

# Articular Chondroprogenitor Cells Maintain Chondrogenic Potential but Fail to Form a Functional Matrix When Implanted Into Muscles of SCID Mice

Cartilage  
2014, Vol. 5(4) 231–240  
© The Author(s) 2014  
Reprints and permissions:  
sagepub.com/journalsPermissions.nav  
DOI: 10.1177/1947603514541274  
cart.sagepub.com  


Paula Marcus<sup>1</sup>, Cosimo De Bari<sup>2</sup>, Francesco Dell'Accio<sup>3</sup>, and Charles W. Archer<sup>1,4</sup>

## Abstract

**Objective.** Articular cartilage is a complex tissue comprising phenotypically distinct zones. Research has identified the presence of a progenitor cell population in the surface zone of immature articular cartilage. The aim of the present study was to determine the *in vivo* plasticity of articular cartilage progenitor. **Design.** Chondroprogenitor cells were isolated from bovine metacarpalphalangeal joints by differential adhesion to fibronectin. Cells were labeled with PKH26 and injected into the thigh muscle of severe-combined immunodeficient (SCID) mice. After 2 weeks, the muscles were dissected and cryosectioned. Sections were stained with safranin O and labeled for sox9 and collagen type II. Polymerase chain reaction analysis was carried out to determine plasticity for a number of tissue-specific markers. Full-depth chondrocytes acted as a control. **Results.** Fluorescent PKH26 labeled cells were detected after 2 weeks in all samples analyzed. A cartilage pellet was present after injection of freshly isolated chondrocytes. After injection with clonal and enriched populations of chondroprogenitors, no distinct pellet was detected, but diffuse cartilage nodules were found with regions of safranin O staining and Sox9. Low levels of collagen type II were also detected. Polymerase chain reaction analysis identified the presence of the endothelial cell marker PECAM-1 in one clonal cell line, demonstrating phenotypic plasticity into the phenotype of the surrounding host tissues. **Conclusions.** The bovine articular cartilage progenitor cells were able to survive *in vivo* postimplantation, but failed to create a robust cartilage pellet, despite expressing sox9 and type II collagen. This suggests the cells require further signals for chondrogenic differentiation.

## Keywords

chondrocytes, stem cells, articular cartilage, rodent, cartilage repair

## Introduction

The smooth movement between skeletal elements is achieved through articular cartilage, a connective tissue that covers the ends of bones and provides a low friction, high load-bearing surface to minimize stress on the underlying bone. The seemingly simple, yet complex cartilaginous matrix is produced and maintained by chondrocytes. Chondrocytes are largely cytoplasmically isolated from neighboring cells<sup>1</sup> and this, combined with the avascular and aneural nature of articular cartilage, means that the tissue has a limited capacity for repair after damage or injury. Prolonged growth of chondrocytes *in vitro* has also been shown to result in their dedifferentiation or, more correctly, their phenotypic modulation.<sup>2,3</sup> Alternative cell sources may be required for the biological repair of larger chondral lesions where low levels of native cartilage remain, or a very extensive chondrocyte expansion procedure is needed.<sup>4</sup> Again, we await an expansion procedure where phenotypic expression is maintained.

The *in vivo* plasticity of mesenchymal progenitor and stem cells (MSCs) isolated from a variety of connective tissue sources has already been established. These cells have been shown to maintain the ability to differentiate into many different tissue types including myocardium, skeletal muscle, cartilage, tendon, and neural cell lineages.<sup>5</sup> It has been found that the surface zone of bovine articular cartilage contains a population of stem/progenitor cells that are required for the appositional growth of the tissue.<sup>6,7</sup> When grown in culture, the progenitor cells exhibit the similar

<sup>1</sup>Cardiff School of Bioscience, Cardiff University, Cardiff, UK

<sup>2</sup>Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK

<sup>3</sup>Centre for Experimental Medicine and Rheumatology, Queen Mary University of London, London, UK

<sup>4</sup>School of Medicine, Swansea University, Swansea, UK

## Corresponding Author:

Paula Marcus, Cardiff School of Bioscience, Cardiff University, Museum Avenue, Cardiff CF10 3AX, UK.  
Email: pmarcus@uhnresearch.ca

properties as MSCs isolated from the bone marrow (BMSC). They have an extended cell cycle time and increased ability to differentiate into differing cells types, although only along the connective tissue lineage. Importantly, unlike BMSCs, these cells are not overtly endochondral in nature.<sup>8</sup> These cells express the cell surface signaling molecule Notch-1, which has been shown to regulate the clonality of these chondroprogenitor cells. Although not all of the surface zone cells that express Notch-1 are progenitor cells, progenitor status requires Notch-1. Knock down by siRNA of Notch-1 and by the pan-Notch inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester) abolishes the clonality of these cells.<sup>6</sup>

The ability of chondroprogenitor cells isolated from the surface of articular cartilage to differentiate into adipogenic, osteogenic, and chondrogenic tissue has already been demonstrated *in vitro*<sup>9</sup> and *in ovo*.<sup>6</sup> Clonally derived labeled progenitor cells were injected into the distal and proximal limbs of 3-day-old chick embryos (stage 19-21) and tracked *in ovo* for a minimum of 1 week.<sup>6</sup> Lac-z positive cells were detectable around the injection site after 1 day, and after 1 week cells could be detected in multiple tissues types including cartilage, bone, tendon, and muscle. It was also found that these progenitor cells had formed articular fibrocartilage, perimysium, tendon, and bone, thus suggesting functional engraftment.

The use of stem/progenitor cells in cartilage repair procedures may avoid some of the constraints of expanded mature chondrocytes such as dedifferentiation and variable cell quality and potency. In addition, using stem/progenitor cells from the tissue in question may improve the structural characteristics of the repair tissue as these cells may have the appropriate developmental repertoire to recapitulate the appropriate morphogenesis. The aims of this study were to assess the responses of bovine articular cartilage progenitor cells to implantation within an ectopic location (muscle) and test their abilities to differentiate into cartilage or redifferentiate into surrounding tissue types.

## Materials and Methods

### Chondrocyte Isolation and Differential Adhesion Assay

Petri-dishes (35 mm) were coated overnight at 4°C with 10 µg/mL bovine serum fibronectin (FN; Sigma, UK) in 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub> (PBS+). Chondrocytes were isolated from the surface zone (SZ) and full-depth (FD) of 7-day bovine metacarpal-phalangeal (MCP) joints by fine dissection as described by Dowthwaite *et al.*<sup>6</sup> The chondrocytes were released from their matrix by sequential enzyme digestion, initially in 3.17 units/mL pronase (Boehringer Mannheim, Germany) in Dulbecco's modified Eagle medium F12 (high glucose DMEM/F12; GIBCO BRL, Life

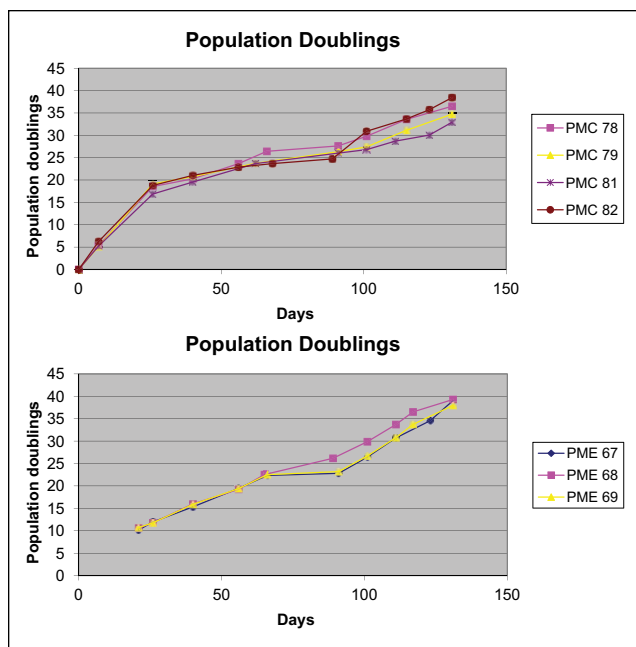
Technologies Ltd., UK) containing 5% fetal calf serum (FCS) and supplemented with 50 µg/mL ascorbic acid (Sigma), 50 µg/mL Gentamycin (GIBCO), and 1 mg/mL glucose for 3 hours on a roller at 37°C. The pronase containing media were removed and replaced with supplemented DMEM/F12 + 5% FCS with 0.12 units/mL collagenase type I (Sigma) and incubated overnight at 37°C on a roller. After digestion, the cells were passed through a 40 µm mesh cell strainer (Falcon), and chondrocytes were resuspended at 4,000 cells/mL in DMEM/F12 without FCS, seeded onto FN coated 35-mm Petri-dishes, and incubated at 37°C for 20 minutes. After 20 minutes, the media and nonadherent cells were removed and dishes filled with 2 mL DMEM/F12 plus 10% FCS.

### Isolation of Clones

Clones were isolated from colonies comprising more than 32 cells derived from the differential adhesion assay. Colonies were defined as comprising over 32 cells, that is, more than 5 population doublings in order to negate mature chondrocytes or the transient amplifying population. Enriched populations were also used, where no colonies had been isolated. Both clonal and enriched cell lines were grown to between 30 and 40 population doublings. Sterile polystyrene 6.4 mm cloning rings (Sigma) were dipped into sterile "Vaseline" using sterile forceps. The ring was then placed over the marked colony with gentle pressure and 200 µL trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; GIBCO) added. The dish was then incubated at 37°C for 2 to 5 minutes. Cells were then aspirated by gentle pipetting and added to 1 mL DMEM/F12 plus 10% FCS in a 24-well plate. The cells were then expanded in culture. For comparison, some of the plates used for differential adhesion were allowed to grow until confluence. This procedure produced a chondroprogenitor-enriched population. When the cells in the 35 mm dishes were confluent, the cells were dissociated using TrypLE express trypsin with phenol red (Invitrogen), counted, and then placed into T75 flasks. All cells lines were fed every 2 days. In all cases, at each passage, population doublings were calculated (**Fig. 1**).

### Cell Labeling and In Vivo Implantation

Cells were grown to between 30 and 40 population doublings (5<sup>8</sup> to 5<sup>11</sup> cells). Cells were trypsinized and resuspended at 2 × 10<sup>7</sup> cells/mL in a polypropylene tube. They were then washed in serum-free media and centrifuged at 400 × *g* for 5 minutes. Cells were then labeled with PKH26 red fluorescent cell marker as per the manufacturer's guidelines (Sigma). Four clonal cell lines and three enriched clonal cell populations were used for this study. Freshly isolated bovine chondrocytes were used as a positive control with late passage human chondrocytes as an unlabelled negative control.



**Figure 1.** Graph highlighting population doublings for the four clonal (PMC78, PMC79, PMC81, and PMC82) and three enriched (PME67, PME68, and PME69) cell lines used during *in vivo* experiments. Population doublings were similar between the different clonal and enriched cell lines used.

### Muscle Injections

Eight-week-old severe-combined immunodeficiency (SCID) mice were used for this study, and all animal procedures were approved by a local ethics committee. Labeled cells were transferred to an Eppendorf tube and resuspended in 50  $\mu$ L PBS (Sigma). Finally 25  $\mu$ L ( $5 \times 10^5$  cells) were injected intramuscularly into the thighs of two different mice. After 2 weeks, the mice were expired by cervical dislocation and the muscles were dissected to determine the presence of a cartilage pellet. If no pellet was found, the fibers were pooled together and frozen for cryosectioning and polymerase chain reaction (PCR) analyses. Once the samples were sectioned, they were examined using an Olympus BX61 fluorescent microscope to determine the presence of fluorescently labeled cells.

### Histology

Sections were stained for glycosaminoglycans using both toluidine blue and safranin O. Whereas both stain for glycosaminoglycans, the staining pattern can be different for different cartilage types.<sup>10</sup> Samples were fixed in 10% normal buffered formalin solution (10%; Sigma) for 10 minutes before being rinsed in running water for 2 minutes. For toluidine blue staining, sections were placed in 0.001 mg/mL toluidine blue (Sigma) for 1 minute and rinsed in

running water for 5 minutes. Excess water was removed with filter paper and cover-slips were placed over the sample and mounted with DPX (RA Lamb).

For safranin O staining, sections were placed in hematoxylin (RA Lamb, UK) for 2 minutes and then washed again in water for 5 minutes. They were then placed into 0.1% safranin O solution (BDH, UK) for a further 2 minutes before being washed for 30 seconds in water. Finally, the sections were passed through a series of increasing industrial methylated spirit concentrations (IMS; Fisher Scientific; 70% to 100%) for 2 minutes at each concentration. The slides were then cleared in xylene for 2 minutes ( $\times 2$ ). Cover-slips were placed over the sample and mounted with DPX (RA Lamb). Slides were then viewed and images taken using a Leica DMRB light microscope.

### Immunocytochemistry

To perform immunolabeling for the chondrogenic transcription factor Sox9, sections were circled with a DAKO pen (Dako, UK) fixed in 2% paraformaldehyde/PBS (both Sigma) for 2 minutes and blocked with swine serum (Dako) for 20 minutes at room temperature (serum used at 1:20). The anti-Sox9 rabbit polyclonal antibody (Abcam, UK) was diluted to 10  $\mu$ g/mL in 0.1% PBS/Tween. Rabbit IgG (Dako) was used as an isotype negative control. The excess blocking serum was tipped off and the antibody added to the slides. The rabbit IgG and a slide with 0.1% PBS/Tween instead of a primary antibody were used as controls. The sections were then incubated overnight at 4°C. Mouse limb sections were used as a positive control. Sections were washed in 0.1% PBS/Tween for 5 minutes ( $\times 3$ ) and then incubated with swine anti-rabbit FITC (Dako) in 0.1% PBS/Tween at 10  $\mu$ L/mL for 1 hour at room temperature, which was washed off in 0.1% PBS/Tween for 5 minutes ( $\times 3$ ). Finally, sections were mounted using one drop of “Vectashield” with DAPI (Vector Laboratories, UK) and a cover-slip placed over the top. Slides were then imaged using an Olympus BX61 fluorescent microscope.

For Collagen Type IIa immunolabeling (CIICI), sections were circled with a “Dako” pen, fixed, and washed as described for the Sox9 immunolabeling. The endogenous hydrogen peroxidase activity was blocked with 0.3%  $H_2O_2$  in methanol for 30 minutes. Sections were then blocked with 2 mL 0.1% PBS/Tween plus 1 drop of blocking serum (Universal quick kit; Vector Labs) for 10 minutes at ambient temperature. The antibody diluent buffer was prepared and then used to dilute the CIICI antibody (DSHB, USA) and mouse IgG control (Dako) to 2.5  $\mu$ g/mL. Excess blocking serum was tipped off and the slides were incubated with the primary antibody overnight at 4°C. A negative control was also set up using antibody diluent only. Sections were washed in 0.1% PBS/Tween before the biotinylated secondary antibody (universal horse anti-rabbit/goat; Vector

**Table 1.** Polymerase Chain Reaction Standard Primers.

Standard Primers	Forward Primer	Reverse Primer	Annealing Temperature	Product Size
$\beta$ -Actin	TGT ATG CCT CTG GTC GTA CCA C	GAG GAC TCG CGT TCA TGA GAC	58°C	596
Sox9	CTC AAG GGC TAC GAC TGG AC	CGT TCT TCA CCG ACT TCC TC	60°C	313
Coll Type II (Valcourt et al., 1999)	GCC TCG CGG TGA GCC ATG ATC	CTC CAT CTC TGC CAC GGG GT	60°C	IIA: 472; IIB: 268

All primers are shown 5'-3' with corresponding annealing temperature and product size.

Laboratories) was added, and a control was set up with no secondary antibody. Slides were then washed in 0.1% PBS/Tween ( $\times 3$  for 5 minutes). After washing, the slides were incubated with Streptavidin complex (as per the manufacturer's instructions) for 5 minutes at room temperature. The peroxidase substrate solution (Nova Red Peroxidase Quick Kit; Vecta Laboratories) was prepared as per the manufacturer's guidelines. The slides were then passed through a round of increasing concentration of IMS (70%, 95%, and 100% twice) for 2 minutes at each concentration to rehydrate the samples. The slides were then cleared in xylene for 4 minutes before mounting with DPX. Images were taken using a Leica DMRB light microscope.

### RNA Extraction and cDNA Synthesis

Small biopsies (between 2 mm and 5 mm in diameter) were taken from each sample and the tissue was powdered by dismembration (Braun Biotech). Samples were placed in the dismembrator for 90 seconds at 2,000 rpm  $\times$  g with 200  $\mu$ L TRizol reagent (Invitrogen). After three cycles in the dismembrator, the powder was collected and placed in a 1.5 mL RNase free tube (Alphalabs, UK) with 800  $\mu$ L TRizol. The samples were then incubated at room temperature for 5 minutes and 250  $\mu$ L of chloroform was added. The samples were then vortexed and left for a further 30 minutes. Tubes were centrifuged at 12,000  $\times$  g for 15 minutes at 4°C, resulting in the formation of an upper aqueous layer. This layer was removed and placed in a clean RNase free tube. An equal volume of 2-propanol (Sigma) was added and the samples were precipitated at -20°C overnight.

After precipitation, samples were centrifuged at 12,000  $\times$  g for 10 minutes at 4°C and the supernatant removed. The pellet was then washed in 1 mL of 75% ethanol and centrifuged at 7,500  $\times$  g for 5 minutes at 4°C. The supernatant was then removed and the pellet left to air-dry. When dry, the pellet was resuspended in 89  $\mu$ L Sigma molecular biology water. The samples were then DNase treated to remove genomic DNA using DNA-free kit (Ambion, UK) according to the manufacturer's instructions. Reverse transcription (RT) was carried out using the SuperScript III reverse transcriptase kit (Invitrogen). The cDNA was transcribed

from 10.5  $\mu$ L of RNA with 0.25  $\mu$ g Random primers and 0.8  $\mu$ M of each dNTP (both Promega).

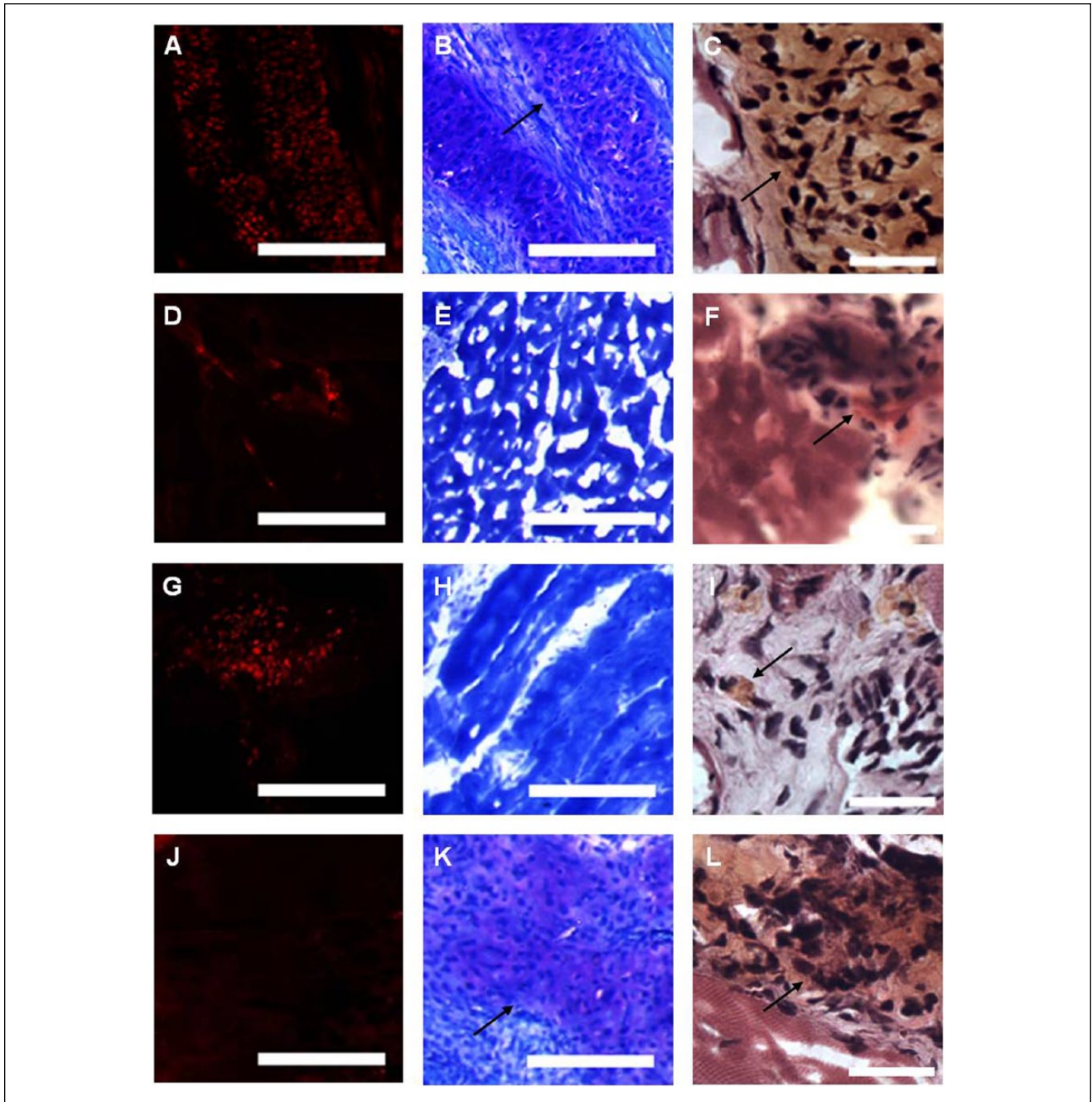
### Polymerase Chain Reaction

Primers for PCR were designed using the Primer 3 software and synthesized by MWG (UK). Each 12.5  $\mu$ L PCR reaction contained 1  $\mu$ L cDNA, 200  $\mu$ M of each dNTP, 0.24  $\mu$ M of each primer (0.2  $\mu$ M of  $\beta$ -actin primers), 10  $\mu$ L 5 $\times$  buffer (GoTaq Flexi DNA polymerase kit; Promega), 1.25 to 1.5  $\mu$ M MgCl<sub>2</sub>, and 4 units GoTaq DNA polymerase (Promega). Initial denaturation occurred for 1 minute at 94°C, with amplification comprising 35 to 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C to 60°C (see Table 1), and 30 seconds at 72°C. A final extension step of 5 minutes at 72°C then occurred. Amplification was carried out in a Techne FTGENE2D thermocycler (Techne, UK). The products were then run on a 1% agarose gel stained with ethidium bromide (both Promega). No PCR product was detected when either RNA was replaced with water at the RT reaction or the cDNA was replaced with water at the PCR stage. To determine if RNA extraction had been successful, PCR was carried out using primers for  $\beta$ -actin.

### Results

Chondroprogenitor cells were injected into SCID mice intramuscularly to determine the stability of the chondroprogenitor phenotype within a myogenic milieu and their capacity to form a stable cartilage at an ectopic site. After 2 weeks, fluorescent PKH26 labeled cells were detected in all of the samples (**Fig. 2A, D, G, J**), suggesting the chondroprogenitor cells and full-depth chondrocytes are able to survive within the muscle tissue *in vivo*.

A distinct cartilage pellet was present after intramuscular injection of freshly isolated full-depth bovine chondrocytes that could be removed as an entity by dissecting out the surrounding muscle mass. The pellet was cryosectioned for analysis and sections stained with toluidine blue and safranin O to determine the presence of highly sulfated glycosaminoglycans, a core component of a cartilage matrix. The pellet stained intensely with both toluidine blue and



**Figure 2.** Intramuscular injection of PKH26 labeled chondrocytes into SCID mice. Images showing PKH26 positively labeled cells extract from mouse muscle fibers following 2-week implantation *in vivo* from bovine full-depth (A), clonal (D), and enriched cell populations (G). Unlabelled human full-depth cells were used as a negative control (J), with no red labeling found in any samples. Scale = 140  $\mu$ m. Samples were stained with toluidine blue (B, E, H, K) and safranin O (C, F, I, L) to determine the presence of glycosaminoglycans in regions adjacent to PKH26 labeled cells in bovine full-depth (B, C), clonal (E, F), enriched (H, I), and human full-depth (J, K) cells. Regions of positive toluidine blue staining was seen in control samples as indicated by arrows. Positive safranin O staining was found in all samples as indicated by arrows. Sections stained with safranin O were counterstained with hematoxylin. Scale = 50  $\mu$ m.

safranin O (Fig. 2B, C), and the regions of intense staining corresponded to areas with a high proportion PKH26 labeled cells (Fig. 2A). Cells within this pellet were rounded and compact, typical of a chondrogenic phenotype during

cartilage development. Although there was no detectable pellet in the sample containing passaged human unlabelled chondrocytes, some regions of positive safranin O and toluidine blue staining were found, suggesting the presence of



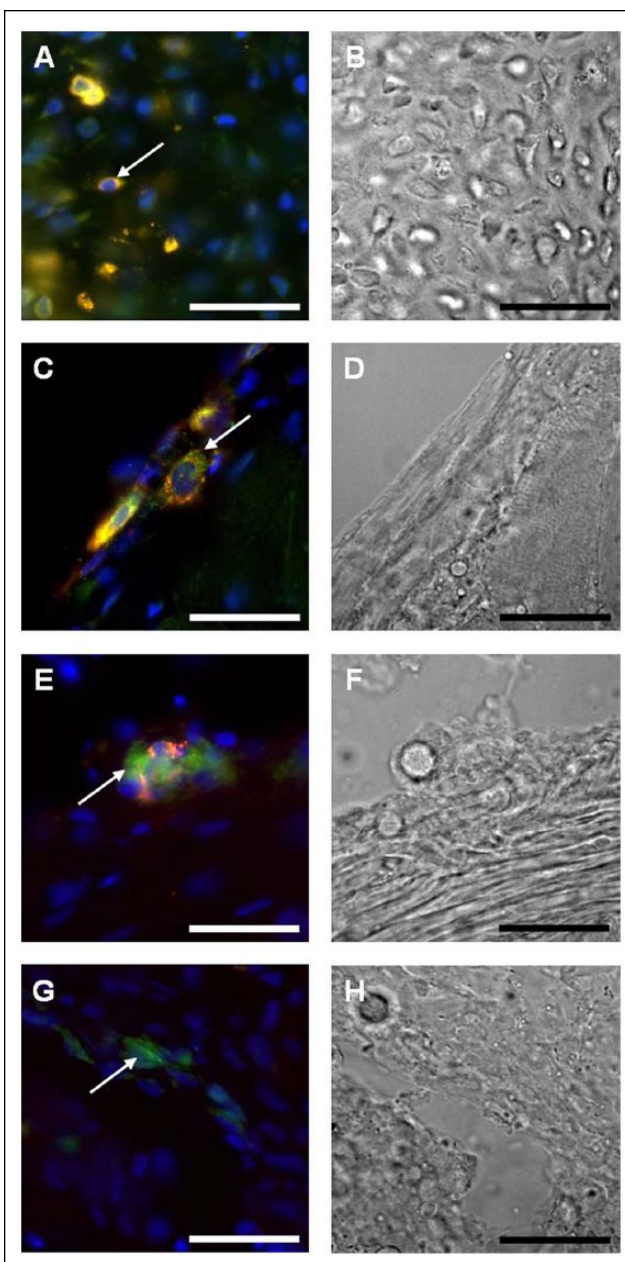
cartilage nodules within the muscle fibers with condensed areas of cells (**Fig. 2J, K, L**). More safranin O positive regions were identified within the sections, and safranin O is known to stain intensely in both articular and meniscal cartilage.<sup>10</sup> However, fluorescently labeled cells were not detected in any section. This result can be anticipated as one would not expect to generate a robust cartilage pellet from passaged human chondrocytes within 2 weeks and especially within a nonpermissible environment.

In both the clonal and enriched progenitor populations, small cartilage nodules were found that were positive for safranin O staining (**Fig. 2F, I**), and these regions were also positive for alcian blue (data not shown), although they were negative for toluidine blue staining (**Fig. 2E, H**). These regions corresponded to areas with fluorescently labeled cells (**Fig. 2D, G**), although the cells appeared fibroblastic, suggesting that they may be depositing a more fibrocartilagenous matrix. The levels of staining observed after injection with chondroprogenitor cells were much weaker than for the full-depth cell lines. Furthermore, these could not be dissected free from the surrounding tissue. Although the cells appeared slightly rounded, they were not as condensed as those observed in the full-depth cell pellet. Some of the labeled progenitor cells had a more elongated morphology, but there was no apparent difference in the morphological appearance of the cells from both the clonal and enriched populations.

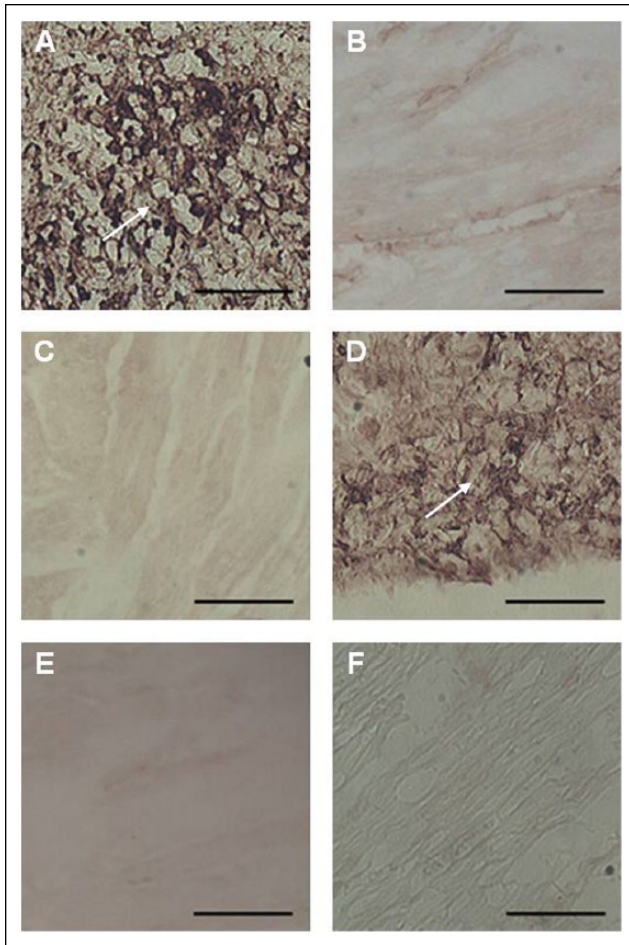
Sections were then labeled for the transcription factor Sox9 and collagen type II. Full-depth chondrocytes were found to express high levels of Sox9, with intense labeling seen within the pellet (**Fig. 3A**). This pellet also labeled positively for type II collagen (**Fig. 4A**). The human full-depth chondrocytes were also positive for both Sox9 and type II collagen (**Figs. 3G and 4D**). After injection, with bovine clonal and enriched populations, no type II collagen could be detected by immunocytochemistry in any of the sections analyzed (**Fig. 4B, C**). Despite this absence, Sox9 was detected in some of the PKH26 positive cells, whereas other cells appeared negative (**Fig. 3C, E**).

Both Sox9 and collagen type II mRNA were present in all samples analyzed by PCR (**Fig. 5**). In the case of type II collagen, both the mature (type IIB) and immature (type IIA) forms were detected in full-depth, enriched, and clonal populations. To ensure that this collagen was from the progenitor cells and not the mouse tissue, primers were designed specifically for bovine type II collagen. Bovine collagen was found in all the treated samples but was not present in a mouse control sample.

*In vitro*, and when the cells were implanted into an embryonic *in vivo* model (chick), the progenitor cells showed plasticity.<sup>6</sup> To ascertain if the cells were capable of differentiating into muscle or blood vessels, bovine-specific primers were designed (**Table 2**). To determine if the cells



**Figure 3.** Immunolabeling of implanted chondrocytes with Sox9 antibody. Immunolabeled muscle samples from mice injected with bovine full depth (**A, B**), clonal (**C, D**), and enriched (**E, F**) after 2 weeks. Extensive Sox9 labeling was seen in the full-depth pellet as indicated by the arrows (**A**). In both the clonal and enriched chondroprogenitor populations isolated cells labeled positively for Sox9. These areas corresponded to regions with PKH26 positive cells (**C and E**). Corresponding bright field images are shown (**B, D, and F**). Human unlabeled full-depth cells were used as a negative control (**G, H**). Positive Sox9 labeling could be seen in these samples even in the absence of PKH26. Cells were labeled with PKH26 (red), Sox9 (green), and nuclear stain (blue). Phase contrast images were taken of corresponding regions. Scale = 65  $\mu$ m.



**Figure 4.** Immunolabeling of implanted chondrocytes with collagen type II antibody. Immunolabeling was carried out on samples from mice injected with full-depth bovine (A), clonal (B), enriched (C), and human full-depth (D) cell populations to determine the presence of type II collagen. Both the full-depth bovine and human chondrocytes expressed type II collagen as seen by the high levels of labeling (A and D). In the case of the bovine chondrocytes this labeling was restricted to the cell pellet. Neither the samples containing clonal nor enriched progenitor cells expressed collagen type II. The substrate used was nova red and controls were set up using mouse IgG (E) and PBS only (F), which were both clear of any positive labeling. Scale = 20  $\mu$ m.

were forming muscle, primers to the muscle marker MyoD were used. There was no MyoD mRNA detected in the full-depth, clonal, or enriched populations (Fig. 5). It was, however, detected in bovine muscle controls, but was absent in mouse tissue. The marker used to assess for differentiation into endothelial cells was platelet-endothelial cell adhesion molecule (PECAM-1; CD31). As for MyoD, CD31 was absent in the full-depth and enriched populations, but it was present in one of the clonal populations of cells examined (Fig. 5). As with MyoD, PECAM-1 was present in bovine muscle samples but not detected in mouse controls.

## Discussion

The current experiments show for the first time that bovine articular cartilage progenitor cells are able to survive within the muscle mass after injection into a SCID mouse. The presence of the fluorescently labeled cells, but without cartilage matrix elaboration, after 2 weeks *in vivo* suggests that the progenitor cells are unable to fully differentiate within this inappropriate environment and may require further signals to undergo complete differentiation. In contrast, mature chondrocytes were able to elaborate a cartilaginous matrix within the muscle over the same time-span.

The *in vitro* differentiative potential, and the chondroprogenitor cells' ability to continue to undergo chondrogenic differentiation after extended time in culture, has already been demonstrated,<sup>6,11</sup> but this is the first study assessing their *in vivo* differentiation potential within a mammalian model. In order to determine the ability of chondroprogenitor cells to form cartilage-like tissue *in vivo*, immunohistochemical analysis was carried out for type II collagen and the chondrogenic transcription factor Sox9. Sox9 is a regulator of chondrogenic differentiation that is expressed constitutively by chondroprogenitors and differentiated chondrocytes, where it has been shown to have a role in the initiation of chondrogenesis.<sup>12</sup> Type II collagen is a key component of the matrix of articular cartilage and Sox9 is known to bind to the collagen type II promoter.<sup>13</sup> The freshly isolated full-depth chondrocytes appeared to be able to maintain a chondrogenic phenotype when injected intramuscularly as shown by the presence of a defined cell pellet (Figs. 2 and 4) and extracellular matrix that was positive for both type II collagen and sulfated glycosaminoglycans. Analysis of the type II collagen of the cell pellets indicated that one of the full-depth pellets had not yet expressed the mature form of type II collagen, type IIB. It is possible that this FD pellet was not as well developed as the remaining cell pellets.<sup>14</sup>

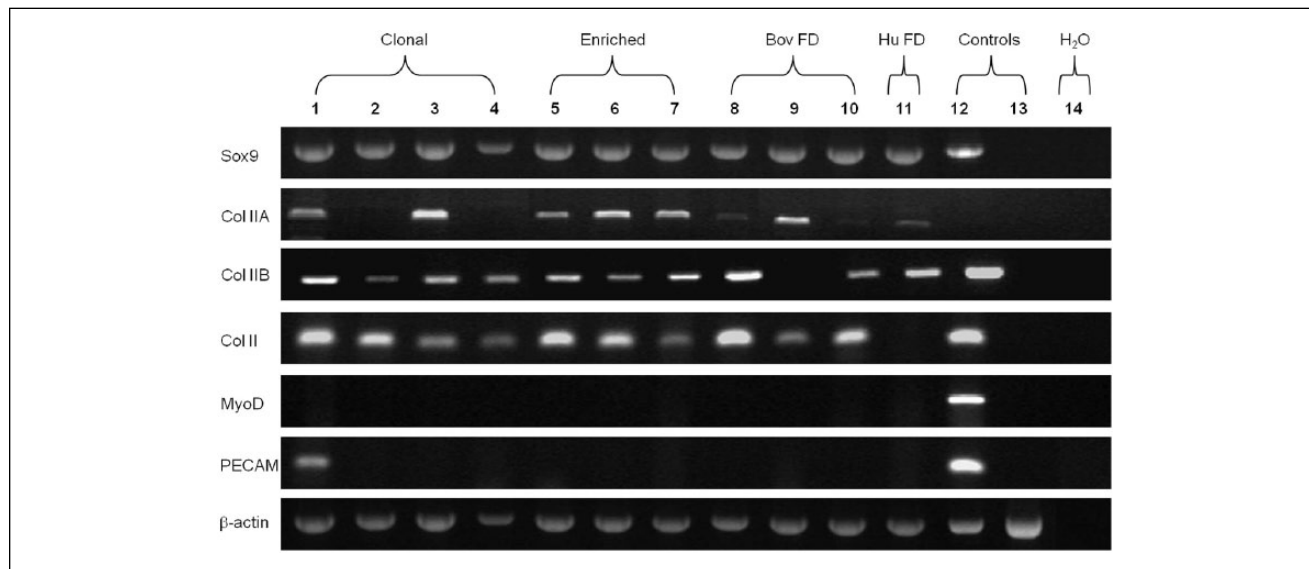
Despite expressing Sox9 and type II collagen (Fig. 5), the chondroprogenitor cells failed to form an identifiable cartilaginous pellet. Interestingly, all samples from either the clonal or enriched cell lines expressed the type IIB collagen, which is synthesized by mature chondrocytes.<sup>14,15</sup> However, two of the clonal cell lines also continued to express type IIA, which is known to play a key role in chondrogenesis.

It has already been shown that Sox9 expression alone is not enough to generate stable cartilage formation. Sox9 is required for the initiation of cartilage formation, whereas two other Sox transcription factors, L-Sox5 and 6, are required for the maintenance of the phenotype<sup>16-18</sup> and collagen type II expression.<sup>19,20</sup> We believe that the inability of the chondroprogenitor cells to form a robust cartilage pellet is not due to dedifferentiation of the cells,<sup>11</sup> but instead the absence of the extra signals needed to allow complete

**Table 2.** Polymerase Chain Reaction Bovine Specific Primers.

Bovine Specific	Forward Primer	Reverse Primer	Annealing Temperature	Product Size
Coll Type II	ACG TCC AGA TGA CCT TCC TG	TGG ATG AGC AGA GCC TTC TT	60°C	127 bp
PECAM-1	CTG GAG TCT TCA GCC ACA CA	TAT AAC CCG CTG TCC CAC TC	60°C	164 bp
MyoD	GCT ACT TCG CGA CCA GGA C	GGC GCC TCG CTG TAG TAA GT	60°C	219 bp

All primers are shown 5'-3' with corresponding annealing temperature and product size.



**Figure 5.** PCR showing expression of differentiation markers. PCR results showing markers for chondrogenic (Sox9 and collagen type II), bovine muscle (MyoD), and bovine endothelial (PECAM) cell differentiation. Cell populations used were clonal (lanes 1-4), enriched (lanes 5-7), bovine full-depth (lanes 8-10), and human chondrocytes (11). Positive controls used were cartilage explants for Sox9 and type II collagen, and bovine muscle for MyoD and PECAM (lane 12). Mouse limbs were used as a negative control (lane 13) along with a water control (lane 14). Sox9 was seen in all bovine implanted cell lines examined along with bovine-specific type II collagen. The bovine collagen type II was not seen in either mouse tissue or samples containing human injected cells. There was no MyoD seen in any of the tested cell lines, whereas PECAM-1 was seen in a single clonal cell population. Both PECAM-1 and MyoD mRNA were present in the bovine muscle samples and not the murine, showing that the primers were bovine specific. The cDNA was correctly extracted as indicated by the presence of  $\beta$ -actin in all samples analyzed.

chondrogenic differentiation to occur. Previous work by Dell'Accio *et al.*<sup>21</sup> showed that if chondrocytes had been expanded extensively (dedifferentiated), or if early passage cells were implanted into the muscle, human muscle markers were found, demonstrating the importance environmental factors on cell phenotype.

Skeletal muscle differentiation is associated with a group of transcription factors including MyoD.<sup>22</sup> This transcription factor was found in none of the clonal cell lines analyzed, indicating that none of the populations of cells are capable of undergoing myogenic differentiation. It was also possible that the cells were engrafting into blood vessels within the muscle. Consequently, we used the expression of PECAM-1 as a marker of endothelial differentiation.<sup>23</sup> Interestingly, PECAM-1 was detected in a single clonal population of progenitor cells, suggesting that this cell line may have the potential to form endothelial cells (Fig. 5). We

do not know why only one clonal cell line should switch lineage. However, we do know that the chondrogenic capacity of these clonal cell lines is not equivalent. This suggests that the clones may vary in how "mature" they are, suggesting that there may be a more immature population with greater plasticity.

When injected into the developing chick embryo it has been shown that reprogramming of the progenitor cells is possible,<sup>6</sup> presumably due to extensive cell signaling and abundance of growth factors occurring during development. This has also been identified in MSCs, where differing environmental and growth factors have been shown to have an effect on differentiation both *in vitro*<sup>24,25</sup> and *in vivo*. Our data show that all of the bovine cell lines survive *in vivo* and maintain chondrogenic potential, suggesting that chondrogenesis is the default differentiation pathway of the cells. However, the progenitor cells inability to



produce a cartilage-like tissue shows that the cells do not spontaneously produce cartilage *in vivo* in a mature system. Intramuscular implantation of progenitor cells into a mature system allows for the plasticity of cells to be determined without the wide variety of signals acting on the cells that would be present during development.<sup>21</sup> It may be predicted that the injection of these cells into a more favorable environment would lead to true chondrogenesis.

The rapid growth rate of these progenitor cells and their ability to undergo many population doublings makes them an ideal cell source to investigate for tissue engineering. They can be easily grown in culture, maintain chondrogenic potential, and have been shown to retain the ability to differentiate along multiple cell lineages even after extensive growth in culture.<sup>15</sup> The use of progenitor/stem cells in cartilage repair procedures may avoid some of the constraints of expanded mature chondrocytes such as dedifferentiation and variable cell quality and potency. The identification of a similar cell population in human articular cartilage<sup>8</sup> allows for the possibility to treat larger defects that require the generation of large numbers of cells capable of undergoing chondrogenic differentiation.

### Acknowledgments and Funding

This work was funded by BBSRC. Paul Marcus was also co-funded by Smith & Nephew. We would also like to thank Dr. Emma Blain and Dr. Sophie Gilbert for their assistance with PCR.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Ethical Approval

All animal procedures were approved by a local ethics committee.

### References

1. Archer C, Francis-West P. The chondrocyte. *Int J Biochem Cell Biol.* 2003;35:401-4.
2. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell.* 1982;30:215-24.
3. Chen G, Sato T, Ushida T, Hirochika R, Tateishi T. Redifferentiation of dedifferentiated bovine chondrocytes when cultured *in vitro* in a PLGA-collagen hybrid mesh. *FEBS Lett.* 2003;542:95-9.
4. Vukicevic S, Luyten FP, Reddi AH. Stimulation of the expression of osteogenic and chondrogenic phenotypes *in vitro* by osteogenin. *Proc Natl Acad Sci U S A.* 1989;86:8793-7.
5. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol.* 2004;36:568-84.
6. Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJ, *et al.* The surface of articular cartilage contains a progenitor cell population. *J Cell Sci.* 2004;117:889-97.
7. Hayes AJ, MacPherson S, Morrison H, Dowthwaite G, Archer CW. The development of articular cartilage: evidence for an appositional growth mechanism. *Anat Embryol (Berl).* 2001;203:469-79.
8. Williams R, Khan IM, Richardson K, Nelson L, McCarthy HE, Anabetsi T, *et al.* Identification and clonal characterization of a progenitor cell sub-population in normal human articular cartilage. *PLoS One.* 2010;5:e13246.
9. Thornemo M, Tallheden T, Sjogren Jansson E, Larsson A, Lovstedt K, Nannmark U, *et al.* Clonal populations of chondrocytes with progenitor properties identified within human articular cartilage. *Cells Tissues Organs.* 2005;180:141-50.
10. Naumann A, Dennis JE, Awadallah A, Carrino DA, Mansour JM, Kastenbauer E, *et al.* Immunohistochemical and mechanical characterization of cartilage subtypes in rabbit. *J Histochem Cytochem.* 2002;50:1049-58.
11. Khan IM, Bishop JC, Gilbert S, Archer CW. Clonal chondroprogenitors maintain telomerase activity and Sox9 expression during extended monolayer culture and retain chondrogenic potential. *Osteoarthritis Cartilage.* 2009;17:518-28.
12. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrughe B. Sox9 is required for cartilage formation. *Nat Genet.* 1999;22:85-9.
13. Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, *et al.* SOX9 directly regulates the type-II collagen gene. *Nat Genet.* 1997;16:174-8.
14. Hering TM, Wirthlin L, Ravindran S, McAlinden A. Changes in type II procollagen isoform expression during chondrogenesis by disruption of an alternative 5' splice site within Col2a1 exon 2. *Matrix Biol.* Epub 2014 Apr 13.
15. Zhu Y, Oganessian A, Keene DR, Sandell LJ. Type IIA procollagen containing the cysteine-rich amino propeptide is deposited in the extracellular matrix of prechondrogenic tissue and binds to TGF-beta1 and BMP-2. *J Cell Biol.* 1999;144:1069-80.
16. Ikeda T, Kamekura S, Mabuchi A, Kou I, Seki S, Takato T, *et al.* The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. *Arthritis Rheum.* 2004;50:3561-73.
17. Lefebvre V, Li P, de Crombrughe B. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. *EMBO J.* 1998;17:5718-33.
18. Stokes DG, Liu G, Dharmavaram R, Hawkins D, Piera-Velazquez S, Jimenez SA. Regulation of type-II collagen gene expression during human chondrocyte de-differentiation and recovery of chondrocyte-specific phenotype in culture involves Sry-type high-mobility-group box (SOX) transcription factors. *Biochem J.* 2001;360:461-70.
19. Lefebvre V, Behringer RR, de Crombrughe B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. *Osteoarthritis Cartilage.* 2001;9 Suppl A: S69-75.
20. Lefebvre V, Crombrughe BD. Toward understanding Sox9 function in chondrocyte differentiation. *Matrix Biol.* 1998;16:529-40.
21. Dell'Accio F, Bari CD, Luyten FP. Microenvironment and phenotypic stability specify tissue formation by human articular cartilage-derived cells *in vivo*. *Exp Cell Res.* 2003;287: 16-27.

22. Yanagisawa M, Suzuki N, Mitsui N, Koyama Y, Otsuka K, Shimizu N. Effects of compressive force on the differentiation of pluripotent mesenchymal cells. *Life Sci.* 2007;81:405-12.
23. Bogdanov AA Jr, Lin CP, Kang HW. Optical imaging of the adoptive transfer of human endothelial cells in mice using anti-human CD31 monoclonal antibody. *Pharm Res.* 2007;24:1186-92.
24. Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M. Isolation and multilineage differentiation of bovine bone marrow mesenchymal stem cells. *Cell Tissue Res.* 2005;319:243-53.
25. Fukumoto T, Sperling JW, Sanyal A, Fitzsimmons JS, Reinholz GG, Conover CA, *et al.* Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periosteal mesenchymal cells during chondrogenesis in vitro. *Osteoarthritis Cartilage.* 2003;11:55-64.
26. Valcourt U, Ronziere M. C, Winkler P, Rosen V, Herbage D, Mallein-Gerin F. (1999). Different effects of bone morphogenetic proteins 2, 4, 12, and 13 on the expression of cartilage and bone markers in the MC615 chondrocyte cell line. *Exp Cell Res* 251, 264-74.