implantation of *ex vivo*-expanded cells back into the same host.

Early experimental work showed that *ex vivo*-expanded chondrocytes can be successfully transplanted into a growth plate injury site and are able to form the growth plate-like columnar structures in a rabbit model (Bentley & Greer 1971). Similarly using rabbit models, Lee *et al.* (1998) showed that transplantation of *ex vivo*-expanded chondrocytes to the injury site can eliminate growth arrest and angulation deformities in treated limbs compared with the injured, untreated control limbs. In addition, they observed similar effects when the chondrocytes were either implanted immediately after the growth plate injury, or implanted following surgical removal of an existing bone bridge formed following a growth plate injury induced earlier (Lee *et al.* 1998). More recently, Jin *et al.* (2006) and Li *et al.* (2013) also in a rabbit growth plate injury model reported success in restoring the growth plate to its original form, preventing bone bridge formation and in possibly preventing early ossification and closure of the growth plate following transplantation of chondrocytes (Jin *et al.* 2006) or chondrocytes that were microencapsulated (Li *et al.* 2013) using a semipermeable membrane

shown to be able to reduce potential transplantation-associated immunological reactions (Koo *et al.* 2008, Revell & Athanasiou 2009).

Although many studies (including the above) have shown some degree of success in repairing the injured growth plate, it is interesting to highlight that the majority of these were done in rabbit growth plate injury models. However, one earlier study by Foster *et al.* (1990) performed in a larger ovine model suggested that chondrocytes directly isolated from the growth plate may possibly be used as an interpositional material after growth plate injury since the implanted chondrocytes prevented bone bridge formation (Foster *et al.* 1990). However, although the concept of chondrocyte transplantation may be promising and may be potentially useful for many situations requiring cartilage repair such as in arthritis, this technique's suitability remains questionable realistically in inducing regeneration of injured growth plate cartilage in humans. This is due to the source limitations with chondrocyte harvest, the time taken for the collection, and *ex vivo* expansion followed by the re-implantation into the injury site (Miura *et al.* 2002). By the time the chondrocytes are ready to use, it is likely that the growth plate injury site has already formed the undesirable bony repair. Thus an alternative source of cells or a different approach of

therapy is much needed for the prevention of the undesirable bony repair and regeneration of growth plate cartilage following growth plate injury in humans.

MSCs for growth plate injury repair

Owing to the limited feasibility of utilising chondrocyte transplantation techniques for the regeneration of growth plate cartilage, more recent studies have focussed on the suitability of utilising adult MSCs. Adult MSCs are capable of differentiating into cells of many tissues such as cartilage, bone, and fat. In addition, MSCs can be isolated from many different sources, including the periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, as well as the bone marrow (Xian & Foster 2006, McCarty *et al.* 2009). Interestingly, previous studies have observed that synovium-derived MSCs in particular had the greatest capability to enhance the chondrogenic differentiation potential when compared with any other mesenchymal tissue-derived cells (Sakaguchi *et al.* 2005). However, other studies have reported that bone marrow-derived MSCs (BMMSCs) are most suitable for cartilage tissue engineering, as they possess higher proliferation rates and higher levels of expression of cartilage-specific genes, when compared with MSCs derived from other tissues (Park *et al.* 2006, Schmitt *et al.* 2012). In addition, due to their location and abundance, BMMSCs in particular seem ideal for growth plate cartilage regeneration (Caplan 1991, Provot & Schipani 2005, Xian & Foster 2006). More recently, MSCs were successfully isolated directly from murine epiphysis. Cheng *et al.* (2012) suggest that this novel type of MSCs could potentially be better than BMMSCs as they have shown greater capacities in growth and differentiation potential as well as possessing immunosuppressive and anti-inflammatory properties (Cheng *et al.* 2012).

In the last decade, studies have looked into the possibility and suitability of applying MSCs for inducing growth plate cartilage regeneration. Chen *et al.* (2003) observed that MSCs derived from the periosteum were able to completely correct bone angulation as well as regenerate the growth plate in a rabbit model (Chen *et al.* 2003). Hui *et al.* (2005) also reported some success in utilising MSCs to decrease limb length discrepancy in a rabbit model (Hui *et al.* 2005). Similarly, Yoshida *et al.* (2012) found less bone bridge formation and reduced limb length discrepancies following implantation of synovium-derived MSCs into a rabbit growth plate injury model (Yoshida *et al.* 2012). In addition to the above smaller animal models, Planka *et al.* (2012) also recently implanted allogeneic MSCs into a

miniature pig growth plate injury model and found that some growth disturbances were able to be prevented in addition to a decrease in bone angulation deformities. Planka *et al.* (2012) also observed repair tissue at the injury site being similar to hyaline cartilage with possible growth plate-like column structures. In a larger ovine growth plate injury repair model, McCarty *et al.* (2010) studied the efficacy of autologous MSC transplantation in repairing the injured growth plate. Following the transplantation of autologous MSCs into a proximal tibial growth plate injury site, McCarty *et al.* (2010) observed no increase in cartilage tissue formation 5 weeks post-transplantation. Instead, there was an increase in fibrous tissue formation in the animals treated with MSCs. The authors, however, highlighted that the transplantation did not increase the amount of osteogenesis or undesirable bony repair tissue. So far, although there have been some advances in utilising MSCs for growth plate regeneration, attenuation of undesirable bony repair, and/or prevention of the associated angulation and limb length deformities, these have been achieved mainly in the rabbit models. The lack of cartilage regeneration induced by autologous MSCs in the ovine growth plate injury model (McCarty *et al.* 2010) has highlighted that more work is needed to explore the potential of MSCs for growth plate regeneration in large animal models.

Challenges and future prospects for growth plate regeneration using MSC-based therapy

Similar to most tissue engineering/cell-based therapies for bone/articular cartilage repair, more work is required to optimise strategies to solve the three major requirements for successful growth plate cartilage regeneration: optimal sources of MSCs and their expansion; optimal signal molecules that induce chondrogenesis; and matrix scaffold that supports cartilage regeneration and recovery of growth plate zonal structure. Although limited, growth plate cartilage regeneration-specific studies have shown some success in various animal models when used in combination with cells such as MSCs, chondrogenic growth factors and supporting scaffolds (Table 1).

MSCs and their sources for growth plate regeneration

Specific for growth plate regeneration, the suitability of the MSCs seems to rely heavily on their source of isolation. Using a rabbit growth plate injury model, Hui *et al.* (2005) compared the effectiveness of MSCs of different sources for growth plate regeneration and found that MSCs isolated from both the bone marrow and periosteum were able to

correct the angulation and prevent further bony repair and to differentiate into organised growth plate-like chondrocytes at the injury site. On the other hand, treatment with MSCs that were isolated from adipose tissue formed disorganised chondrocytes within the growth plate injury site of the rabbits (Hui *et al.* 2005). Furthermore, Planka *et al.* (2008) examined the differences in suitability and efficiency of both autologous and allogeneic transplanted MSCs and found no significant differences between the two types of MSC transplantations in correcting limb length discrepancy and angulation as well as phenotype of the cartilage repair tissues formed within the growth plate injury site in rabbits.

In addition to finding the most appropriate source of MSCs for growth plate regeneration, another clinical challenge involves getting sufficient amounts of cells for implantation. As chondrogenic differentiation favours a dense and packed cell environment, MSC transplantation of a lesser cell density may not create a favourable environment for optimal growth plate regeneration. Current animal models showing significant repair effects on angulation, length discrepancy and chondrocyte regeneration have utilised cell numbers between 1.6×10^6 and 4×10^6 cells depending on the size of defects and types of the animal models (Ahn *et al.* 2004, Hui *et al.* 2005, McCarty *et al.* 2010). However, finding an optimal cell density and optimising conditions for MSC *in vitro* expansion to achieve a clinically relevant number of cells for implantation still need to be thoroughly investigated for successful growth plate cartilage regeneration.

Growth factors or their combination for growth plate regeneration using MSCs

MSCs are heavily influenced by and require signals (particularly growth factors) for their migration, proliferation and differentiation. In regard to growth plate cartilage repair, ideally, a combination of growth factors would firstly assist with optimal expansion of MSCs and then with the induction of chondrogenic differentiation. Some of the growth factors that have previously been studied for their MSC mitogenic properties include PDGF, IGF1, FGF2 and TGF α (McCarty *et al.* 2009), and growth factors with known chondrogenic properties include FGF2, TGF β 1, TGF β 3, BMP7 and IGF1 (Lennon *et al.* 1995, Yamaguchi 1995, Johnstone *et al.* 1998, Worster *et al.* 2000, 2001, McCarty *et al.* 2009). FGF2 has been shown to have potent mitotic properties as well as encouraging MSCs differentiating towards a chondrogenic differentiation (Solchaga *et al.* 2005). Moreover, rat BMSCs that were initially treated with FGF2 during cell expansion underwent chondrogenesis once placed in a 3D

agarose gel together with TGF β 1 (Coleman *et al.* 2007, 2013). On the other hand, cells that were not treated with FGF2 resulted in no production of cartilaginous matrix even with the addition of TGF β 1 (Coleman *et al.* 2013). Fukumoto *et al.* (2003) found that TGF β was able to induce a 20% increase in chondrogenesis in MSCs in an *in vitro* chondrogenic assay. TGF β 3 has been shown to stimulate chondrogenic differentiation as well as increase the expression of various cartilage matrix molecules in MSCs (Indrawattana *et al.* 2004, Tang *et al.* 2009). Ahn *et al.* (2004) reported success in reducing angular deformity in a rabbit growth plate injury model using TGF β 3 alongside MSCs embedded in Gelfoam. However, when repeated in a larger animal model, these reported chondrogenic effects of MSCs and TGF β 1 were not found in an ovine growth plate injury model (McCarty *et al.* 2010).

In order to elicit a more potent chondrogenic differentiation response from MSCs, the combinational use of growth factors can often increase chondrogenesis. Fukumoto *et al.* (2003) showed that by combining TGF β 1 and IGF1, a significant 20% increase in cartilage production was observed when compared with treatment with either growth factor alone. This enhancing effect is thought to be related to the fact that while IGF1 is essential for the differentiation and maturation of growth plate chondrocytes, TGF β is more useful in inducing chondrogenesis (Indrawattana *et al.* 2004, Jaklenec *et al.* 2008). McCarty *et al.* (2009) also found that the combination of BMP7 with TGF β 1 produced larger chondrogenic pellets when compared with those treated with TGF β 1 alone. Overall however, more studies are needed to further identify the most optimal mixture of growth factors as well as their delivery to better induce growth plate cartilage regeneration from MSCs.

Matrix scaffolds for growth plate regeneration using MSCs

In addition to growth factors, another challenge for growth plate regeneration is to find the most appropriate carrier support systems (matrix scaffolds) for MSC transplantation and their chondrogenic differentiation. A matrix scaffold essentially acts as a support structure for MSCs, as a delivery and stability vehicle for growth factors, as well as influencing the eventual differentiation of these cells. Ideally, they should be biodegradable, biocompatible, promoting cell attachment, as well as allowing for tissue formation (Hutmacher 2000, Drury & Mooney 2003). Planka *et al.* (2012) embedded MSCs into a chitosan and collagen combined scaffold. Following the implantation of this

construct into the growth plate injury site of a miniature pig model, Planka *et al.* (2012) observed some success in preventing limb length discrepancy as well as minimising angulation deformities. In addition, the injury sites also showed signs of hyaline-type cartilage repair (Planka *et al.* 2012). An earlier study by Li *et al.* (2004) in a rabbit growth plate injury model also found some success with MSCs embedded in chitosan gel in correcting angulation deformity and length discrepancy in their rabbit growth plate model following the removal of the initial bone bridge. Furthermore, Schmitt *et al.* (2012) observed that following sequential additions of both TGF β and BMP2, BMSCs in an agarose scaffold formed differentiated chondrocytes *in vitro* with zonal alignment, which mimic growth plate-like zonal structure. However, more studies are still required to find the optimal combination of MSCs, growth factors, and supporting matrix scaffold to induce regeneration of injured growth plate cartilage with proper zonal structure and functional recovery.

Potential *in situ* approach for growth plate regeneration mobilising endogenous MSCs

Although autologous or allogeneic MSC transplantations offer a clear advantage over direct chondrocyte transplantation for potential growth plate regeneration, there are still many limitations with their use, including laborious isolation and *ex vivo* expansion, time required to have sufficient numbers of cells for transplantation, the need for ingredients such as foetal calf serum, associated expenses and potential immunoregulatory issues following implantation (Xian & Foster 2006). Since bone formation and bony repair occur rapidly within the growth plate injury site (starting after 1 week following the injury), there is the possibility that the long length of time it takes for MSC harvest, *in vitro* expansion and implantation could limit the usefulness of this *in vitro* MSC expansion/transplantation cell-based therapy for growth plate regeneration initially after growth plate injuries. Ultimately, potential mobilisation and use of a patient's own endogenous MSCs to regenerate the growth plate injury site immediately following injury would be ideal with the development of an *in situ* endogenous progenitor cell therapy, which can potentially circumvent all these limitations of the *in vitro* MSC approach (De Bari *et al.* 2003, Evans *et al.* 2007) as well as the need for the surgical removal of the formed bony repair tissue later on. However, more work in future on this area is needed to identify the most optimal signals and support to mobilise sufficient amounts of endogenous MSCs into the growth

plate injury site and promote their expansion and cartilage regeneration at the growth plate injury site.

More studies in large animal models

While tissue engineering/cell therapy has become a promising way of enabling growth plate regeneration in growth plate injury repair models in rabbits, more studies are needed to be carried out in large animal models that would mimic human growth plate anatomy, regeneration potentials and clinical outcomes more closely. Currently, there are only very limited studies that have examined the effects of chondrocyte transplantation as well as MSC transplantation using the ovine growth plate injury model, which saw limited success in growth plate regeneration using implanted chondrocytes or autologous MSCs (Foster *et al.* 1990, McCarty *et al.* 2010). Although direct transplantation of growth plate chondrocytes elucidated some positive effects including the prevention of bony repair tissue, the cellular survival rates of the implants were unpredictable, making them not ideal for clinical use (Foster *et al.* 1990). On the other hand, following autologous MSC transplantation in the presence of exogenous TGF β 1 in this ovine growth plate injury model, McCarty *et al.* (2010) observed undesirable fibrous tissue formation rather than cartilage regeneration. Overall, the above studies in the larger animal models have not resulted inasmuch success and positive outcomes as observed in similar growth plate injury models in rabbits. This highlights the need for the use of larger animal models to evaluate tissue engineering constructs/MSC-based cell therapies in regenerating injured growth plate cartilage before an optimal MSC-based cell therapy can be developed for human usage.

Conclusions

Injuries to the growth plate are common occurrences and in many cases will result in undesirable bony repair at the injury site causing bone growth defects and lifelong orthopaedic problems. While the currently used surgical techniques can to some extent 'correct' some associated orthopaedic issues, they are highly invasive and often ineffective requiring repeated procedures. In the last 15 years, experimental studies with animal models such as the rat growth plate injury model have been able to observe the phases of growth plate injury repair responses leading to the undesirable bony repair at the injured growth plate. In addition, recent studies in a rat growth plate injury remodel have uncovered the important roles of PDGF as a potent chemotactic agent

promoting infiltration/proliferation of mesenchymal progenitor cells, osteoprogenitors and/or chondroprogenitors into the injury site as well as the critical role of VEGF as a key angiogenic factor involved in new blood vessel formation important for the bony repair of the injured growth plate. Furthermore, recent work has identified up-regulation of both BMP and Wnt/B-catenin signalling pathways accompanying the osteogenesis response at the injured growth plate and blockage of B-catenin signalling has been shown to inhibit the undesirable bone bridge formation. The findings of these important signals for bony repair represent some advances in the understanding of the mechanisms for growth plate bony repair. They, in combination with recent cartilage tissue engineering advances, could also potentially be explored in future studies for the development of an *in situ* cell-based therapy for growth plate regeneration. In addition, these new findings of the signals important for growth plate bony repair can also be explored by the current MSC-based tissue engineering approach for identifying potential 'optimal growth factors and osteogenesis/angiogenesis inhibitors' so to enhance the likely success of the implanted exogenously expanded MSCs in regenerating the injured growth plate. Further work is required to study the molecular signals that lead to growth plate bony repair. Additional studies are required to find the appropriate and most optimal combination of chemotactic growth factors and scaffolds that will aid in devising an *in situ* biological strategy mobilising endogenous MSCs for blocking bony repair and inducing growth plate regeneration. Furthermore, although there have been many promising results in smaller growth plate injury models (such as the rabbit), more studies in larger animals are required to investigate the therapeutic effectiveness of MSC-based therapies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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