

Informing future cartilage repair strategies: a comparative study of three different human cell types for cartilage tissue engineering

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Abstract A major clinical need exists for cartilage repair and regeneration. Despite many different strategies having been pursued, the identification of an optimised cell type and of pre-treatment conditions remains a challenge. This study compares the cartilage-like tissue generated by human bone marrow stromal cells (HBMSCs) and human neonatal and adult chondrocytes cultured on three-dimensional (3D) scaffolds under various conditions *in vitro* and *in vivo* with the aim of informing future cartilage repair strategies based upon tissue-engineering approaches. After 3 weeks *in vitro* culture, all three cell types showed cartilage-like tissue formation on 3D poly (lactide-co-glycolide) acid scaffolds only when cultured in chondrogenic medium. After 6 weeks of chondro-induction, neonatal chondrocyte constructs revealed the most cartilage-like tissue formation with a

prominent superficial zone-like layer, a middle zone-like structure and the thinnest fibrous capsule. HBMSC constructs had the thickest fibrous capsule formation. Under basal culture conditions, neonatal articular chondrocytes failed to form any tissue, whereas HBMSCs and adult chondrocytes showed thick fibrous capsule formation at 6 weeks. After *in vivo* implantation, all groups generated more compact tissues compared with *in vitro* constructs. Pre-culturing in chondrogenic media for 1 week before implantation reduced fibrous tissue formation in all cell constructs at week 3. After 6 weeks, only the adult chondrocyte group pre-cultured in chondrogenic media was able to maintain a more chondrogenic/less fibrocartilaginous phenotype. Thus, pre-culture under chondrogenic conditions is required to maintain a long-term chondrogenic phenotype, with adult chondrocytes being a more promising cell source than HBMSCs for articular cartilage tissue engineering.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ACI	Autologous chondrocyte implantation
ALPSA	Alkaline-phosphatase-specific activity
CCA	Compressive cell adhesion
CLSM	Confocal laser scanning microscopy
DPX	p-Xylene bis-pyridinium bromide
ECM	Extracellular matrix
FCS	Fetal calf serum
FDA	Food and Drug Administration
HBMSCs	Human bone marrow stromal cells

MSCs	Mesenchymal stem cells
NBF	Neutral buffered formaldehyde
PLGA	Poly (lactide-co-glycolide) acid
TGF- β 3	Transforming growth factor beta 3
α -MEM	Alpha-modified minimum essential medium

Introduction

Normal diarthrodial joint function requires the presence of healthy articular cartilage capable of bearing mechanical load. Damage to articular cartilage is of clinical concern because of the limited ability of the tissue to self-repair spontaneously (LeBaron and Athanasiou 2000a; Saha et al. 2011; Tuli et al. 2003). A number of clinical strategies have been adopted over the years to tackle this problem, e.g., arthroscopic lavage and debridement, periosteum and osteochondral transplantation, autologous chondrocyte implantation (ACI) and free toe and joint to hand transfer but each of these methods has its own disadvantages limiting their use (Gu et al. 1997; Redman et al. 2005; Ringe and Sittering 2009; Tuan et al. 2003; Yang and Gu 2000). Over the last decade, cell-based therapies have shown much promise for articular cartilage regeneration; for example, Brittberg et al. (1994) have reported the successful repair of cartilage defects following autologous chondrocyte transplantation. In 1995, Genzyme employed this technique to develop “Carticel”, a Food and Drug Administration (FDA)-approved ACI treatment for patients with cartilage injuries of the knee. A major drawback of ACI is that it is a two-step procedure in which cells are first harvested to allow for cell expansion followed by a surgical procedure for the transplantation of the expanded cells. The adverse effects of ACI include arthrofibrosis, graft overgrowth and chondrosis (Vasiliadis et al. 2010). Other associated problems with chondrocyte transplantation are limited cell availability, loss of re-differentiation capability *in vitro* and a risk of donor site morbidity (Baksh et al. 2007). Moreover, no direct evidence has been presented to date to suggest that ACI treatment is superior to alternative surgical options such as microfracture or mosaicplasty. Studies comparing the cost effectiveness of ACI against that of mosaicplasty or microfracture found insufficient difference of any significance between the three procedures (Clar et al. 2005; Rodriguez-Merchan 2012; Vasiliadis et al. 2010). On the basis of these comparisons, experts have voiced the need for more basic science research into determining factors that help differentiate and maintain stem cells in a chondrogenic phenotype (Clar et al. 2005). Thus, efforts are now focused on stem cell therapy. Because of ethical issues and potential problems in the regulation of cell differentiation *in vivo*, the application of embryonic stem cells has been limited (Lin et

al. 2005). Wei et al. (2012) have reported the use of induced pluripotent stem cells for chondrogenic differentiation. However, their reprogramming may have the risk of generating germ cell tumours (Grad et al. 2011). Adult mesenchymal stem cells (MSCs) have become a popular alternative for the treatment of cartilage injuries. MSCs are said to have extensive self-renewal, long-term cell viability and multi-lineage potential together with a lack of or minimal immunogenicity (Lin et al. 2005). Adult stem cells derived from a myriad of sources (bone marrow, fat pad, synovial fluid, dental pulp and periosteum) have demonstrated chondrogenic potential *in vitro* (Emans et al. 2005; Estes et al. 2006; Johnstone et al. 1998; Shintani and Hunziker 2007; Yanai et al. 2005). Some of these stem cells have been used clinically to regenerate bone but their clinical application in regenerating human cartilage is either undocumented or in the early stages (Horwitz et al. 2002; Wakitani et al. 2002). Whereas the numerous different types of adult stem cells isolated have been documented to possess the typical stem cell markers (adherence to tissue culture plastic; expression of CD105, CD73 and CD90; differentiation along chondro/osteo/adipogenic lineages), these stem cell populations have diverse proliferation rates and dissimilar differentiation potentials and respond differently to growth factors (Chen and Tuan 2008; Dominici et al. 2006). The method of cell isolation, culture, medium, growth factors, interaction with scaffold material and substrate topography are all known to affect cell proliferation and differentiation. However, different protocols are used by different research groups, making it a challenge to compare between different studies; thus, the optimum cell source and culture conditions for use in cartilage tissue repair is yet to be determined (Chen and Tuan 2008). To date, human bone marrow MSCs (HBMSCs) remain the most studied and best understood stem cell source used in cartilage tissue engineering (Chen et al. 2005; Johnstone et al. 1998; Mackay et al. 1998; Penick et al. 2005; Ponticciello et al. 2000; Wayne et al. 2005; Yanai et al. 2005)

The fabrication of *ex vivo* three-dimensional (3D) cartilage constructs by using functional tissue-engineering approaches has been used in an attempt to solve clinical problems of repair/regeneration of articular cartilage. The outcomes of such studies have however yielded inconsistent results (LeBaron and Athanasiou 2000a). We have previously compared HBMSCs, human adult chondrocytes and human neonatal articular chondrocytes under various two-dimensional (2D) culture conditions simulating the *in vitro* stage of articular chondrocyte implantation therapy and have reported intrinsic differences in the microenvironment of the three cell types studied (Saha et al. 2010). In the present study, we have seeded cells on the cartilage phase of a TruFit CB (Smith & Nephew) scaffold. This scaffold is an FDA-approved resorbable biphasic implant constituted

predominantly from polylactide-co-glycolide copolymer (PLGA), calcium sulphate and polycolide and has a mean pore diameter of 250 μm (<http://www.ortovit.eu/ortopedie/special/doc/TruFit%20CB%20Plugs.pdf>). The scaffold used has been documented in the literature as successfully supporting chondrocyte migration and the synthesis of chondrogenic ground matrix and has yielded promising clinical results (Agrawal and Stinson 2007; Williams and Gamradt 2008). The present study builds on our previous work and compares the genesis of 3D cartilage-like tissue in vitro and in vivo by using the same three cell types as before in an attempt to illustrate intrinsic differences between the chondrogenic potential of a progenitor cell type and immature and mature chondrocytes, which represent the three different life stages of a cartilage-forming cell and also to identify an optimum cell type for use in cartilage tissue engineering, thereby informing future cell therapy strategies for cartilage repair.

Materials and methods

Passage 1 HBMSCs (two donors aged 38 and 40 years) and primary adult chondrocytes (two donors aged 35 and 40 years) from haematologically normal donors were obtained from Lonza (UK). Primary neonatal articular chondrocytes (Passage 0) were provided by Smith & Nephew (UK) with appropriate ethical approval and consent for use in commercial and collaborative research. Alpha-modified minimum essential medium (α -MEM) and fetal calf serum (FCS) were from Lonza.

Cell culture

Cells were expanded in basal media (α -MEM with 10% FCS) and the medium was changed every 3 days. The cells were passaged at 70%–75% confluency up to a maximum of passage 4. To induce chondrogenesis, a serum-free chondrogenic medium consisting in α -MEM supplemented with 10 ng/ml transforming growth factor beta 3 (TGF- β 3; Peprotech, USA), 10^{-8} M dexamethasone, 100 μM ascorbate-2-phosphate and $1\times$ insulin transferrin selenium supplement (Johnstone et al. 1998; Saha et al. 2010) was used in place of the basal medium.

Dynamic seeding of cells on 3D scaffolds

In order to visualise cell attachment and spreading on/within the scaffolds, the cells were labelled with 50 μg CellTracker Green 5-chloromethylfluorescein diacetate (Invitrogen) in 5 ml α -MEM for 45 min (Yang et al. 2006). The cells were trypsinised and 2.5×10^5 cells of each cell type were seeded dynamically onto the separated

ultra-violet-sterilised cartilage phase of TruFit CB Plugs ($3\times 1.5\text{ mm}^3$) by using a dynamic seeding apparatus designed in house (rotational speed: 17 rpm) for 24 h. Optical sections of the cell-scaffold construct (up to a depth of 300 μm) were obtained by confocal laser scanning microscopy (CLSM; Leica). Images were stacked and 3D reconstruction was carried out by using Leica confocal software enabling us to visualise cell viability up to a considerable depth within the scaffold.

In vitro static culture of cells on 3D scaffolds

After 24 h of dynamic seeding, the constructs were transferred to 24-well plates and cultured in vitro in either basal or chondrogenic medium under static conditions at 37°C in a humidified atmosphere of 5% CO_2 ($n=4$). After 3 or 6 weeks, the constructs were fixed in 10% neutral buffered formaldehyde (NBF) and embedded in paraffin. Sections (5 μm thick) were prepared by using a Leica microtome.

In vivo intra-peritoneal implantation

All the in vivo studies were carried out under UK Home Office project license and were approved by the University of Leeds ethical committee. Briefly, cells were dynamically seeded onto scaffolds as described above. Constructs were then cultured in vitro in basal or chondrogenic media ($n=4$) for a further 7 days and then sealed into diffusion chambers (Millipore, UK) prior to intra-peritoneal implantation in male immuno-compromised nude mice (MF1-Nu/Nu, 4–5 weeks old; Partridge et al. 2002; Yang et al. 2004). After 3 or 6 weeks, the mice were killed in compliance with UK Home Office regulations and the chambers removed and fixed in 10% NBF at 4°C. Samples were then processed in the same way as the in vitro samples.

Alcian Blue/Sirius Red staining

Alcian Blue/Sirius Red staining was used to visualise the presence of sulphated glycosaminoglycans and collagen within the constructs. Briefly, the sections were stained with Weigert's haematoxylin for 15 min prior to being stained with 0.5% Alcian Blue for 10 min. After a 20-min treatment with freshly prepared 1% molybdophosphoric acid, samples were stained for up to 1 h in 0.3% Sirius Red. The sections were then mounted in p-xylene bis-pyridinium bromide (DPX) and observed under an Olympus BX50 microscope by using SPOT software.

Immunohistochemical staining

Immunohistochemistry was carried out in order to assess the expression of Sox9 (rabbit polyclonal anti-SOX9; Abcam)

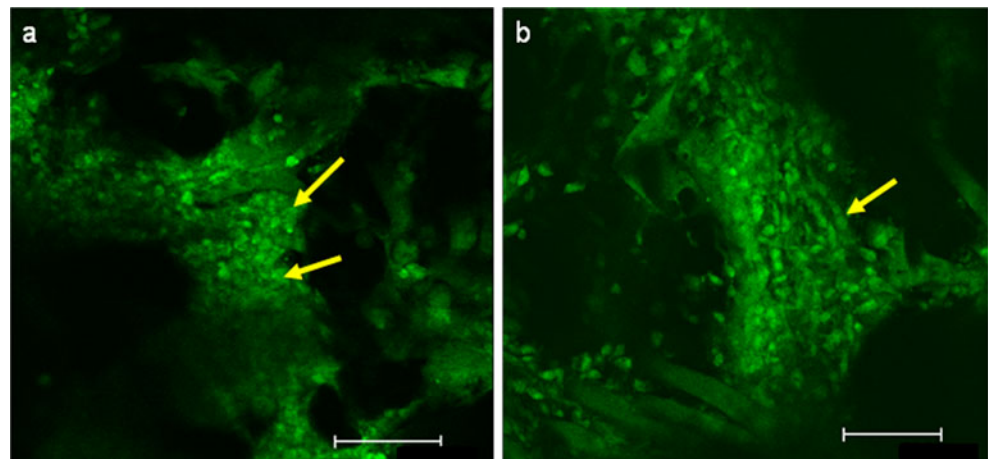
and aggrecan (mouse monoclonal anti-aggrecan; Abcam) in 5- μm -thick paraffin sections of the constructs retrieved after culture *in vitro* and *in vivo*. Endogenous peroxidase activity was blocked by using 3% hydrogen peroxide and an antigen retrieval step was carried out by using an antigen unmasking vector solution (Vector Laboratories, Calif., USA). Samples were incubated in primary antibody (1/50 dilution of anti-Sox9; 1/100 dilution of anti-aggrecan) for 1 h at room temperature. The EnVision Kit, which contained the secondary antibody (Dako), was employed to detect peroxidase activity. All samples were processed simultaneously to ensure homogeneity in the staining procedure. Samples tested for the presence of Sox9 were counterstained in Fast Green for 30 s. Negative controls (minus the primary antibody) were also carried out for each sample. All samples were dehydrated, cleared in xylene, mounted in DPX and observed under an Olympus BX50 microscope by using SPOT software. For any given 3D construct, consecutive paraffin sections were used to assess the expression of the different chondrogenic markers in order to minimise any depth-dependent variations that may have arisen. A qualitative assessment of the intensity of any immunostaining observed was made.

Results

Cell attachment and spreading on 3D scaffolds

After 24 h of dynamic seeding, CLSM 3D reconstruction images showed that the majority of cells of each cell type were viable and had attached on all sides of the scaffolds (Fig. 1). Cell morphology appeared to be stretched with elongated processes clearly visible indicating cell spreading. Cells were also observed to have penetrated into the central part of the scaffold (ingrowth). No obvious differences were noted between any of the cell types with respect to cell viability, attachment and spreading.

Fig. 1 Confocal images of human bone marrow stromal cells seeded onto TruFit scaffolds. The cells were labelled with live/dead fluorescent markers (green viable cells, yellow arrows attachment of viable cells onto all available areas of the 3D scaffold). Bars 94.34 μm (a), 72.62 μm (b)



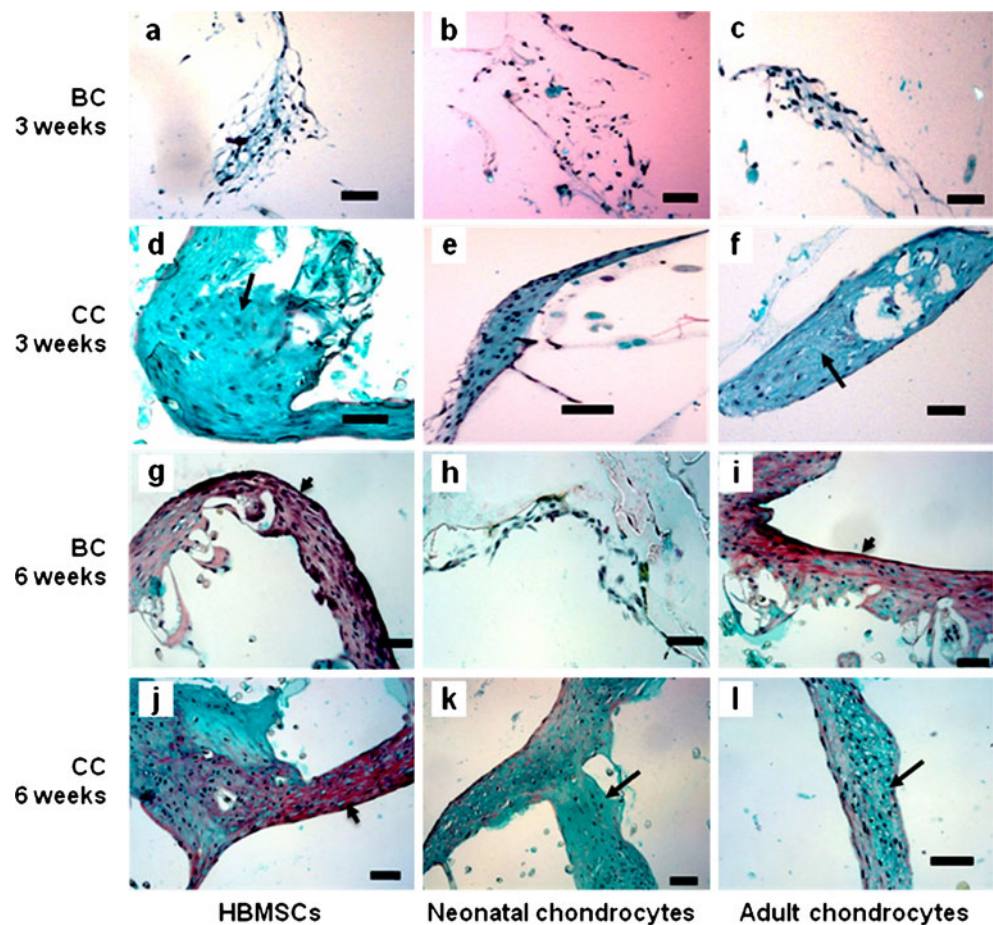
Alcian Blue/Sirius Red staining of *in vitro* 3D tissue-engineered constructs

Constructs cultured for 3 weeks in basal media *in vitro* revealed only limited cell proliferation and extracellular matrix (ECM) formation, confirmed by extremely faint Alcian Blue staining for all three cell types (Fig. 2a–c). In contrast, intense Alcian Blue staining (but not Sirius Red staining), together with the appearance of cells aligned parallel to the surface edge of the scaffold, was seen in the matrix of constructs following chondrogenic culture. Again, this was similar for all three cell types (Fig. 2d–f). After 6 weeks in basal medium, HBMSCs and adult chondrocyte constructs contained areas that stained intensely for Sirius Red (Fig. 2g, i) revealing a fibrous capsule formation in the outermost layer of the constructs. However, the matrix in the neonatal articular chondrocyte constructs cultured in the same group failed to stain intensely with Alcian Blue/Sirius Red because of limited tissue formation (Fig. 2h). Under chondrogenic culture conditions, constructs for all three cell types were more cellular, with the presence of cells growing both parallel and perpendicular to the construct surfaces. A thick fibrous capsule was seen in the HBMSC constructs, which stained strongly with Sirius Red staining (Fig. 2j). In comparison, only limited Sirius Red staining was observed in adult chondrocyte constructs, with neonatal chondrocyte constructs having the thinnest fibrous capsule of all three cell types observed (Fig. 2k, l).

Immunohistochemical analysis of 3D constructs cultured *in vitro*

Sox9 was detected in all basal and chondro-induced constructs after both 3 weeks (Fig. 3a–f) and 6 weeks *in vitro* (Fig. 3g–l) with chondro-induced adult chondrocytes appearing to have visibly more Sox9-positive cells after 6 weeks in culture, although no further quantification was performed to confirm this observation (Fig. 3l). Similar

Fig. 2 Alcian Blue/Sirius Red staining of in vitro static cultured cell-scaffold constructs. Histological appearance of constructs following basal culture for 3 weeks (a–c) and 6 weeks (g–i) compared with that of chondro-inductive culture for 3 weeks (d–f) and 6 weeks (j–l). The constructs cultured in chondrogenic media at 3 and 6 weeks stain more intensely for Alcian Blue (arrows) in comparison to constructs cultured for similar time points in basal media. Constructs cultured for 6 weeks are much more cellular than those cultured for 3 weeks (red arrowheads Sirius Red staining, BC basal culture, CC chondrogenic culture, HBMSCs human bone marrow stromal cells). Bars 1 mm



immunostaining intensities for aggrecan were detected for all three cell types cultured in either basal or chondrogenic medium for 3 weeks. After 6 weeks in basal or chondro-induced medium, all constructs appeared to have increased amounts of aggrecan as evidenced by their higher staining intensities compared to the respective 3-week constructs (Fig. 4). The highest staining intensity for aggrecan was observed at 6 weeks in the constructs containing adult chondrocytes following chondro-induction, with a similar pattern to that seen for Sox9 expression.

Alcian Blue/Sirius Red staining of in vivo 3D tissue-engineered constructs

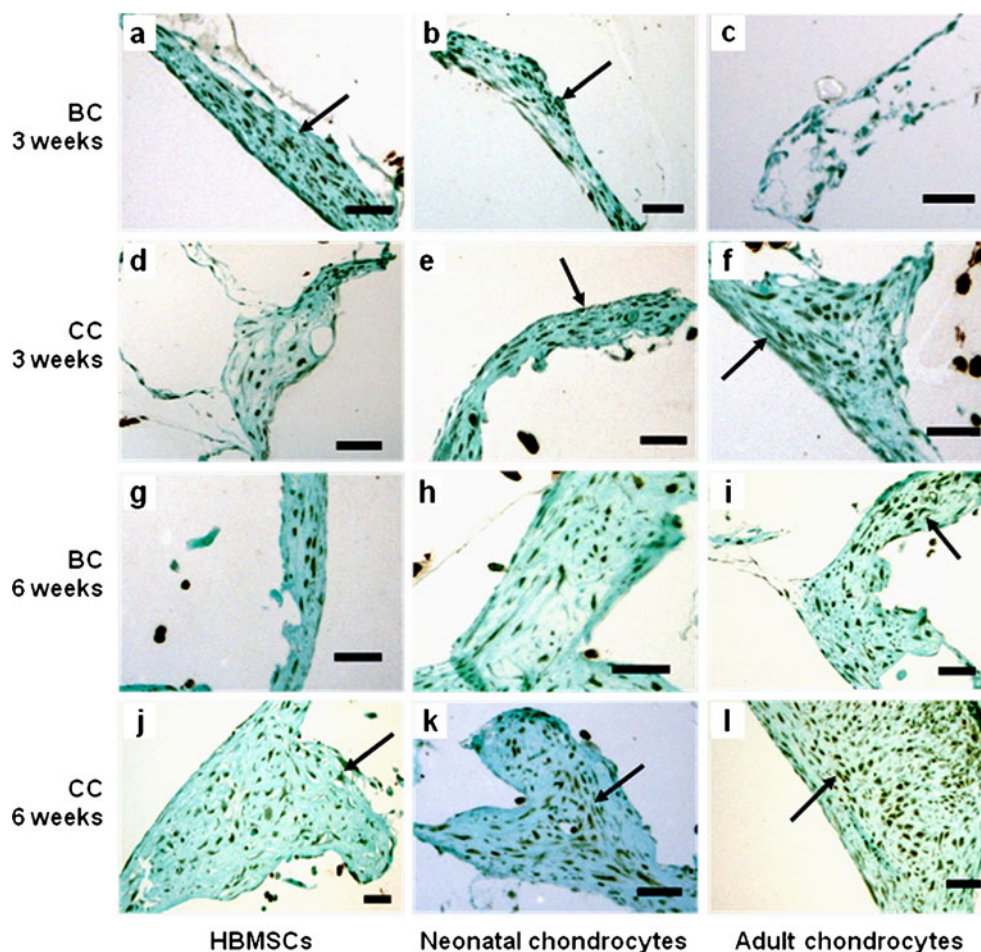
After 3 weeks of in vivo implantation, histological examination revealed that HBMSCs and adult and neonatal chondrocytes formed a more compact neo-tissue (Fig. 5a–f) compared with similar constructs generated in vitro (Fig. 2a–f). However, constructs pre-cultured in basal medium for a week before implantation had a dense anionic matrix indicative of mucopolysaccharides accompanied by fibrous capsule formation (Sirius Red positive) for all cell types, with constructs containing neonatal articular chondrocytes having formed the least amount of neo-tissue

(Fig. 5a–c). In contrast, pre-chondro-induced constructs for all three cell types revealed the formation of a cartilage-like neo-tissue with mainly Alcian Blue staining and little Sirius Red staining at week 3 (Fig. 5d–f). After 6 weeks of in vivo implantation, HBMSC and neonatal chondrocyte constructs pre-cultured in basal medium appeared to be fragmented with a limited amount of tissue being apparent (Fig. 5g, h). Constructs containing adult chondrocytes did not appear fragmented but had copious amounts of Sirius-Red-positive staining (Fig. 5i). For the constructs pre-cultured in chondrogenic medium, cartilage-like structures were seen after 6 weeks in vivo for all three cell types; however, a prominent fibrous capsule had formed in the constructs containing HBMSCs and neonatal articular chondrocytes (red staining in Fig. 5j, k). In contrast, chondro-induced adult chondrocyte constructs had little Sirius Red staining and lacked any evidence of a prominent fibrous capsule after 6 weeks of in vivo implantation (Fig. 5l).

Immunohistochemical analysis of 3D constructs cultured in vivo

Sox9 was detected in both chondro-induced and non-chondro-induced constructs after 3 and 6 weeks culture in

Fig. 3 Sox9 immunohistochemical staining of in vitro statically cultured cell-scaffold constructs. Sox9 immunohistochemical staining of constructs following basal culture for 3 weeks (a–c) and 6 weeks (g–i) compared with that of chondro-inductive culture for 3 weeks (d–f) and 6 weeks (j–l). All sample constructs stained positively for Sox9 (arrows, dark brown) with qualitatively the highest Sox9-positive cell staining being observed for constructs cultured in chondrogenic media for 6 weeks (BC basal culture, CC chondrogenic culture, HBMSCs human bone marrow stromal cells). Bars 1 mm



vivo (Fig. 6). Qualitatively, for each cell type, more Sox9-positive cells appeared to be present in constructs pre-cultured statically in chondrogenic medium than in those pre-cultured in basal medium. Fewer Sox9-positive cells were seen in the chondro-induced constructs for all cell types after 6 weeks in vivo compared with chondro-induced constructs cultured for 3 weeks in vivo. Strong immuno-staining for aggrecan was evident in all of the in vivo constructs with the highest staining intensities being observed for chondro-induced neonatal and adult chondrocyte constructs implanted in vivo for 6 weeks (Fig. 7).

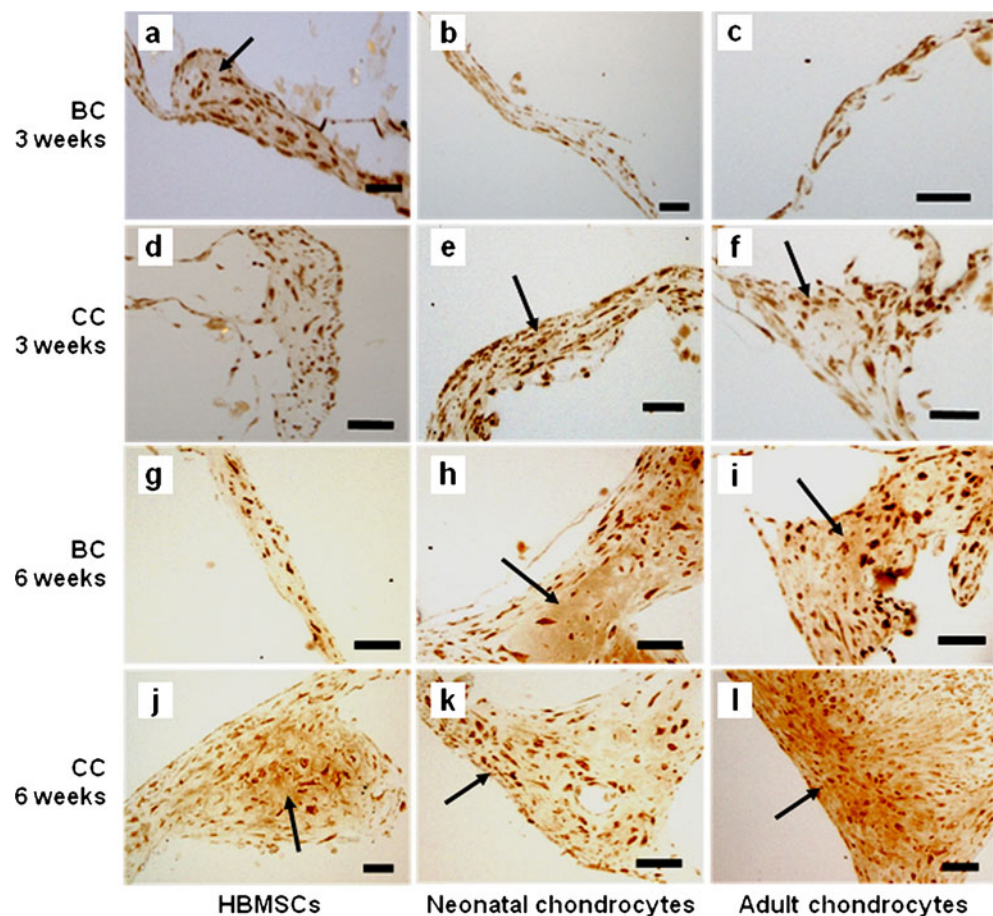
Discussion

Successful integration of engineered neocartilage with host cartilage is the ultimate goal for cartilage repair. The hypothesis behind functional cartilage tissue engineering is that successful articular cartilage repair/regeneration will involve the *ex vivo* synthesis of cartilaginous tissue (LeBaron and Athanasiou 2000a). Differences in methodologies used by researchers to investigate cartilage repair have led to conflicting results. No agreed consensus has been reached concerning the ideal scaffold/cell source/regulatory

factors (mechanical stimuli and growth factors) best suited for cartilage regeneration (Caplan and Dennis 2006; LeBaron and Athanasiou 2000b) and the specific signalling pathways that induce chondrogenesis remain generally unknown (Kolf et al. 2007).

The success of any cell-based therapy depends upon the selection of an appropriate cell source. Chondrocytes are an obvious choice for de novo engineering of articular cartilage and are used routinely in the clinic for ACI. The main drawback to this approach lies in the need to produce a homogeneous ECM that can theoretically be induced via the application of appropriate biochemical and biomechanical signals (Klein et al. 2009). In order to obtain a functionally relevant tissue-engineered construct, we need to understand tissue histogenesis occurring in vivo. In our previous study, biochemical assays and gene expression analysis of the cells in 2D culture were carried out under conditions mimicking current cell processing methodologies for ACI therapies in the clinic (Saha et al. 2010). In the present study, we have compared the chondrogenic potential of three human cell types, namely HBMSCs, neonatal articular chondrocytes and adult chondrocytes, in 3D constructs in vitro and in vivo. Our primary goal has been to engineer tissues with histological structures resembling those seen in

Fig. 4 Aggrecan immunohistochemical staining of *in vitro* statically cultured constructs. Aggrecan immunohistochemical staining of constructs following basal culture for 3 weeks (a–c) and 6 weeks (g–i) compared with that of chondro-inductive culture for 3 weeks (d–f) and 6 weeks (j–l). All sample constructs stained positively for aggrecan (arrows, brown) with qualitatively the highest staining intensity being observed in chondro-induced adult chondrocyte samples cultured for 6 weeks (BC basal culture, CC chondrogenic culture, HBMSCs human bone marrow stromal cells). Bars 1 mm



native cartilage, given the apparent importance of morphologically distinct zones in the functioning of healthy articular cartilage (Klein et al. 2009). Ultimately, this work should help to determine the optimum cell source and pre-treatment conditions for *ex vivo* cartilage generation.

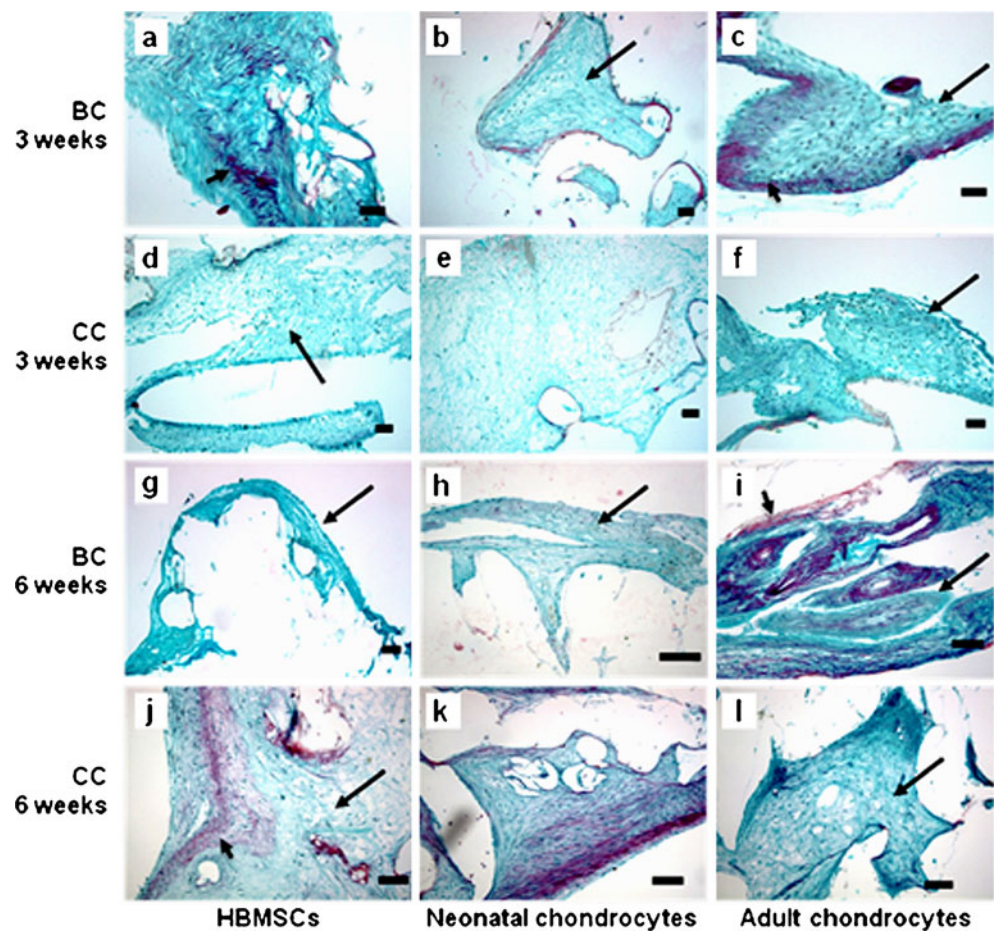
We are aware that the use of neonatal articular chondrocytes as a cell source for cartilage tissue engineering has potential ethical issues. We are not proposing their use as an alternative cell source but have included them in this study because little is known about the behaviour of neonatal articular chondrocytes in culture. Furthermore, we feel that studying these cells alongside mature cartilage cells and stromal cells will help improve our understanding of the biology of chondrocytes and illustrate key differences in the factors that are involved in the differentiation/maintenance of a chondrogenic cell type. In our previous study, for example, we showed differences in temporal gene expression between neonatal articular chondrocytes and the mature cells. In experiments that mimicked current cell processing methodologies in the clinic, our previous work comparing neonatal articular chondrocytes and the mature cell types indicated that signalling events occurred during the first few days of chondrogenic culture prior to the deposition and organisation of a hyaline cartilage ECM. We also showed that the chondrogenic phenotype, which is a

balance between proteoglycan content and collagen integrity, was regulated in a sequential/cyclic manner (Saha et al. 2010).

In order to induce chondrogenesis *in vitro*, we used serum-free α -MEM chondrogenic medium containing 10 ng/ml TGF- β 3 based upon its known chondro-inductive properties (Alhadlaq et al. 2004; Darling and Athanasiou 2003; Pittenger et al. 1999; Tare et al. 2005). All three cell types were seeded onto the cartilage phase of TruFit CB Plug scaffolds of 3 mm in diameter. Unlike most synthetic scaffolds, TruFit CB Plug scaffolds can support cell attachment and growth without surface modification and promote the repair of osteochondral defects *in vivo* (Agrawal and Stinson 2007; Williams and Gamradt 2008), including the successful repair of an articular cartilage defect in the human femoral condyle (Carmont et al. 2009).

Static seeding of cells onto the scaffold can result in a clustering of cells at the periphery with an extremely low cell density at the centre (Vunjak-Novakovic et al. 1998; Wendt et al. 2003). A standard dynamic seeding apparatus (17 rpm) designed and manufactured in-house was therefore employed to seed the cells onto the TruFit CB Plug scaffolds and the results confirmed uniform cell viability and cell density throughout the scaffolds for all three cell types used. Following dynamic seeding, the constructs

Fig. 5 Alcian Blue/Sirius Red staining of cell-scaffold constructs in vivo. After 1 week in vitro culture in basal (a–c, g–i) and chondro-inductive medium (d–f, j–l), the cells and scaffolds were sealed in diffusion chambers that were implanted intra-peritoneally in nude mice for 3 (a–f) or 6 weeks (g–l). Sirius Red staining (arrowheads, red) indicates collagenous matrix formation. Alcian Blue staining (arrows, blue) indicates the presence of mucopolysaccharides in the matrix. After 3 weeks of in vivo implantation, the basal group shows more fibrocartilage-like tissue formation than samples pre-cultured in chondro-inductive medium. At 6 weeks, fibrocartilage-like tissue formation is observed in both HBMSCs and neonatal chondrocyte constructs pre-incubated in chondro-inductive medium. In contrast, adult chondrocytes of the same culture group seem to retain their cartilage-like tissue structure (BC basal culture, CC chondrogenic culture, HBMSCs human bone marrow stromal cells). Bars 1 mm



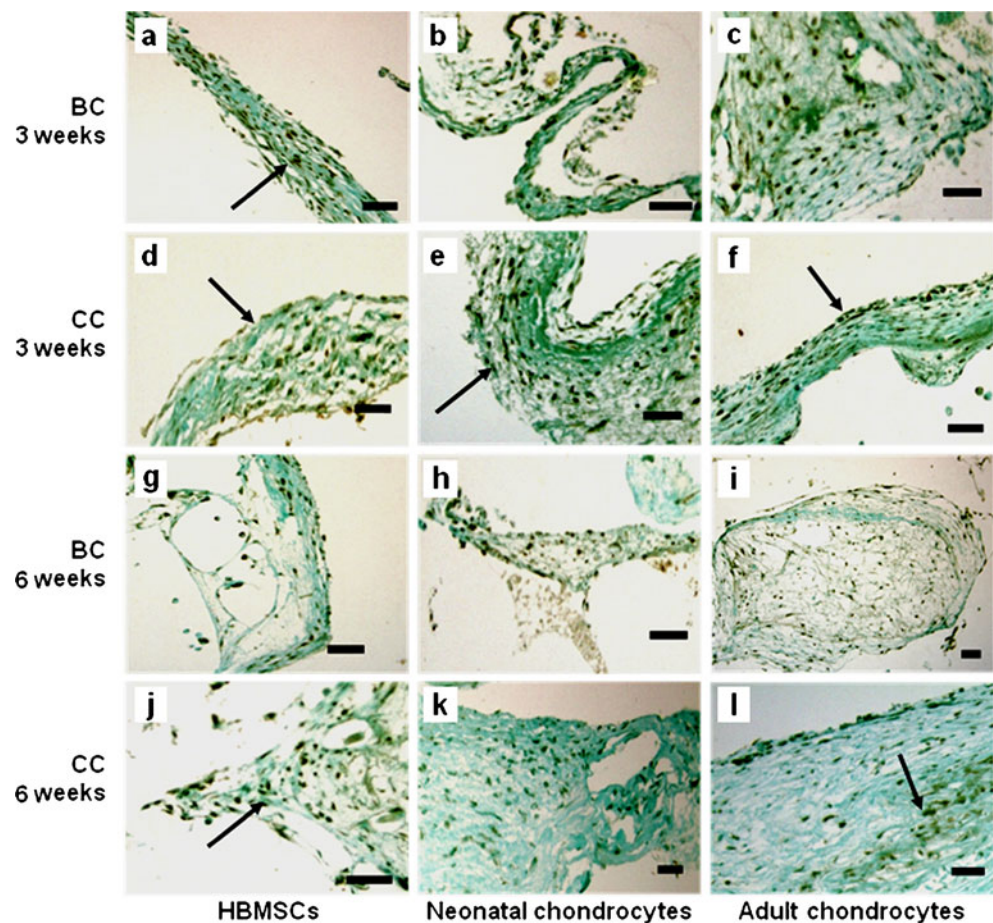
were cultured statically to encourage compressive cell adhesion (CCA).

After 3 weeks of in vitro culture in chondro-induced medium, individual cells embedded in lacunae arranged parallel to the periphery of the scaffolds were observed in all of the constructs further supporting a cartilage-like phenotype. After 6 weeks in vitro, 3D chondro-induced constructs for all three cell types visually appeared to be more cellular compared to their 3-week counterparts. Intense Alcian Blue/Sirius Red staining and pronounced zonal organisation similar to that seen histologically for native articular cartilage (cells appeared flattened and parallel to the surface, with larger rounder cells in the middle-to-base area being aligned perpendicular to the surface) were observed in constructs for all three cell types in chondrogenic medium without any exogenous stimuli or perfusion/diffusion of nutrients. Our results contradict those reported by Seda Tigli and colleagues (2009) who detected faint positive Safranin-O staining for all chondrocyte and MSC constructs over 4 weeks of static chondrogenic culture on chitosan and silk scaffolds, indicative of incomplete chondrogenesis. In the current study, positive Alcian Blue staining characteristic of glycosaminoglycans was seen for all our cell constructs as early as 3 weeks in static chondrogenic

culture, indicating that a combination of any of the three cell types with TGF- β 3 and the PLGA TruFit CB Plug scaffold would be suitable for the induction/short-term maintenance of chondrogenesis. The most interesting observation was that, of all the three cell types studied in vitro, neonatal articular chondrocytes appeared to have formed the thinnest fibrous capsule after 6 weeks of chondrogenic culture compared with the mature cell types. However, the highest staining intensity for Sox9 and aggrecan was observed in the constructs containing chondro-induced adult chondrocytes at 6 weeks.

Most in vivo models used to study chondrogenesis are implant models with the main drawback being apoptosis of almost 85% of the implanted cells over a 4-week period (Mierisch et al. 2003; Ostrander et al. 2001). The constructs need first to undergo a period of CCA before in vivo implantation, although highly mature constructs might fail to integrate with the native tissue (Obradovic et al. 2001) and a determination of the extent of in vitro maturation before the 3D construct is placed in vivo is important. In this study, after dynamic seeding onto the scaffold, the 3D constructs were allowed to undergo a week of CCA in static culture in either basal or chondro-induced medium before implantation within diffusion chambers intra-peritoneally in

Fig. 6 Sox9 immunostaining of cell-scaffold constructs in vivo. Positive staining for Sox9 (arrows, brown) was seen in constructs after in vivo implantation intra-peritoneally in nude mice for 3 (a–f) or 6 weeks (g–l). Constructs pre-cultured in chondrogenic media stained more intensely for Sox9 compared with constructs pre-incubated in basal media. In vivo constructs were qualitatively observed to stain less intensely for Sox9-positive cells compared with the staining observed for constructs cultured in vitro (BC basal culture, CC chondrogenic culture, HBMSCs human bone marrow stromal cells). Bars 1 mm



Nu/Nu mice. This time period was based on results from real-time polymerase chain reaction analysis (Saha et al. 2010), which had previously indicated that signalling events occur during the first few days of chondrogenic culture prior to the deposition and organisation of an ECM of hyaline-like cartilage.

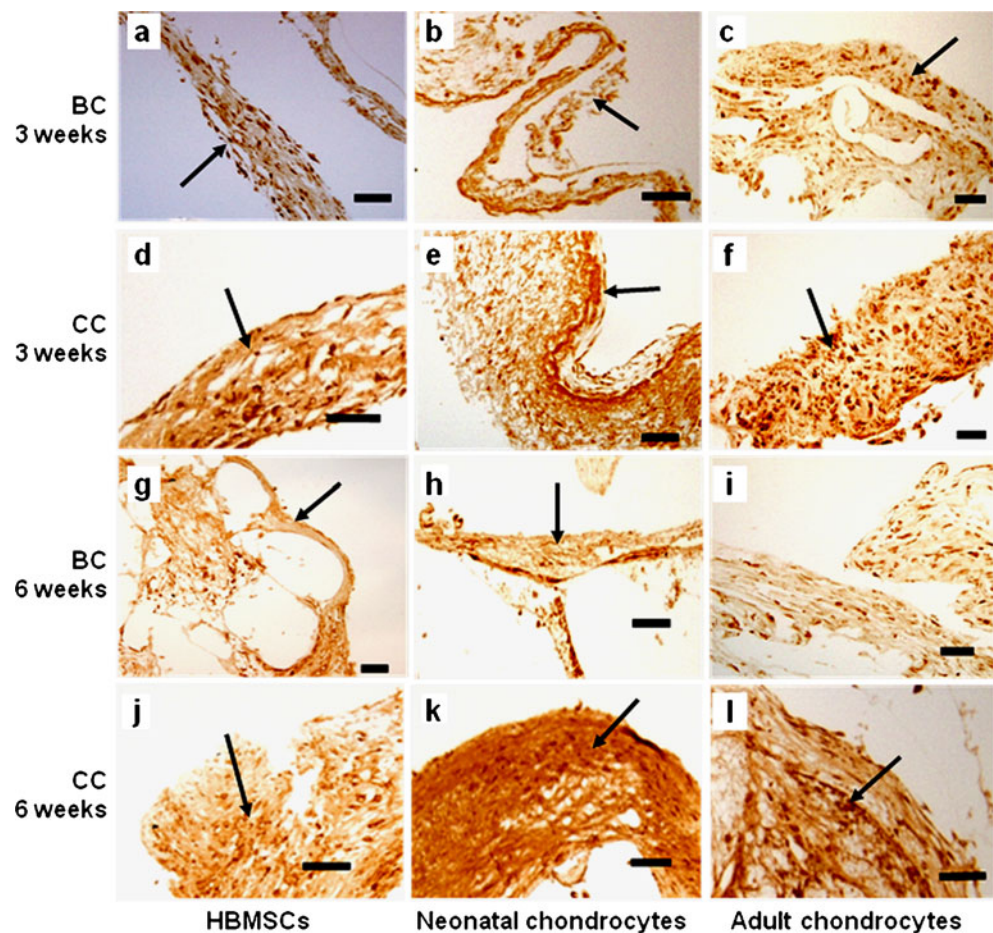
The diffusion chamber model provides an enclosed space within the host animal for studies of cellular proliferation/differentiation without host versus donor tissue rejection (Partridge et al. 2002; Yang et al. 2004). This model approaches an in vivo bioreactor, since the chambers allow the diffusion of nutrients within the peritoneal space similar to articular cartilage in a joint, together with possible (albeit low level) biomechanical loading. In this study, 3D construct samples of all three cell types that were pre-incubated in basal medium formed comparable fibrocartilage-like tissue within 3 weeks of in vivo implantation. After 6 weeks, the adult chondrocyte construct group showed the formation of highly organised fibrocartilage structures. Interestingly, the neo-tissue generated by HBMSCs and neonatal articular chondrocytes under similar conditions lacked any obvious structural organisation and the disorganised tissue was limited. However, pre-incubation in chondrogenic medium was seen to improve the cartilage-like characteristics of the neo-tissue generated by all three cell types, with no fibrous tissue

formation discernable in any of the constructs at 3 weeks. Pre-incubation in chondrogenic medium for 1 week might therefore help maintain cell signalling pathways post-implantation. Fibrocartilage-like tissue formation was, however, observed after 6 weeks in vivo for both chondro-induced HBMSCs and neonatal chondrocytes. In contrast, adult chondrocyte constructs lacked evidence of any significant fibrous tissue formation and the formed tissue tended to be more compact and had the smallest fibrous capsule (as shown by Sirius Red staining).

The importance of stem cell condensation and homing is now appreciated, making it imperative to be able to recapitulate fully natal and postnatal development for a successful tissue-engineering approach. In vitro chondrogenic induction has been documented to improve the quality of cartilage genesis in vivo (Liu et al. 2007). A 7-day pre-incubation in chondro-induced media has been observed to be sufficient to sustain the chondrogenic phenotype for long-term culture in adult chondrocytes. Perhaps prolonging the pre-incubation period in vitro to 10–14 days before in vivo implantation in the case of HBMSCs and neonatal articular chondrocytes would yield a more hyaline-like tissue, even after a long-term culture period.

We have built extensively on our previous research of comparing and contrasting the chondrogenic potential of

Fig. 7 Aggrecan immunostaining of in vivo diffusion-chamber-cultured constructs. Positive staining for aggrecan (arrows, brown) was observed in all samples with varying intensities after in vivo implantation intra-peritoneally in nude mice for 3 (a–f) or 6 weeks (g–l). Constructs pre-cultured in chondrogenic media stained more intensely for aggrecan compared with constructs pre-incubated in basal media. Similarly high staining intensities were observed in chondro-induced neonatal and adult chondrocyte constructs implanted in vivo for 6 weeks (BC basal culture, CC chondrogenic culture, HBMSCs human bone marrow stromal cells). Bars 1 mm



HBMSCs, neonatal articular chondrocytes and adult chondrocytes for indications of optimal chondrogenic potential (Table 1). We have previously documented the three cell types as having different doubling times with the immature cell type (neonatal articular chondrocytes) being the most proliferative of the three cell types and

HBMSCs having the longest doubling time. In order to be in tune with current cell processing methodologies in the clinic, a cell type with a short doubling time is preferred in order to prevent de-differentiation during monolayer expansion (Saha et al. 2010). High alkaline-phosphatase-specific activity (ALPSA) levels in chondrocytes indicate a shift from

Table 1 Comparing and contrasting chondrogenic potential across three human cell types. A rapid visual summary of the observations for a number of specific parameters indicating optimal chondrogenic potential, which is scored qualitatively: a score of +, ++ and +++

indicates least, moderate and most ideal cell type, respectively, for the particular parameters being analysed (HBMSCs human bone marrow stromal cells, ALPSA alkaline-phosphatase-specific activity, qPCR quantitative polymerase chain reaction, 3D three-dimensional)

Parameters (reference)	HBMSCs	Neonatal articular chondrocytes	Adult articular chondrocytes
Doubling time (Saha et al. 2010)	+	+++	+++
ALPSA (Saha et al. 2010)	++	+	+++
Aggrecan; qPCR and immunohistochemistry (Saha et al. 2010)	+	+++	++
Alcian Blue/Sirius Red staining of 3D constructs cultured in chondrogenic media in vitro			
• 3 weeks	+++	+	++
• 6 weeks	+	+++	++
Alcian Blue/Sirius Red staining of 3D constructs pre-incubated in basal media and cultured in vivo			
• 3 weeks	+	+	+
• 6 weeks	–	–	+
Effect of iron oxide incorporation on chondrogenic potential after long-term culture (Saha et al. 2012)	+++	+	+++

the proliferative/pre-hypertrophic phase to a hypertrophic differentiation state. Under ordinary basal culture, HBMSCs (default osteogenic potential) exhibit high ALPSA levels, whereas adult chondrocytes have the lowest ALPSA levels. For the purpose of cartilage cell therapy, a cell type with a low default ALPSA is preferred.

Human neonatal cartilage, unlike mature cartilage, is known to contain two types of chondrocytes (Hwang 1978). A lack of understanding exists with respect to the purpose of the two types of chondrocytes in immature cartilage but we have shown there to be considerable differences in chondrogenic gene regulation mechanisms between immature and mature human chondrocytes (Saha et al. 2010, 2012). Neonatal articular chondrocytes cultured in chondrogenic medium have been seen to have the highest level of collagen type II gene expression (a key chondrogenic marker) compared with the other two cell types. This difference in their gene expression might be related to the differences observed at a histological level as, in a 3D construct *in vitro*, these cells reveal the thinnest fibrous capsule (indicative of a more hyaline-like tissue formation) of the three cell types.

Before *in vivo* implantation, the samples were allowed 1 week for CCA, with one group additionally being provided with chondro-inductive cues. After *in vivo* implantation, only the mature chondrocyte constructs formed a cartilage-like tissue with the thinnest fibrous capsule. In the absence of chondro-inductive cues, only the adult chondrocyte group was capable of forming a tissue, although it was fibrous in nature. This *in vivo* outcome is most likely to have occurred because differentiated mature chondrocytes are able to maintain their chondrogenic/pre-hypertrophic phenotype with chondro-inductive cues provided by TGF- β 3 in the culture medium. We have previously shown that the addition of TGF- β 3 in our chondrogenic culture medium decreases cartilage catabolic effects and helps maintain a pre-hypertrophic chondrogenic phenotype while inducing chondrogenesis in HBMSCs (Saha et al. 2010). Evidence has been presented that aggrecan is co-expressed with Sox9 across all three cell types (Saha et al. 2010; Sengers et al. 2007; Shukunami et al. 1997; Tare et al. 2005). In this study, we have confirmed these data via immunohistochemistry in 3D cultures, both *in vitro* and *in vivo*. This is a direct consequence of Sox9 promoting the gene expression of cartilage-type ECM by binding to the promoter elements of aggrecan (Saha et al. 2010).

The TGF- β 3 in the culture medium only helps to differentiate the de-differentiated immature chondrocytes and undifferentiated HBMSCs. It is unable to maintain the chondrogenic phenotype of these two cell populations during long-term culture *in vivo*. In a controlled *in vitro* environment, e.g., after 6 weeks culture in chondrogenic medium, neonatal chondrocyte constructs successfully form

structures that mimic native articular cartilage, with minimal fibrous tissue formation, to a greater extent than is seen for the other cell types. However, under *in vivo* conditions, even chondro-induced neonatal articular chondrocytes fail to retain a hyaline-like structure.

In the field of cartilage tissue engineering, confusion still surrounds the difference between chondrogenic induction and the maintenance of the chondrocyte phenotype. Differences in the ability of TGF- β 3 to maintain a chondrogenic phenotype in HBMSCs and neonatal articular chondrocytes can be attributed to a difference in the pattern of the temporal gene expression of key chondrogenic markers when the cells are cultured with this growth factor (Saha et al. 2010) or when they are cultured with medium containing metal ions (Saha et al. 2012). Smad2/3 has been reported to bind to Sox9 in a manner dependent upon TGF- β to form transcriptional complexes that enhance chondrogenesis (Furumatsu et al. 2005). The threshold level of TGF- β that impacts on relevant chondrogenic gene markers is not known and, possibly, higher concentrations of the growth factor help maintain the chondrogenic phenotype of neonatal articular chondrocytes and HBMSCs for a longer duration.

Our research highlights the need for a better understanding between chondro-inductive factors and those factors that support the maintenance of a chondrogenic phenotype. Furthermore, our results emphasise the importance of sustained growth factor incorporation for the maintenance of a chondrocyte-like phenotype in immature chondrocytes or undifferentiated HBMSCs.

In conclusion, this study has provided evidence that (1) the chondrogenic medium used enhances cartilage matrix formation *in vitro* and *in vivo* but, although supporting the differentiation of HBMSCs, is unable to maintain their chondrogenic phenotype in long-term cultures; (2) the same inductive medium is capable of maintaining the chondrogenic phenotype of adult chondrocytes in long-term cultures but not of neonatal chondrocytes; (3) immature (neonatal) chondrocytes appear to be highly dependent on chondro-inductive growth factors for the formation and maintenance of the cartilage phenotype, both in short-term and in long-term cultures. Thus, under chondrogenic culture conditions, adult chondrocytes appear to be a more promising cell source than HBMSCs for use in articular cartilage tissue engineering.

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