European Cells and Materials Vol. 37 2019 (pages 277-291) DOI: 10.22203/eCM.v037a17 ISSN 1473-2262

UNDERSTANDING TISSUE-ENGINEERED ENDOCHONDRAL OSSIFICATION; TOWARDS IMPROVED BONE FORMATION

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Abstract

Endochondral ossification (EO) is the process by which the long bones of the body form and has proven to be a promising method in tissue engineering for achieving cell-mediated bone formation. The present review centred on state-of-the-art research pertaining to mesenchymal stem cells (MSCs)-mediated endochondral bone formation, focusing on the role of donor cells, extracellular matrix and host immune cells during tissue-engineered bone formation. Possible research avenues to improve graft outcome and bone output were highlighted, as well as emerging research that, when applied to tissue-engineered bone grafts, offers new promise for improving the likelihood of such grafts transition from bench to bedside.

Keywords: Endochondral ossification, bone tissue engineering, regenerative medicine, mesenchymal stem cell differentiation, extracellular matrix, vascularisation, collagen type X, immune system.

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List of Abbreviations

ALP	alkaline phosphatase
ANG1	angiopoietin 1
BMP2	bone morphogenetic protein 2
CCN2	cellular communication network factor 2
COLX	collagen type X
CTACK	cutaneous T-cell attracting chemokine
CTGF	connective tissue growth factor
DCs	dendritic cells
ECM	extracellular matrix
EO	endochondral ossification
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GDF5	growth and differentiation factor 5
hPLAP	human placental alkaline phosphatase
HMGB1	high mobility group box 1
HSPG	heparin sulphate proteoglycan
Ihh	Indian hedgehog homologue
IL	interleukin
IMO	intramembranous ossification
KO	knock-out
MHC	major histocompatibility complex
MMPs	matrix metalloproteinases
MSC	mesenchymal stem cell/marrow stromal cell
NK	natural killer
PDGFA	platelet-derived growth factor subunit A
PLGA	poly(D,L-lactic-co-glycolic acid)
PRP	platelet-rich plasma
PTHrP	parathyroid hormone-related protein
RANKL	receptor activator of nuclear factor kappa-B ligand

Runx2	runt-related transcription factor 2
SMCD	Schmid metaphyseal chondrodysplasia disorder
TERM	tissue engineering and regenerative medicine
Tg	transgenic
TGF	transforming growth factor
TNFα	tumour necrosis factor alpha
VEGF	vascular endothelial growth factor
WNT	wingless-type MMTV integration site family

Introduction

Bone has an inherent ability to repair itself following small injuries (Schindeler *et al.*, 2008). However, when a critical-size defect exists, or is created following surgery, the bone regenerative capacity is exhausted, making clinical intervention necessary. As a result, bone is one of the most commonly transplanted tissues in the world (Petite *et al.*, 2000). Autologous bone grafts are the current gold-standard treatment option for such defects as they are a natural osteoinductive/osteoconductive material (Frohlich *et al.*, 2008; Lieberman and Friedlaender, 2005) with low risk of immune rejection (Bauer and Muschler, 2000). Although roughly 90 % of autologous grafts are considered to be successful (Bauer and Muschler, 2000; Hayden *et al.*, 2012), their use is limited due to the availability of harvestable material, uncertain integration following implantation and risk of donor site morbidity (Bauer and Muschler, 2000). Although allogeneic and xenogeneic grafts are available, they are associated with other risks, including disease transfer or immunological rejection (Gómez-Barrena *et al.*, 2015). Common complications associated with bone grafts – regardless if autologous, allogeneic or xenogeneic – include insufficient vascularisation at the implant site, leading to poor nutrient/oxygen delivery, cell death and core necrosis (Frohlich *et al.*, 2008; Lieberman and Friedlaender, 2005). This highlights a clear and present need for new suitable graft alternatives.

TERM-based approaches to bone repair vary greatly (Grskovic *et al.*, 2012). Bioactive or inert materials (Table 1), which should enhance bone regeneration by guided tissue regeneration, are currently being developed. Although promising, many of these materials and other TERM approaches also rely on the use of iliac crest bone, which does not address the many issues surrounding the use of autologous bone. The use of various adult progenitor cells to create cell-based alternatives recapitulating one of the developmental pathways of bone formation to achieve bone regeneration and repair of critical-size bone defects has received much attention in recent decades. This review focuses on the state-of-the-art strategies implemented in cell-based TERM and on considerations for improved bone regeneration and output.

Cell-based strategies for bone repair; endochondral *vs.* intramembranous ossification

Bone develops through either IMO (Kim *et al.*, 2011) or EO (Shapiro, 2008; Yang *et al.* 2009). IMO involves the direct differentiation of mesenchymal cells to osteoblasts, the process by which most facial bones are formed (Thompson *et al.*, 2002). IMO can be achieved in TERM by either direct differentiation or through the combination of MSCs with biomaterials (including, but not limited to, tricalcium phosphate or collagen sponges) (Meijer *et al.*, 2008). Although promising, this approach has not reached its full potential due to insufficient vascularisation of the implant, resulting in core necrosis (Chatterjea *et al.*, 2010; Meijer *et al.*, 2008). Vascularisation is crucial for graft survival and is required for proper integration with the patient's existing bones. Instead, EO is a more promising model for bone formation as it naturally induces vascularisation at the implant site (Cervantes-Diaz *et al.*, 2017; Gawlitta *et al.*, 2010; Mackie *et al.*, 2008; Medici and Olsen, 2012; Thompson *et al.*, 2015; Yeung Tsang *et al.*, 2014).

EO relies on the establishment of a cartilage template, which is achieved by condensation and differentiation of mesenchymal cells (Mackie *et al.* 2008). Chondrocytes within the template exhibit a zonal distribution, with clear divisions between the different stages of chondrocyte differentiation within the template. Resting chondrocytes display a seemingly sporadic distribution and are thought to maintain a population of cells which, when triggered, give rise to the more organised, disk-like proliferating chondrocytes (Schrier *et al.*, 2006; Yang *et al.*, 2003). Proliferating chondrocytes contribute to longitudinal bone growth and are regulated by a complicated feedback loop, including factors such as TGFβ, PTHrP and Ihh (Chen *et al.*, 2008; Kronenberg *et al.*, 2003). These factors are also involved in initiating hypertrophic differentiation. When hypertrophic differentiation starts, chondrocytes secrete factors to recruit other cell types critical for successful EO (Ballock *et al.*, 2003; Kronenberg *et al.*, 2003) (Fig. 1). For example, factors such as ANG-1, PDGFA and VEGF will aid in the recruitment of the nearby vasculature to the cartilage template (Colnot *et al.*, 2001), which will ultimately result in the delivery of pre-osteoblastic cells to the cartilage template (Maes *et al.*, 2010). Factors released by the hypertrophic chondrocytes, including MMPs and other proteolytic enzymes, will contribute to early matrix remodelling (Nishimura *et al.*, 2012); release of RANKL and VEGF will recruit osteoclasts, which further contribute to proper matrix remodelling (Engsig *et al.*, 2000). Together, osteoblasts delivery through the invaded vasculature, trans-differentiation of chondrocytes in the cartilage template and osteoblast invasion from the surrounding bone collar calcify the cartilage matrix and bone formation occurs (Maes *et al.*, 2010; Yang *et al.*, 2014). The coordination of these events with cell/vascular recruitment ultimately controls effective

Table 1. Bone-graft-related terminology and definitions/examples.

Term	Definition	Reference
Osteoinductive material	Can induce osteogenic differentiation of primitive cells; induces bone formation. Process that is observed during bone repair (healing).	Finkemeier, 2002 Lee, 2016
Osteoconductive material	Causes bone formation on the surface of a material; induces migration of bone-forming cells to the surface of the material; observed regularly on bone implants; examples: hydroxyapatite, tricalcium phosphate.	Finkemeier, 2002 Lee, 2016
Inert material	Not chemically active; material does not join/integrate directly with bone; example: titanium, steel.	LeGeros, 2008 Roselló Llabrés *et al.*,
Bioactive material	Causes a biological response allowing for tissue bonding to the material; surface reactivity influences ability to bond to bone; example: bioactive glass and ceramics.	Ducheyne *et al.*, 1999
Allogeneic graft	Tissue or cells obtained from donor material of same species as recipient; osteoinductive and osteoconductive; can be fresh or frozen.	Roselló Llabrés *et al.*, 2014
Autologous graft	Tissue or cells obtained from patient receiving treatment; osteoinductive and osteoconductive.	Roselló Llabrés *et al.*, 2014
Xenogenic graft	Tissue or cells obtained from a non-human source; example: bovine, porcine.	Roselló Llabrés *et al.*, 2014

bone formation in EO. This can be recapitulated in TERM by differentiating MSCs chondrogenically and implanting them subcutaneously either as pellets or seeded in scaffolds (Knuth *et al.*, 2017; Scotti *et al.*, 2010; Tonnarelli *et al.*, 2014; van der Stok *et al.*, 2014). This seems to mirror developmental EO and shows excellent integration within the host tissue (Chan *et al.*, 2009). Tissue-engineered EO, utilising MSCs, has been proven as a viable method to achieve bone formation (Dickhut *et al.*, 2009; Hennig *et al.*, 2007; Janicki *et al.*, 2010; Jukes *et al.*, 2008; Karoliina *et al.*, 2008) (Fig. 2). Huang *et al.* (2006) demonstrated the ability of chondrogenically-primed MSCs, loaded into a hyaluronan/gelatine scaffold, to form bone. Bahney *et al.* (2014) and van der Stok *et al.* (2014), each independently, demonstrated how these chondrogenic MSCs could also be used to partially repair a critical-size defect even without a biomaterial support. Interestingly, this has been shown to be specific for chondrogenically-differentiated MSCs, as chondrocytes following expansion and differentiation will not form bone or bone marrow *in vivo* despite similar culture characteristics. Whether this is associated with the developmental origin of these cells or their expression of specific proteins, such as COLX – a hypertrophy-associated collagen not expressed by culture-expanded chondrocytes – is unknown (Hellingman *et al.*, 2011; Pelttari *et al.*, 2006; Pleumeekers *et al.*, 2014). It is also possible that chondrocytes do not interact with cells of the host in a similar fashion. In order to develop better TERM approaches to bone defect repair recapitulating EO, understanding how MSC-mediated EO occurs and the kinetics of the process is necessary.

The donor's role: recruitment of the host and long-term involvement

Induction of vascular invasion, *de novo* formation of a marrow cavity and osteoclast activity observed in tissue-engineered constructs have demonstrated that endogenous host cells have a role in new-bone formation (Farrell *et al.*, 2011; Tasso *et al.*, 2009; Tasso *et al.*, 2010; Tortelli *et al.*, 2010). Donor MSCs directly contribute to bone-forming cell populations in TERM EO. Using cell-labelling methods, implanted chondrogenically-differentiated MSCs have been shown to persist within the bone matrix and contribute directly to bone formation (Bahney *et al.*, 2014; Farrell *et al.*, 2011; Scotti *et al.*, 2013). Farrell *et al.* (2011) suggested that the initial bone formation is mediated by donor MSCs. Using immunocompetent transgenic rats overexpressing hPLA, donor cells were tracked following implantation into syngeneic wild type rats. A mixed population of both positive and negative hPLAP cells found embedded within the bone matrix demonstrated that cells were of both donor and host origin. Scotti *et al.* (2013) further suggested that donor cells that persist in the newly formed bone may have undergone trans-differentiation to osteoblast-like cells. They reported that donor and host bone have a zonal distribution. Host cells contribute to bone formation in the outer periphery of the implant and donor cells in the central portion (Scotti *et al.*, 2013). Although Scotti *et al.* (2013) hypothesised that over time these donor cells would be replaced by host cells, Bahney *et al.* (2014) suggested that most of the bone formation is donor-derived. This research contrasts with the developmental situation where it is

believed that following hypertrophic differentiation of chondrocytes, apoptosis is their only fate, as shown in previous avian-based research (Gibson *et al.*, 1995). Recently, this theory has been challenged. Studies in development, fracture repair and TERM showed that hypertrophic chondrocytes do not all undergo apoptosis. Rather, a subset of them are plastic and capable of transdifferentiating into osteoblasts or osteoblast-like cells, further aiding in the process of bone formation (Bahney *et al.*, 2014; Yang *et al.*, 2014; Zhou *et al.*, 2014). From a developmental point of view, Yang *et al.* (2014) showed that these transdifferentiated hypertrophic chondrocytes persist throughout development, being present not only in foetal bone but also in the bone of adult mice. These finds have changed how researchers view bone homeostasis in development and in TERM since chondrocytes do contribute to bone formation. In tissue engineering, there is a trend towards development of acellular grafts which are, indeed, attractive from a clinical perspective. However, knowing that implanted cells play an important role in bone formation, it may be necessary to rethink such approaches in order to maximise bone output. Certainly, in more challenging clinical situations.

The role of the ECM in MSC-mediated EO

During chondrogenic differentiation of MSCs, a bioactive matrix is produced which can greatly influence EO *in vivo*. Studies have suggested that the quality of the pre-implantation matrix influences *in vivo* bone formation. Scotti *et. al* (2010) reported that after longer priming, more chondrogenic induction and GAG production is achieved, resulting in better bone formation following implantation. Knuth *et al.* (2017) reported how stronger chondrogenic induction can influence *in vivo* bone formation; however, they hypothesised that a more GAG-rich matrix delays bone marrow formation due to delayed remodelling. Perhaps this indicates that parameters, using ECM components produced by chondrogenically-differentiated MSCs, can be set to assess bone formation; nevertheless, performing this without destroying the pellet would be difficult. Recently, Correa *et al.* (2015) have suggested that chondrogenic potential can be influenced through the addition of certain FGFs which modulate TGFβ receptors, in turn altering GAG concentration. If so, researchers could utilise this approach to alter GAG production within the pre-implantation constructs. However, research in this area has yielded conflicting data and how TGFβ receptor modulation influence ECM production by MSCs is still an area of ongoing investigation (Correa *et al.*, 2015; de Kroon *et al.*, 2015).

When trying to further understand how the ECM influences EO, valuable insight comes also from the use of chondrogenically-differentiated MSCs, not to achieve EO but as a tissue engineering cartilage replacement. Chondrocytes formed through differentiation of MSCs as compared to native chondrocytes exhibit clear differences in structure, ECM deposition, cellular phenotypes and mechanical properties (as reviewed by Somoza *et al.*, 2018). Researchers are investigating how they can prevent tissue-engineering MSC cartilage constructs from forming bone *in vivo*. For instance, suppression of canonical WNT signalling during chondrogenic differentiation results in less hypertrophic constructs, containing less COLX in the ECM, which has a negative effect on bone formation *in vivo* (Narcisi *et*

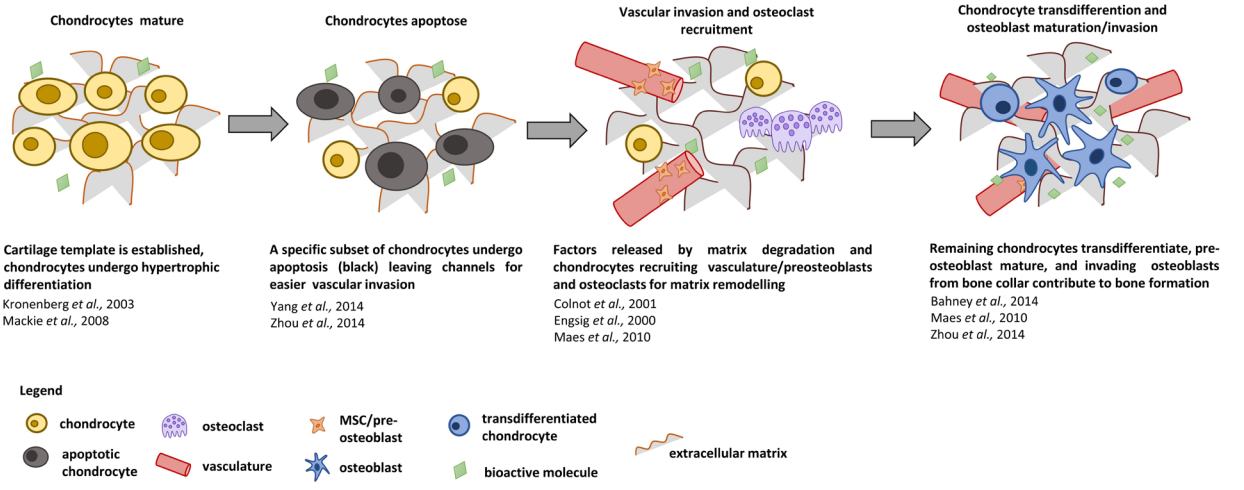

Fig. 1. Snapshot of cellular invasion and behaviour during developmental EO. Following the establishment of the cartilage template, a specific subset of hypertrophic chondrocytes undergoes apoptosis. This creates space for the nearby vasculature to invade and release bioactive molecules within the matrix. At the same time, pericytic-like pre-osteoblasts, attached to the side of the vasculature, invade it by passive migration. Factors released from the degraded ECM further aid in the recruitment of matrix-remodelling osteoclasts. The non-apoptotic chondrocytes found within the matrix are capable of trans-differentiating into osteoblast-like cells that, in combination with mature osteoblasts, contribute to bone formation.

al., 2015). This may indicate that, for improved bone formation, the enhancement of the WNT signalling pathway during chondrogenic differentiation would have a beneficial effect on ECM and cell behaviour for bone repair. Importantly, Narcisi *et al.* (2015) also highlighted the importance of hypertrophic differentiation for the induction of bone formation with MSC-based endochondral grafts.

Developmentally, hypertrophic differentiation precedes mineralisation and, during this phase, 45 % of the collagens produced is COLX (Luvalle *et al.*, 1992). COLX adds to the structural stability in the surrounding pericellular network of hypertrophic chondrocytes (Schmid and Linsenmayer, 1985; Shen, 2005) but, from a bone formation stand point, its role can be more clearly seen in Tg and KO studies. In such studies, perinatal death is reported in the absence of COLX (around 25 % in Tg mice and 10 % in KO mice), with the surviving mice exhibiting a range of phenotypes including dwarfism, skeletal abnormalities, defective haematopoiesis or normal phenotype (Campbell *et al.*, 2004; Jacenko *et al.*, 2001; Jacenko *et al.*, 2002; Kwan *et al.*, 1997). The absence of COLX has an impact on the normal skeletal development in mice, but the exact mechanisms contributing to each of these abnormalities needs to be further explored to truly understand how COLX contributes to bone formation and the supportive role it plays during the process. In the absence of COLX, abnormal GAG distribution and decreased HSPG content around hypertrophic chondrocytes is reported (Jacenko *et al.*, 2001). Proper proteoglycan distribution throughout the remodelled matrix is essential as it not only plays a role in stabilising the ECM, but also regulates the availability of growth factors trapped within the matrix that are crucial for EO, contributing to induction of blood vessel invasion and attraction of matrix remodelling cells, such as osteoclasts, in a timely manner (Kim *et al.*, 2011; Yang *et al.*, 2012). Proper ECM arrangement is not only important with regards to the above-mentioned aspects but also for proper placement of smaller structures, such as matrix vesicles.

Matrix vesicles are small structures that bud from the membrane of chondrocytes, osteoblasts and other cells. These structures carry with them, among other things, a collection of bioactive enzymes, proteins and phospholipids, specific to the cell they are produced from, that are important in the initiation of calcification (Anderson *et al.*, 2005; Golub, 2009; Lohan *et al.*, 2017). Matrix vesicles become entrapped in the ECM and help attract cells through their content (*i.e.* VEGF to attract blood vessels, BMPs to attract osteoblasts, *etc.*), making their point of anchoring and zonal distribution crucial for proper cell recruitment to the correct area (Kirsch *et al.*, 2000; Nahar *et al.*, 2008). Research has focussed on the interactions between COLX and annexin V, which is found on matrix vesicles. Annexin V facilitates calcium influx into matrix vesicles, which is important for the initiation of biomineralisation within the vesicles, in turn influencing matrix mineralisation and bone formation. COLX can selectively bind to annexin V, initiating this influx of calcium into the matrix vesicles (Kirsch and Pfäffle, 1992; Wu *et al.*, 1991). Others reported that when COLX is absent, vesicle distribution throughout the matrix is disrupted and subsequent bone formation is stunted (Kwan

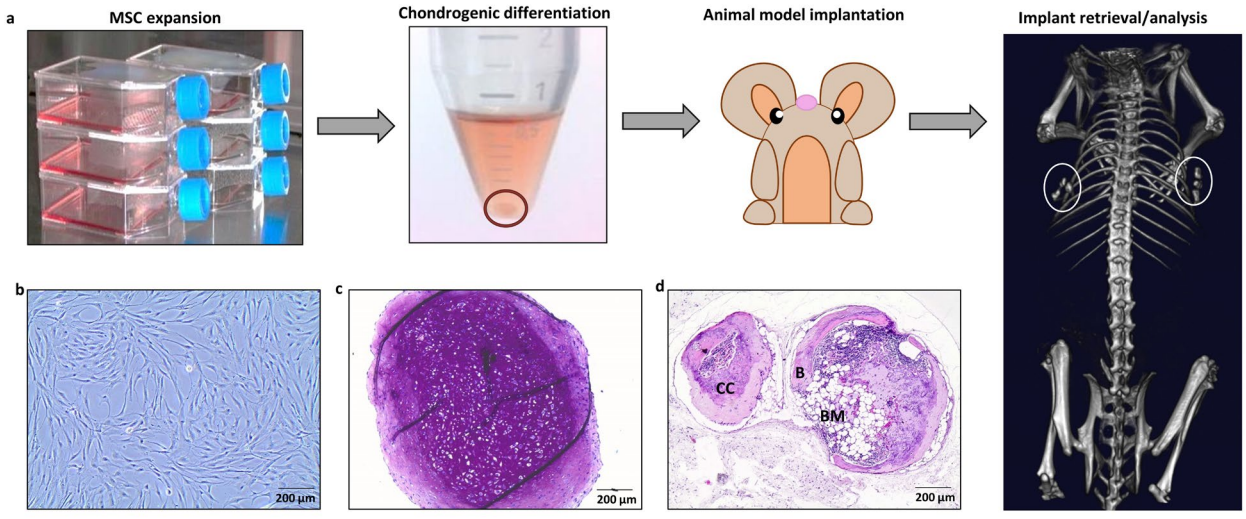

Fig. 2. Achieving tissue engineered EO. (**a**) MSCs are expanded to reach the required cell number through cell passage. Then, MSCs are chondrogenically-differentiated, usually through the addition of TGFβ, dexamethasone and vitamin C (here a chondrogenic pellet is shown in the red circle). Following differentiation, the resulting chondrocytes are implanted in an animal model for a predetermined time. Following implantation, the resulting construct can be retrieved and analysed (constructs in white circles). (**b**) Representative MSCs during expansion phase. (**c**) A representative thionine staining of MSCs chondrogenically-differentiated for 21 d trough pellet culture. (**d**) Haemotoxylin and eosin staining showing representative bone formed from chondrogenically-differentiated MSCs after 8 weeks of subcutaneous implantation in nude mice (B-bone, CC-calcified cartilage, BM-bone marrow).

et al., 1997; Wu *et al.*, 1991). This is alarming and shows that proper placement of matrix vesicles is required for cell attraction to the proper site of bone formation. However, this conclusion is challenged by others in the field who found that knocking out annexin V results in no change in mineralisation or bone formation (Grskovic *et al.*, 2012). Although initially these results appear to be contradictory, there could be a simple explanation. COLX plays a role in supporting and maintaining ECM proteoglycan and collagen organisation. When COLX is absent, these are no longer properly organised. Matrix vesicles also associate with the hyaluronic-acid-binding region found in proteoglycans, which can also result in calcium influx (Wu *et al.*, 1991). If COLX is absent, matrix vesicles can possibly associate more strongly with proteoglycans, allowing them to be entrapped in the matrix, possibly no longer specifically, at the border of the chondro-osseous junction, but still able to initiate mineralisation, thus allowing bone formation to still take place.

EO also influences proper development of the bone marrow niche and proper haematopoiesis, which studies have suggested is also partially regulated by COLX. Cytokines, chemokines and growth factors bind and interact with HSPG, in part regulating or controlling an immune response (Sweeney *et al.*, 2008; Wu *et al.*, 1991). COLX decrease correlates with a decrease in HSPG and a dysregulation of the immune system of Tg mice. Additionally, an increase in factors that play a role in regulating immune responses – including IL-4, IL-12, CTACK and leptin, which all bind to HSPG – and major changes to the immune system itself are shown. Mice with defective or missing COLX often have a severely decreased immune cell count. Although the immune cells that remain in the mouse often function properly, the immune response they elicit cannot be controlled, ultimately leading to death in immune-challenge studies (Sweeney *et al.*, 2008). When mice with defective/missing COLX are challenged with an opportunistic parasite, they can initially clear the parasitic infection but do not recover and ultimately die. *Post-mortem* investigation shows enlarged livers and increased parasite cysts in the brain, liver and lungs, both indicative of a malfunctioned immune response (Sweeney *et al.*, 2008). With a decreased HSPG count and an increased production of immune factors, the body is unable to regulate the response properly. Researchers have argued over the importance of COLX in regulating the immune response, as conflicting results have been shown (Kwan *et al.*, 1997; Rosatil *et al.*, 1994). However, the differences observed between researchers may also come down to the genetic profile of the models they used.

Osteoimmunology from a tissue engineering perspective

In large bone defects, the cells of the immune system play an important role. The complex interaction between cells of the skeletal system and the immune system is critical for successful bone repair and is initiated by an inflammatory response to the damaged tissue (Dar *et al.*, 2018; Kolar *et al.*, 2010; Mountziaris and Mikos, 2008; Pape *et al.*, 2010). This leads to the secretion of pro-inflammatory cytokines, including TNFα, IL-6 and IL-1β (Gerstenfeld *et al.*, 2003; Mountziaris and Mikos, 2008). These cytokines can induce angiogenesis and attract cells of the innate immune response (monocytes, macrophages, DCs, neutrophils and NK cells). Subsequently, those cells release specific cytokines and growth factors which attract cells of the adaptive immune system (T and B cells) (Kovach *et al.*, 2015). Immune cells are not the only cells attracted during this inflammatory

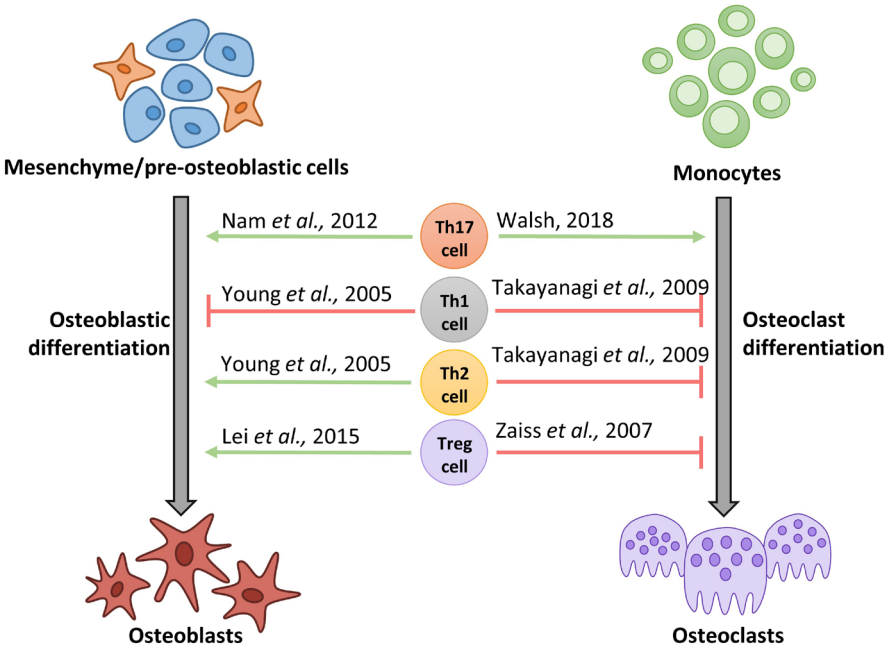

Fig. 3. T cells can influence osteoblastic and osteoclastic maturation. The release of cytokines and various growth factors during bone formation and fracture repair results in the recruitment of various immune cells which can influence bone formation and remodelling (green arrow: positive influence, red bar line: negative influence).

response. Bone-specific growth factors such as TGFβ and BMP-2 are also secreted, leading to the recruitment of osteoprogenitor cells (including MSCs) to the site of inflammation (Kovach *et al.*, 2015). The combined expression of growth factors with secretion of inflammatory mediators induces the proliferation and differentiation of osteoprogenitor cells to osteoblasts (Dimitriou *et al.*, 2011; Loi *et al.*, 2016; Mizuno *et al.*, 1990). IMO and EO are the two processes by which osteoprogenitor cells can differentiate to osteoblasts. Unlike in IMO, during EO, the secretion of TGFβ2 or 3, BMPs and other signalling molecules, each of which can be influenced by immune cells, leads to the formation of a cartilage template that is replaced by woven bone (Brighton, 1984; Einhorn and Gerstenfeld, 2015; Gerstenfeld *et al.*, 2003; Kolar *et al.*, 2010; Kuntzman, 2010). Most fractures heal through EO and previous studies have demonstrated the importance of the immune system during the repair process; lymphocytes, in particular, are crucial for fracture healing (Schindeler *et al.*, 2008). During bone remodelling, infiltrating T and B cells into the fracture callus are negatively involved in the bone repair process (Konnecke *et al.*, 2014; Young *et al.*, 2005). During bone remodelling, Th1, Th2 and regulatory T cells negatively influence osteoclast maturation; however, Th17 cells show a positive effect on osteoclast formation (Takayanagi, 2009; Walsh *et al.*, 2018; Zaiss *et al.*, 2007). Mice lacking T and B cells appear to have accelerated fracture healing as compared to those with a fully competent immune system (Toben *et al.*, 2011). More specifically, CD8 T cells inhibit fracture repair (Reinke *et al.*, 2013). However, on the other hand, other T cells have varying effects on bone formation/regeneration depending on the studied subtype (Lei *et al.*, 2015; Nam *et al.*, 2012; Young *et al.*, 2005). Collectively, the complex interaction between the immune system and the cells of the skeletal system is critical for the outcome of the bone repair/regeneration as the manipulation of a specific subset of immune cells could greatly impact bone formation.

The use of autologous cells for bone regeneration is ideal due to the lack of immune rejection upon implantation. However, autologous cells have the drawback of the limited quantity of material that can be obtained. Moreover, the material that is obtained is usually of poor quality. This is due to the fact that autologous cell transplantation is generally needed in elderly and diseased patients and, therefore, having cells with poor proliferative and differentiation capacities as compared to those that could be obtained from healthy individuals (Mueller and Glowacki, 2001). Furthermore, treating patients with their own cells can cause a major delay in treatment timetables due to *in vitro* cell manipulations (*e.g.* expansion and quality control) before cells can be administered back into the patient. Taking this into consideration, new and improved TERM-based approaches to bone repair need to be developed. The use of allogeneic cells would be preferable as there would be an immediate approved stock of cells ready to treat a patient. This advantage has led to an increased research interest aiming at using allogeneic cells for TERM applications. Research on allogeneic MSCs has demonstrated that they are somewhat immunoevasive, due to low surface expression of costimulatory molecules (*e.g.* CD80 and CD86) and MHC class II (Aggarwal and Pittenger, 2005; Ankrum *et al.*, 2014; Le Blanc and Ringden, 2007; Nauta and Fibbe, 2007). Such an aspect is advantageous as MSCs will be implanted into an inflammatory environment during fracture repair (Ankrum *et al.*, 2014; Asari *et al.*, 2009; Corcione *et al.*, 2006; Djouad *et al.*, 2007; Jiang *et al.*, 2005; Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2009; Spaggiari *et al.*, 2008; Zhao *et al.*, 2012; Zheng *et al.*, 2008). In normal situations, implantation of allogeneic cells would lead to cell rejection by the adaptive immune system. However, allogeneic MSCs can evade the immune response and in some instances avoid rejection upon implantation (Ankrum *et al.*, 2014; Ryan *et al.*, 2005). In studies focussed on the use of allogeneic MSCs for bone repair, the immune response has been shown to play an important role in the process. Bone regeneration induced by allogeneic MSCs is negatively impacted by Th1 cells through the inhibition of osteogenesis-specific gene expression (osteocalcin, Runx2 and ALP) (Dighe *et al.*, 2013). On the other hand, osteogenesis is promoted by Th2, Th17 and regulatory T cells (Kovach *et al.*, 2015; Liu *et al.*, 2011; Omar *et al.*, 2011). While there have been numerous studies on allogeneic undifferentiated MSCs, little investigation has been carried on how the immune system responds to allogeneic MSCs when they are pre-differentiated into another tissue type prior to implantation. Allogeneic undifferentiated MSCs have been shown to be non-immunogenic (Aggarwal and Pittenger, 2005; English, 2013; Gao *et al.*, 2016; Hoogduijn *et al.*, 2010; Nauta and Fibbe, 2007). Due to their immunoevasive nature, they can modify the immune system to their desired purpose. Few studies have investigated the effects of allogeneic chondrogenic MSCs on the immune system. Thus far, results have been conflicting, with reports demonstrating allogeneic chondrogenic MSCs to be both immunogenic (Mukonoweshuro *et al.*, 2014; Ryan *et al.*, 2014) and non-immunogenic (Adkisson *et al.*, 2010; Kiernan *et al.*, 2016; Le Blanc *et al.*, 2003; Zheng *et al.*, 2008).

The contradictory results were highly dependent on how the co-culturing work was performed during the experiments. Even in the *in vivo* setting, little is known about the effects of these pre-differentiated MSCs on the immune system. Kiernan *et al.* (2016) have recently detailed the various studies that have focussed on the interactions between the immune system and allogeneic differentiated MSCs in the context of bone tissue engineering. More recently, the immune-privileged nature of allogeneic MSCs has been called into question. As reviewed by Griffin

et al. (2013) and Lohan *et al.* (2017), host responses vary in response to the presence of allogeneic MSCs from minor inflammation to right out rejection. The idea that allogeneic MSCs could be recognised and targeted by the host is concerning for many in the field of tissue engineering. It is clear from these studies that there is more research that needs to be conducted to determine how pre-differentiated MSCs interact with the immune system in an allogeneic setting before these cells can be used clinically. However, it appears increasingly unlikely that MSCs or differentiated MSCs are truly incapable of completely evading the immune system. The question to be answered is whether this is an issue to be concerned about.

Further considerations, towards improved bone output

MSC-mediated endochondral bone formation has yielded some promising results in animal model defect repair; however, treatment of large bone defects is still problematic. Although Harada *et al.* (2014) showed how chondrogenically-primed rat MSCs could heal a critical-size defect, no other group has demonstrated such large bone defect repair. Although MSC-mediated EO can form bone *in vivo*, the quantity usually formed, outside Harada *et al.* (2014) study, is insufficient to treat large bone defects. From a translational perspective, the volume of chondrogenic MSCs required to properly heal critical-size defects would require unmanageable cell numbers, incubator space, reagents and time to maintain, which would make the cost of such constructs astronomical (Penick *et al.*, 2005; van der Stok *et al.*, 2014). In order to treat large defects, scale-up approaches are necessary to improve bone output.

When considering scaled-up bone formation, the need for successful vascularisation to maintain cell health during regeneration must be considered. As most cells of the body are rarely more than 100-200 μm away from a capillary, due to diffusion limits influencing their behaviour (Biederman-Thorson *et al.*, 2013; Ko *et al.*, 2007), proper vascularisation in TERM constructs is critical. Although chondrocytes are thought to be well suited to survive in the initial defect site, as their true environment is also hypoxic and avascular (Pfander and Gelse, 2007), remodelling, vessel invasion and bone formation introduce new cells with variable oxygen/nutrient requirements into the defect site (Ko *et al.*, 2007), making vascularisation crucial to ensure these cells' survival. In small defect repair, vascularisation occurs rapidly enough to allow graft survival and integration; however, with a large defect, natural vascularisation rates may not be sufficient. Therefore, vascularisation must be induced or compensated for in the initial implanted construct to prevent cell death. Pre-vascularisation of chondrogenic grafts pre-implantation has shown more promising results (Ng *et al.*, 2017; Yousefi *et al.*, 2016). Freeman *et al.* (2015) showed that the pre-vascularisation of chondrogenic MSCs can result in accelerated vascularisation, host cell survival and ossification *versus* non-vascularised counterparts. These constructs were implanted for only 4 weeks but it would be interesting to see how constructs perform following longer *in vivo* implantation or in immunocompetent animals. These studies are promising but special care must be taken when selecting endothelial cell sources since the phenotype of the cell differs depending on the tissue type they are isolated from (Chi *et al.*, 2003; Garlanda and Dejana, 1997). Takigawa (2013) investigated how the addition of biologically relevant compounds known to influence endothelial cell behaviour, such as VEGF, could be utilised for improving graft vascularisation. However, high doses of VEGF result in uncontrollable bone formation, indicating further research is required to make this a more viable option (Maes *et al.*, 2010). By accelerating processes which are known to be important for *in vivo* bone formation, such as vascularisation, it could be possible to, not only improve graft performance, but also increase bone formation, as bone-forming osteocytes can invade the cartilage template migrating through the vasculature (Maes, 2013; Maes *et al.*, 2010). From a TERM approach, pre-vascularising grafts or inducing faster vascularisation is advantageous to not only tackle the issue of poor vascularisation but also to increase bone formation in the process.

Given the complications associated with cell-based approaches for tissue regeneration, there is the attempt to find possible cell-free approaches, which could circumvent these limitations. MSCs used in endochondral tissue engineering bone grafts directly contribute to the bone-forming population (Bahney *et al.*, 2014; Farrell *et al.*, 2011; Mendes *et al.*, 2018; Scotti *et al.*, 2013). Implanted chondrogenically-differentiated MSCs persist within the bone matrix and contribute directly to bone formation, instructing host bone formation throughout the process (Bahney *et al.*, 2014; Farrell *et al.*, 2011; Mendes *et al.*, 2018; Scotti *et al.*, 2013). These studies have suggested that implanted cells are essential for proper bone formation; however, devitalised grafts derived from chondrogenically-differentiated MSCs can also form endochondral bone *in vivo* (Bourgine, 2013; Bourgine *et al.*, 2014a; Bourgine *et al.*, 2014b; Cunniffe *et al.*, 2015). Bourgine *et al.* (2013; 2014a; 2014b) created decellularised grafts which maintain bone formation potential once implanted. They utilised immortalised cell lines – eliminating many of the culture-induced issues associated with MSCs – which are decellularised through activation of an engineered death-inducible receptor within the cell. Once decellularised and implanted, these constructs show promising bone formation ability. Moreover, these immortalised cells could be further manipulated to overexpress factors known to improve bone formation, such as BMP2, which, in turn, would be

incorporated in the ECM and could further improve bone output. Cunniffe *et al.* (2015), following this same line of research, showed that matrices produced specifically by hypertrophic chondrogenically-differentiated MSCs produced significantly more bone than non-hypertrophic matrices, indicating that something not yet identified but produced specifically during hypertrophy could be the key to improved bone formation. Although bone formed by acellular grafts produced significantly less bone volume than cellularised counterparts, these cell-free grafts are still able to recruit host vasculature and cells required for proper bone formation (Bourgine, 2013; Bourgine *et al.*, 2014a). With further optimisation, they could be a promising alternative to current autologous bone grafts. Although decellularised grafts and off-the-shelf treatment options are an ideal solution for tissue engineering, it remains that current cell-based approaches yield better bone formation than acellular counterparts. As such, a popular scale-up approach consists of using growth factors combined with novel biomaterials. Growth factors important for developmental induction of EO, such as BMP-2 (Decambron *et al.*, 2017; Penick *et al.*, 2005; Stüdle *et al.*, 2018), TGFβ (Mendes *et al.*, 2018; Stüdle *et al.*, 2018), VEGF (Carlevaro *et al.*, 2000; Gerber *et al.*, 1999), PRP (Janssen *et al.*, 2013) as well as potentially novel factors (Fahmy-Garcia *et al.*, 2017) are being characterised to determine if their use in combination with MSCs would improve bone output. The use of these factors has shown variable results, performing as well as, better or worse than iliac crest bone (Janssen *et al.*, 2013). Two drawbacks associated with this approach are that these factors are extremely expensive and are used at supraphysiological levels, which is associated with additional risk. For example, high doses of BMP-2 can cause soft tissue swelling (Shahlaie and Kim, 2008), abnormal excessive bone formation (Zara *et al.*, 2011) and increased cancer risk (Carragee *et al.*, 2013). As such, researchers are also investigating other compounds which are known to be involved in EO and could possibly be used at more physiologically acceptable doses. This includes GDF5. This protein is well known for its role in joint formation, chondrogenesis and hypertrophic differentiation and is also a member of a subgroup of BMPs (Coleman *et al.*, 2013). Other proteins which are more recently identified as being important during EO, including CTGF (also known as CCN2) and HMGB1, have also been investigated as possible additions to improve bone formation as they have shown positive results *in vitro* for improved cell recruitment, vascularisation and osteogenesis (Khattab *et al.*, 2015; Kubota and Takigawa, 2011; Takigawa, 2013; Taniguchi *et al.*, 2007). Such proteins could be used at more physiologically relevant doses as compared to BMP-2 (Fahmy-Garcia *et al.*, 2017). However, even when supraphysiological doses are required, researchers are looking for ways to possibly decrease the effective dosage required to prevent these unwanted side effects. By coupling or crosslinking factors to matrices, the concentration of these compounds can be reduced to something more physiologically acceptable (Mumcuoglu *et al.*, 2018; Quinlan *et al.*, 2015). Further research into the identification of new biologically relevant compounds is also useful. Recent studies have identified new stem cell populations which are activated in response to acute skeletal injury (Chan *et al.*, 2018). By studying the secreted profile of these cells in comparison with controls, new relevant targets could be identified which may not even require supraphysiological doses to be effective. Additionally, the use of organs-on-a-chip and other computational models, which have been proven promising to identify/validate targets and have improved screening methods (Esch *et al.*, 2015), could accelerate results and research.

Conclusions

Modelling grafts in tissue engineering after EO has been an active area of research in bone tissue engineering for years. The initial cartilage graft is well suited to survive in an avascular environment and it can induce, on its own, the migration of all the previously mentioned cell types. Although it is a reproducible method for bone formation, progress to enhance the bone-forming capacity of these constructs, to properly fill large bone defects, is moving slowly. However, as the understanding of the interactions that take place improves – not only between donor and host cells but also those of a fully functioning immune system – a better appreciation of how to improve such grafts will follow. As research in the field continues, graft vascularisation, integration and bone output will improve, making these tissue-engineered endochondral grafts a viable alternative to autologous bone graft substitutes in the future.

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Discussion with Reviewer

Diego Correa: Taking into consideration the pericytic phenotype of MSCs and their description as osteoprogenitors during bone formation and fracture healing (Maes *et al.*, 2010), what would be the real contribution of such cells (as perivascular) during the engineering of bone grafts?
Authors: The idea that perictyic MSCs attach to the side of the vasculature, in a way effectively and passively migrating to the site of bone formation, is one of our favourite theories in bone tissue engineering. Although Maes *et al.* (2010) show that these cells undergo osteoblastic differentiation and contribute to bone formation, we believe they could also contribute to a number of crucial processes, considering the many bioactive molecules they encounter in this environment which can influence their behaviour. It would be interesting to see if they also contribute to the stabilisation of the bone marrow niche microenvironment or somehow further influence the migration of the vasculature into the centre of the cartilage template. We fully believe they contribute to bone formation, but as the field digs deeper into this area, they may find these cells play an even bigger role in bone formation.

Diego Correa: If such a phenomenon results critical, how do the authors envision to enhance it?
Authors: If it proves critical that pericytic MSCs, specifically the subset that co-migrate with the invading vasculature, are crucial for bone formation, it could be advantageous to enhance or accelerate the migration of the vasculature. Alternatively, determining what factor(s) this specific subset of MSCs is attracted to would be better; then, this(these) could be incorporated into a tissue-engineered construct or bound to a biomaterial to enhance migration of this specific MSC subtype which would have the potential to improve or accelerate bone formation.

Editor's note: The Scientific Editor responsible for this paper was Martin Stoddart.