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DEVELOPMENTAL PLASTICITY OF HUMAN FOETAL FEMUR-DERIVED CELLS IN PELLET CULTURE: SELF ASSEMBLY OF AN OSTEOID SHELL AROUND A CARTILAGINOUS CORE

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Abstract

This study has examined the osteogenic and chondrogenic differentiation of human foetal femur-derived cells in 3-dimensional pellet cultures. After culture for 21-28 days in osteogenic media, the pellets acquired a unique configuration that consisted of an outer fibrous layer, an osteoid-like shell surrounding a cellular and cartilaginous region. This configuration is typical to the cross section of the foetal femurs at the same age and was not observed in pellets derived from adult human bone marrow stromal cells. Time course study showed that after 7-14 days, the cells of the inner cellular region were viable, proliferated rapidly, and were immuno-positive for c-myc, as well as for bone sialoprotein and type I collagen. After 21-28 days, the cells accumulated at the inner edge of the osteoid shell. The direction of osteoid formation thus differed from that of periosteal bone formation. Following micro-dissection of the human foetal femurs into epiphyses, bone cylinder and hypertrophic cartilage, epiphyseal chondrocytes and osteoblasts both gave rise to osteoid-shell forming cells. These studies demonstrate the developmental plasticity of human foetal skeletal and epiphyseal chondrocytes and suggest that the microenvironment modulates lineage commitment and matrix formation. Furthermore, this *ex vivo* model offers a new approach to delineate human bone development as well as a model with potential application for evaluation of therapeutic compounds for bone formation.

Keywords: Human foetal cells, stem cells, differentiation, bone development, pellet culture, bone model, tissue engineering, self assembly, 3D model.

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Introduction

Cultured cells derived from human foetal femurs can differentiate into osteogenic, chondrogenic and adipogenic lineages (Mirmalek-Sani *et al*., 2006). The femur of a 10 week old foetus consists of two large cartilaginous epiphyses that contain chondrocytes and a diaphysis with a hypertrophic cartilage within a thin bone collar (Mirmalek-Sani *et al*., 2006). At 10 weeks, vascular invasion of the diaphysis has not occurred and the isolated cells constitute a mixed population of epiphyseal and hypertrophic chondrocytes as well as osteogenic cells derived from the bone collar. The earliest phase of long bone development in the embryo consists of a rod-shaped condensation of mesenchymal cells which differentiate along the chondrogenic lineage centrally, while the peripheral cells become flattened and form the perichondrium of the so-called cartilage 'anlage' (Sandell and Adler, 1999; Hall and Miyake, 2000; Goldring *et al*., 2006). Subsequent bone formation involves two phases. The initial phase intramembranous bone formation, occurs outside the mid-shaft region of the cartilage rod. Cells from the perichondrium differentiate into osteoblasts and deposit osteoid onto the cartilage anlage and chondrocytes located within the central part of the cartilage rod differentiate to hypertrophic chondrocytes. The second phase, endochondral ossification, takes place with vascular invasion. Endochondral ossification involves apoptosis of terminal chondrocytes, invasion of vascular cells into the vacated lacunae, resorption of transverse septa and deposition of bone onto non-resorbed calcified longitudinal septa.

In the last decade tremendous advances have been made in identifying the signals, transcriptional regulators and altered gene expression patterns that accompany the various stages of endochondral ossification (Kronenberg, 2003; Goldring *et al*., 2006). However, to date, the cellular and molecular events that occur during initial membranous bone formation, in particular which events are obligatory (*vs*. associated) remain unclear. Caplan (Caplan, 1987) identified a layer of 4-6 flattened 'stacked cells' as the cells that differentiate to the osteoblasts that produce the first bone collar at the mid-diaphysis. Bianco *et al.* (Bianco *et al*., 1998; Riminucci *et al*., 1998) demonstrated that both osteoblasts differentiating from the osteogenic perichondrium and chondrocytes facing these osteoblasts (vis-à-vis chondrocytes) contribute to the pool of cells that form the initial bone collar.

We have previously reported on the phenotype, multipotentiality and osteogenic (bone forming capacity of human foetal derived skeletal cells in monolayer culture (Mirmalek-Sani *et al*., 2006) as well as osteogenic matrix formation by human bone marrow stromal cells in pellet culture (El-Serafi *et al*., 2011). The current study has examined the differentiation potential of human foetal femur derived cells in 3D pellet culture to determine if this will provide a new approach to examine and inform bone formation and to delineate the mechanisms therein. 3D culture is known to enhance cell differentiation and function, in comparison to human bone marrow stromal cells. Matrix formation in the pellets derived from human foetal femur derived skeletal cells was observed to be non-uniform with a central core of cells within an indistinct matrix surrounded by a distinct shell of osteoid-like matrix beneath an outer fibrous layer of flattened cells reminiscent of the initial bone collar in the original foetal femur. These findings were not observed in human bone marrow stromal cell pellets.

The current approach provides a unique model to i) examine, delineate and interrogate normal human bone development including an approach to interrogate intramembranous bone formation in 3D, ii) bone tissue regeneration through intervention of the development process and mechanisms therein and, iii) a model to further examine the developmental plasticity of foetal skeletal cells indicating the importance of the microenvironment in modulating lineage commitment and differentiation.

Materials and Methods

Tissue culture reagents, α-Minimum Essential Medium (α-MEM), dexamethasone, Insulin Transferrin Selenium (ITS) solution, staining solutions, and all biochemical reagents were obtained from Sigma-Aldrich, Poole, Dorset, U.K., unless otherwise stated. Foetal Calf Serum (FCS) was purchased from Invitrogen Ltd (Paisley, U.K.).

Cell isolation

Human bone marrow stromal cells (HBMSCs) were isolated from bone marrow samples obtained from patients undergoing routine hip arthroplasty with appropriate consent and ethical approval (Southampton and South West Hants Local Regulatory Ethics Committee 194/99). Human foetal femurs were obtained from women undergoing termination of pregnancy, with informed consent and ethical permission from the Southampton and South West Hants joint LREC; gestational ages were estimated by stage according to the Carnegie classification or foot length. Femurs were obtained from three foetus samples with femurs form each individual representing one sample. Femurs were dissected and all attached muscles and ligaments were removed. Femurs were incubated overnight with 2 mL collagenase B (5 mg/mL, Roche Diagnostics (Welwyn Garden City, Hertfordshire, U.K.), in α-MEM at 37 °C (Mirmalek-Sani *et al*., 2006). After centrifugation at 250 g for 4 min, cells were separated from the remaining tissue using a 70 μm cells strainer (VWR, Radnor, PA, USA). The cells were expanded in monolayer culture in basal media (α-minimal essential medium (MEM), 10 % FCS and 100 U/mL penicillin and streptomycin), then frozen in 10 % dimethylsulphoxide (DMSO) in foetal calf serum (FCS) and stored at -80 °C. In addition, femurs were micro-dissected into three parts: cartilaginous epiphyses, bone collar and hypertrophic cartilage and cells extracted as detailed above.

Cell culture

Skeletal cells were expanded in monolayer in basal medium under 5 % CO_2 at 37 °C in humidified air. At confluence, cells were washed twice with phosphate buffered saline (PBS) and incubated at 37 °C with collagenase IV (0.2 mg/mL) for 45 min and with trypsin for 5 min to allow isolation of individual cells. For pellet cultures typically $5x10^5$ cells were transferred to 30 mL concave-end polystyrene universal tubes (Greiner Bio-One, Stonehouse, Gloucestershire, U.K.) in 1 mL of medium, centrifuged at 400 g for 10 min and then incubated at 37 °C under 5 % CO_2. The cells were observed to form a pellet within 24 to 48 h and media in these studies changed three times per week. Pellets were cultured in either osteogenic or chondrogenic media. Osteogenic medium consisted of basal medium with ascorbate-2-phosphate (100 μM) and dexamethasone (10 nM). Chondrogenic medium consisted of basal medium, ascorbate-2-phosphate (100 μM), dexamethasone (10 nM), TGF-β3 (10 ng/mL; VWR) and ITS supplement (1.0 mg/mL insulin from bovine pancreas, 0.55 mg/mL human transferrin and 0.5 μg/mL sodium selenite) in the absence of FCS.

Histology

At the end of the culture period, pellets were fixed in 4 % paraformaldehyde, processed through graded ethanol and chloroform and embedded in paraffin wax. 7 μm sections were stained with Alcian blue/Sirius red to distinguish bone from cartilage matrix. Alcian blue (0.5 % in 1 % acetic acid) stains proteoglycans, which typically stain an indistinct light blue in connective tissues, whereas the aggrecan of cartilage matrix stains a typical turquoise blue. Sirius red (1 % in 33 % saturated picric acid) stains fibrous collagens type I and II. In normal cartilage, the staining sites for type II collagen are covered up by aggrecan and only Alcian blue staining is observed. However, if the concentration of proteoglycans is low, type II collagen of the cartilage matrix also stains with Sirius red (Smith *et al*., 2003), although less brightly than bone matrix. Images presented are representative of the five pellets form each group examined.

The organic *bone* matrix contains predominantly type I collagen and some non-collagenous proteins (e.g., osteocalcin, osteonectin, osteopontin, bone sialoprotein). The organic *cartilage* matrix contains almost equal amounts of type II collagen and aggrecan. Immunostaining was carried out for collagens type I and II, bone sialoprotein and osteonectin. Bone sialoprotein was selected as a bone matrix component and as a marker for the onset of osteogenesis (Bianco *et al*., 1993) whilst, osteonectin was selected as this protein is one of the most abundant non-collagenous proteins in bone (Delany *et al*., 2003). In addition, sections were immuno-stained

for proliferating cell nuclear antigen (PCNA), a marker of cells in the S-phase of the cell cycle, and c-myc, an oncogenic differentiation factor (Cole and McMahon, 1999) associated with pluripotency and reprogramming (Singh and Dalton, 2009). The antibody to Type I collagen (LF67; 1:300; (Fleischmajer *et al*., 1990)) and the antibody to osteonectin (LF8; 1:200; (Pacifici *et al*., 1990)) were kindly provided by Dr Larry Fisher (National Institutes of Health (NIH), Bethesda, MD, USA). Type II collagen antibody was obtained from Calbiochem (Leicestershire, UK; 1:500). The antibody to c-myc was purchased from abcam (9E11, 1:100; Cambridge, UK); PCNA (clone PC10) was obtained from Dako (Cambridgeshire, UK; 1:100). Antigen retrieval was undertaken for PCNA and collagen II by heating the slides in Target Retrieval Solution® (Dako) at 90 °C for 10-20 min.

After quenching endogenous peroxidase activity with 3 % H_2O_2 and blocking non-specific antibody binding with 1 % bovine serum albumin (BSA) in PBS, sections were incubated with the relevant primary antibody at 4 °C overnight, followed by washing and incubation with the appropriate biotinylated secondary antibody (1 h). Visualisation of the immune complex was by the avidin-biotin method linked to peroxidase and 3-amino-9-ethylcarbazole (AEC) providing a dark brown antigen colour. For negative controls, the primary antibody was replaced with PBS. In all cases, Alcian blue served as a counter stain.

Live/dead staining: Cell Tracker Green/Ethidium homodimer-1

Prior to fixation, pellets were incubated for 2 h at 37 °C in culture medium containing Cell Tracker Green (10 μg/mL, Ex_λ 492 nm, Em_λ 517 nm) and ethidium homodimer-1 (5 μg/mL, Ex_λ 568 nm, Em_λ 580-620 nm) (Molecular Probes, Invitrogen). Cell Tracker Green identifies metabolically active cells, while the latter labels apoptotic and necrotic cells. Pellets were fixed and processed as detailed above and sections were viewed with a confocal microscope.

Results

Pellet structure is dependent on cell type and culture media

Pellets derived from adult HBMSCs and cultured in osteogenic media (Fig. 1A) consisted of small cells located within the connective tissue displaying discrete regions of Sirius red-positive matrix. Pellets cultured in chondrogenic medium (Fig. 1B), displayed as expected, extensive cartilage formation as evidenced by Alcian blue staining and the presence of cells within lacunae. In contrast, the structure of pellets derived from foetal skeletal cells differed significantly from that of HBMSCs-derived pellets. After 21 d, the size of the foetal pellets was observed to have increased by ~50 %, whereas pellets formed from HBMSCs failed to increase in size. In addition, the foetal pellets, cultured in osteogenic media were observed to form a distinct 'shell', which stained strongly with Sirius red (Fig. 1C) surrounded by an outer fibrous/cellular layer whilst the centre of the pellet was composed of small, compact cells within loose connective tissue. Foetal skeletal cells cultured in chondrogenic media displayed (Fig. 1D) extensive matrix formation throughout the pellet, as indicated by Sirius red staining (Fig. 1D) however no shell layer was observed. The shell layer observed under osteogenic culture conditions, expressed type I collagen, (Fig. 1E), but no expression of type II collagen was observed (Fig. 1G). Von Kossa staining for mineral deposits proved negative (data not shown) and this non-mineralised bone shell matrix was referred to as an 'osteoid shell'. In contrast, the collagen types of pellets cultured in chondrogenic media were a mixture of both type I and II collagen (Figs. 1F and 1H).

Osteoid shell formation is dependent on cell density

To determine whether pellet size could influence osteoid shell formation, the number of cells used for pellet formation was varied from $0.25x10^6$ to $8x10^6$ cells per pellet. An osteoid shell was found in all pellets formed with $0.25x10^6$ (Fig. 2A), $0.5x10^6$ (Fig. 1C) and $1x10^6$ (Fig. 2B) cells per pellet. However an osteoid shell was not observed in pellets formed from a higher number of cells. The structure of pellets formed from $4x10^6$ (Fig. 2C) and $8x10^6$ (not shown) cells consisted of small dense cells with negligible matrix formation evidenced by minimal Sirius red positive collagen fibres. The optimum number of cells for osteoid shell formation was observed to be less than $2x10^6$ cells.

Cells modulating osteoid shell formation

The foetal femurs used in these studies contain three cell types: (1) immature epiphyseal chondrocytes, (2) osteoblasts and osteoblast progenitors in the bone collar of the diaphysis, and, (3) hypertrophic chondrocytes in the central part of the diaphysis. To determine the contribution of each cell type to the morphology of the osteoid shell-containing pellets, femurs from a 9-week old foetus were micro-dissected into three parts (Fig. 2D). Group one consisted of the two epiphyses; group two included the bone collar and group three the central (hypertrophic) cartilage. The epiphyses comprised predominantly of epiphyseal chondrocytes with cells of the surrounding perichondrium. Given the size of the bone shaft, group two would also contain some hypertrophic chondrocytes. For cell isolation, the cells from each group were incubated overnight with collagenase B at 37 °C. Groups 2 and 3 yielded less than 50,000 cells, whereas group 1 yielded over 150,000 cells indicating epiphyseal chondrocytes were the predominant cell type. The low proliferative capacity of the hypertrophic chondrocytes precluded formation of a pellet.

The typical osteoid shell was formed in pellets derived from cells present in the bone collar (group 2) (Fig. 2E), as well as epiphyseal chondrocytes (Fig. 2F) with pellets derived from osteogenic cells displaying an intense stained osteoid shell, thicker than that observed in pellets from epiphyseal chondrocytes or in pellets derived from the whole femur. These results indicate the potential of osteogenic cells to form an osteoid shell as well as epiphyseal chondrocytes (Fig. 2E).

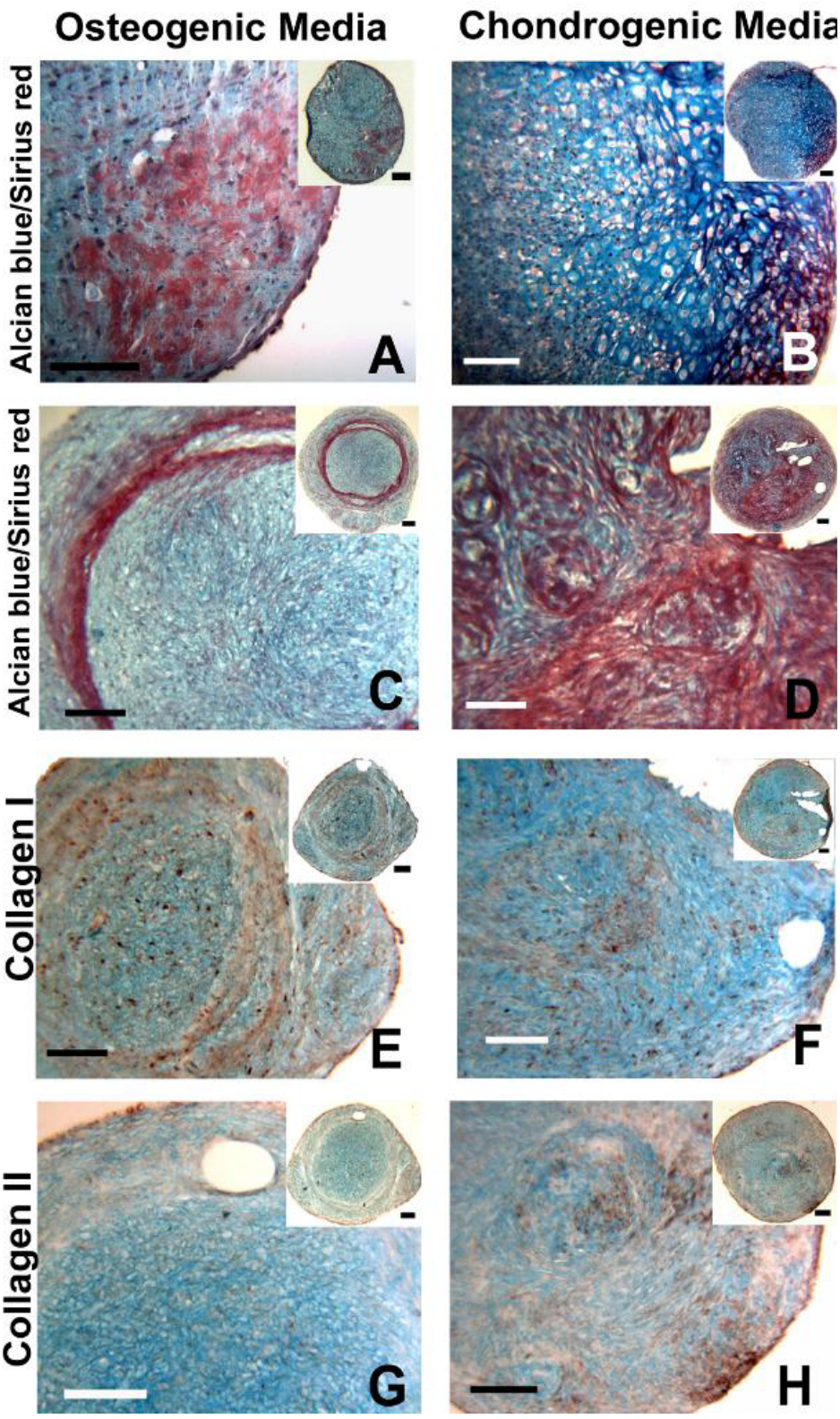

Fig. 1. **(A-D)** Alcian blue/ Sirius red staining of 21 d pellets formed from human bone marrow stromal cells (hBMSCs) **(A-B)** and foetal femur-derived cells **(C-D)**. **(A)** In hBMSCs cultured in osteogenic media a discrete region of Sirius-red stained matrix was present. **(B)** When cultured in chondrogenic media, hBMSCs display the morphology of chondrocytes within a cartilage matrix. The foetal cells cultured in osteogenic **(C)** media formed a Sirius red positive shell around a central core. Foetal cells cultured in chondrogenic media showed extensive collagenous matrix formation with little proteoglycan **(D)**. **(E-H)** Immuno-staining of the pellets formed from foetal femur-derived cells for type I **(E,F)** and type II **(G,H)** collagen in osteogenic **(E,G)** and chondrogenic **(F,H)** media. The osteoid-like shell was intensely positive for collagen I **(E)** and negative for collagen II **(G)** while the matrix of foetal cells cultured in chondrogenic media was a mixture of collagen I **(F)** and collagen II **(H)**. Representative images of 3-5 pellets in each group. Scale Bar = 100 μm.

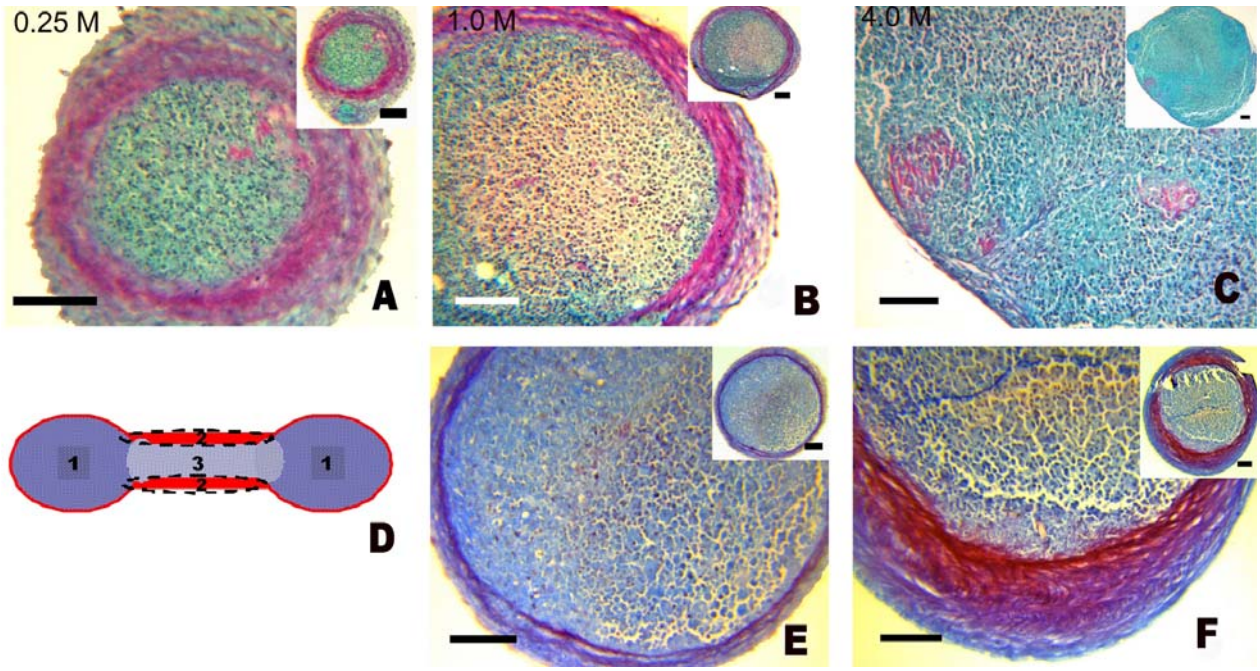

Fig. 2. Osteoid shell formation under different conditions. **(A-C)** Effects of pellet size. An osteoid shell only developed in pellets formed from **(A)** 0.25x10^6 and **(B)** 1x10^6 cells /pellet. Larger pellets 4x106 **(C)** did not form an osteoid shell, but small random areas of osteoid-like matrix were present. **(D-F)** Microdissection of the foetal femur into three cell types. As shown in the diagram **(D)**, the epiphyses (1) were separated from the bone collar (2) and the hypertrophic cartilage (3). **(E)** Cells derived from the epiphysis formed a well-defined shell structure around central chondrogenic area, which was comparable to the previous results. **(F)** Cells derived from the bone collar formed an intensely stained shell with small undifferentiated cells in the centre. Hypertrophic chondrocytes did not yield enough cells for pellet formation. Alcian blue / Sirius red staining, Scale bars = 100 μm.

Time course of osteoid shell formation

Time course studies at 7, 14, 21 and 28 d were undertaken to attempt to determine the sequence of cellular events leading to osteoid shell formation (summarised in Fig. 3). Discrete, Sirius-red positive fibres were detected at day 7 (Fig. 3A), while the centre of the pellets contained small, dense cells, some of which were immuno-positive for collagen type I (Fig. 3B). Relatively more cells were immuno-positive for bone sialoprotein (Fig. 3C) and osteonectin (Fig. 3D), however no staining for type I collagen, bone sialoprotein (BSP) or osteonectin-positive cells in the outer perimeter was recorded. After 14 d, the shell fibres were thicker (Fig. 3E) and the number of collagen type I (Fig. 3F), BSP (Fig. 3G) and osteonectin (Fig. 3H) positive cells had increased in the centre with negligible cell staining in the outer fibrous layer. After 21 d, a distinct shell could be observed together with the small dense cells (Fig. 3I), immuno-positive for type I collagen (Fig. 3J), BSP (Fig 3K) and osteonectin (Fig. 3L); located at the inner aspect of the osteoid shell as well as cartilage-like cells as evidenced by cells in lacunae with a proteoglycan-rich matrix, located at the core. A comparable configuration was observed at day 28 (Fig. 3M), with an intense shell and immunoreactivity for type I collagen (Fig. 3N), bone sialoprotein (Fig. 3O) and osteonectin (Fig. 3P). The core of the pellet consisted of a matrix with a cartilage phenotype evidenced by intense Alcian blue staining and cells within lacunae, typical features of cartilage.

Proliferation, cell death and reprogramming

Given the transverse section (TS) through a pellet with an osteoid shell superficially resembled a TS of a foetal femur, histology of foetal femurs was carried out. We have previously published images of longitudinal sections of a foetal femur (Mirmalek-Sani *et al*., 2006). As shown in Fig. 4A, a TS through the mid-diaphysis shows the thin bone collar around hypertrophic cartilage. Almost all the cells of the epiphyses and perichondrium of the foetal femurs in the S-phase of the cell cycle also stained for PCNA (Fig. 4B) and c-myc (Fig. 4C), but this was not the case for the hypertrophic chondrocytes (not shown). Similar results had previously been obtained for the growth plates of rabbit (Aizawa *et al*., 1999) and rat (Wang *et al*., 1997) long bones. Expression of *c-myc* in embryonic stem cells is associated with self-renewal and *c-myc* is implicated in the reprogramming of somatic cells to induced pluripotent cells (Knoepfler, 2008; Singh and Dalton, 2009) while the expression of PCNA is associated with DNA synthesis and cell division (Essers *et al*., 2005). The positive staining in epiphyseal chondrocytes of the human foetal femurs suggests that these cells express some progenitor /stem cell-like characteristics. To address this possibility cell proliferation, PCNA and *c-myc* expression and cell viability were examined during pellet culture. After 7 d (Fig. 4D), cells throughout the centre of the pellet were observed to be viable, however a layer of necrotic cells was observed near the perimeter of the pellets, at the location where the

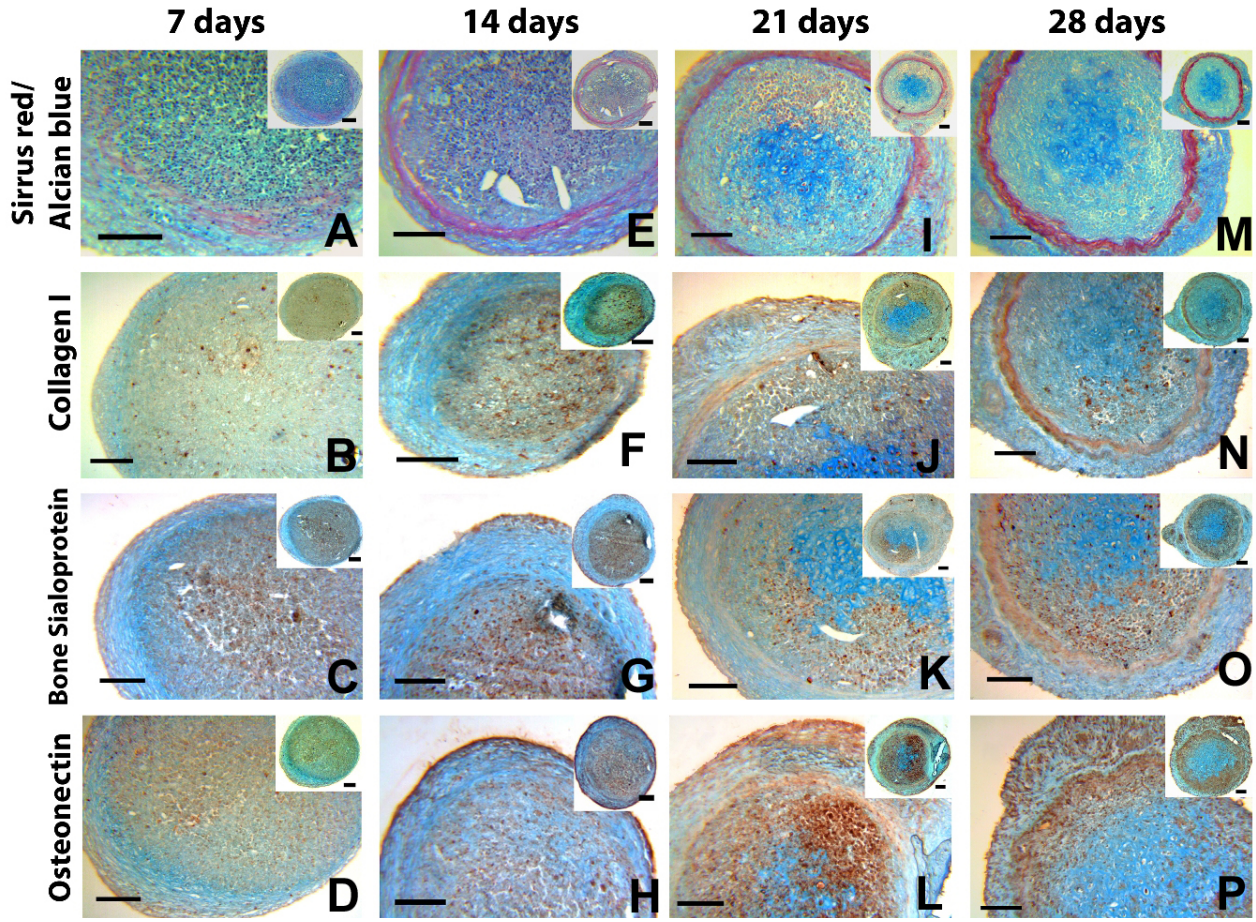

Fig. 3. Time course of foetal pellets cultured in osteogenic media and fixed after 7 d **(A-D),** 14 d **(E-H)**, 21 d **(I-L)** and 28 d **(M-P)**. The width and the intensity of the staining of the osteoid shell gradually increased with time. Cartilage matrix was observed in the centre of the pellets after 21 d **(I)** and the proteoglycan density was increased at 28 d **(M)**. Immuno-localisation of type I collagen **(B, F, J, N)** bone sialoprotein **(C, G, K, O)** and osteonectin **(D,H,L,P)** with time in culture. After 7 d **(B,C)**, some cells located throughout the centre were positive for collagen I and almost all cells, except those in the perimeter, were positive for bone sialoprotein, an osteogenic marker. After 14 d **(F,G)**, the absence of staining in outer shell was even more distinct. After 28 d **(M-P)**, the matrix of the osteoid shell stained with increased intensity, collagen I, bone sialoprotein and osteonectin. Bone sialoprotein-immunopositive cells were present at the inner aspect of the shell, while the cartilage matrix expanded in the core. Scale bars = 100 μm.

osteoid shell was observed 14-28 d later. At this stage, a high number of proliferating cells were present throughout the pellet, stained for PCNA (Fig. 4E) although, staining for c-myc; in the centre of the pellet was too intense to permit resolution of individual cells (Fig. 4F). After 28 d, fewer proliferating cells stained with PCNA were present (Fig. 4G) and the cells that stained intensely for c-myc were concentrated at the inner aspects of the osteoid shell (Fig. 4H), co-localising in part with the BSP-positive cells (Fig. 3O). Furthermore, the core of the pellet contained cartilage matrix consistent with a loss of cell proliferation and emergence of a differentiated phenotype.

Discussion

Human foetal skeletal cells isolated at 8.5-10 weeks post-conception, typically consist of epiphyseal chondrocytes (>70 %), hypertrophic chondrocytes (~20 %), and osteogenic cells located at the bone collar. During the initial expansion in monolayer culture, cell division of epiphyseal chondrocytes was maintained, whereas cells isolated from the bone shaft and in particular, from the hypertrophic cartilage displayed limited proliferation. The foetal cell population at the start of pellet culture consisted, therefore, predominantly of cells from the epiphyses, with a small contribution of osteogenic cells. Over a period of 28 d cells from this population produced a shell of osteoid-like matrix near the outer perimeter and, in time, cartilage cells at the pellet core, which resembled the cross section of the foetal femur. Immunostaining showed that the osteoid matrix consisted of type I, but not type II, collagen as well as expression of osteonectin and bone sialoprotein, both typical non-collagenous proteins of the bone matrix. No osteoid shell formation was observed in HBMSC cultures under the same conditions. There are

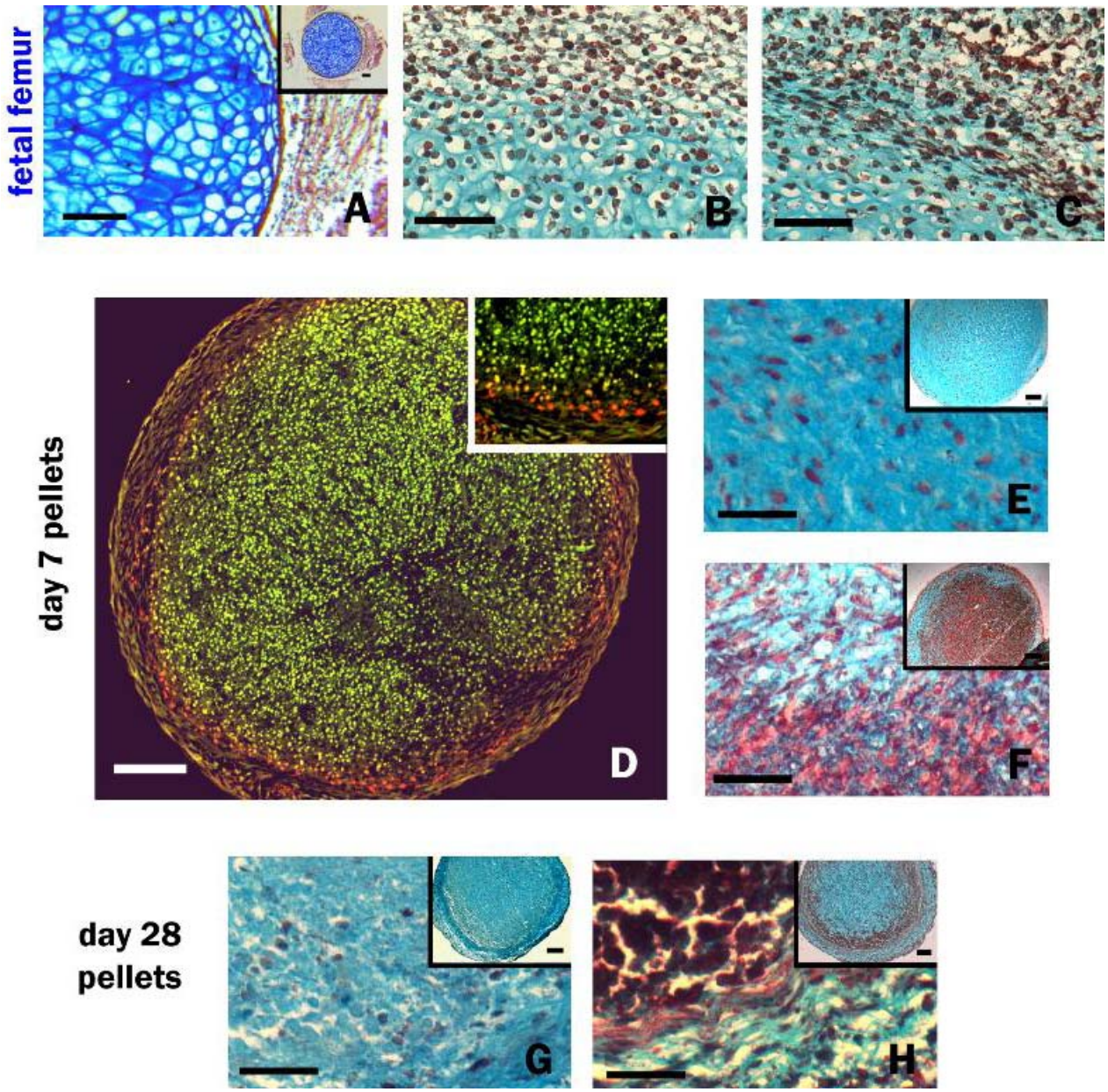

Fig. 4. Localisation of proliferating cell nuclear antigen (PCNA) and c-Myc in foetal femurs **(B,C)** before culture and in pellets after 7 d **(E,F)** or 28 d **(G,H)** of culture. **(A)** A transverse section through a foetal femur, stained with Alcian blue/Sirius red. A very thin bone collar (red) surrounds hypertrophic chondrocytes. **(B,C)** Epiphyseal chondrocytes of the foetal femur adjacent to the perichondrium. Almost all cells are proliferating (**B**, PCNA) and positive for c-Myc **(C)**. **(D)** Live-dead staining identifies a shell of dying cells (red) at the location where the osteoid shell forms after 14-28 d, whereas the small undifferentiated cells throughout the pellet are viable. **(E)** PCNA immunostaining demonstrates a high degree of cell proliferation, while **(F)** the density of c-Myc positive cells is very high in the inner part of the pellet. After 28 d, the number of cells positive for PCNA **(G)** was approximately 30 % of that at day 7. c-myc expression was also noted to be reduced at 28 d in contrast to day 7 samples and are localised at the inner aspect of the osteoid shell

several possible hypotheses as to how this *in vivo* like structure may arise and there is evidence in support for all four hypotheses: i) Foetal epiphyseal chondrocytes and osteoblasts "memorise" or retain their phenotype and migrate to a location that approximates their position *in vivo;* ii) Epiphyseal chondrocytes de-differentiate in culture followed by re-differentiation to either bone or cartilage cells depending on environmental cues; iii) Epiphyseal chondrocytes of human foetal femurs retain progenitor cell-like characteristics and subsequently differentiate to bone or cartilage cells and iv) Epiphyseal chondrocytes may trans-differentiate to bone-forming cells, but only close to the perimeter of the pellets, at the precise location of the osteoid ring.

(i) Pellets derived from the micro-dissected bone shaft displayed the broadest osteoid shell indicating the presence

of cells differentiated along the osteogenic lineage. Memory of tissue origin has been reported in connection with regeneration of the axolotl limb, where cartilage-derived blastema cells exhibit positional memory (Kragl *et al*., 2009). It is possible that bone shaft derived osteogenic cells as well as epiphyseal chondrocytes contributed to the osteoid-forming cells.

(ii) Chondrocytes in monolayer culture de-differentiate to a fibroblastic phenotype (Dessau *et al*., 1978). Stokes *et al*. (Stokes *et al*., 2002) reported human foetal epiphyseal chondrocytes in monolayer displayed up-regulation of genes associated with the undifferentiated pre-chondrocyte phenotype including COL1A1, tenascin, cadherin 11 and TWIST. In contrast, three-dimensional culture can often reverse de-differentiation (Dessau *et al*., 1978; Stokes *et al*., 2001). In the current study, the de-differentiation that probably occurred in monolayer culture was reversed during 3D pellet culture and may, in part, explain cartilage formation in the central core of the pellet after 21-28 d; although not the formation of the osteoid shell.

(iii) The high rate of cell division and of *c-myc* expression in the epiphyseal chondrocytes supports the notion that the cells retain progenitor cell-like characteristics. C-myc is present in proliferating growth plate chondrocytes, but not in hypertrophic chondrocytes (Wang *et al*., 1997; Aizawa *et al*., 1999). Over expression has been shown to maintain chondrocytes in a proliferative state, whereas decreased *c-myc* expression was required for chondrocyte maturation (Iwamoto *et al*., 1993). C-myc proteins have multiple roles, regulating cell division, transcription and transformation (Adhikary and Eilers, 2005), inducing ES cell self-renewal, pluripotency (Cartwright *et al*., 2005; Knoepfler, 2008; Singh and Dalton, 2009) or apoptosis (Hoffman and Liebermann, 2008) as well as modulating gene activation through DNA interactions (Knoepfler *et al*., 2006; Lin *et al*., 2009; Varlakhanova and Knoepfler, 2009). The presence of c-myc protein in the foetal epiphyseal chondrocytes, as found in the present study, together with the increased expression in 7-day pellets suggests a potential role in the modulation of gene expression of the cells.

(iv) The explanation for apoptotic or necrotic cells near the perimeter of the pellets is unclear. If diffusion constraints had caused cell death, then dying cells would be observed in the centre of the pellet. Apoptosis of chondrocytes does take place in hypertrophic chondrocytes of the growth plate, prior to vascular invasion (Aizawa *et al*., 1997; Fujita *et al*., 1997; Roach *et al*., 2004; Shapiro *et al*., 2005; Thomas *et al*., 2007). However, apoptosis has been shown to be involved in the trans-differentiation of chondrocytes to bone-forming cells (Descalzi *et al*., 1992; Roach *et al*., 1995; Erenpreisa and Roach, 1996; Scammell and Roach, 1996) involving asymmetric cell division. It is unclear if the environment around the perimeter would be conducive as a consequence of mechanical alterations or altered oxygen tension (higher at surface) influencing lineage commitment. An understanding of which of these hypotheses predominate (foetal cells de-differentiation/ re-differentiation or transdifferentiation and the mechanisms controlling such a process) has implications for tissue regeneration paradigms, bone pathologies and stem cell ontogeny.

There are parallels in the transverse section of the osteoid-containing pellet to a transverse section of a foetal femur. Thus it is possible formation of the osteoid-like shell involves processes similar to the membranous bone formation at the diaphysis of cartilaginous long bones. During normal development, the cartilaginous anlage is surrounded by a perichondrium. In response to as yet unidentified signals, cells from the perichondrium differentiate into osteoblasts and lay down osteoid, first in the mid-diaphysis, then proximally and distally along the cartilage anlage. This transforms the perichondrium into a periosteum, which consists of two layers, an outer fibroblastic layer and an inner osteogenic layer. The inner layer contains pools of stem or progenitor cells that differentiate into osteoblasts and also have chondrogenic potential (Colnot, 2009). Perichondral membranous bone formation thus proceeds from the outer periosteum to the bone cylinder. In the pellets, a fibrous/cellular layer similar to the periosteum surrounded the osteoid shell although, in contrast to the periosteum proper, this layer contained virtually no cells positive for PCNA, c-myc or osteogenic markers (type I collagen, osteonectin or BSP). The latter, in particular, is a good marker of newly differentiated osteoblasts and indicative of new bone formation (Chen *et al*., 1992; Bianco *et al*., 1993). By contrast, PCNA, c-myc, and the osteogenic markers were found in cells located in the centre of pellets. Thus, the direction of osteoid formation is in marked contrast to that of periosteal bone formation *in vivo*. However, the osteoid shell formed suggests the osteoid-producing cells can be located either on the inside or outside of the membranous osteoid. Riminucci *et al*. (Riminucci *et al*., 1998) provided an *in vivo* example of cells inside the future bone cylinder contributing to first bone formation: Some epiphyseal chondrocytes, which the authors termed vis-à-vis cells and which were located on the inside of the future bone cylinder opposite to periosteal osteoblasts, differentiated into bone-forming cells.

Muraglia (Muraglia *et al*., 2003) previously reported chondro-osteogenic differentiation in pellet cultures, maintaining HBMSCs for four weeks in chondrogenic medium, followed by 1-3 weeks in osteogenic medium. After 14 d in chondrogenic medium, BSP and Collagen I-immunopositive cells were seen around the perimeter of the pellets and after additional culture in osteogenic media, a bone-like matrix had formed although the matrix was not a distinct shell. The present study demonstrates that cells, including epiphyseal chondrocytes from the foetal femur, have the capacity to form an osteoid shell near the perimeter and cartilage pellet core. This 3D *ex vivo* model will be useful to i) investigate the factors that trigger osteogenic or chondrogenic differentiation, ii) possible mechanisms involved in the reprogramming of epiphyseal chondrocytes to osteoid-forming cells and, iii) offers a new *ex vivo* model for evaluation of therapeutic compounds for bone formation. The application of this culture model with other types of cells, application of mechanical forces, addition of growth factors or additional culture conditions

may aid our understanding of other associated events, such as neo-vascularisation. This 3D model offers a facile *ex vivo* innovative approach to approach to enhance our understanding of skeletal development and formation.

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References

Adhikary S, Eilers M (2005) Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol **6**: 635-645.

Aizawa T, Kokubun S, Tanaka Y (1997) Apoptosis and proliferation of growth plate chondrocytes in rabbits. J Bone Joint Surg Br **79**: 483-486.

Aizawa T, Kokubun S, Kawamata T, Tanaka Y, Roach HI (1999) c-Myc protein in the rabbit growth plate. Changes in immunolocalisation with age and possible roles from proliferation to apoptosis. J Bone Joint Surg Br **81**: 921-925.

Bianco P, Riminucci M, Silvestrini G, Bonucci E, Termine JD, Fisher LW, Robey PG (1993) Localization of bone sialoprotein (BSP) to Golgi and post-Golgi secretory structures in osteoblasts and to discrete sites in early bone matrix. J Histochem Cytochem **41**: 193-203.

Bianco P, Cancedda FD, Riminucci M, Cancedda R (1998) Bone formation via cartilage models: the "borderline" chondrocyte. Matrix Biol **17**: 185-192.

Caplan AIPDG (1987) The cellular and molecular embryology of bone formation. BoneMin Res **5**: 117-183.

Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S (2005) LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development **132**: 885-896.

Chen J, Shapiro HS, Sodek J (1992) Development expression of bone sialoprotein mRNA in rat mineralized connective tissues. J Bone Miner Res **7**: 987-997.

Cole MD, McMahon SB (1999) The Myc oncoprotein: a critical evaluation of transactivation and target gene regulation. Oncogene **18**: 2916-2924.

Colnot C (2009) Skeletal cell fate decisions within periosteum and bone marrow during bone regeneration. J Bone Miner Res **24**: 274-282.

Delany AM, Kalajzic I, Bradshaw AD, Sage EH, and Canalis E. 2003. Osteonectin-null mutation compromises osteoblast formation, maturation, and survival. Endocrinology **144**: 2588-2596.

Descalzi CF, Gentili C, Manduca P, Cancedda R (1992) Hypertrophic chondrocytes undergo further differentiation in culture. J Cell Biol **117**: 427-435.

Dessau W, Sasse J, Timpl R, Jilek F, von der Mark K (1978) Synthesis and extracellular deposition of fibronectin in chondrocyte cultures. Response to the removal of extracellular cartilage matrix. J Cell Biol **79**: 342-355.

El-Serafi AT, Oreffo RO, Roach HI (2011) Epigenetic modifiers influence lineage commitment of human bone marrow stromal cells: Differential effects of 5-aza-deoxycytidine and trichostatin A. Differentiation **81**: 35-41.

Erenpreisa J, Roach HI (1996) Epigenetic selection as a possible component of transdifferentiation. Further study of the commitment of hypertrophic chondrocytes to become osteocytes. Mech Ageing Dev **87**: 165-182.

Essers J, Theil AF, Baldeyron C, van Cappellen WA, Houtsmuller AB, Kanaar R, Vermeulen W (2005) Nuclear dynamics of PCNA in DNA replication and repair. Mol Cell Biol **25**: 9350-9359.

Fleischmajer R, Macdonald ED, Perlish JS, Burgeson RE, Fisher LW (1990) Dermal collagen fibrils are hybrids of type-I and type-III collagen molecules. J Struct Biol **105**: 162-169.

Fujita I, Hirata S, Ishikawa H, Mizuno K, Itoh H. (1997) Apoptosis of hypertrophic chondrocytes in rat cartilaginous growth plate. J Orthopaed Sci **2**: 328-333.

Goldring MB, Tsuchimochi K, Ijiri K (2006) The control of chondrogenesis. J Cell Biochem **97**: 33-44.

Hall BK, Miyake T (2000) All for one and one for all: condensations and the initiation of skeletal development. Bioessays **22**: 138-147.

Hoffman B, Liebermann DA (2008) Apoptotic signaling by c-MYC. Oncogene **27**: 6462-6472.

Iwamoto M, Yagami K, Lu Valle P, Olsen BR, Petropoulos CJ, Ewert DL, Pacifici M (1993) Expression and role of c-myc in chondrocytes undergoing endochondral ossification. J Biol Chem **268**: 9645-9652.

Knoepfler PS (2008) Why myc? An unexpected ingredient in the stem cell cocktail. Cell Stem Cell **2**: 18-21.

Knoepfler PS, Zhang XY, Cheng PF, Gafken PR, McMahon SB, Eisenman RN (2006) Myc influences global chromatin structure. EMBO J **25**: 2723-2734.

Kragl M, Knapp D, Nacu E, Khattak S, Maden M, Epperlein HH, Tanaka EM (2009) Cells keep a memory of their tissue origin during axolotl limb regeneration. Nature **460**: 60-65.

Kronenberg HM (2003) Developmental regulation of the growth plate. Nature **423**: 332-336.

Lin CH, Lin C, Tanaka H, Fero ML, Eisenman RN (2009) Gene regulation and epigenetic remodeling in murine embryonic stem cells by c-Myc. PLoS One **4**: e7839.

Mirmalek-Sani SH, Tare RS, Morgan SM, Roach HI, Wilson DI, Hanley NA, Oreffo ROC (2006) Characterization and multipotentiality of human fetal femur-derived cells: Implications for skeletal tissue regeneration. Stem Cells **24**: 1042-1053.

Muraglia A, Corsi A, Riminucci M, Mastrogiacomo M, Cancedda R, Bianco P, Quarto R (2003) Formation of a chondro-osseous rudiment in micromass cultures of human bone-marrow stromal cells. J Cell Sci **116**: 2949-2955.

Pacifici M, Oshima O, Fisher LW, Young MF, Shapiro IM, and Leboy PS. 1990. Changes in Osteonectin Distribution and Levels Are Associated with Mineralization of the Chicken Tibial Growth Cartilage. Calcif Tissue Int **47**: 51-61.

Riminucci M, Bradbeer JN, Corsi A, Gentili C, Descalzi F, Cancedda R, Bianco P (1998) Vis-a-vis cells and the priming of bone formation. J Bone Miner Res **13**: 1852-1861.

Roach HI, Aigner T, Kouri JB (2004) Chondroptosis: a variant of apoptotic cell death in chondrocytes? Apoptosis **9**: 265-277.

Roach HI, Erenpreisa J, Aigner T (1995) Osteogenic differentiation of hypertrophic chondrocytes involves asymmetric cell divisions and apoptosis. J Cell Biol **131**: 483-494.

Sandell LJ, Adler P (1999) Developmental patterns of cartilage. Front Biosci **4**: D731-D742.

Scammell BE, Roach HI (1996) A new role for the chondrocyte in fracture repair: endochondral ossification includes direct bone formation by former chondrocytes. J Bone Miner Res **11**: 737-745.

Shapiro IM, Adams CS, Freeman T, Srinivas V (2005) Fate of the hypertrophic chondrocyte: microenvironmental perspectives on apoptosis and survival in the epiphyseal growth plate. Birth Defects Res C Embryo Today **75**: 330-339.

Singh AM, Dalton S (2009) The cell cycle and Myc intersect with mechanisms that regulate pluripotency and reprogramming. Cell Stem Cell **5**: 141-149.

Smith JO, Oreffo RO, Clarke NM, Roach HI (2003) Changes in the antiangiogenic properties of articular cartilage in osteoarthritis. J Orthop Sci **8**: 849-857.

Stokes DG, Liu G, Coimbra IB, Piera-Velazquez S, Crowl RM, Jimenez SA (2002) Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. Arthritis Rheum **46**: 404-419.

Stokes DG, Liu G, Dharmavaram R, Hawkins D, Piera-Velazquez S, Jimenez SA (2001) 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 **360**: 461-470.

Thomas CM, Fuller CJ, Whittles CE, Sharif M (2007) Chondrocyte death by apoptosis is associated with cartilage matrix degradation. Osteoarthritis Cartilage **15**: 27-34.

Varlakhanova NV, Knoepfler PS (2009) Acting locally and globally: Myc's ever-expanding roles on chromatin. Cancer Res **69**: 7487-7490.

Wang Y, Toury R, Hauchecorne M, Balmain N (1997) Expression and subcellular localisation of the Myc superfamily proteins: c-Myc, Max, Mad1 and Mxi1 in the epiphyseal plate cartilage chondrocytes of growing rats. Cell Mol Biol (Noisy-le-grand) **43**: 175-188.

Editor's Note: Since all questions/comments by the reviewers were answered by text changes, there is no "Discussion with Reviewers" section.