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AUTHOR(S)

Emmet M. Thompson, Amos Matsiko, Eric Farrell, Daniel J. Kelly, Fergal O'Brien

CITATION

Thompson, Emmet M.; Matsiko, Amos; Farrell, Eric; Kelly, Daniel J.; O'Brien, Fergal (2015): Recapitulating endochondral ossification: a promising route to in vivo bone regeneration.. Royal College of Surgeons in Ireland. Journal contribution. <https://hdl.handle.net/10779/rcsi.10766621.v1>

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Recapitulating endochondral ossification: A promising route to *in vivo* bone regeneration

Emmet M. Thompson^{1, 2, 3}, Amos Matsiko^{1, 2, 3}, Eric Farrell⁴, Daniel J. Kelly^{2, 3, 5}, Fergal J. O'Brien^{1, 2, 3}

¹ Tissue Engineering Research Group, Dept. of Anatomy, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland.

² Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland.

³ Advanced Materials and Bioengineering Research (AMBER) Centre, RCSI & TCD

⁴ Department of Oral and Maxillofacial Surgery, Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands.

⁵ Department of Mechanical and Manufacturing Engineering, Trinity College Dublin, Dublin 2, Ireland.

Abstract

Despite its natural healing potential, bone is unable to regenerate sufficient tissue within, critical-sized defects resulting in a non-union of bone ends. As a consequence, interventions are required to replace missing, damaged or diseased bone. Bone grafts have been widely employed for the repair of such critical-sized defects. However, the well-documented drawbacks associated with autografts, allografts and xenografts have motivated the development of alternative treatment options. Traditional tissue-engineered constructs typically attempt to direct *in vitro* bone-like matrix formation prior to implantation into bone defects, mimicking the embryological process of intramembranous ossification (IMO). Commonly, tissue-engineered constructs developed using this approach inevitably fail once implanted due to poor perfusion leading to avascular necrosis and core degradation. Due to such drawbacks, an alternative tissue engineering strategy based on endochondral ossification (ECO) has begun to emerge which involves the use of *in vitro* tissue-engineered cartilage as a transient biomimetic template to facilitate bone formation within large defects. This is driven by the hypothesis that hypertrophic chondrocytes can secrete angiogenic factors and alkaline phosphatase which play pivotal roles in both the vascularization of constructs *in vivo* and the deposition of a mineralized extracellular matrix with resulting bone deposition. In this context, this review will focus on current strategies taken to recapitulate ECO using a range of distinct cells, biomaterials and biochemical stimuli in order to facilitate *in vivo* bone formation.

Keywords: Bone; intramembranous ossification; endochondral ossification; hypertrophy; mesenchymal stem cells

1.1. Introduction

Bone, the second most commonly transplanted tissue worldwide after blood products (Shegarfi and Reikeras, 2009), is a highly vascularized tissue, unique in its capacity to self-regenerate without the formation of a fibrotic scar (Marsell and Einhorn, 2011). Despite its natural healing potential, native bone is not always able to repair large-scale defects which can result in permanent bone loss and fracture non-union. Consequently interventions such as bone grafting are required to replace damaged or diseased bone resulting in more than 1 million bone repair surgeries and between 500,000 to 600,000 bone-grafting procedures carried out annually in the United States (Cheung, 2005, Marino and Ziran, 2010). Autogenous bone grafting, also known as autografting, represents the clinical “gold standard” due to its high immuno-compatibility and naturally osteoinductive, osteoconductive and osteogenic properties (Khan et al., 2005). However, the drawbacks associated with this approach include donor site morbidity, limited graft supply, a decrease in the regenerative capacity of graft material with an increase in donor age, as well as donor-to-donor variability. Allografting donor tissue from another individual is less frequently undertaken due to the risk of immune rejection, disease transmission and the potential for chronic inflammation. Xenografting of tissues or organs derived from a non-human animal source into a human recipient is also associated with similar drawbacks such as infection, rejections and chronic inflammation. These limitations have motivated the development of alternative treatment options (Grabowski and Cornett, 2013, Muller et al., 2013).

The generation of tissue-engineered autologous constructs using a combination of cells derived from a patient and an optimized biomaterial would avoid many of the drawbacks associated with autografting and allografting. Despite numerous efforts in the area of bone tissue engineering

(TE), a mechanically competent, vascularized construct that is capable of supporting defect repair with fully functional regenerated bone tissue has yet to be developed (Johnson et al., 2011). Bone graft substitutes represent an alternative approach for the treatment of bone loss. However, commercially available bone graft substitutes have shown limited clinical success in human trials ensuring autogenous bone grafting approaches continue to account for the majority of bone graft procedures, regardless of the significant associated risks and costs (Grabowski and Cornett, 2013).

Traditional tissue-engineered constructs are produced by culturing cells on biodegradable biomaterial scaffolds in media containing osteogenic factors to stimulate *in vitro* bone-like matrix formation prior to implantation into bone defects *in vivo*, mimicking the embryological process of intramembranous ossification. However, extensive surface matrix deposition *in vitro*, diffusion limitations and the lack of a vascular network hamper nutrient delivery and waste removal from the centre of such TE constructs, resulting in poor perfusion, avascular necrosis and core degradation *in vivo* (Lyons et al., 2010, Kelly and Prendergast, 2005, Tremblay et al., 2005, Ko et al., 2007, Phelps and Garcia, 2009). As a result, traditional tissue-engineered constructs which appear to demonstrate great potential *in vitro* often fail once implanted. This creates a major challenge in the field of TE and several strategies to improve osteogenesis and construct vascularization have been investigated including the delivery of angiogenic biomolecules (Fuchs et al., 2012, Rivron et al., 2012, Kanczler et al., 2010), hypoxic culture (Wise et al., 2013, Zou et al., 2012), flow perfusion (Barron et al., 2012, Keogh et al., 2011) and *in vitro* vascularization of scaffolds (Liu et al., 2012, Duffy et al., 2011, Tsigkou et al., 2010, McFadden et al., 2013). However, an optimal approach has yet to be clearly identified.

Recently, there has been a move towards recapitulating the process of endochondral ossification in the field of TE through the use of engineered cartilage as transient templates to promote healing in bone defects ([Fig. 1](#)). Bone repair strategies based on endochondral priming may be able to harness this progression to provide the structural, cellular, angiogenic and osteogenic factors required to facilitate vascularization, bone regeneration and fracture healing (Kanczler and Oreffo, 2008, Dickson et al., 1995, Trueta and Amato, 1960). In this context, such advances in bone tissue engineering that aim to recapitulate the process of endochondral ossification in the repair of bone defects will be the focus of this review.

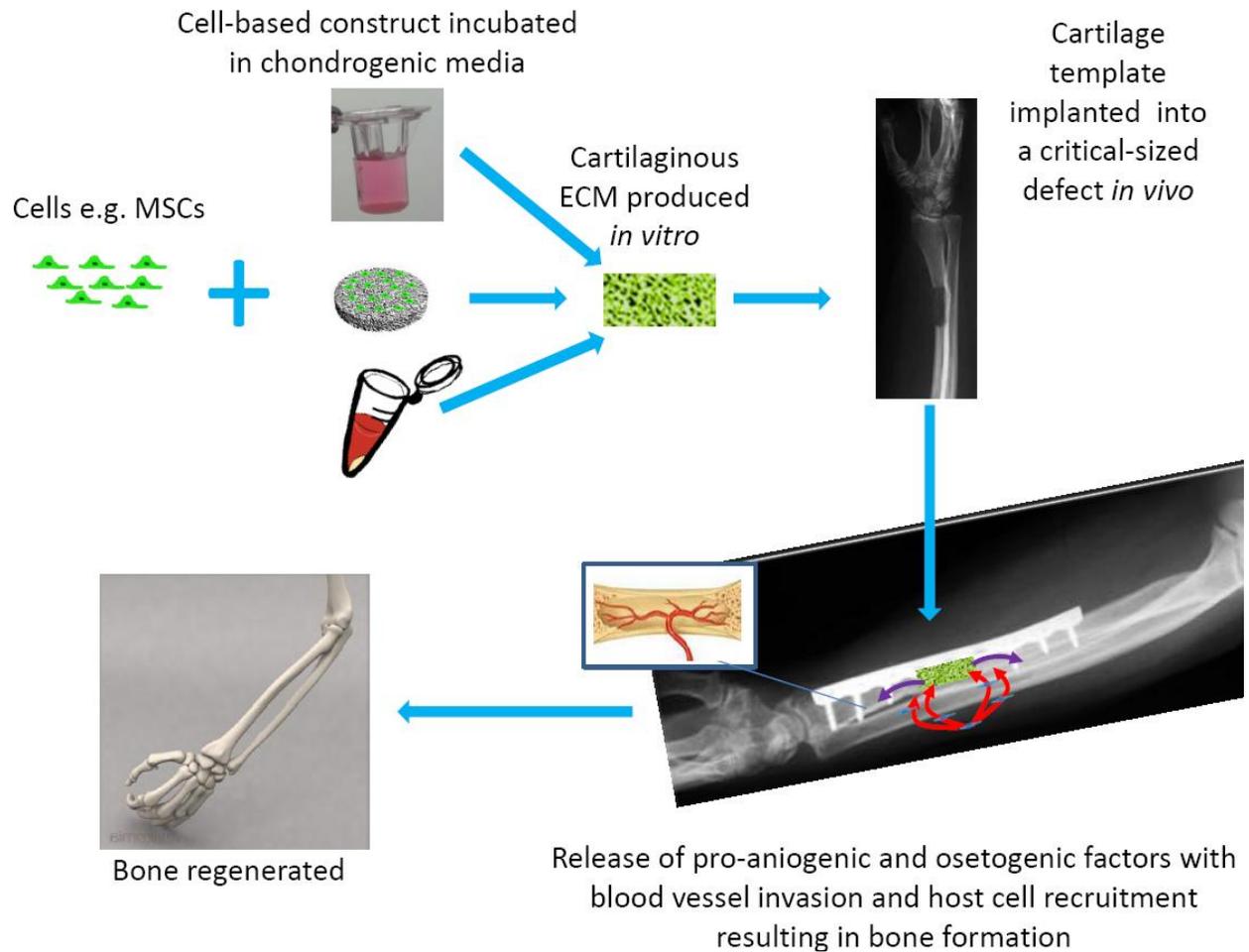


Figure 1. Endochondral tissue engineering strategies for bone regeneration. Tissue engineered cartilage can be generated *in vitro* to act as a transient biomimetic template capable of secreting trophic biomolecules beneficial to bone formation. Current approaches to *in vitro* cartilage production include self-assembly systems using transwell plates, cell seeding onto biomaterial scaffolds and cell pellet culture. Following implantation into skeletal defects, this cartilage templates can provide the structural, cellular and angiogenic stimuli required for the formation of nascent vascularized bone capable of restoring tissue form and function to its pre-injury state.

1.2. Embryonic bone development and fracture healing: the role of endochondral ossification

Bone development occurs by two distinct processes: intramembranous ossification (IMO) and endochondral ossification (ECO). IMO forms most of the bones that make up the craniofacial skeleton. This process is characterized by mesenchymal stem cells (MSCs) differentiating directly into osteoblasts and eventually producing bone. Unlike IMO, ECO is characterized by a cartilaginous template anlage and is responsible for the formation of long bones as well as their elongation during growth and fracture healing (Gerstenfeld et al., 2003). This dynamic process involves several stages, many of which occur concurrently, both temporally and spatially. ECO begins with MSCs undergoing condensation and differentiating into chondrocytes which secrete an extracellular matrix (ECM) that forms a hyaline cartilage template ([Fig. 2](#)). The template grows interstitially and cells derived from the inner aspect of the perichondrium undergo direct osteoblastic differentiation and secrete mineralized tissue that forms the bone collar, subsequently becoming cortical bone. Meanwhile, chondrocytes in the cartilage template proliferate, mature and undergo hypertrophy secreting collagen type X matrix, angiogenic factors such as vascular endothelial growth factor (VEGF), as well as the enzyme alkaline phosphatase with resulting mineralized tissue deposition. Matrix metalloproteinase activity is required at this stage to degrade the cartilaginous template thereby making it permissive of blood vessels which invade the diaphysis. These vessels deliver osteoclasts and osteoprogenitor cells thereby forming the primary ossification centre (POC). The calcified cartilage template is partially resorbed by recruited osteoclasts and woven bone is laid down. While the diaphysis is remodeled to form a medullary cavity, a secondary ossification centre (SOC) is formed in the epiphyses, with the periphery of the bone ends maintaining the stable superficial hyaline articular cartilage tissue

seen within diarthroidal joints. Between the primary and secondary ossification centers, an epiphyseal/growth plate persists and is composed of cartilage tissue that continuously grows and is replaced by bone thereby regulating elongation of bones.

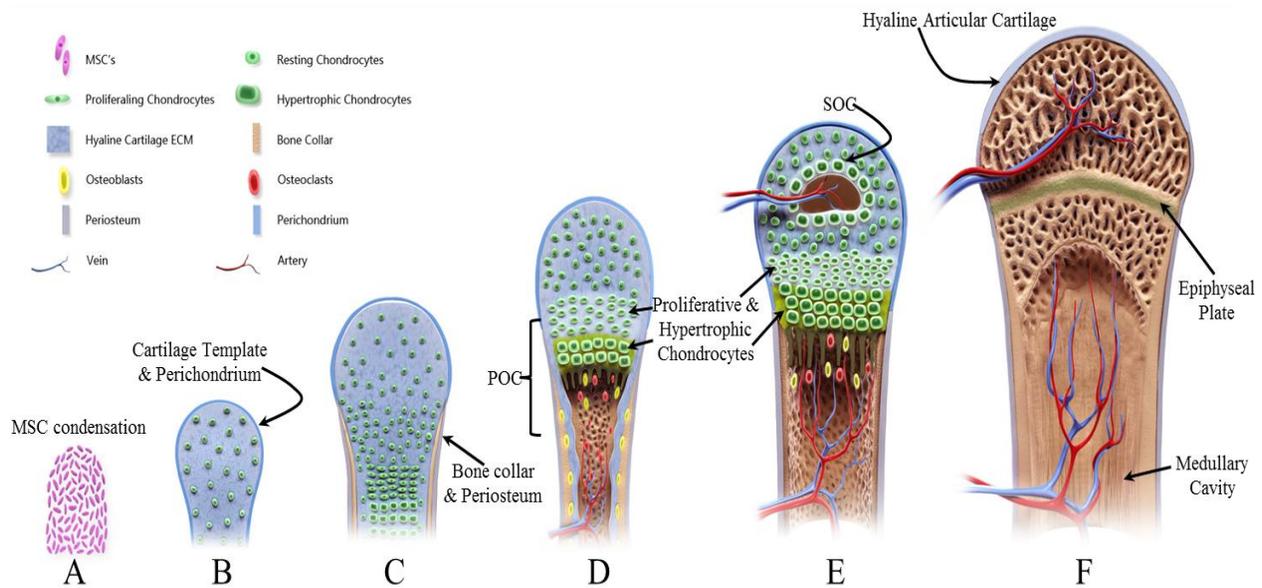


Figure 2. Overview of endochondral bone development. (A) At the beginning of bone formation, mesenchymal stem cells condense together before (B) undergoing chondrogenic differentiation to form the hyaline cartilage template of future long bones. MSCs located on the periphery form the perichondrium. (C) Cells in the centre of template undergo hypertrophy while cells located on the periphery undergo direct osteoblastic differentiation to form an encircling bone collar. (D) Hypertrophic cells initiate mineralisation of the cartilage rudiment. The diaphysis is invaded by the ossification front of blood vessels and influxing cells resulting in the formation of the primary ossification centre (POC). Osteoclasts remodel the calcified cartilage template while osteoprogenitor develop into osteoblasts and lay down the osteoid of developing bone. (E&F) In early postnatal life the developing bone ends, the epiphyses, are invaded by blood vessels leading

to the formation of the secondary ossification centre (SOC) with the periphery maintaining a stable cartilage phenotype resulting in hyaline articular surfaces seen within joints. The epiphyseal growth plate persists between the POC and SOC. It is the site longitudinal bone growth before ossifying in adulthood.

From a clinical perspective, the majority of bone fracture healing occurs by secondary/indirect healing in which a transient soft callus composed of a cartilaginous template is an important intermediate for normal repair, analogous to endochondral bone formation (Ito and Perren, 2007). Following the initial inflammatory phase involving blood vessels disruption, macrophage and poly-morphonuclear neutrophils release and migration into the fracture site, the fracture hematoma is replaced by granulation tissue with osteoclasts degrading necrotic bone. Progenitor cells that originate from the periosteum differentiate into osteoblasts thereby stimulating appositional growth and enveloping the defect. The callus is invaded by MSCs that differentiate into chondrocytes and synthesize a cartilaginous ECM which undergoes ECO to form calcified tissue that results in woven bone. Therefore, TE strategies that can mimic the natural progression of bone growth and healing could provide a therapeutic option due to their production of trophic and pro-angiogenic factors that can stimulate cell migration and vascularization in areas bone loss resulting in enhanced regeneration.

1.3. Regulation of remodeling and neo-vascularization in endochondral ossification

As previously mentioned two critical steps during endochondral ossification are the vascular invasion and remodeling of the cartilage anlage. A functional vascular network is essential to overcome diffusion limitations in the enlarging cartilage template that would result in death of

the inner cell populations (Lovett et al., 2009). Moreover, vessel ingrowth allows osteoclasts and osteoprogenitor cells access to inner aspect of the developing bone and marks the beginning of ossification. As chondrocytes undergo hypertrophy, they enlarge in size with a 5- to 10-fold increase in mean cell volume (Hunziker et al., 1999, Noonan et al., 1998) ([Fig. 3](#)). To compensate for this increase in size, hypertrophic chondrocytes secrete MMP-13 which degrades the ECM surrounding the cells that is rich in fibrillar collagen (Mackie et al., 2008). MMP-9 is also secreted by the cells to degrade aggrecans (Cawston and Wilson, 2006). This remodeling of the matrix by MMP activity is essential to the invasion of blood vessels as it removes transverse cartilage septae that would otherwise prevent longitudinal vessel ingrowth. Moreover, matrix removal provides space for chondrocytes to expand into as they undergo hypertrophy. In addition to this degradation, osteoclasts are also recruited alongside blood vessels and play a crucial role in the resorption of the mineralized matrix, thereby helping to create the medullary cavity found in long bone a diaphysis (Mackie et al., 2008).

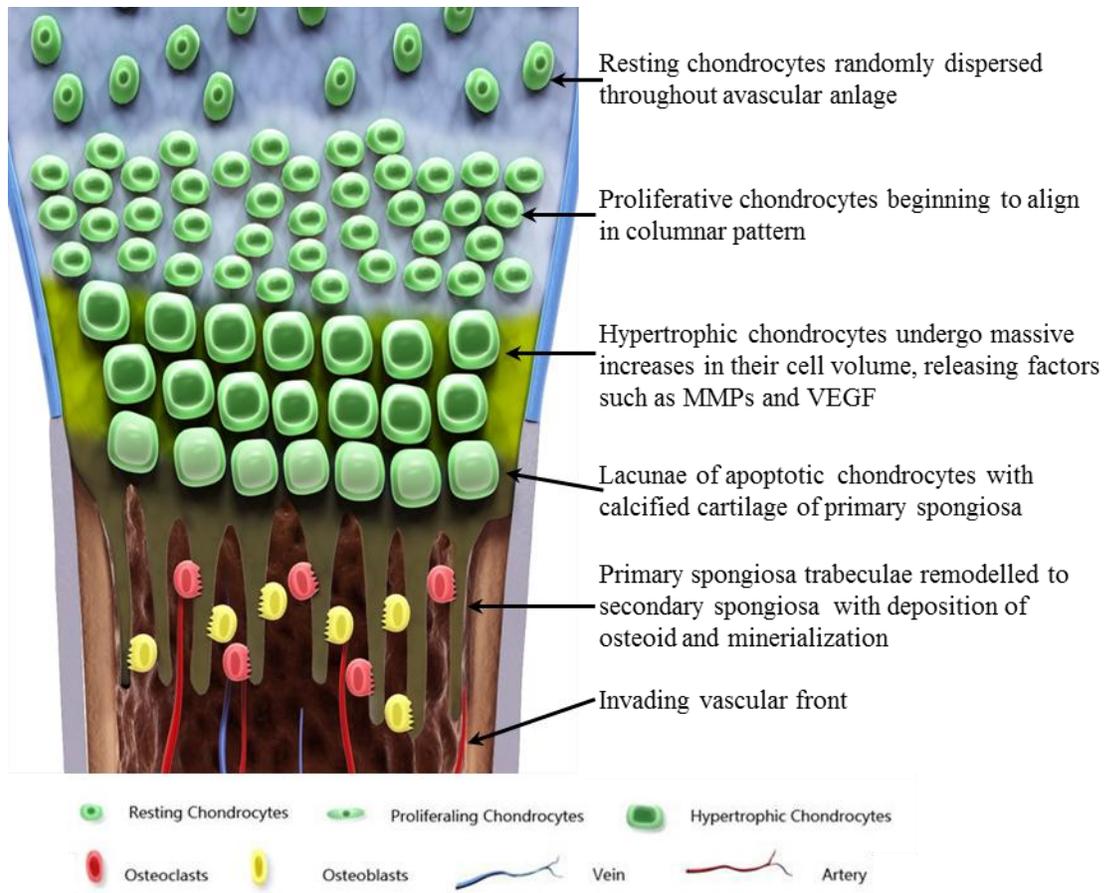


Figure 3. Zonal chondrocyte arrangement at growth plates. Chondrocytes adopt morphologically distinct maturational zones and maintain a spatially fixed position allowing post-natal limb lengthening by driving continued expansion of the primary and secondary ossification centres. From distal to proximal, in relation to the invading ossification front, the pattern consists of resting chondrocytes, flattened proliferating chondrocytes in a columnar orientation that will dictate the longitudinal direction of bone growth, expanded hypertrophic chondrocytes and apoptotic chondrocytes. Finally the calcified cartilage and the primary spongiosa mark the chondroosseous junction.

The second key process during endochondral ossification is vascularization of the developing bone. VEGF, is one of the key pro-angiogenic factors involved in ECO and its role during bone development has been widely study both *in vitro* and *in vivo* (Dai and Rabie, 2007). It is fundamental in the recruitment of blood vessels and studies have also shown that it may also mediate recruitment of osteoclasts (Nakagawa et al., 2000). This growth factor is expressed in at least 6 isoforms which are either bound to the ECM or found in soluble form. Murine VEGF-120, one of the soluble forms, diffuses into the local environment to set up a concentration gradient that is responsible for epiphyseal vascular invasion as well as chondrocyte survival. The VEGF-164 isoform is highly expressed by chondrocytes and is found in a soluble form as well as bound to the cartilage ECM. Influxing osteoclasts and locally released MMPs degrade the hypertrophic cartilage matrix to release this heparin-bound form of VEGF-making it bioavailable (Vu and Werb, 2000). The higher local concentration of VEGF created by this release perpetuates the VEGF gradient emanating from the hypertrophic zone and causes the purposeful directional recruitment of the ossification front. It also helps to mediate chondrocyte growth and survival on the perichondrium. The VEGF-188 isoform is also bound to the ECM and is responsible for vascular invasion of the metaphysis (Goldring et al., 2006). Angiopoietins are another set of angiogenic factors that are involved in neo-vascularization. Angiopoietin-1 and -2 are responsible for vascular invasion during fracture healing. The angiopoietin receptors: Tie-1 and -2 are also known to be up-regulated during the fracture healing (Goldring, 2006, Gerstenfeld et al., 2003, Lehmann et al., 2005). Overall, further information is still needed in order to mimic the natural occurrence of these pro-angiogenic and pro-remodeling factors for TE construct degradation. However, the development of systems that promote their own degradation

and vascularisation would be advantageous, particularly in large defects requiring voluminous grafts which are at an increased risk of avascular necrosis and construct failure.

1.4. Recapitulating embryological bone development for bone defect repair

Recently, the concept of recapitulating embryological processes has been adopted in the field of TE in what has been coined as ‘developmental engineering’ to produce paradigms for tissue repair (Lenas et al., 2009) In particular, strategies have been employed to mimic the process of ECO for bone defect repair application (Oliveira et al., 2009a, Oliveira et al., 2009b, Gawlitta et al., 2010, Farrell et al., 2011, Huang et al., 2006). The primary question centered among such studies is whether cells driven towards late stage hypertrophy following chondrogenesis *in vitro* can support mineralized tissue formation with features of native bone subsequent to implantation in an *in vivo* environment. Varieties of cell sources, biomaterials, as well as soluble biochemical factors have been widely investigated to reproduce this process both *in vitro* and *in vivo* and will be explored in the following sections.

1.4.1 Cell sources for endochondral bone tissue engineering strategies

Several cell types are being investigated in the development of tissue-engineered cartilage templates *in vitro* with subsequent *in vivo* application for bone formation. Adult Mesenchymal stem cells (MSCs) are considered as a promising source of cells in TE and numerous sources such as those derived from adipose tissue, infrapatellar fat pad, bone marrow and synovium have shown success in bone TE {Buckley, 2010 #281; Meyer, 2010 #236; Vinardell, 2012 #79}. Such success can be attributed to their multi-potency, ability to secrete a range of cytokines and growth factors which mediate cellular activity and suppress immunological response through

inhibition of TNF- α and IFN- γ secretion (Devine et al., 2001; Beyth et al., 2005; Pittinger et al., 1999; Caplan, 2007). However, MSCs derived from the bone marrow have been the most frequently investigated source of progenitor cells for *in vivo* endochondral tissue engineering to-date. This is in part due to their potential to differentiate into chondrocytes and subsequently progress towards a hypertrophic phenotype *in vitro* (Hellingman et al., 2010). Furthermore, these cells are naturally found in the bone marrow microenvironment in a perivascular niche and are released locally or recruited systemically during trauma, thus, they are more likely to respond favorably to signals associated with tissue damage, inflammation and ultimately bone repair (Caplan and Correa, 2011). MSCs may also alleviate the need for use of additional growth factors and cytokines due to their well-documented capacity to secrete bioactive molecules that mediate differentiation and immunological responses (Zhu and Huangfu, 2013, Hellingman et al., 2010, Thomson et al., 1998, Pittenger et al., 1999). Moreover their low immunogenic activity and immunosuppressive properties are of significant advantage for clinical translation (Law and Chaudhuri, 2013, De Miguel et al., 2012).

A number of other cell sources have been investigated for endochondral bone TE including both chondrocytes and chondrocyte-like cells. However, the results observed with adult chondrocytes for bone formation have not been as encouraging as those seen with MSCs. Primary porcine chondrocytes seeded onto polycaprolactone (PCL) / BMP-2scaffolds have shown the ability to produce cartilage matrix with mRNA expression of the hypertrophic marker COL X (Jeong et al., 2012). However, *in vivo* s/c assessment of this system did not result in significant formation with ossification being limited to the external aspect of the scaffolds. Human articular chondrocytes (HACs) have also been driven towards a hypertrophic phenotype with the use of

micromass culture (Narcisi et al., 2012). However, these cells also synthesized only poor levels of bone tissue when implanted *in vivo* and their use in 3D endochondral bone tissue engineering may be limited by their stable phenotype (Vacanti et al., 1995), the risk of arthritic development subsequent to procurement (Boyce et al., 2013, Schindler, 2011) and slow *ex vivo* expansion (Lin et al., 2008, Oshin et al., 2007). As an alternative, chondrogenic cells such as the ATDC5 cell line isolated from mouse teratocarcinoma fibroblastic cells (Weiss et al., 2012) and chick embryo sternal chondrocytes with a transient phenotype (Oliveira et al., 2009a, Oliveira et al., 2009b) have been used to engineer cartilage *in vitro* resulting in subsequent bone formation *in vivo*. These cells therefore represent valuable models that will allow in-depth exploration of the process of ECO in tissue-engineered cartilage constructs and also the *in vivo* response to such systems.

Embryonic stem cell-derived embryoid bodies have also shown ability to produce cartilage for endochondral bone formation following seeding onto ceramic particles and culture in serum-free chondrogenic differentiation medium for 21 days (Jukes et al., 2008). Upon subcutaneous implantation of these constructs bone formed in areas surrounding hypertrophic chondrocytes and mineralized cartilage matrix. However, ethical and moral objections, tumorigenicity and teratoma formation, challenges related to scaling up production of large cell numbers, genetic instability and immunogenic difficulties make them a less viable option in the short to medium term (Jung et al., 2012, Puri and Nagy, 2012). A novel source of autologous cells for endochondral tissue formation in the future might be fracture haematoma-derived cells, previously shown to contain multilineage progenitor cells with similar cell-surface antigen profiles to BMSCs (Oe et al., 2007). These cells can be easily retrieved during surgery for

fracture repair and have recently been shown to contain a cell population with the potential to undergo chondrogenesis, hypertrophy and calcification *in vitro* (Koga et al., 2013). Despite the potential shown by several cell types for endochondral bone formation, the optimal cell source has yet to be identified.

1.4.2 Biomaterials used for endochondral bone tissue engineering strategies

Biomaterial scaffolds are used to create a three-dimensional environment that acts as a template to support cell interaction, proliferation and differentiation with maintenance of a desired phenotype and function. To achieve this, a biomaterial should possess appropriate cues, based on the native tissue, such as biochemical, architectural and mechanical characteristics that facilitate these desired cell responses (Mahmoudifar and Doran, 2012). However, having the ideal equilibrium between these characteristics has proven complex. An optimal biomaterial composition should provide suitable cues in order to enhance cellular attachment and subsequent differentiation. In particular, the ligand binding sites with specific RGD sequences provided by a particular material may affect cellular functions such as adhesion, spreading and differentiation (Sukmana, 2012). Scaffold architecture is another well recognized determinant of cellular function such as adhesion, migration and matrix deposition and may significantly affect construct vascularisation (Boccaccini et al., 2012, Sukmana, 2012, Tian and George, 2011). Therefore, scaffold architecture may play a key role in the success of tissue-engineered endochondral constructs by allowing vessel invasion *in vivo*. Whilst hydrogels have been widely used in bone TE, their success may be affected by lack of a porous nature subsequently resulting in limited perfusion and reduced cellular migratory capacity. To overcome this, researchers have attempted to incorporate channels within the hydrogels in order to improve perfusion (Sheehy et

al., 2011). Scaffold mechanical properties such as substrate stiffness are known to affect *in vitro* lineage specification such as MSC chondrogenesis and osteogenesis (Steward et al., 2013, Murphy et al., 2012, Kelly and Jacobs, 2010, Engler et al., 2006). Indeed it has been shown that more compliant scaffolds support differentiation towards a chondrogenic lineage *in vitro*, while stiffer substrates support development of a hypertrophic phenotype both *in vitro* and *in vivo* (Bian et al., 2013). Therefore, such properties need to be taken into consideration when developing scaffolds suitable for endochondral bone tissue engineering.

A number of distinct biomaterials have been developed using a range of naturally-derived and synthetic polymeric materials and have been shown to support MSC chondrogenesis *in vitro* (Table 1). In our laboratory, collagen typically is the primary constituent of scaffolds for tissue regeneration including bone and cartilage (O'Brien et al., 2005; Tierney et al., 2009; Matsiko et al., 2012). Similarly, other collagen-based scaffolds have been widely used for both *in vitro* and *in vivo* tissue engineering and in particular endochondral bone formation with varying success (Scotti et al., 2013, Glatt et al., 2012, Rivron et al., 2012, Weiss et al., 2012, Farrell et al., 2011, Farrell et al., 2009, Oliveira et al., 2009b, Yu et al., 2010). Part of their success could be attributed to their biocompatibility, biodegradability, presence of functional motifs that facilitate tissue regeneration and vessel development (Duffy et al., 2011, Tian and George, 2011, Sukmana, 2012, Yannas et al., 1989, Burke et al., 1981).

Biomaterial	Cell Source	Evaluation	Reference
Alginate	Human, bone marrow derived	Gene expression, histology, SEM, GAG content	(Wang et al., 2009)
	Human	Gene expression	(Xu et al., 2008)
Alginate (RGD-modified)	Human, bone marrow derived	Biochemical analysis, gene expression, histology	(Re'em et al., 2010)
Agarose	Porcine, bone marrow	Biochemical analysis, histology, immunohistochemistry micro-computed tomography, <i>in vivo</i>	(Sheehy et al., 2013)
	Porcine, bone marrow, infrapatellar fat pad and synovial membrane derived	Biochemical analysis, histology, immunohistochemistry, micro-computed tomography mechanical properties, <i>in vivo</i>	(Vinardell et al., 2012)
	Bovine, bone marrow derived	Biochemical analysis, histology, mechanical properties	(Mauck et al., 2006)
Chitosan	Human, adipose and placenta derived	Biochemical analysis, gene expression, histology	(Huang et al., 2011)
	Human, bone marrow and adipose derived	Gene expression, histology, SEM	(Seda Tigli et al., 2009)
Collagen type I	Human, bone marrow derived	Biochemical analysis, gene expression, histology, micro-computed tomography, <i>in vivo</i>	(Scotti et al., 2013)
	Human, umbilical cord derived	Biochemical analysis, gene expression, histology	(Chen et al., 2013)
Collagen type I / GAG	Rat, bone marrow derived	Biochemical analysis, gene expression, histology, immunohistochemistry, mechanical properties	(Matsiko et al., 2012)
	Human, bone marrow derived	Gene expression, histology, immunohistochemistry, micro-computed tomography, <i>in vivo</i>	(Farrell et al., 2011)
	Human, bone marrow derived	Histology, immunohistochemistry, <i>in vivo</i>	(Farrell et al., 2009)
Collagen type II	Human, bone marrow derived	Histology	(Chang et al., 2007)
Fibrin	Human, bone marrow derived	Biochemical analysis, gene expression, histology	(Ho et al., 2010)
	Human, bone marrow derived	Biochemical analysis, gene expression, histology	(Dickhut et al., 2008)
Hyaluronic acid	Human, bone marrow derived	Biochemical analysis, gene expression, histology, mechanical properties, <i>in vivo</i>	(Bian et al., 2013)
	Goat, bone marrow derived	Biochemical analysis, gene expression, histology, mechanical properties	(Toh et al., 2012)
Hyaluronan / Gelatin	Rabbit, bone marrow derived	Histology, immunohistochemistry, <i>in vivo</i>	(Huang et al., 2006)
Matrigel	Human, bone marrow derived	Biochemical analysis, gene expression, histology	(Dickhut et al., 2008)
Poly lactic-co-glycolic acid (PLGA) / Poly(ϵ -caprolactone) (PCL) nanofiber scaffold	Rat, bone marrow derived	Biochemical analysis, histology, SEM, <i>in vivo</i>	(Yang et al., 2013)
Poly(ethylene glycol) (PEG)	Human, bone marrow derived	Biochemical analysis, histology, immunohistochemistry	(Buxton et al., 2007)
Poly(ethylene oxide) diacrylate (PEODA) with collagen mimetic peptides	Goat, bone marrow derived	Biochemical analysis, gene expression, histology	(Lee et al., 2008)
Polyglycolic acid (PGA)	Rabbit, bone marrow derived	Histology, immunohistochemistry	(Zhou et al., 2008)
Poly(L-lactic acid) (PLLA) nanofiber scaffolds	Human, bone marrow derived	Gene expression, histology, mechanical properties	(Janjanin et al., 2008)
Silk fibroin scaffold	Human, bone marrow and adipose derived	Biochemical analysis, histology	(Seda Tigli et al., 2009)
Sulfonate-coated polyacrylamide (S-PAAm) gels	Mouse, bone marrow derived	Gene expression, histology	(Kwon, 2013)

Table 1. The table shows a list of scaffolds that have shown potential to support cartilage-like matrix deposition and may be suitable candidates for the development of cartilage templates for

endochondral bone formation. Modified and reprinted from J Biomec, Vol 43(1), Huang AH, Farrell MJ, Mauck RL, Mechanics and mechanobiology of mesenchymal stem cell-based engineered cartilage, Pages No. 128-36, Copyright 2010, with permission from Elsevier

Other approaches for the fabrication of scaffolds for ECO applications in the future may include the use of decellularized constructs that can maintain the native 3D architecture, porosity and importantly the ECM components with appropriate cues that facilitate biological signaling (Arenas-Herrera et al., 2013). This technique has already led to clinical translation for human airway tissue repair applications (Macchiarini et al., 2008). Recently, decellularized matrices have been used as platforms for vascular graft development (Sheridan et al., 2012) and reported to support human articular chondrocytes and human bone marrow-derived MSC (hBMSC) chondrogenic differentiation and synthetic activity for cartilage regeneration (Giavaresi et al., 2013).

1.5 *In vitro* incubation periods to promote maturation and hypertrophy for endochondral bone tissue engineering

One of the major challenges to ECO-based strategies for bone formation is identifying the optimal *in vitro* cultivation period and conditions prior to implantation. Research to date shows the heterogeneous approaches taken in terms of *in vitro* incubation periods in order to facilitate MSC-derived chondrocytic maturation and hypertrophy prior to implantation (Table 2). 21 days of culture in TGF- β -supplemented chondrogenic differentiation media alone has been shown to stimulate MSCs differentiating towards late stage chondrogenesis, hypertrophy and subsequent bone formation *in vivo* (Sheehy et al., 2013, Vinardell et al., 2012, Pelttari et al., 2006, Huang et

al., 2006). However, studies have shown that either an additional 7 days of culture in the presence of β -glycerol-phosphate (β -GP) only (28 days in total) (Farrell et al., 2011) or an additional 14 days of culture in the presence of β -GP and thyroxine (35 days in total) (Mumme et al., 2012, Scotti et al., 2013, Scotti et al., 2010) have also stimulated bone formation *in vivo*.

Depending on the culture regime and *in vitro* incubation period used, differing stages of cartilage maturity and hypertrophy at the time of implantation can be achieved. A recent study investigated the impact of cartilage maturation on subsequent bone formation following implantation using three distinct groups (Scotti et al., 2010). Pre-hypertrophic and early hypertrophic groups were formed from hBMSC transwell constructs which were cultured in chondrogenic media for 1 and 2 weeks respectively. The late hypertrophic group was formed from the same hBMSC transwell constructs but had undergone 3 weeks of culture in chondrogenic media followed by an additional 2 weeks of culture in thyroxine-supplemented media. The culture periods showed progressively more mature cartilage formation *in vitro* with respect to culture length. Moreover, following subcutaneous implantation, template maturation correlated with bone formation and similar gene profiles to those seen in embryonic long bone development such as IHH, PTHrP and BMPs (Scotti et al., 2010). However, delayed implantation or prolonged *in vitro* culture may fail to take advantage of such genes and proteins that facilitate endochondral bone formation (Jeong et al., 2012). Furthermore, another study demonstrates the potential risk of using more mature, metabolically active hypertrophic cells with a shorter bioactive lifespan compared to immature pre-hypertrophic cells with lower nutritional and oxygen requirements (Gawlitta et al., 2010). Taken together, it is clear that the *in*

vitro culture duration prior to implantation needs to be standardised in order to facilitate enhanced chondrocyte hypertrophy and subsequent bone formation through ECO.

1.5.2 *In vitro* regulation of hypertrophy for endochondral bone tissue engineering

A wide range of cytokines and growth factors have utilized by a number of studies in order to drive MSCs towards a hypertrophic phenotype. However, there is a general lack of standardization with regards to the ideal culture conditions prior to *in vivo* implantation. The bone marrow contains a plethora of growth factors, cytokines and cells that are crucial in the homeostasis of bone and cartilage tissue. Some of these growth factors include bone morphogenetic proteins (BMPs). Whilst isoforms of BMP such as BMP-2 are fundamental in early stage mesenchymal stem cell chondrogenic differentiation and maintenance of a chondrocytic phenotype, such growth factors are also involved in regulating terminal differentiation (Goldring, 2006). A different isoform, BMP-6 is also involved in mediating chondrocyte hypertrophy. Treatment of chondrocytes with BMP-6 has been widely shown to stimulate gene expression of COLX, demonstrating the role of such a growth factor in ECO (Grimsrud et al., 1999, Grimsrud et al., 2001). The use of other supplemental growth factors *in vitro* to promote chondrocyte hypertrophy will be discussed below.

Transforming growth factor-beta 1, 2 and 3 (TGF- β 1, TGF- β 2 and TGF- β 3), members of the TGF- β super family, have also been shown to play crucial roles in cartilage and endochondral bone formation during fracture healing (Marsell and Einhorn, 2011, Gerstenfeld et al., 2003). *In vitro* exposure to such factors can induce chondrogenesis, proliferation and matrix deposition in MSCs with eventual progression towards hypertrophy (Mueller et al., 2010, Johnstone et al.,

1998). This is mediated through complex interactions with members of the fibroblast growth factor (FGF) and wingless-type (Wnt) protein families (Cleary et al., 2013). TGF- β subtypes in the presence of additional factors, such as thyroid hormone have been used to accelerate MSC-derived chondrocytes towards hypertrophy following only 14 days of chondrogenic-priming (Mueller et al., 2010, Mueller and Tuan, 2008). However, (Farrell et al., 2009) and (Pelttari et al., 2006) have shown that MSCs incubated in TGF- β 2 and TGF- β 3 respectively could promote the expression of genes associated with chondrogenesis and hypertrophy as well as factors critical to vascularization such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) without the need for other additional factors such as thyroid hormones. Furthermore, the different TGF- β subtypes have been shown to influence the level of mineralization in pellet culture without significant differences in collagen type X staining or VEGF secretion within media samples (Cals et al., 2012). Significantly higher levels of mineralization were observed *in vitro* in hypertrophic hBMSC-derived chondrocyte pellets cultured in TGF- β 3 with the addition of β -GP than in a TGF- β 1/ β -GP treated group (Cals et al., 2012), while TGF- β 2 chondrogenically-primed hBMSCs underwent mineralization *in vitro* in the presence of β -GP, without significant differences in the expression of hypertrophic markers compared to untreated groups (Farrell et al., 2009).

Another type of growth factor involved in mediating hypertrophy is insulin growth factor (IGF). IGF-1 and -2 are required in chondrocyte proliferation and hypertrophy. A study utilizing IGF-receptor1 knockdown mice demonstrated that their growth was significantly stunted (Baker et al., 1993). It is also now known that IGF-1 is involved in growth hormone activity. Growth hormone is responsible for longitudinal bone growth. A study elegantly demonstrated that a

reduction in IGF-1 levels correlated with interstitial growth of long bones in mice (Yakar et al., 2002). It is suggested that growth hormone activity is dependent on localized IGF-1 production (Nilsson et al., 2005).

Thyroid hormone works in synergy with the growth hormone and is also involved in hypertrophy and growth of bones. It has been widely used both *in vitro* and *in vivo* and has been shown to support MSC chondrogenesis and maturation. The thyroid hormone derivatives triiodothyronine (T₃) and thyroxine (T₄) have been shown to stimulate enlargement of chondrocytes, thereby regulating hypertrophy and secretion of collagen type X and alkaline phosphatase activity (Ahmed et al., 2007). The T₄ isoform of thyroid hormone has also been used to enhance hypertrophy of MSC-derived chondrocytes following 21 days of chondrogenic-priming with TGF-β1 (Scotti et al., 2013, Mumme et al., 2012, Scotti et al., 2010).

Trans-retinoic acid, a metabolite of vitamin A, has been shown to support maturation, hypertrophy and cartilaginous tissue formation by chick embryo chondrocytes on both collagen and chitosan scaffolds *in vitro* (Palmer et al., 2010, Oliveira et al., 2009a, Oliveira et al., 2009b, Oliveira et al., 2010, Teixeira et al., 2006), possibly through its action on retinoic acid receptors (RAR) causing down regulation of Indian Hedgehog (IHH) and upregulation of RARγ, MMP13 and Runx2 gene expression to promote hypertrophy and bone formation (Williams et al., 2010, Koyama et al., 1999). Although this system has not been assessed with MSC-derived chondrocytes, RAR-γ is highly expressed by MSC-derived chondrocytes under T₃ hypertrophic conditions with β-GP (Mueller and Tuan, 2008) and could offer an alternative culture regime for endochondral bone tissue engineering. Other approaches including adenoviral vectors encoding

for IHH (Steinert et al., 2012), bone morphogenetic protein-2 and -4 (BMP-2, BMP-4) and TGF- β 1 (Palmer et al., 2005) have all shown the ability to induce chondrogenesis in hBMSC pellets as early as 3 weeks *in vitro*, while soluble mediators such as vitamin D3 (Dreier et al., 2008, Gerstenfeld and Shapiro, 1996, Boyan et al., 1992) and leptin (Wang et al., 2012, Wang et al., 2011) have also been shown to favorably influence *in vitro* chondrocyte hypertrophy.

The use of pro-inflammatory cytokines such as interleukin-1 beta (IL-1 β) may be beneficial in the process of endochondral bone formation and template remodeling (Scotti et al., 2013, Mumme et al., 2012). MSC-seeded collagen constructs cultured with IL-1 β led to *in vitro* up-regulation of genes associated with chondrogenesis and hypertrophy (Scotti et al., 2013). In addition to increased MMP-13 activity and tartrate-resistant acid phosphatase (TRAP) activity compared to controls, bone formation was enhanced *in vivo*. Overall, these data imply enhanced endogenous and host mediated-ECM degradation in response to IL-1 β which could be beneficial in the degradation of larger constructs needed to facilitate clinically relevant bone regeneration *in vivo*. Furthermore, encouraging a hypertrophic phenotype in a cartilage template for bone formation may promote implant degradation as these hypertrophic cells can release lysosomal hydrolases involved in growth plate cartilage degradation *in vivo* (Bastow et al., 2012).

1.6 Current *in vivo* models of endochondral ossification

Following encouraging *in vitro* results involving several different biomaterials, a number of studies have examined the *in vivo* potential of tissue engineered cartilage templates for ECO bone formation ([Table 2](#)). In addition, recent work in our laboratory has demonstrated chondrogenesis with subsequent bone formation *in vivo* using alginate-based constructs in a

subcutaneous model ([Fig. 4](#)). Indeed, the majority of studies published thus far, have utilized subcutaneous models to determine the *in vivo* efficacy of their constructs for endochondral bone formation (Farrell et al., 2009, Sheehy et al., 2013, Farrell et al., 2011, Pelttari et al., 2006). The ectopic subcutaneous environment offers a reproducible, easily accessible location for *in vivo* validation. However, in order to fully determine the potential of constructs for endochondral ossification, osseous defect models should be utilized. One of the earliest studies to assess the *in vivo* potential of cartilage constructs for bone formation used a distinct *in vivo* model with the excision of an entire bone (Huang et al., 2006). The authors investigated the potential of an autologous cartilage construct derived from MSCs for carpal bone reconstruction following lunate excision in the rabbit forelimb. This was the first time that whole-bone repair was attempted and the study demonstrated that the cartilage construct was capable of supporting woven bone tissue formation following 12 weeks of implantation. Moreover, the study also demonstrated presence of neo-vascularization following implantation demonstrating the potential of such cartilage-based systems to promote blood vessel ingrowth. To further translate the potential of ECO-based strategies for therapeutic applications more investigation into clinically relevant bone defect models is required. There are now a number of well recognized weight-bearing segmental bone defects models established on small animals which should be adopted for the validation and assessment of ECO-based strategies. Our laboratory has recently adopted a rat femoral segmental defect model and the evaluation of a number of biomaterials for endochondral bone formation is currently underway.

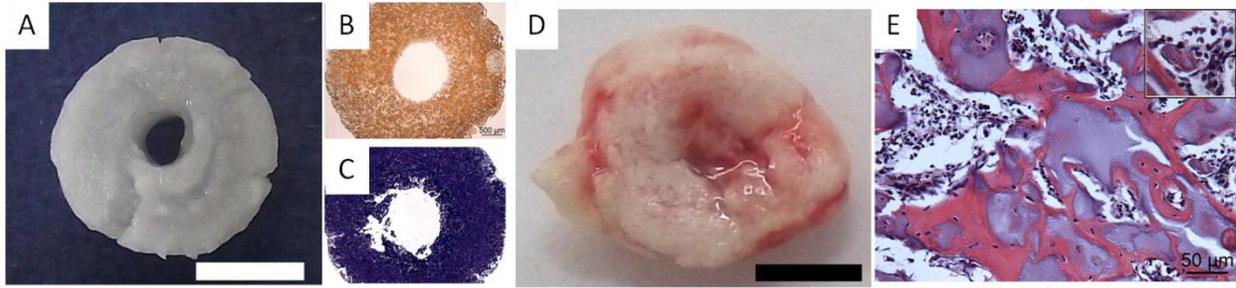


Figure 4. (A) Macroscopic image, (B) collagen type II immunohistochemistry and (C) aldehyde fuschin - alcian blue histology of chondrogenically primed MSC seeded alginate hydrogels pre-implantation. (D) Macroscopic image and (E) haematoxylin and eosin histology of alginate constructs 6 weeks post implantation. Macroscopic scales bars 3 mm. Prior to subcutaneous implantation constructs had undergone robust chondrogenesis, as indicated by intense staining for collagen type II and sGAG (B and C). Post-implantation, H&E staining (E) demonstrated the presence of trabecular bone struts, bone marrow foci, and blood vessel infiltration (inset). Images courtesy of E. Sheehy.

There has been a wide range of distinct approaches adopted for *in vivo* bone formation based on ECO in terms of biomaterials used, cell sources, soluble biochemical factors as well as *in vitro* culture duration, as shown on [Table 2](#). However, one similarity observed in these studies is the use of immune-incompetent mice to assess *in vivo* potential of the constructs thus far. Whilst this is widely accepted, host immunological responses are well known to play a key role in remodeling and neo-tissue formation following biomaterial implantation (Badylak and Gilbert, 2008, Brown et al., 2012). Therefore, the immunological processes that participate in mediating the host response to tissue-engineered constructs post-implantation need to be investigated in order to validate their potential for clinical translation.

Construct	Cell type	Culture conditions	<i>In vivo location & time points</i>	Reference
3D cell pellet	hBMSC	4-5*10 ⁵ cells / pellet, 3-7 weeks in TGF-β2 supplemented media	Subcutaneous (SCID mice), 4-6 weeks	(Pelttari et al., 2006)
Agarose Hydrogel (single layer and bilayered)	BMSC and HAC, porcine	20*10 ⁶ cells / ml, 21 days in TGF-β3 supplemented media	Subcutaneous (nude mice), 4weeks	(Sheehy et al., 2013)
Electrospun PLGA/PCL polymer fiber scaffold and 3D cell pellet	BMSC, rat	1*10 ⁶ cells / scaffold, 28 days in TGF-β2 and BMP-2 supplemented media	Subcutaneous (nude mice), 8 weeks	(Yang et al., 2013)
Chitosan scaffold	Chondrocytes, chick embryo sternum	20*10 ⁴ cells / scaffold, 10 days in hyaluronidase and ascorbic acid supplemented media followed by: - 10 days with the addition of all-trans retinoic acid	Subcutaneous (nude mice), 1, 2, 3, 4, and 5 months	(Oliveira et al., 2009b)
Collagen-GAG scaffold	hBMSC	30*10 ⁴ cells / scaffold, 21 days in TGF-β2 supplemented media	Subcutaneous (nude mice), 4 weeks	(Farrell et al., 2009)
	hBMSC	1*10 ⁶ cells / scaffold, 21 days in TGF-β2 supplemented media followed by: - 7 days in TGF-β2 media or - 7 days in TGF-β2 media with the addition of β-glycerophosphate	Subcutaneous (nude mice), 8 and 14 weeks	(Farrell et al., 2011)
Collagen type I mesh	hBMSC	40*10 ⁶ cells / cm ³ , 21 days in TGF-β1 with IL-1β supplemented media followed by: - 14 days in hypertrophic media without TGF-β1 but supplemented with thyroxine, β-glycerol phosphate, dexamethasone and L-ascorbic acid-2-phosphate	Subcutaneous (nude mice), 5 and 12 weeks	(Mumme et al., 2012)
	hBMSC	70*10 ⁶ cells / cm ³ , 21 days in TGF-β1 supplemented media followed by 14 days in hypertrophic media without TGF-β1 but supplemented with thyroxine, β-glycerol phosphate	Subcutaneous (nude mice), 5 and 12 weeks	(Scotti et al., 2013)
Hyaluronan / Gelatin	BMSC, rabbit	1*10 ⁵ cells/ μl, 21 days in TGF-β1 supplemented media	Lunate excision arthroplasty (rabbit), 6 and 12 weeks	(Huang et al., 2006)
Polylactic acid coated polyglycolic acid scaffold	hBMSC	2.5*10 ⁶ cells / scaffold, 4, 8 and 12 weeks in TGF-β1 supplemented media	Subcutaneous (nude mice), 12 and 24 weeks	(Liu et al., 2008)
Transwell plate self assembly	hBMSC	5*10 ⁵ cells / insert: - 7 days in TGF-β1 supplemented media or - 14 days in TGF-β1 supplemented media or - 21 days in TGF-β1 supplemented media followed by 14 days in hypertrophic media without TGF-β1 but supplemented with thyroxine, β-glycerol phosphate	Subcutaneous (nude mice), 4 and 8 weeks	(Scotti et al., 2010)

Table 2. Examples of tissue-engineered cartilage that have resulted in *in vivo* bone formation following an endochondral progression including construct type, cell type, culture conditions, additional supplements to promote ECO, implantation site and *in vivo* incubation periods

An important aspect of *in vivo* assessment is the contribution of donor and host cells to endochondral bone formation following implantation of constructs for ECO bone formation. Until recently, there have been few studies investigating the retention of implanted cells within endochondral grafts and their contribution to neo-tissue formation in such systems. However, it now appears that MSCs used in endochondral TE not only play a pivotal role in contributing to early tissue formation directly but also act to recruit specific host cell populations and secrete trophic factors that influence the process of vascularization and bone formation. Using MSC-derived cartilage templates, (Farrell et al., 2011) showed that implanted MSCs play a direct role in early osteogenesis, with host osteoblasts acting as the main contributors to continued bone formation. Using *in situ* hybridization for human DNA sequences (human *Alu* repeats), studies have also shown that following implantation of MSC-derived cartilage precursors, MSCs initially intersperse with host cells and contribute directly to bone formation *in vivo* (Scotti et al., 2013, Scotti et al., 2010, Peltari et al., 2006). Moreover, implanted donor cells have been shown to further co-localize specifically to inner regions of endochondral bone formation with osteoblast- and osteocyte-like cells (Scotti et al., 2013). Conversely, the outer regions of these constructs were observed to be characteristic of cortical-like bone and contained only host cells (Scotti et al., 2013). MSCs are known to recruit host cells and this was demonstrated by (Tortelli et al., 2010) using MSC-seeded scaffolds which stimulated bone and blood vessel formation via an endochondral-like process by recruiting host-derived CD31+ endothelial cells. Moreover,

compared to osteoblast-seeded scaffolds, the MSC-seeded scaffolds stimulated greater neo-vascularisation following implantation. Furthermore, analysis of this process was carried out by (Tasso et al., 2010) who investigated the nature of recruited host cells and demonstrated consecutive recruitment of two distinct waves of host cells: (i) CD31+ endothelial progenitors capable of tubule formation and (ii) CD146+ cells that displayed characteristics of MSCs. Their findings suggest that donor MSCs can act as both a cell source for chondro- and osteogenesis, as well as a co-ordinator for angiogenic-osteogenic coupling needed for bone generation and may help to explain the results from the above studies.

1.7 Future directions and concluding remarks

The evolution of strategies to repair bone defects using a tissue engineering approach has led to an ever-growing interest in recapitulating the embryological process of endochondral ossification in what is now coined as ‘developmental engineering’. This concept has been motivated by the hypothesis that cells driven through a chondrogenic lineage can secrete cartilaginous tissue that can act as a template for bone formation. In particular, hypertrophic chondrocytes can secrete angiogenic factors and alkaline phosphatase which play pivotal roles in both the vascularization of constructs *in vivo* and the deposition of a mineralized extracellular matrix with resulting bone deposition. Ultimately, this conveys great significance with regards to overcoming the drawbacks associated with a multitude of tissue-engineered constructs characterised by poor perfusion and avascular necrosis. However, several pivotal questions still remain to be answered including the optimal cell source, biomaterial scaffold and *in vitro* culture conditions required to produce robust chondrogenesis and hypertrophy *in vitro* with subsequent bone formation *in vivo*. Taken together, recapitulating endochondral ossification to develop such constructs for bone

defect repair is still in its nascency but it offers an attractive alternative to traditional bone tissue engineering strategies.

Acknowledgements

We acknowledge funding from the Health Research Board of Ireland (HRA_POR/2011/27). We also acknowledge Eamon Sheehy (TCBE, TCD) for providing macroscopic and histological *in vivo* images.

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