

Cellular methods in cartilage research: Primary human chondrocytes in culture and chondrogenesis in human bone marrow stem cells

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Abstract

Work in our laboratory has focused on the *in vitro* culture of both human articular chondrocytes and human mesenchymal stem cells to understand what controls their ability to synthesise an appropriate cartilage-like extracellular matrix containing a predominantly collagen type II fibrillar network embedded in an aggrecan-rich ECM. This review focuses on the methodologies that we have found to be successful with cartilage and bone marrow sources of human cells and comments on the many factors which may enable improved phenotypic performance once the cells are in a fully chondrogenic environment.

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1. Introduction

Loss of cartilage at the articulating surface during degenerative joint diseases leads to pain and immobility, which is presently treated by pain relief and eventually by replacement of the diseased joint with an implant. Whilst the outcomes of joint arthroplasty are often favourable, an increased incidence of prosthesis failure over time limits the usefulness of this procedure for individuals below the age of 50. Therefore for younger people a desirable treatment for cartilage damage would be the resurfacing of the joint with a functional replacement tissue. Initially, research has focused on the repair of small, acute, localised defects in articular cartilage that may occur as a result of sports injury. Whilst the incidence of patients needing such treatment is significantly less than for those with arthritis, the localised defect presents an achievable first step in articular surface regeneration.

Cell-based strategies for cartilage repair vary, but generally contain a number of common features. A source of chondrocytes needs to be identified; normally autologous primary chondrocytes, although autologous bone marrow derived mesenchymal stem cells have become an interesting alternative. The cells usually require expansion and when enough cells are obtained they are delivered into the wound site and retained there by some means. The phenotypic state of these cells when injected and their potential to be fully chondrogenic will no doubt have a profound effect on their ability to direct functional tissue restoration. Many studies both *in vivo* and *in vitro* have examined how to promote the retention of the chondrocyte phenotype and its potential benefits.

2. Primary chondrocytes

2.1. Isolation and culture of articular chondrocytes

Chondrocytes from both human and animal sources can be isolated from both post-operative and post-mortem cartilage tissue. The cartilage cell biologist does have

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more leeway in matters of the freshness of the tissue than those who study other cell types. Due to the avascular, hypoxic nature of cartilage it is possible to successfully isolate cells even from refrigerated stored tissue. This may be particularly useful with human tissue where the supply can be erratic. We have generally isolated cells from human cartilage following total-knee replacement, on the day of the operation, but at times when this was not possible, tissue was refrigerated overnight and still gave satisfactory yields of chondrocytes. We have not explored longer times of storage, although there are studies that indicate that articular cartilage can be stored for several days without significant reduction in cell viability [1].

The following protocol has given satisfactory chondrocyte yields from human articular cartilage:

1. Cartilage was cut from the subchondral bone using a sterile sharp scalpel blade and placed in serum free Dulbecco's Modified Eagle's Medium (DMEM). We have routinely used DMEM containing 4 mM L-glutamine (Lonza, Slough, UK—Catalogue No. BE12-741F). Once all the cartilage was obtained, a new sharp scalpel blade was used to dice the cartilage as finely as possible. Dicing the tissue finely improved the efficiency of the subsequent enzymatic digestion. For human tissue it is important that the person carrying out the dissections has had appropriate vaccinations e.g. Hepatitis B.

2. The exact methods for the enzymatic isolation vary between laboratories. For many years we performed a 1 h digestion in trypsin (Lonza) in serum-free DMEM followed by an overnight digestion in 0.08% bacterial collagenase type II (Invitrogen, Paisley, UK) in DMEM + 5% foetal bovine serum (FBS). Other laboratories substitute the initial trypsin digestion with other treatments, such as digestion with hyaluronidase or Pronase. The rationale for this initial step is that the tissue is “loosened” by the pre-digestion, allowing better access of the collagenase to the collagen fibrillar network. However, we have now dispensed with this step, and simply digest the tissue overnight in collagenase (as above), with no apparent loss in the efficiency of the digestion.

3. Once digested, the cell suspension was strained through commercially available cell strainers (100 μ m pore size) and centrifuged (600g for 5 min) to obtain a cell pellet. This pellet was washed twice in DMEM containing 10% FBS and the cells counted. For standard monolayer cultures, the cells are plated on tissue culture plastic at a density of 20,000 cells/cm². Where retention of phenotype is important with short-term cultures, higher densities may be desirable to minimise cell spreading and suppress cell division of the chondrocytes.

4. The chondrocytes are then grown to confluence in DMEM containing 10% FBS, which typically takes around 10 days with media changes every 2 days. They are then split at a 1:2 ratio following trypsinisation.

It is important to bear in mind that from the moment the chondrocytes begin to attach to tissue culture plastic, spread out and begin to divide, they change their gene expression and go on to progressively lose their chondrogenic phenotype over several passages in culture. This is associated with a loss of rounded shape, reduced extracellular matrix synthesis, loss of expression of chondrocyte marker genes (e.g. COL2A1, which encodes the collagen type II alpha 1 chain; and the transcription factor SOX9) and increased expression of fibroblastic genes (e.g. COL1A1 which encodes the collagen type I alpha 1 chain) [2,3]. Fig. 1 shows this process in detail. COL1A1 is increased over several orders of magnitude to maximal levels in just two passages of culture. COL2A1 decreases over the same period to a similar extent but takes 3–4 passages to reach minimal levels whilst SOX9 decreases for up to six or seven passages. Some of these changes in gene expression are reversible and can be demonstrated by the culture of the cells in an environment that favours cell rounding. Culture in alginate beads [4] or in suspension on poly-HEMA coated dishes [5] can lead to re-expression of COL2A1 and SOX9 by articular chondrocytes expanded in monolayer. Experiments in our laboratory have shown that reduction of cell spreading was also able to control SOX9 expression. Chondrocytes were grown in multiwell plates coated with decreasing concentrations of fibronectin blocked with bovine serum albumin. This caused a progres-

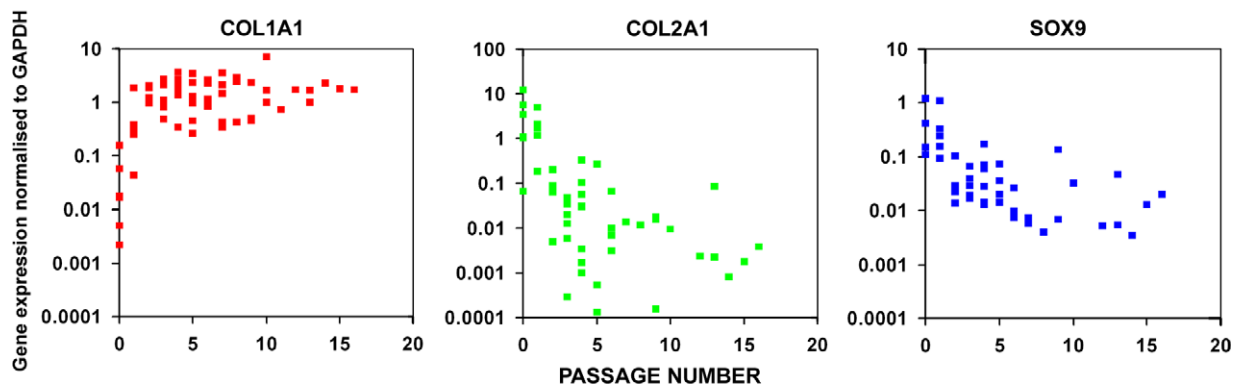


Fig. 1. Gene expression of COL1A1, COL2A1 and SOX9 in human articular chondrocytes from passage 0 (freshly isolated cells) to passage 17. Data generated by real time PCR analysis and each point represents the gene expression of an individual culture. Note the logarithmic scale on the y-axis.

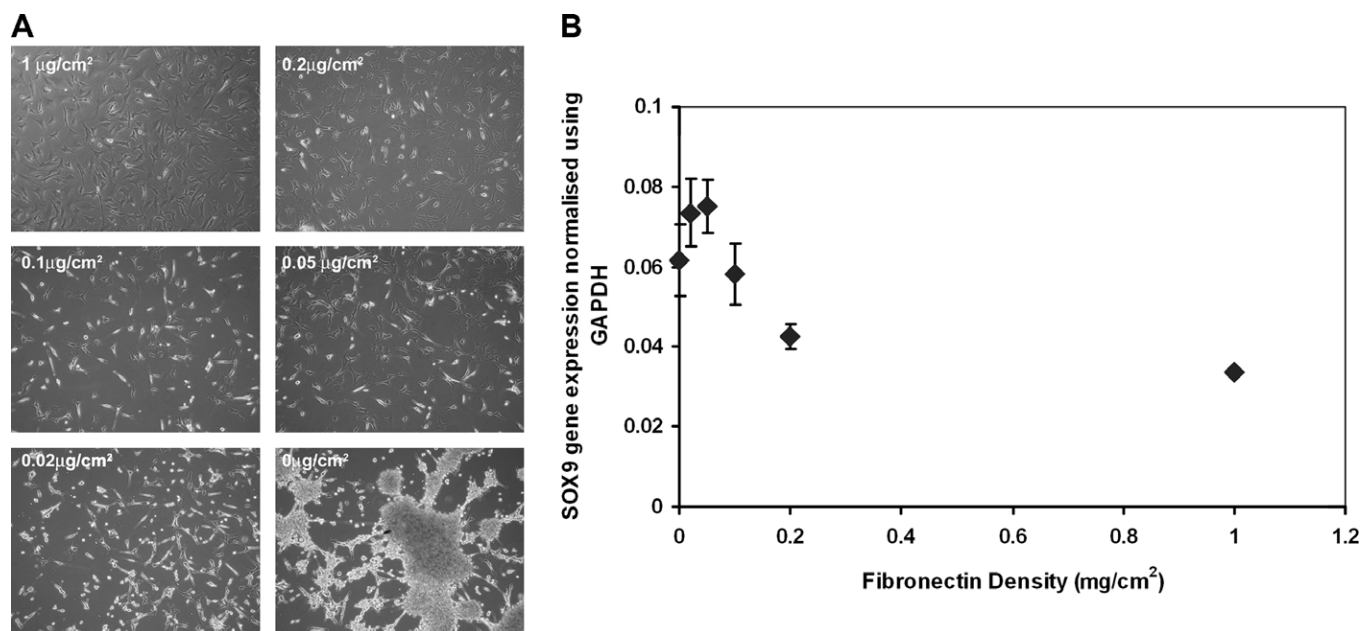


Fig. 2. The effect of cell spreading on SOX9 expression in human articular chondrocytes. Passage 2 chondrocytes were allowed to attach to cell culture dishes treated with decreasing concentrations of human fibronectin. The surfaces were blocked with bovine serum albumin. (A) Phase contrast images showing increased cell spreading at higher fibronectin concentrations. (B) Real time PCR analysis of SOX9 gene expression in chondrocytes grown on different concentrations of fibronectin.

sive reduction in cell spreading and a corresponding increase in SOX9 mRNA (Fig. 2). Other studies by us and others have also demonstrated that re-expression of chondrocyte marker genes, including SOX9, by primary and passaged chondrocytes depends on the prevention of actin stress fibre formation. Initially these studies were carried out with fungal derived actin depolymerising reagents such as the cytochalasins [6], but more recent work has shown similar results by specifically preventing actin stress fibre formation through inhibitors of RhoA effector kinases 1 and 2 [7,8]. Our experience has shown that effective re-expression of chondrocyte phenotype by simple transfer to cell-rounded 3D culture conditions can be obtained with chondrocytes that have been expanded up to passage 4 at a 1:2 split ratio. However at later passage the cells lose this ability to recover a cartilage extracellular matrix-forming phenotype and this clearly reflects a progressive change in phenotype, which currently appears difficult to reverse *in vitro* by simple physiological means. The addition of growth factors to the culture medium of human chondrocytes as described by Barbero et al. [9] with PDGF-BB, TGF β 1 and FGF2, can be very helpful, by greatly increasing the rate of cell proliferation, thus producing more cells more quickly, and also giving some increase in the recovery of phenotype in subsequent chondrogenic culture [10].

2.2. Growth factor stimulation of cartilage matrix formation in adult chondrocytes

Many studies have reported on the treatment of mature articular chondrocytes with a variety of growth factors to

stimulate ECM synthesis. This is related to an even wider literature that is focused on understanding the mechanisms driving degenerative joint diseases. The observations show many inconsistencies that appear not simple to explain, but may relate to cell species, tissue source, passage in culture and the chondrogenic environment under which tests are reported (e.g. growth factors have been reported to have differential effects depending on whether the cells are cultured as monolayers, or in a three-dimensional environment). TGF β family members (TGF β 1, 2, 3) are able to stimulate matrix synthesis in articular chondrocytes promoting collagen and GAG synthesis [11–13]. Insulin like Growth Factor-1 (IGF-1) causes upregulation of cartilage extracellular matrix production by mature chondrocytes in organ culture [14,15] and *in vivo* cartilage repair [16]. In monolayer culture of human articular chondrocytes, IGF-1 had little effect on aggrecan and collagen type II gene expression in defined media, but was able to increase the expression of these genes synergistically when combined with TGF β -1 [17]. With rat chondrocytes, the expression of these genes was increased by IGF-1, but only in cells from younger animals [18]. Fibroblast Growth Factor-2 (FGF-2) is a potent mitogen in many cells and at low concentrations can act as an enhancer of collagen and GAG synthesis in cartilage explant cultures [15]. Interestingly, at higher concentrations there is an inhibitory effect on ECM synthesis. Monolayer cultures of rabbit chondrocytes seem to respond to FGF-2 by retaining a greater degree of differentiation plasticity. In its presence they were reported to proliferate at a faster rate, produce more ECM upon reaching confluence and were more receptive to re-expression of a chondrogenic phenotype in three-dimensional culture [19,20].

Interestingly human articular chondrocytes do not respond to FGF-2 alone during monolayer expansion, but with the addition of TGF β -1 and Platelet Derived Growth Factor-BB (PDGF-BB) they also show stronger proliferation and retention of phenotype as noted above [9].

2.3. Stimulation of cartilage matrix formation using gene therapy

With advances in methods of gene transduction have come new routes to promote cartilage matrix formation by articular chondrocytes and stem cells [21–23]. The advent of safe, non-replicating adenoviral, retroviral and lentiviral vectors has allowed a broad range of primary cells to be efficiently transduced. Whilst there is still debate about the efficacy of viral vectors in therapeutic applications [24], their use has become routine to help answer key questions in research. In chondrocytes, adenoviral vectors provide transduction efficiencies of up to 95% [25,26], which starkly contrasts with the low efficiencies using non-viral lipid based methods or electroporation. Adenoviral transductions lead to transient expression of genes for up to 1–2 weeks, which is adequate for some purposes. For more sustained expression retroviruses and lentiviruses incorporate themselves into the host cell genome and are now available at high titre to transduce chondrocytes with high efficiency [26–28].

TGF β -1 has been transduced into articular chondrocytes, *in vitro*, using an adenovirus and resulted in increased cartilage ECM synthesis [29]. Increased matrix formation has also been obtained when either IGF-1 or BMP-7 were transduced into chondrocytes [30,31]. Auricular chondrocytes transduced *in vivo* by infection of adenovirus containing FGF-18, divided at an increased rate and exhibited upregulated type II collagen and proteoglycan deposition [32]. Mesenchymal progenitors have also been reported to be guided down a chondrogenic pathway if transduced with BMP-13 [33], without leading to endochondral ossification.

Our laboratory has investigated in some detail the consequences of transducing chondrocytes with the transcription factor SOX9, which is essential for the full expression of the chondrocyte phenotype [26,34,35]. It has been shown to bind to enhancer regions within the cartilage collagen genes COL2A1 [36], COL9A1 [37] and COL11A2 [38] and upregulates gene expression, an effect which is further enhanced by SOX5 and SOX6. During monolayer expansion of human articular chondrocytes, SOX9 gene expression is down regulated in parallel with the expansion of COL2A1 [3,39]. Furthermore, its expression recovers if passaged chondrocytes are placed into suspension or alginate culture [5]. Over expression of SOX9 in chondrocytic cell lines was shown to stimulate COL2A1 gene expression. Our initial experiments examined the effect of SOX9 transduction on primary human articular chondrocytes isolated from OA cartilage at total knee replacement. These cells had been repeatedly subcultured (>6 passages) and had thus lost their intrinsic ability to regain a chondrogenic phenotype. A cDNA encoding FLAG tagged SOX9 was transduced into

the cells using an RKAT retrovirus [26]. High transduction efficiency was made possible by increasing the rate of cell proliferation by means of a cocktail of the growth factors PDGF-BB, TGF β -1 and FGF-2 (Sigma, Poole, UK), referred to above, as retroviruses require cell division for transduction and integration [9]. The transduced chondrocytes displayed a 10- to 20-fold increase in SOX9 expression, which in turn led to a 10-fold increase in expression in COL2A1 in monolayer cultures [26]. This was a modest increase in COL2A1 as its expression was much lower at passage 6 than when the cells were in cartilage before isolation. Taking the SOX9 transduced cells and placing them in a cell pellet or alginate bead culture led to a further major increase in COL2A1 expression and increased cartilage matrix deposition [26]. Stimulation of these pellet cultures with TGF β -3 and IGF-1 then led to even greater levels of ECM deposition and to higher levels of COL2A1 gene expression [10]. There was also high levels of collagen type II protein distributed throughout the pellet suggesting a uniform level of chondrogenic expression. Deposition of proteoglycan was also increased substantially. Control pellets of cells transduced with retrovirus lacking SOX9, showed only a very weak chondrogenic response to culture. The cumulative effects of pellet culture, growth factors and SOX9 transduction resulted in a 90-fold increase in COL2A1 gene expression compared to control cells grown in monolayer. Co-culture of SOX9 transduced cells and control cells within pellets at a 50:50 ratio resulted in intermediate levels of matrix formation and gene expression, demonstrating that there is no significant localised paracrine signalling, which could enhance SOX9 expression in the cells transduced without SOX9.

These results showed that the SOX9 transduced cells were not strongly chondrogenic in monolayer culture, but the SOX9 transduction made them more responsive to chondrogenic stimuli, such as pellet culture and added growth factors, which were additive in their effects. The effect that SOX9 has on these passaged human articular chondrocytes was specific, as SOX9 transduction of human dermal fibroblasts did not make them chondrogenic, as they could not form cartilage matrix or respond to growth factors in a chondrogenic pellet culture system [10]. The passaged human articular chondrocytes thus retained the specific responses of chondrocytes that were potentiated by SOX9 and the results showed that boosting SOX9 expression made the chondrocytes more sensitive to cartilage matrix forming stimuli.

2.4. Modulation of SOX9 in articular chondrocytes

The use of SOX9 transduction clearly showed the importance of this transcription factor in supporting matrix gene expression and matrix assembly by articular chondrocytes. There is also evidence that a SOX9 based gene therapy approach with intervertebral disc cells gave protection in a rabbit disc degeneration model [40]. Enhancing endogenous SOX9 expression would provide a more easily applicable and potentially safer means of driv-

ing the matrix-forming phenotype in chondrocytes. However, the regulation of SOX9 gene expression at the molecular level is not yet well understood. Studies of campomelic dysplasia, a genetic disease caused by haploinsufficiency of SOX9, have identified mutations in DNA sequences up to one megabase upstream of the coding region of the SOX9 gene, which are able to interfere with its expression and show its gene to have a very large regulatory region [41,42]. In addition to chondrogenesis, SOX9 has a role in a number of developmental processes as diverse as sex determination [43], heart formation [44], neural crest initiation [45] and gastrointestinal formation [46]. Therefore, regulation of this gene is likely to be complex and may involve many different tissue specific regulatory elements. As discussed above, SOX9 expression in chondrocytes is altered by changes in cell shape and actin cytoskeleton integrity [7,8,26,47]. These results implicate cell shape and the disruption of actin stress fibres directly in the control of SOX9 gene expression and may provide part of the mechanism for the re-expression of the matrix-forming phenotype on transfer of cells to 3D culture. During chondrogenic differentiation SOX9 can also be upregulated by BMP-2 in mesenchymal derived mouse cell lines [48] by sonic hedgehog in chick presomitic mesoderm [49] and by dexamethasone in newborn murine costal chondrocytes [50]. Recent work from our laboratory has also shown that chondrocytes can regulate SOX9 post-transcriptionally through a p38 MAPK dependent mechanism [8] and others have shown that constitutive activation of the p38 MAPK pathway in mouse chondrocytes results in a dwarfism phenotype due to insufficient maturation of growth plate chondrocytes. Interestingly, this phenotype was similar to that observed in mice with a chondrocyte specific SOX9 over-expression [51]. Activators of the p38 MAPK pathway are therefore attractive targets for the discovery of external modulators of SOX9 expression. Our ongoing work has now revealed that induction of this pathway by osmotic stress can also control SOX9 mRNA stability (Simon Tew and Timothy Hardingham, unpublished data). The effects of osmotic change on chondrocyte function have been previously described and lead to alterations in extracellular matrix synthesis. These results provide exciting evidence of a chondrocyte “osmosensing” mechanism, which could have a physiological role in regulating cartilage ECM gene expression. Further work in this area may be able to generate new ways to stimulate and retain high levels endogenous SOX9 gene expression in chondrocytes and stem cells, thereby promoting cartilage extracellular matrix formation and a stable chondrocyte phenotype.

3. Mesenchymal stem cells

3.1. Three-dimensional chondrogenic cultures of human mesenchymal stem cells in Transwells

The ability to differentiate adult stem cells or other uncommitted precursor cells along specific lineage path-

ways including chondrogenesis has identified a new cell source with which to tackle cartilage regeneration. The use of cells other than chondrocytes for cartilage repair in the joint relieves the problems of tissue harvesting and donor site morbidity. It also eases to some extent the challenge of generating sufficient cells, as bone marrow-derived mesenchymal progenitors can be expanded to considerable numbers *in vitro* whilst retaining differentiation capacity [52,53]. Under appropriate culture conditions, these cells can be driven down different differentiation pathways of the mesenchymal lineage, including chondrogenesis, and to deposit an extracellular matrix having some of the key features of hyaline cartilage. Attempts to use these cells in the clinical setting have been restricted to implantation in human knees in a carrier gel [54,55] or in animal models, where direct injection into the articular space has limited targeting to the articular cartilage [56]. With these characteristics an important application of these cells has been for the *in vitro* study of chondrocyte differentiation. The most commonly used method in this field established over many years has involved culturing the cells in chondrogenic medium as cell aggregates, often referred to as pellet culture, which was originally developed using rabbit MSC and later with human bone marrow derived stem cells [57,58]. Recently we have modified this method with a different format for the culture employing a porous membrane support for the cells that creates initially a shallow multilayer of stem cells, which then differentiate and grow a disc of cartilage-like tissue. This method results in a more uniform differentiation of the MSC and more efficient production of matrix by the cells [59]. The efficient chondrogenesis in the system may be largely due to much improved mass-transport in the shallow disc geometry with permeable access from above and below and it results in almost double the matrix production from the same number of cells in a cell aggregate format.

Preparation of pellet and Transwell chondrogenic cultures with hMSC

1. Monolayer expanded hMSC are grown in medium (typically MSCGM from Lonza) supplemented with FGF2 at 5 ng/ml (R&D Systems; Sigma). Split at a ratio of 1:3, we use these cells at up to passage 3 or 4 (where passage 1 is when the initial adherent colonies reach near confluence).

2. Remove cells from flasks with trypsin/EDTA, neutralize with serum-containing medium and count the cells. Each disc or pellet is made with 500,000 cells (pellets can also be made with 250,000 cells).

3. Spin the appropriate number of cells to make all the pellets and/or discs needed for the experiment (240g for 5 min.). Drain all medium well. It is critical that as much medium is removed as possible—any serum present will interfere with the disc formation and/or the chondrogenic differentiation. To ensure more complete serum removal it may be found preferable to resuspend the cells in serum-free medium and respin to give them a wash before transfer to the next step.

4. Resuspend the cells in the appropriate volume of differentiation medium:

High glucose DMEM with sodium pyruvate
TGF β 3 10 ng/ml (stock in 4 mM HCl; 1% bovine serum albumin)

Dexamethasone 100 nM (stock in 100% ethanol)

Ascorbic acid-2-phosphate 50 μ g/ml (stock in H₂O)

Proline 40 μ g/ml (stock in H₂O)

1 \times ITS + 1 premix (Insulin, transferrin, selenium, linoleic acid) (we use Sigma)

This medium composition is the variation of the original formulation [57] developed by Mackay et al. [60]. It differs from the original recipe by the substitution of TGF β 3 for TGF β 1 [61] and by the addition of the proline.

For pellets, 1 ml of medium is used for 500,000 cells and 0.5 ml for 250,000 cells. For discs, 100 μ l medium is used per 500,000 cells for a 6.5-mm diameter Transwell culture.

5. For pellets, 1 ml of cell suspension is added to 15 ml polypropylene tubes. Spin at 240g for 5 min. Transfer to a tissue culture incubator with caps loosened. In 24 h, the cells in each tube should contract into a small pellet, and after 5–7 days (donor dependent) will expand in size. Re-feed every 2–3 days with differentiation medium.

6. For discs, pipette dropwise 100 μ l of cell suspension evenly onto the dry filter inserts of Transwells in a multi-well plate (6.5 mm diameter inserts, 0.4 μ m pore size polycarbonate membrane, 24-well plate Corning Catalogue No. 3413). Be careful not to puncture the membrane. Transfer the plate to a centrifuge equipped with a rotor suitable for 96-well plates and spin at 200g for 5 min. A very small amount of medium may come through the membrane. Transfer the filter inserts to a well of a 24-well plate con-

taining 0.5 ml of differentiation medium. Incubate as for pellet cultures; the medium will probably need to be changed every 2 days with 0.5 ml of medium in the well. This time can be extended by adding larger amounts of medium to the well (up to 1 ml). The medium in the culture insert (100 μ l) is also carefully changed at each feed.

Dependent on the donor, the discs should be coherent by 4–5 days, be self-supporting at 7 days of culture and be translucent, flexible and deformable discs of hyaline-like tissue by day 14 (Fig. 3).

3.2. Human mesenchymal stem cell hints and tips

The addition of FGF2 to the medium in which the MSCs are expanded prior to differentiation has been shown to have a significant effect on the ability of hMSC to undergo chondrogenesis [52,53,62,63]. With hMSC expanded without the addition of FGF2, chondrogenesis can be variable, whereas in our hands it is more or less universally successful in preparations expanded with FGF2 in MSCGM (Lonza).

A high cell density and intimate cell–cell contact appear to provide strong signals in the initial stages of both *in vivo* and *in vitro* chondrogenesis. Differentiation of high density monolayer MSC cultures (as measured for example by the upregulation of collagen type II mRNA levels) occurs to some extent, but is very inefficient. There is also some evidence of differentiation in alginate/collagen/agarose hydrogels with relatively monodisperse cells [64–66], though the initial cell density is probably important here. However, to achieve the rate and extent of ECM deposition observed in chondrogenic pellet cultures and particularly in the

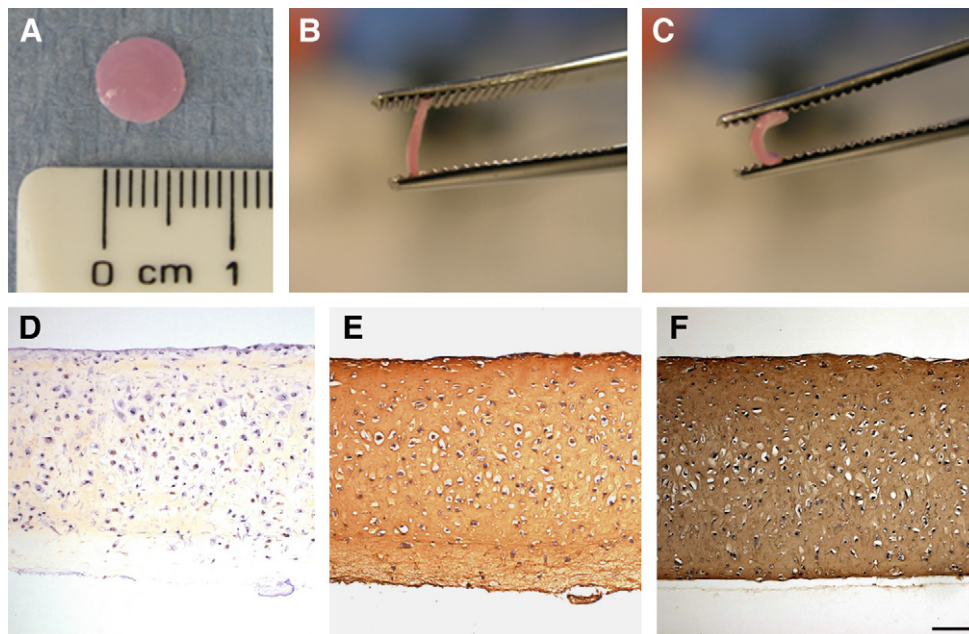


Fig. 3. Formation of scaffold free cartilaginous tissue using human mesenchymal stem cells. After 14 days of chondrogenic culture, human MSC elaborate an extensive ECM to form cartilaginous discs conforming to the diameter of the Transwell insert (A) and of uniform thickness (B). The discs are flexible (C) and whilst 5 μ m paraffin-embedded sections immunostain weakly for collagen type I (D), they stain strongly and uniformly with antibodies against collagen type II (E) and the proteoglycan aggrecan (F). Bar in (F) = 100 μ m.

membrane supported Transwell cultures [59], a 3D cell mass with cell–cell contact appears to be an important feature of the culture system.

We routinely use expanded populations of hMSC at passage 3 or 4. The addition of FGF2 can, however, extend the ability of MSC cultures to divide and retain differentiation potential. In our hands this can extend at least beyond passage 7 and has been reported to prolong differentiation capacity to as many as 70 population doublings [53].

In the Transwell culture the cells initially should stay attached to the membrane in a thin multicellular layer. If this procedure is carried out with primary human chondrocytes, they fail to stay as shallow multilayer, but contract to form a smaller diameter cell mass. With hMSC there is much less tendency to contract, but contraction may result from the effects of residual serum left with the cells before resuspension in chondrogenic medium and rough handling of the inserts following the centrifugation of the insert/multiwell plate assembly. To help alleviate this, an extra wash of the hMSC in serum-free medium may be helpful, and from day 0 of culture (steps 2–6, above), the culture medium on both discs and pellets should not be changed until day 3, to avoid disturbing the cultures, but should be changed every 2 days thereafter.

Assessing chondrogenesis in pellet and Transwell cultures can be most easily done by weighing the tissue formed. This is because chondrogenesis not only involves an increase in cell number due to proliferation, but also the deposition of a highly hydrated cartilage-like ECM gives a major increase in wet mass. A pellet culture of 0.5 million hMSC thus typically weighs 8–10 mg after 2 weeks and a Transwell culture starting with the same number of cells, about 20–25 mg. More substantial evidence of chondrogenesis can be obtained by determining collagen type II gene expression, especially as COL2A1 gene expression is extremely low in hMSC and increases 100- to 1000-fold during chondrogenesis. The deposition of collagen type II in the ECM is highly selective for chondrogenic cells. The generation of a proteoglycan-rich extracellular matrix is also a hallmark of chondrogenesis and histochemical staining of sections with safranin-O, toluidine blue, or alcian blue is a confirmatory adjunct to immunolocalisation of collagen type II. These primary measures can be supported by the determination of the expression of a number of other cartilage matrix proteins [59].

The standard size culture insert that we routinely use is the 6.5 mm diameter Transwell insert from Corning. Other size inserts can also be used with the number of cells applied to the membrane scaled to retain the same cell density per unit area. The advantages offered by the Transwell culture of hMSC for chondrogenesis is that it results in the rapid and efficient production of cartilage-like tissue construct without any scaffold or other support. It illustrates the importance of a good nutrient supply for differentiation and for maximal matrix production. The Transwell tissue produced is also more uniform than is achieved in a pellet, with no marginal collagen type I expression, and an even

staining of the matrix for collagen type II and proteoglycan. The system is thus ideal for further study of all the factors controlling matrix assembly and as a model cartilage system for investigating matrix turnover and remodelling.

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