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REVIEW ARTICLE

Skeletal tissue engineering using embryonic stem cells

Jojanneke M. Jukes, Clemens A. van Blitterswijk and Jan de Boer*
MIRA Institute for Biomedical Technology and Technical Medicine, Department of Tissue Regeneration, University of Twente, Enschede, The Netherlands

Abstract

Various cell types have been investigated as candidate cell sources for cartilage and bone tissue engineering. In this review, we focused on chondrogenic and osteogenic differentiation of mouse and human embryonic stem cells (ESCs) and their potential in cartilage and bone tissue engineering. A decade ago, mouse ESCs were first used as a model to study cartilage and bone development and essential genes, factors and conditions for chondrogenesis and osteogenesis were unravelled. This knowledge, combined with data from the differentiation of adult stem cells, led to successful chondrogenic and osteogenic differentiation of mouse ESCs and later also human ESCs. Next, researchers focused on the use of ESCs for skeletal tissue engineering. Cartilage and bone tissue was formed *in vivo* using ESCs. However, the amount, homogeneity and stability of the cartilage and bone formed were still insufficient for clinical application. The current protocols require improvement not only in differentiation efficiency but also in ESC-specific hurdles, such as tumourigenicity and immunorejection. In addition, some of the general tissue engineering challenges, such as cell seeding and nutrient limitation in larger constructs, will also apply for ESCs. In conclusion, there are still many challenges, but there is potential for ESCs in skeletal tissue engineering. Copyright © 2009 John Wiley & Sons, Ltd.

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Keywords embryonic stem cells; bone; cartilage; tissue engineering; chondrocyte; osteoblast; chondrogenic differentiation; osteogenic differentiation

1. Tissue engineering of cartilage and bone

Tissue engineering aims at repairing or replacing damaged or diseased tissue. Cells, scaffold materials or both form the basis of this approach. Tissue engineering is used for tissues that have a poor self-healing capacity, such as cartilage, or when defects are too big for the body's capacity to heal itself, such as large bone defects.

For instance, in autologous chondrocyte transplantation (ACT) (Brittberg *et al*., 1994), articular cartilage is harvested from a minor load-bearing region of the knee. Chondrocytes are isolated and expanded *in vitro* and subsequently injected under a periosteal flap sutured over the defect. Since the first operation in 1987, patients have been followed-up continuously and good results have been achieved, especially regarding the durability of the repair (Brittberg *et al*., 2001; Peterson *et al*., 2002). An alternative approach for the delivery and retention of cells in the defect site is the use of a scaffold, which in addition can provide mechanical support directly after surgery, thereby reducing the rehabilitation time. ACT and scaffold-based therapies for the repair of cartilage defects generally require millions of chondrogenic cells (Tallheden *et al*., 2005). In order to obtain these cell amounts, *in vitro* cell expansion is required. It is well known that chondrocytes dedifferentiate in monolayer culture (von der Mark *et al*., 1977). Furthermore, in osteoarthritis, cartilage degenerates, due to a disturbed chondrocyte metabolism or mechanical failure. As such, chondrocytes might not be the ideal cell source to repair the defect.

Current therapies for bone substitution include the use of autografs, allografts and synthetic materials. In the first

*Correspondence to: Jan de Boer, MIRA Institute for Biomedical Technology and Technical Medicine, Department of Tissue Regeneration, University of Twente, PO Box 217, 7500 AE Enschede, The Netherlands. E-mail: j.deboer@tnw.utwente.nl

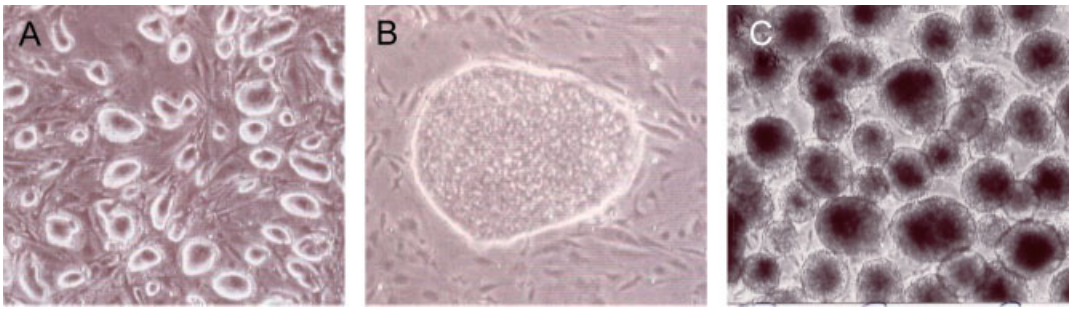

Figure 1. Embryonic stem cells and embryoid bodies. (A) Mouse ESC colonies on a feeder layer of mouse embryonic fibroblasts. (B) Human ESC colony on a feeder layer of mouse embryonic fibroblasts. (C) Mouse EBs in suspension

strategy, often referred to as the 'golden standard', healthy bone is harvested from the patient and implanted into the bone defect. Good results have been obtained, but donor site morbidity and limited availability within the patient's body are drawbacks of the technique. More bone is available for allograft treatments, for which bone is harvested from a human cadaver. The bone has to have all components removed that might induce an immune response, including cells. However, the risk of disease transmission cannot be ruled out, and allografts are less efficient than autografts. When autografts or allografts are not practicable, materials such as titanium alloys for mechanical support, or ceramic materials or polymers to support bone growth, have been used for hip replacement and other defects. However, poor long-term integration with the surrounding bone, due to mechanical and biological incompatibility, results in a need for bone graft substitutes. Cell-based bone tissue engineering might result in better bone healing and several cell types have been investigated.

1.1. Adult stem cells for tissue engineering

The use of mature cell types such as chondrocytes and osteoblasts is associated with several drawbacks. The limited availability, donor site morbidity, dedifferentiation and limited proliferative capacity urged researchers to study other cell types, particularly stem cells. The ability of stem cells to self-renew and differentiate into various cell types makes them an interesting source for tissue engineers. The best known example are bone marrow-derived stromal cells (Pittenger *et al*., 1999), also known as mesenchymal stem cells (MSCs), which can be differentiated into adipocytes, chondrocytes and osteoblasts. Other cells that have been differentiated into the chondrogenic or osteogenic lineage include mesenchymal stem cells isolated from adipose tissue derived after liposuction (Zuk *et al*., 2002), fetal blood and liver (Campagnoli *et al*., 2001), peripheral blood (Zvaifler *et al*., 2000), umbilical cord (Romanov *et al*., 2003), placenta (Fukuchi *et al*., 2004), amniotic fluid (De Coppi *et al*., 2007; Kunisaki *et al*., 2006) and human embryonic germ cells (Kim *et al*., 2005b). The low frequency, limited proliferative capacity and high variability on differentiation potential are drawbacks of adult stem cells.

1.2. Embryonic stem cells for tissue engineering

Another potentially interesting cell type for tissue engineering are embryonic stem cells (ESCs). In 1981, mouse ESCs were isolated (Evans and Kaufman, 1981; Martin, 1981) (Figure 1A), followed by their human counterparts in 1998 (Thomson *et al*., 1998) (Figure 1B). By definition, ESCs can self-renew and are pluripotent. Therefore, ESCs supply an infinite number of cells that can be differentiated into all cell types of the human body, in contrast to adult stem cells, which are at best multipotent and generate limited numbers of cells *in vitro*. Stem cell biologists have developed protocols to proliferate the ESCs indefinitely while remaining undifferentiated. Equally challenging is the development of defined protocols to differentiate ESCs into the desired cell type *in vitro* and *in vivo*. The first step in most ESC differentiation protocols is the formation of embryoid bodies (EBs) (Figure 1C). EBs are spontaneously forming free-floating aggregates of ESCs, in which cells differentiate at random into lineages of all three germ layers – endoderm, mesoderm, and ectoderm. These intact EBs, or single cells derived from these aggregates, so-called EB cells, are used in the subsequent directed differentiation steps.

The ability of ESCs to form cartilage or bone is well known, as these tissues are readily observed in teratomas. A teratoma, a benign tumour in which tissue from all three germ layers can be recognized, develops when undifferentiated ESCs are injected into various organs of an animal. However, the directed and controlled chondrogenic and osteogenic differentiation of ESCs had to be determined. Strategies for the differentiation of MSCs and ESCs into the chondrogenic and osteogenic lineage were reviewed in 2004, but data on ESC was limited at that time (Heng *et al*., 2004a, 2004b). The strategies include culturing in defined media to which chondro- or osteoinductive cytokines, growth factors and chemical factors have been added, the use of instructive substrata, such as ECM components, co-culturing and the addition of conditioned media, the application of physical stimuli, and genetic manipulation. Many of these protocols are based on protocols established for adult stem cells, chondro- and osteoprogenitors, or mature chondrocytes and osteoblasts.

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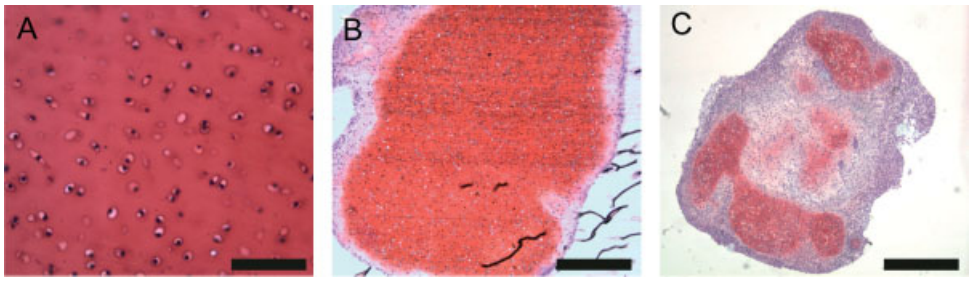

Figure 2. Cartilage. (A) A cross-section of bovine articular cartilage in which the chondrocytes can be recognized in their lacunae, surrounded by ECM. Glycosaminoglycans are stained pink by safranin O. Scale bar = 100 μm. (B) After 21 days in pellet culture, bovine chondrocytes, which were harvested from the cartilage in (A), secreted cartilage ECM. Scale bar = 500 μm. (C) After 21 days in pellet culture in serum-free chondrogenic medium, mouse ESCs have formed cartilage nodules. Scale bar = 500 μm

ESCs have the potential to become all cell and tissue types of the body, and during EB formation cells differentiate at random. During embryonic development, chondrogenesis and osteogenesis are closely intertwined. Therefore, it should not be surprising that many of the chondrogenic supplements are also involved in osteogenic differentiation. In fact, many of the cytokines, growth factors and signalling pathways described for chondro- and osteogenesis play a role in the development of many tissues. A well-defined differentiation protocol is required to direct all ESCs into the chondrogenic or osteogenic lineage.

A decade ago, researchers started to differentiate ESCs into chondrogenic and osteogenic lineages. The aim of many papers was to study chondrogenesis and osteogenesis, and not specifically the use of ESCs for tissue engineering or regenerative medicine applications. Using ESCs, the early steps of chondrogenesis and osteogenesis can be studied, for which adult stem cells and chondrocytes or osteoblasts are too mature. Another advantage is the relative ease of stably transfecting ESCs and obtaining high cell numbers of transfected cells. By generating *Sox9*$^{+/-}$ and *Sox9*$^{-/-}$ ESC lines, Sox9 was identified as the first transcription factor that is essential for chondrocyte differentiation and cartilage formation (Bi *et al*., 1999). Gene knockout or overexpression helped in elucidating the influence of transcription factors and genes on chondrogenesis and osteogenesis (Hargus *et al*., 2008; Kim *et al*., 2005a; Tai *et al*., 2004, 2005; Woei Ng *et al*., 2007). By creating chimaeric mice, the function of these genes can be studied in the developing embryo or living animals. In other studies, mouse ESCs were used as a screening system to identify factors that are both necessary and sufficient for cartilage and bone formation (Ikeda *et al*., 2004; Ohba *et al*., 2007). The lessons learned from ESCs as a model to study cartilage and bone development can result in improved protocols for more successful application of ESCs in cartilage and bone tissue engineering.

First studies were performed using mouse ESCs, and differentiation approaches were translated to human ESCs. After establishing the chondrogenic and osteogenic potential of ESCs, tissue engineers focused on the formation of cartilage and bone in combination with scaffolds. In this review, we further comment on the current challenges that have to be solved before ESCs can be used in the clinic, such as optimization of culture protocols, tumourigenicity of ESCs and immune rejection of allogeneic ESCs.

2. Chondrogenic differentiation of ESCs

To determine whether ESCs have indeed differentiated into the chondrogenic linage, markers that characterize cartilage were investigated. Articular cartilage is composed of chondrocytes, the extracellular matrix (ECM) they secrete, and water. The major components of the cartilage ECM are collagen type II and the proteoglycan aggrecan. Two chondrocyte-specific transcription factors have been identified, Sox9 (Bi *et al*., 1999) and scleraxis (Cserjesi *et al*., 1995). Cartilage has some distinct histological features: round cells in a lacuna surrounded by large areas of ECM (Figure 2A).

2.1. Spontaneous chondrogenic differentiation of mouse ESCs

In the first studies, intact mouse EBs were cultured on tissue culture plastic in basic ESC proliferation medium, and in the outgrowth of the attached EBs cartilaginous regions were observed (Kramer *et al*., 2000). Various stages of chondrogenesis, such as condensed mesenchymal cells, chondroprogenitor cells, mature and hypertrophic chondrocytes, could be recognized by analysing the expression pattern of mesenchymal and cartilage-related transcription factors and genes (Kramer *et al*., 2000, 2003) and electron-microscopic analysis (Kramer *et al*., 2005b). Thus, mouse ESCs can differentiate into the chondrogenic lineage, not only in teratomas but also *in vitro*, although with varying efficiency between several mouse ESC lines (Kramer *et al*., 2005a).

2.2. Chondrogenic differentiation of ESCs in a co-culture system

The formation of tissues in the developing embryo is heavily influenced by factors that are secreted by,

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or through direct cell–cell interactions with, the surrounding cells. This can be mimicked *in vitro* in a co-culture system, in which ESCs are differentiated into the chondrogenic lineage in the direct or indirect presence of chondroprogenitor cells or mature chondrocytes.

Antibiotic-resistant mouse ESCs were combined with progenitor cells derived from the limb buds of the developing embryo in micromass culture. After 4 days, antibiotic treatment allowed only the ESCs to survive. These cells continued to differentiate, and cartilaginous nodules were formed. Direct cell–cell contact was important in this system, as no chondrogenic differentiation was observed when conditioned medium from limb bud progenitor cells was added to mouse ESCs (Sui *et al*., 2003). In an indirect co-culture system, human ESCs were separated from primary human nasal chondrocytes (Vats *et al*., 2006) or bovine chondrocytes (Hwang *et al*., 2008c) by a well insert. Factors that were secreted by the mature chondrocytes were able to diffuse through the membrane and stimulate the chondrogenic differentiation of the human ESCs.

2.3. Growth factor-induced chondrogenic differentiation

Some of the chondrogenic factors that are secreted by the surrounding cells have been identified. Rather than adding the more mature cells, the isolated factors can be added to the ESC cultures. In the following experiments, the basic proliferation medium was supplemented with growth factors, such as members of the transforming growth factor β (TGFβ) superfamily, to direct and enhance chondrogenic differentiation. TGFβ1 alone did not enhance, but actually slightly reduced, the amount of cartilage nodules derived from mouse ESCs (Kramer *et al*., 2000). The addition of TGFβ3 and parathyroid hormone under low-serum conditions after retinoic acid treatment of EBs favoured chondrogenic differentiation (Kawaguchi *et al*., 2005). When bone morphogenetic protein (BMP)-2 or -4 was added early in the differentiation protocol when EBs were in suspension, chondrogenesis was induced (Kramer *et al*., 2000). The chondrogenic effect of BMPs was time-dependent. Rather than chondrogenic differentiation, BMP4 induced ESCS into the osteogenic lineage when added to retinoic acid-treated EBs during the entire differentiation procedure (discussed below) (Kawaguchi *et al*., 2005). The addition of BMP2 and TGFβ1 during EB formation, followed by the addition of insulin, ascorbic acid (AA) and BMP2 during the differentiation period, resulted in the most profound stimulation of cartilage-specific gene expression (zur Nieden *et al*., 2005). Thus, promotion of the chondrogenic lineage was dependent on the combination of the growth factors, hormones and other supplements, their concentrations and time of application.

2.4. Effect of cell morphology on chondrogenic differentiation

Cartilage tissue can easily be recognized by the round-shaped chondrocytes embedded in the ECM. When chondrocytes are isolated from cartilage and grown in monolayer culture, the cell morphology changes from round to fibroblast-like and the cytoskeleton reorganizes from a cortical distribution towards bundled stress fibres of actin filaments. As a consequence, chondrocytes dedifferentiate and switch from collagen type II to collagen type I expression. Dedifferentiated chondrocytes can redifferentiate by culturing them in pellets or in a hydrogel in which cell–cell or cell–hydrogel contact is present. Cell morphology can also be influenced by culturing cells in the presence of a cytoskeleton-disrupting agent such as cytochalasin, which has previously been used to induce chondrogenesis of mesenchymal limb bud cells (Zanetti and Solursh, 1984). When ESCs were treated with cytochalasin, cell morphology changed from fibroblast-like towards round, the stress fibres were disrupted and actin filaments reorganized. Cytochalasin-treated EB-derived cells expressed more Sox9 and collagen type II after 4 h. After 5 days, the treated cells stained positive for the collagen type II protein, but the expression of the collagen type II gene returned to the level of control cultures (Zhang *et al*., 2006). Thus, cytochalasin treatment and the resulting change in cell morphology had an effect on chondrogenesis of ESCs. However, we should realize that a transient rise in gene expression does not necessarily mean that significantly more extracellular matrix will be secreted by the cells. Therefore, additional experiments will have to be performed to see the long-term effect of changing cell morphology on cartilage formation.

2.5. Effect of aggregation on chondrogenesis

In the studies described above, the ESCs were cultured on tissue culture plastic. This two-dimensional (2D) environment does not seem to be the most logical choice to form a three-dimensional (3D) tissue. One of the earliest steps in chondrogenesis is the formation of a high cell density aggregate, which allows cell–cell interactions between the mesenchymal cells and with the surrounding cells. Whereas cartilage tissue does not consist of a single cell layer but is a 3D structure, the flat EB plating culture results in flat cells and does not allow extensive cell–cell interactions. Mesenchymal or precartilage condensation can be mimicked in a micromass or pellet culture system (Figure 2B), in which the cells are cultured in a high cell density aggregate. Cartilage formation of mesenchymal cell lines and MSCs was enhanced in pellets (Johnstone *et al*., 1998). The influence of aggregation on chondrogenic differentiation of mouse ESCs was further investigated. Chondrogenic differentiation of mouse ESCs on tissue culture plastic was mainly observed in regions where cells clustered into

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3D-like chondrogenic nodules. Rather than waiting for the cells to form the aggregates themselves, micromass culture or pellets of EB cells were formed in basic proliferation medium and compared with plated EBs. Chondrogenesis was indeed enhanced in the 3D cultures (Tanaka *et al*., 2004). In addition, pellets allow analysis of the cartilage morphology. By making cross-sections of the pellets, round cells could be recognized in ECM. Thus, aggregation enhanced chondrogenesis of ESCs and created a 3D tissue, which is a feature of cartilage.

2.6. Cartilage formation in serum-free chondrogenic medium

Instead of culturing in ESC basic proliferation medium supplemented with cartilage-specific growth factors, ESCs can also be differentiated in medium used to drive adult stem cells or primary cells into the chondrogenic lineage. Chondrogenic differentiation of rabbit and human MSCs can be achieved in serum-free medium containing proline, AA, sodium pyruvate, insulin–transferrin–selenious acid (ITS+), dexamethasone (Dex) and TGFβ1 or TGFβ3 (Johnstone *et al*., 1998; Mackay *et al*., 1998; Yoo *et al*., 1998). Cartilage nodules were formed in pellets of mouse ESC-derived EB cells cultured in this serum-free chondrogenic medium containing TGFβ3 (Jukes *et al*., 2008b; Nakayama *et al*., 2003) (Figure 2C). We even observed cartilage formation in the absence of TGFβ3 (Jukes *et al*., 2008b) but under both conditions the formed tissue was not homogeneous and other cell types were also present in the pellets.

Chondrogenic differentiation was observed in regions of human EB outgrowth when human ESCs were cultured in serum-free chondrogenic medium containing BMP2. Micromass cultures in the presence of BMP2 again showed the beneficial effect of high cell density on cartilage formation (Toh *et al*., 2007). When human EBs were cultured for 4 weeks in serum-free chondrogenic medium with a cocktail of growth factors, some ECM was produced. These EBs or the dissociated EB cells were cultured for another 4 weeks. EB cells formed pellets and produced more ECM, whereas EBs formed loose aggregates (Koay *et al*., 2007). When these self-assembled constructs were cultured under hypoxia, thereby mimicking the conditions of native cartilage, more collagen type II was produced and the human ESCs displayed better biomechanical functionality (Koay and Athanasiou, 2008).

2.7. Chondrogenic differentiation of human ESC-derived MSCs

Rather than starting the differentiation protocols with undifferentiated human ESCs or cells derived from EBs, some studies have first generated a cell population similar to MSCs. To initiate mesenchymal differentiation, human ESCs were cultured on a feeder layer of murine OP9 stromal cells, which have the potential to induce haematopoietic differentiation of mouse ESCs. After 40 days, cells were sorted by FACS for CD73, a marker expressed in adult MSCs. The CD73-positive fraction expressed a set of surface markers considered to define MSCs, and were negative for haematopoietic markers (Barberi *et al*., 2005). Functional characterization was performed by differentiating the cells into mesenchymal tissues such as fat, cartilage and bone.

During embryonic development, mesenchymal cells arise not only from mesoderm but also from the neural crest. After differentiation of human ESCs into neural rosettes, a CD73-postive fraction could be isolated, which could be differentiated into the adipogenic, chondrogenic and osteogenic lineages (Lee *et al*., 2007). Thus, human ESCs appear to be able to differentiate into mesenchymal-like cells from both mesoderm (Barberi *et al*., 2005) and neural crest (Lee *et al*., 2007). However, both protocols might be rather complicated and lengthy, with low efficiency for tissue-engineering applications.

In a simplified protocol, spontaneously differentiated cells were separated from the undifferentiated human ESC colony by mechanical dissociation. The scraped cells were cultured in basic medium and expressed CD markers similar to those of MSCs (Olivier *et al*., 2006). Multipotency was determined by differentiating the cells into osteogenic and adipogenic, but not chondrogenic, lineages. There are differences in morphology and differentiation capacity between various batches. By using a similar approach, mesenchymal-like cells were derived by passaging the EB outgrowth several times. The cell population became more homogeneous and morphologically similar to MSCs (Hwang *et al*., 2006b). The human EB-derived mesenchymal-like cells expressed some MSC surface markers, but not CD73, the marker used in the above-mentioned studies for selection of the mesenchymal fraction (Barberi *et al*., 2005; Lee *et al*., 2007). Although the derivation of ESC-derived mesenchymal-like cells was simplified, the chondrogenic differentiation capacity has to be optimized.

From the above-described studies, one can conclude that it is possible to direct both mouse and human ESC into the chondrogenic lineage and form cartilage tissue. The positive effect on cartilage formation of some growth factors and supplements and of 3D culture over 2D plating has been confirmed. However, no protocol resulted in the formation of large amounts of homogeneous tissue. Besides cartilage nodules, many other tissue types are also formed. Some protocols are quite complicated and will not be easily transferred into a clinical setting. In conclusion, the efficiency and homogeneity of cartilage formation should be further improved.

3. Cartilage tissue engineering using ESCs

Even though only some requirements for cartilage formation in 2D and small 3D systems had been defined,

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researchers already started culturing and differentiating the ESCs in a tissue engineering set-up. To be able to repair larger defects in clinical practice, the formation of larger constructs is necessary. Generally, this involves the use of a scaffold material. Requirements for successful tissue engineering applications include high cell numbers, homogeneous and high cell density seeding, and good nutrient flow throughout the scaffolds for sustained viability and homogeneous differentiation. The first requirement is often a problem for primary and adult stem cells, but not for ESCs because they can proliferate indefinitely. However, the last prerequisites pose an equal challenge for ESCs.

3.1. Cartilage tissue engineering using polymeric scaffolds

Mouse ESCs that were stimulated into the chondrogenic lineage in basic medium supplemented with AA, insulin and TGFβ were seeded onto polycaprolactone scaffolds. The cells attached to the scaffold and after subcutaneous implantation in mice some Alcian blue-positive cells were observed (Fecek *et al.*, 2008). However, these cells did not form tissue with a typical cartilage morphology. Co-cultured human ESCs were seeded onto poly-D,L-lactide scaffolds and implanted subcutaneously in the backs of immunodeficient mice. After 5 weeks, ECM was produced on the scaffolds and collagen type II was detected by immunostaining (Vats *et al.*, 2006).

When mouse EB cells or intact EBs were seeded onto a poly(ethylene oxide terephthalate)–poly(butylene terephthalate) scaffold and differentiated in serum-free medium containing TGFβ3, cartilage formation was observed. However, the seeding was non-homogeneous and the efficiency was rather low (Jukes *et al.*, 2008b). For homogeneous seeding, the scaffold's pores have to be fully interconnected to allow cells to enter the centre of the scaffold. On the other hand, cells should not wash out by medium flow, or sink to the bottom of the scaffold due to gravitational forces. Optimized scaffold architecture and dynamic seeding and culturing might improve seeding efficiency and homogeneity. However, the seeding of ESCs in chondrogenic medium is complicated by the absence of serum proteins, which result in easy detachment of the cells from the polymeric fibres (Jukes *et al.*, 2008b). Serum proteins enhanced cell attachment on scaffolds, but they inhibited the chondrogenic differentiation of mouse ESCs (Jukes *et al.*, 2008b; unpublished data). A careful balance between medium flow and cell attachment has to be maintained, to successfully seed and differentiate ESCs on scaffolds.

An alternative to direct seeding is using a gel to seed cells on a scaffold. Human EB cells were combined with Matrigel and seeded onto a thin poly(lactic-co-glycolic acid)/poly(L-lactic acid) scaffold. The human ESC proliferation medium was supplemented with TGFβ1 to direct the cells into the chondrogenic lineage. Cartilaginous tissue was indeed formed *in vitro* (Levenberg *et al.*, 2003).

3.2. Cartilage tissue engineering using hydrogels

Hydrogels can be used not only as a seeding method but also as a scaffold. A homogeneous high cell density can be achieved in hydrogels. Gels such as agarose and alginate have been used to study cartilage formation by chondrocytes. Mouse EB cells were encapsulated in various hydrogels and the seeding was indeed homogeneous, but most cells died within 1–7 days (Jukes *et al.*, 2008b), whereas bovine chondrocytes survived the entire 21 day culture period. In Matrigel, only a superficial layer of EB cells survived (Figure 3A) and differentiated into the chondrogenic lineage (Figure 3B). This implies that cell–matrix and cell–cell interactions improve the viability of the EB cells in a hydrogel. Indeed, when intact EBs instead of EB cells were seeded in the hydrogels, the majority of the EBs remained viable

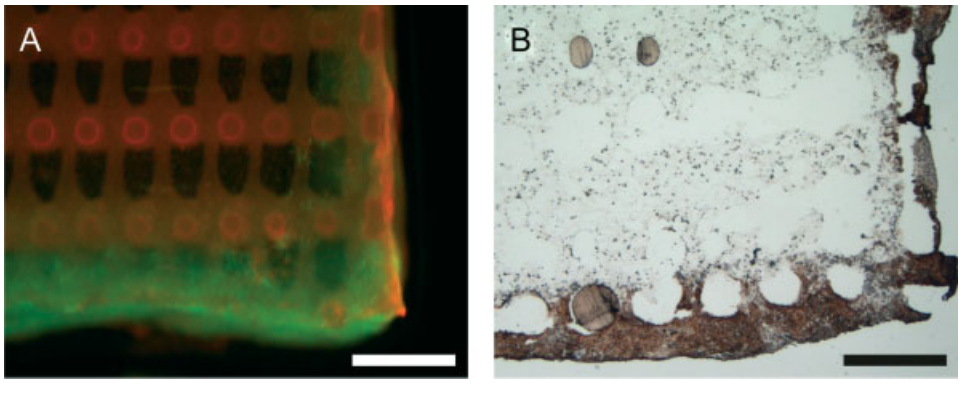

Figure 3. Mouse ESCs in a hydrogel. (A) Mouse ESC-derived EB cells were mixed in Matrigel and then injected into a poly(ethylene oxide terephthalate)–poly(butylene terephthalate) scaffold. After 21 days, a superficial layer of viable cells is present, whereas cells in the centre of the constructs died. Viable cells are indicated by green staining and dead cells by red staining. The scaffold fibres display red background staining. Scale bar = 100 μm. (B) Cartilage formation by mouse ESCs in the viable tissue layer, as seen in (A). Immunostaining for collagen type II is visualized by brown staining. Scale bar = 100 μm

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during the entire 21 day culture period (Jukes *et al*., 2008b).

Next, the chondrogenic differentiation of EBs in gel systems was investigated. When intact mouse EBs were encapsulated in alginate disks, and cultured either in basic proliferation medium or medium supplemented with Dex, chondrogenesis was not enhanced when compared with plated EBs (Tanaka *et al*., 2004). However, when mouse EBs were encapsulated in a poly(ethylene glycol)-based hydrogel and cultured in serum-free chondrogenic medium containing TGFβ1, a significant increase in cartilage markers was observed compared to 2D plating (Hwang *et al*., 2006c). Addition of glucosamine to the chondrogenic medium resulted in improved cartilage matrix production (Hwang *et al*., 2006a). Cartilage formation by EBs was also observed when cells were seeded in either Matrigel or agarose (Jukes *et al*., 2007). When EB-derived cartilage in either Matrigel or agarose was implanted in immunodeficient mice for 3 weeks, some cartilage was observed *in vivo*. However, the implanted cartilage was not very stable. Upon degradation of the gel, most of the cartilage was lost. In addition, teratoma formation was observed in these experiments, even though samples had been pre-cultured *in vitro* (Jukes *et al*., 2007).

Human EB-derived mesenchymal-like cells were seeded in functionalized PEG-based hydrogels and cultured in chondrogenic medium containing TGFβ1. The addition of collagen type I or hyaluronic acid did not result in chondrogenic differentiation, but the addition of RGD-peptides induced the formation of a cartilaginous matrix by human ESC-derived mesenchymal-like cells (Hwang *et al*., 2006b). It is known that mechanical compression can enhance chondrogenic differentiation (Huang *et al*., 2004). When human EB-derived mesenchymal-like cells were encapsulated in a hydrogel and one cycle of mechanical stimulation was applied, the expression of cartilage genes was increased (Terraciano *et al*., 2007). Whether mechanical stimulation of ESCs result in more cartilage formation, and what the optimal loading regime would be, has to be determined.

Co-cultured human ESCs were also seeded in PEG–RGD gels, and cartilage-like tissue was formed and cartilage-specific genes were expressed (Hwang *et al*., 2008c) at higher levels than in PEG hydrogels. When these cell–gel constructs were implanted, cartilage tissue was observed after 12 and 24 weeks *in vivo*.

In conclusion, mouse and human ESCs have been differentiated into the chondrogenic lineage and cartilage was formed not just on plates and in pellets but also in gels and on scaffolds. Some typical tissue-engineering difficulties were encountered, such as limited nutrient diffusion and homogeneous seeding. *In vitro*, regions of cartilage tissue were observed on the scaffolds but still heterogeneous, as also seen in pellets. Few *in vivo* studies were performed, and it is clear that these protocols require major improvement. The regions of cartilaginous tissue were small and in some studies typical characteristics of cartilage were not observed. The efficiency and stability of *in vitro* and *in vivo* cartilage formation has to be improved to obtain clinically relevant amounts of cartilage for tissue-engineering applications.

4. Osteogenic differentiation of ESCs

Bone (Figure 4) is mainly composed of cells, inorganic and organic matrix and water. Three mesodermal-derived cell types constitute bone. Osteoblasts secrete uncalcified matrix called osteoid; the osteocytes reside in little lacunae surrounded by calcified matrix and bone lining cells are in a resting state. The osteoclasts, which originate from haematopoietic precursors, degrade matrix. Their differentiation from ESCs (Duplomb *et al*., 2007a; Goodman *et al*., 2008; Hemmi *et al*., 2001; Okuyama *et al*., 2003; Suzuki and Nakano, 2001; Tsuneto *et al*., 2005a, 2005b; Yamane *et al*., 1997, 2000, 2002) will not be included in this manuscript. Unlike cartilage, bone is a vascularized tissue. Inorganic bone matrix consists of carbonated calcium-phosphate apatite. Collagen type I is the main component of the

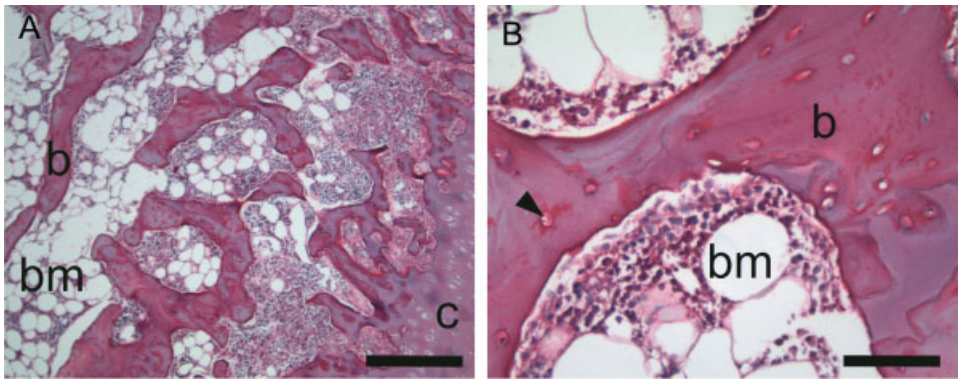

Figure 4. Bone. (A) Haematoxylin and eosin-stained cross-section of bone derived from a bovine knee. The articular cartilage (c) of the femoral head can be seen on the right and the underlying bone (b) and bone marrow (bm) in the marrow cavities on the left. Scale bar = 500 μm. (B) At higher magnification, osteocytes (black arrowhead) can be recognized in their lacunae in the bone matrix (b). Scale bar = 100 μm

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organic matrix and proteoglycans and non-collagenous proteins, such as osteopontin, osteonectin, osteocalcin and bone sialoprotein (BSP), form the remaining fraction. Osteoblasts express alkaline phosphatase (ALP), an enzyme which plays a role in calcification. Two bone-specific transcription factors, osterix (Nakashima *et al*., 2002) and runt-related transcription factor 2/core binding factor α1 (runx2/cbfa1) (Ducy *et al*., 1997), have been identified.

Osteogenic differentiation *in vitro* can be analysed by looking at mineral deposition, ALP activity and osteogenic gene expression. Bone tissue can form *in vivo*, which can be defined histologically by identifying bone lining cells and osteocytes and by looking at the organized structure of lamellar bone. In addition, bone marrow can be found.

4.1. Growth factor-induced osteogenic differentiation of ESCs

Some spontaneous osteogenic differentiation of ESCs was observed in basic medium (Buttery *et al*., 2001). By using similar approaches to those described above for chondrogenic differentiation, the osteogenic fraction could be increased. Growth factors and cytokines widely used to promote osteogenic differentiation of primary osteoblasts, preosteoblasts and adult stem cells (Jaiswal *et al*., 1997) are ascorbic acid (AA), β-glycerophosphate (BGP) and Dex. Supplementing ESC medium with AA and BGP (Buttery *et al*., 2001) or the trio of supplements (Handschel *et al*., 2008; Jukes *et al*., 2008a; Shimko *et al*., 2004; Sottile *et al*., 2003) significantly increased the amount of bone nodules and the expression of osteogenic markers in mouse and human ESC cultures. Osteogenic differentiation was also observed when intact EBs, rather than dissociated EB cells, were cultured in the presence of osteogenic supplements (Cao *et al*., 2005; Chaudhry *et al*., 2004).

Thus, osteogenic protocols could be transferred from adult (stem) cells to ESCs. Researchers also translated a protocol for adipogenic differentiation of mouse ESCs (Dani *et al*., 1997) to osteogenic differentiation (Phillips *et al*., 2001) and later to chondrogenic differentiation (Kawaguchi *et al*., 2005). In this protocol, EBs were treated with retinoic acid (RA) from day 3 to day 5. When RA-treated EBs were cultured in mineralization medium, osteogenic markers were expressed but only few mineralized areas were formed (Phillips *et al*., 2001). Addition of BMP4 (Kawaguchi *et al*., 2005), BMP2 (Phillips *et al*., 2001) or compactin (Phillips *et al*., 2001), a member of the statin family shown to increase bone density and osteoblast number *in vivo* (Mundy *et al*., 1999), greatly enhanced the amount of bone nodules.

Not only biological supplements can induce osteogenic differentiation. Inorganic stimuli derived from bioactive glass could induce the growth and differentiation of primary osteoblasts (Bielby *et al*., 2004b). The soluble ions released from bioactive glass were as effective as Dex at inducing the formation of mineralized nodules, and the addition of bioactive glass extract to Dex-treated cultures resulted in an increased amount of bone nodules (Bielby *et al*., 2005). This suggests that scaffolds of inorganic materials used in bone tissue engineering can enhance osteogenic differentiation.

4.2. Timing of osteogenic supplementation

In previous studies, it was recognized that the effect of many supplements is time-dependent. Late administration of Dex resulted in an increase of bone nodules for both mouse (Bourne *et al*., 2004; Buttery *et al*., 2001) and human ESCs (Bielby *et al*., 2004a). The addition of the calcium-regulating hormone vitamin D3 instead of Dex also increased the number of calcified cells (zur Nieden *et al*., 2003), but only when the osteogenic supplements were added after the EB formation step.

In a microarray study, control ESCs were compared with vitamin D3-treated cells and a change in expression of genes related to pluripotency and osteogenic differentiation was observed (zur Nieden *et al*., 2007). RA was expressed early and BMP2 was expressed at later stages of differentiation. This is in agreement with the observations that RA only enhanced osteogenic differentiation when added during the EB formation phase (Phillips *et al*., 2001), and not when added later during differentiation and mineralization (Buttery *et al*., 2001). Adding osteogenic supplements at the wrong time even resulted in a decrease in osteogenesis, as seen when BMP2 was added during early stages of differentiation (zur Nieden *et al*., 2007). Early administration of BMP4 resulted in chondrogenic differentiation (Kramer *et al*., 2000), whereas continuous stimulation resulted in osteogenesis (Kawaguchi *et al*., 2005).

Thus, by timing the supplementation of RA, BMP2, Dex and vitamin D3, the differentiation of ESCs into the osteogenic lineage could be significantly increased.

4.3. Osteogenic differentiation of ESCs in a co-culture system

Direct and indirect co-culture systems were also investigated for osteogenic differentiation of ESCs. Indirect co-culture of mouse ESCs with primary calvarial osteoblasts isolated from fetal mice resulted in five times more bone nodules, compared to control ESCs in the absence of osteoblasts (Buttery *et al*., 2001). The cell–cell interactions and BMPs secreted by primary bone–derived cells stimulated human ESCs into the osteogenic lineage in a direct co-culture system (Ahn *et al*., 2006). In an alternative co-culture system, the use of bone was avoided. Cell extracts derived from human ESC-derived osteogenic cultures induced undifferentiated human ESCs into the osteogenic lineage (Heng *et al*., 2008).

Rather than inducing cells into the osteogenic lineage, another co-culture aimed at increasing the mesodermal fraction before and during EB formation, by culturing

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mouse ESCs in the presence of medium conditioned by the human hepatocarcinoma cell line HepG2. The most efficient osteogenic differentiation was observed when EBs formed for 1 and 3 days. Longer EB culture times resulted in spontaneous differentiation towards beating cardiomyocytes and reduced osteogenic differentiation (Hwang *et al*., 2006a). Thus, careful timing of the EB formation time of conditioned medium-treated mouse ESCs enhanced osteogenic differentiation.

4.4. Influence of the EB step on osteogenic differentiation

It is unclear whether the EB formation step, a not well characterized step during which the cells differentiate at random, is essential, or whether cells might be directly differentiated into the desired lineage.

HepG2-treated and untreated mouse ESCs still formed bone nodules (Hwang *et al*., 2008b) or cartilage (Hwang *et al*., 2008b) when the EB step was completely omitted, or shortened as described above (Hwang *et al*., 2006a). However, these studies did not compare osteogenic or chondrogenic differentiation without and with EB formation, and therefore one cannot conclude which protocol is more efficient. When the EB formation step was omitted, calcium deposition by human ESCs was slightly delayed (Sottile *et al*., 2003). In contrast, another study concluded that human ESCs formed bone nodules earlier and at higher efficiency without the EB step (Karp *et al*., 2006), as also seen for mouse ESCs (Duplomb *et al*., 2007b). The formation of free-floating EBs was compared with initiation of differentiation in monolayer (Karner *et al*., 2007). All four human ESC lines could be differentiated into mesodermal and osteogenic lineages, although with varying efficiency. In fact, three lines exhibited greater osteogenic potential after monolayer culture, whereas one cell line expressed more bone-specific markers after EB formation (Karner *et al*., 2007).

From the studies described above we can conclude that EB formation is not essential, but we cannot conclude whether the EB formation step is beneficial for osteogenic differentiation.

Even though most researchers based their protocols for osteogenic differentiation of ESCs on protocols established for human MSC, parameters such as the timing of osteogenic supplements and their concentrations varied between the experiments. For example, not all groups used Dex, and when added to the osteogenic medium, the concentration varied from 10^{-4} (Bielby *et al*., 2004b) to 10^{-8} M (Karner *et al*., 2007; Karp *et al*., 2006; Shimko *et al*., 2004), which might have a major influence on osteogenic differentiation. The ESC-specific step of EB formation was different on many points. In some experiments, EB formation was omitted, and when EBs were formed, the duration and the supplements added varied. Later, during differentiation, either intact EBs or EB cells were used. Although the most optimal protocol has not yet been established, mineralization and osteogenic gene expression were observed, although at varying levels.

5. Bone tissue engineering using ESCs

To apply ESCs in the field of bone tissue engineering, the protocols will have to be transferred from tissue culture plastic to scaffolds. The formation of bone tissue by ESCs can be analysed in *in vivo* experiments.

5.1. Osteogenic differentiation of ESCs on scaffolds

In the first study describing 3D osteogenic differentiation of mouse ESCs, RA-treated EBs were first cultured for 3 weeks in 2D on tissue culture plastic in mineralization medium. Next, these differentiated cells were trypsinized and seeded onto polylactic acid scaffolds. Cells attached, grew and, after another 4 weeks in mineralization medium, secreted ECM, which stained positive for mineralization by von Kossa staining, and they expressed osteocalcin and osteopontin. These patterns were similar to the control 2D plates (Chaudhry *et al*., 2004).

To investigate whether the 3D environment enhanced osteogenic differentiation, as described above for chondrogenic differentiation of ESCs, EB cells were encapsulated in a peptide hydrogel, Puramatrix, and cultured in mineralization medium (Garreta *et al*., 2006). Mineralization was observed in both 2D and 3D culture. Slightly higher levels of osteopontin and collagen type I were observed in the 3D hydrogel cultures compared to 2D tissue culture plastic. ALP activity, on the other hand, was higher in the non-osteogenic control than in osteogenic cultures. However, ALP is also expressed by undifferentiated cells, and the number of Oct4-positive cells was higher in this 3D system (Garreta *et al*., 2006). This indicates that the 3D system enhances osteogenic differentiation, but also the undifferentiated state of the cells. All in all, the 3D environment seems to mimic the cell's natural environment, being bone or the stem cell niche, better than 2D culture plastic.

5.2. Osteogenic differentiation of ESCs *in vivo*

Both mouse and human ESCs could be successfully stimulated into the osteogenic lineage *in vitro*. However, for tissue-engineering applications it is essential that the cells can contribute to bone formation *in vivo*. Despite the many *in vitro* studies, no studies have been published about *in vivo* bone formation by mouse ESCs. Our group has observed that stimulation of the cells with compactin, BMP2 or Dex resulted in mineralization *in vitro*, as described above. However, when mouse ESCs were seeded on ceramic scaffold particles, cultured in various osteogenic media for 7 or 21 days and subsequently implanted in subcutaneous pockets of immunodeficient

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mice, no bone was formed (Jukes *et al.*, 2008a). Thus, *in vitro* osteogenic differentiation of mouse ESCs did not lead to bone formation *in vivo* (manuscript submitted).

Human ESC-derived EB cells were cultured in the presence of osteogenic supplements AA and BGP for 14 days, and Dex was added to this medium for another 24 h. These stimulated cells were then seeded onto poly-D,L-lactide scaffolds and implanted subcutaneously onto the back of immunodeficient mice for 5 weeks. Discrete areas of mineralization were observed, and osteocalcin was expressed by the implanted cells (Bielby *et al.*, 2004a). However, the formation of bone in which osteocytes and bone lining cells could be recognized was not observed.

Implantation in the peritoneal cavity of osteogenic and control human ESC-derived EB cells in injection chambers for 11 weeks resulted in the formation of mineralized areas (Tremoleda *et al.*, 2008). However, from the presented data, we conclude that control samples were equally as positive as osteogenic cultures. Apparently, osteogenic supplementation for 4 days before implantation was not long enough to enhance osteogenic differentiation of human ESCs to levels higher than spontaneous osteogenic differentiation. Similar conclusions could be drawn from the *in vitro* data (Sottile *et al.*, 2003).

Human ESCs that were co-cultured *in vitro* for 14 days on a feeder layer of PBDs, as described above (Ahn *et al.*, 2006), were seeded onto porous composite scaffolds using fibrinogen and implanted in immunodeficient mice (Kim *et al.*, 2008). After 4 and 8 weeks *in vivo*, small patches of bone-like tissue were observed between the fibrous connective tissue. More mineralized tissue was observed on ESC-seeded than on empty scaffolds. The addition of BMP2 to the fibrinogen–cell mixture further enhanced osteogenic differentiation.

Thus, some indications of bone-like and mineralized tissue formation by human ESCs *in vivo* were present, but the tissue formed did not resemble bone histologically.

6. Endochondral bone tissue engineering using ESCs

Bone can be formed by two different mechanisms in the embryo. In the first, neural crest-derived mesenchymal cells condense and differentiate into osteoblasts. This direct approach has been used in studies that attempted to differentiate ESCs into bone. In the other mechanism, paraxial and lateral plate mesoderm-derived mesenchymal cells aggregate and form cartilage which matures and calcifies. Blood vessels invade the calcified cartilage, which serves as a template for bone deposition by osteoblasts. As the direct differentiation into the osteogenic lineage did not result in bone formation, the endochondral approach was investigated. The first step in endochondral ossification is the maturation of the cartilage template, which becomes hypertrophic, as indicated by the switch from the expression of collagen type II, a cartilage-specific marker, to collagen type X, expressed by hypertrophic chondrocytes.

6.1. Full chondrogenic maturation of ESCs *in vitro*

Indications of hypertrophic maturation and calcification of ES-derived chondrocytes were observed during *in vitro* chondrogenic differentiation. During spontaneous differentiation of attached mouse EBs, the expression of cartilage markers reduced, which coincided with the appearance of collagen type X, a marker for hypertrophic cartilage, and bone markers (Hegert *et al.*, 2002; Kramer *et al.*, 2003, 2006). Expression of osteogenic markers could also be stimulated by switching from chondrogenic medium to hypertrophic medium (Nakayama *et al.*, 2003) or by adding osteogenic supplements such as vitamin D3, BGP (zur Nieden *et al.*, 2005) or BMP2 (Toh *et al.*, 2007). Thus, in the same culture, several steps of chondrogenic and osteogenic differentiation could be recognized.

6.2. Endochondral bone formation *in vivo*

The initial steps of endochondral ossification can be studied *in vitro*. ESCs are able to produce fully matured chondrocytes and subsequently osteogenesis is observed. However, the actual formation of bone via this indirect approach has to be studied *in vivo*. Therefore, mouse ESCs were seeded on scaffolds and differentiated into the chondrogenic lineage for 21 days in serum-free chondrogenic medium containing TGFβ3. At this time, cartilage tissue was observed and collagen type II was expressed. When these cartilage tissue-engineered constructs were implanted subcutaneously in immunodeficient mice, the cartilage became hypertrophic, calcified, became surrounded by bone tissue and was ultimately replaced by bone within 21 days (Figure 5). The newly formed bone was aligned with osteoblasts, and osteocytes were visible in the mature and mineralized bone tissue. Chondrogenic induction for 3 or 7 days was not sufficient, as bone formation was only observed after induction for 14 and 21 days, which resulted in the formation of a cartilage template. Endochondral bone formation was not just observed in subcutaneous pockets. When ESC-derived cartilage was implanted on a ceramic disc in a critical size cranial defect in an immunodeficient rat, bone was formed as well. Using the endochondral approach, for the first time directed and reproducible *in vivo* bone formation using ESCs was demonstrated in ectopic and orthotopic sites. The process is very robust. Bone was observed in all experiments where an ESC-derived cartilage template was formed *in vitro* (Jukes *et al.*, 2008a). However, when we followed the same approach for human ESCs, the efficiency of cartilage formation was too low to observe endochondral bone formation using human ESCs. Thus, endochondral ossification might be a new approach for bone tissue engineering using ESCs.

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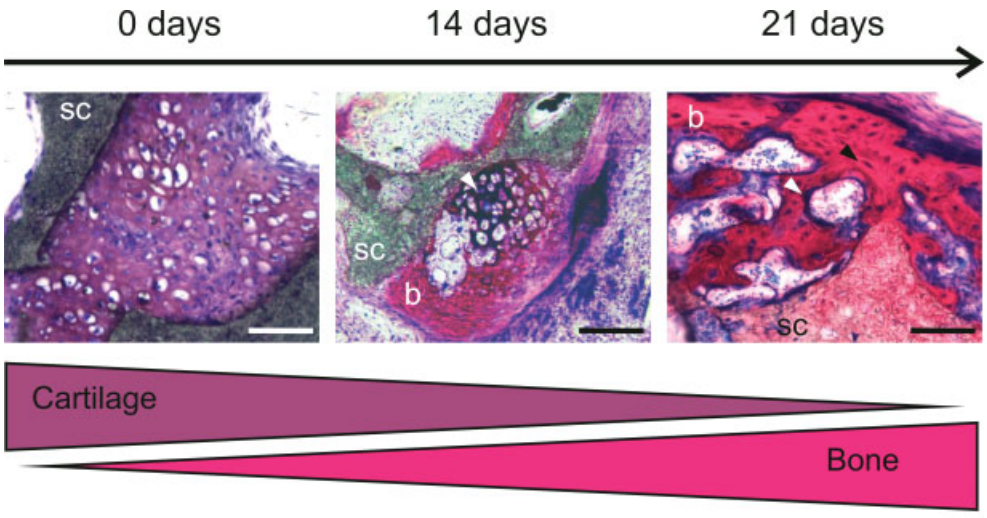

Figure 5. Endochondral bone formation by mouse ESCs. Mouse ESCs were seeded on ceramic scaffolds (sc) and differentiated into the chondrogenic lineage for 21 days. Subsequently, these samples were implanted subcutaneously in nude mice (0 days *in vivo*). After 14 days *in vivo*, hypertrophic cartilage (white arrowhead) can be recognized, surrounded by newly formed bone tissue (b). After 21 days *in vivo*, bone tissue with osteocytes (black arrowhead) and bone lining cells (white arrowhead) can be recognized. Thus, in time, the cartilage matured, served as a template for bone formation and was replaced by bone tissue. Cartilage tissue was stained purplish pink by thionin (0 days), bone tissue was stained reddish pink by basic fuchsin (14 and 21 days). Scale bar = 100 μm

7. Current and future challenges

Some of the basic requirements for cartilage and bone tissue engineering using ESCs have been established. The protocols have been improved on many points, but currently both cartilage and bone formation *in vivo* is not efficient enough for clinical application. Besides enhancing chondrogenic and osteogenic differentiation, some ESC-specific challenges still have to be solved. The first steps to solve some of the challenges have already been addressed and will be described below.

7.1. Upscaling of culture and differentiation of ESCs

The current protocols for ESC maintenance and differentiation are rather time-consuming and labour-intensive, especially protocols for human ESCs. The propagation of human ESCs often involves mechanical dissection by micropipettes, a process that requires concentration, time and experience. Feeder-free culture of human ESCs on normal culture plastics and passaging using trypsin would make upscaling more realistic. In many protocols, growth factors and other cytokines are used. For large-scale culture, efficient use of these expensive supplements will have to be implemented.

To simplify the differentiation of ESCs, osteogenic differentiation without the EB formation step has been described above (Duplomb *et al.*, 2007b; Karner *et al.*, 2007; Karp *et al.*, 2006). A bioreactor system in which maintenance of ESCs, EB formation and differentiation can be combined by simply changing the medium has been described for osteogenic differentiation (Randle *et al.*, 2007). Mouse ESCs were encapsulated in alginate/gelatin beads and cultured in a rotating 50 ml bioreactor vessel in ESC maintenance medium. When the medium was changed to EB medium for a period of 5 days, the proliferating cells formed aggregates, which expressed the mesodermal marker flk-1. Beads were cultured for another 21 days in osteogenic medium and mineralization was observed. ESCs can thus be differentiated in this simplified system, which is amenable for scale-up. ESCs could also be seeded and proliferated on gelatin-based microcarriers in a spinner flask. When proliferation medium was changed to osteogenic medium, indications of osteogenic differentiation were observed (Tielens *et al.*, 2007).

7.2. Animal product-free culture of ESCs

Most isolation, proliferation and differentiation protocols use animal-derived products, which can be contaminated with pathogens. Much attention was drawn to the risk of pathogen transfer to humans when prion diseases such as Creutzfeldt–Jakob, also known as mad cow disease, were discovered. It was also discovered that human ESCs can take up and express non-human sialic acid Neu5Gc from either feeder cells or culture media (Martin *et al.*, 2005), which might provoke an immune response upon transplantation. Therefore, ideally, protocols will be free of animal products before ESCs can be applied in human clinical therapy. This means that ESCs can no longer be isolated and maintained using mouse embryonic fibroblasts as feeder cells, and the medium cannot contain animal serum proteins and animal-derived supplements such as trypsin and growth factors. Several studies have optimized the maintenance protocols by using human feeder cells or ECM as a

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substitute for feeders, and serum-replacement instead of bovine serum. However, most protocols still contain animal products in one of the many steps that are required from isolation to application of ESCs. Full replacement of animal-derived products might result in an undesired high cost of culture and differentiation, which makes the use of ESCs less attractive for clinical application.

7.3. Patient-specific ESCs and ESC-like cells

As ESCs do not exist in the human body, they cannot be isolated from patients. Therefore, immunological rejection upon implantation of scaffolds seeded with allogeneic ESCs can be expected. One approach to circumvent this problem is to match ESCs to the patient, similar to organ transplantations. Since ESCs have an indefinite proliferation capacity, large banks of ESCs can be established, which can be screened to find a potential human leukocyte antigen (HLA) match (Taylor *et al*., 2005). Patients will likely have to take immunosuppressive drugs for the rest of their lives. More radical approaches would be the genetic alteration of ESCs to match the patient, or the very technically and ethically challenging therapeutic cloning.

Recently, patient-specific ESC-like cells have been created. Somatic cells have been reprogrammed to a pluripotent state and are referred to as induced pluripotent stem cells (iPS cells). Transcription factors that are involved in the maintenance of pluripotency, such as *Oct3/4* and *Sox2*, and genes involved in the maintenance and rapid proliferation of ESCs, *c-Myc* and *Klf4*, were introduced into mouse fibroblasts by retroviral transfection (Takahashi and Yamanaka, 2006). These differentiated fibroblasts then reprogrammed into an embryonic-like state, as indicated by their morphology and expression of ESC markers. By improving the protocol, three groups then reported the generation of viable adult chimeras, which indicated that the iPS cells contributed to the formation of all cell and tissue types in the mouse (Maherali 2007; Okita *et al*., 2007; Wernig *et al*., 2007).

Subsequently, human cells have been reprogrammed to pluripotency by the introduction of the four above-mentioned factors in adult human dermal fibroblasts (Takahashi *et al*., 2007), or a combination Oct4, Sox2, Nanog and Lin28 (Yu *et al*., 2007). These human iPS cells expressed ESC markers and could form EBs containing cells of the three germ layers. They could be differentiated into neural and cardiac cells, and after implantation teratomas formed in which many tissues, amongst others cartilage, were observed. The transgenicity of iPS cells might be a drawback for clinical applications, but new protocols using, for example, RNAi or small molecules could avoid viral transduction. Further research will have to be performed to investigate the chondrogenic and osteogenic differentiation of iPS cells for skeletal tissue-engineering applications.

7.4. Tumourigenicity of ESCs

The ability to form benign tumours *in vivo*, so-called teratomas, is a well-known characteristic of ESCs. Undifferentiated mouse ESCs were injected into mouse knee joints or into a subcutaneous pocket and analysed after 1–8 weeks. In total, 22 of 25 mice developed subcutaneous tumours, whereas 8 of 25 knees contained a tumour. The knee tumours were smaller than the subcutaneous tumours but large enough to destroy the knee joint after 8 weeks. Cartilage nodules were observed in both subcutaneous and knee joint tumours (Wakitani *et al*., 2003). When undifferentiated mouse ESCs were implanted into osteochondral defects created in the patellar groove of immunosuppressed rats, no tumours were observed up to 8 weeks. Cartilaginous tissue was observed, which indicated the chondro-inductive effect of the osteochondral defect on ESCs (Wakitani *et al*., 2004). Next, the influence of mechanical stimulation on cartilage and tumour formation by mouse ESCs was investigated. Rats with mouse ESCs in osteochondral defects in the patellar groove had their hind limbs elevated by tail suspension. In one group, free motion of the knee joint was allowed, whereas in the second group the knee joints were immobilized, due to which the patella did not fully cover the defect. Tumours were formed in all the immobilized knee joints, whereas fibrocartilage was amongst the regenerated tissue when knee movement was allowed (Nakajima *et al*., 2008). The confined space outside the defect, created by the movement of the patella, might prevent tumour growth, as also suggested when subcutaneous and knee joint tumours in mice were compared (Wakitani *et al*., 2003). It appears that the environment of the osteochondral defect site has a beneficial effect on osteochondral differentiation and a negative effect on tumour formation by undifferentiated mouse ESCs.

In the experiments described above, undifferentiated ESCs were used, which are known to have tumourigenic potential. Therefore, most researchers differentiate the cells *in vitro* into the desired lineage before they are used in animal studies. However, we observed that 3 weeks of *in vitro* differentiation of mouse ESCs in chondrogenic medium, which resulted in the presence of fully differentiated cartilage tissue, was not sufficient to eliminate the formation of teratomas *in vivo* (Jukes *et al*., 2007). Although the population of undifferentiated cells is greatly reduced upon differentiation, every remaining uncommitted cell can grow uncontrolled when implanted into a defect site. Strategies have to be developed to be absolutely sure that no remaining undifferentiated cells are implanted into the patient, as this can have a detrimental effect. Besides undifferentiated cells, cells that are committed to another lineage are also undesired. For example, cardiomyocytes, which form rather spontaneously during differentiation of ESCs, do not contribute to the repair of a cartilage or bone defect.

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7.5. Homogeneous tissue formation by ESCs

The optimization of differentiation protocols in order to obtain homogeneous tissue can focus on improving the differentiation efficiency or at purifying the heterogeneous cell population. This can either be negative selection by which undifferentiated cells are separated from the rest of the population, or positive selection for chondrogenic or osteogenic markers.

A combination of both optimization and positive selection resulted in more homogeneous cartilage formation by ESCs. To stimulate the formation of mesodermal cells during EB formation, BMP4 was added at this stage. The mesodermal fraction was sorted from the EB cells by selecting for lateral plate mesoderm marker vascular endothelial growth factor receptor 2 (VEGFR-2 or flk-1) and paraxial mesoderm marker platelet-derived growth factor receptor α (PDGFRα) (Nakayama *et al*., 2003). These mesodermal progenitor cells, expressing either flk-1, PDGFRα or both, were capable of forming cartilage nodules in a pellet culture, when cultured in serum-free medium containing TGFβ3. The negative fraction did not form cartilaginous tissue. The PDGFRα-positive fraction was also able to form cartilage nodules in the absence of TGFβ3 (Nakayama *et al*., 2003), as also observed for unsorted cells (Jukes *et al*., 2007). By adding PDGF-BB or BMP4 to the differentiation medium, larger pellets filled with cartilage matrix could be obtained. Thus, the combination of growth factors can stimulate a mesodermal fraction of mouse ESCs towards more homogeneous cartilage formation.

Similarly, osteogenic ESCs were sorted by magnetically activated cell sorting (MACS) using cadherin-11, a cell adhesion molecule expressed at high level during the early stages of osteoblasts differentiation. When the cadherin-11-positive population was cultured for another 2 weeks in mineralization medium, almost homogeneous bone nodule formation was observed, whereas the cadherin-11-negative fraction did not show osteogenic differentiation (Bourne *et al*., 2004).

The choice of selection marker is critical but difficult. By choosing an early marker, the cells might still have the potency to differentiate towards undesired lineages. By choosing a late marker, the cells might already be embedded in their ECM and therefore less accessible for markers and for selection techniques, which mostly require a single cell solution.

8. Conclusion

Differentiation of ESCs into chondrogenic and osteogenic lineages caught the attention of scientists from the beginning of this century. More recently, researchers started to investigate the potential of ESCs in cartilage and bone tissue engineering. The earlier studies merely analysed chondrogenic and osteogenic markers. However, we observed that increased expression of the collage type II gene does not necessarily mean that cartilage tissue will be formed, and mineralization *in vitro* is not a good indicator for *in vivo* bone formation. Some analyses reveal no information on the distribution of cartilage and bone in the sample or tell anything about the heterogeneity, both of which are important parameters.

The search for the optimal protocol continues and, as a result, it is often difficult to compare the results of the articles. Subtle or large differences between differentiation protocols might have a bigger influence on chondrogenesis or osteogenesis than is currently known. In addition, there will be variation between the various mouse and human ESC lines used in various studies. Although there are similarities between mouse and human ESCs, there are even more differences. Not all mouse data can simply be extrapolated to humans ESCs. Last but not least, the amount of *in vivo* data is still limited. The formation of sufficient amounts of functional and stable cartilage and bone requires further research.

Besides increasing the efficiency of cartilage and bone formation, ESC-specific challenges will also have to be addressed. These current disadvantages affect researchers investigating the use of ESCs in any clinical application, and hopefully a combined effort can be made to find the solutions to make the use of ESCs safe.

Progress has been made in the last years, but cartilage and bone tissue engineering using ESCs is still in its infancy. Luckily, this is the period when cartilage and bone develop rapidly in the human body; thus, we can be optimistic about the future perspectives.

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