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Hypertrophy and physiological death of equine chondrocytes *in vitro*

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For further information and explanation of terminology see www.evj.co.uk/glossary

Summary

Reasons for performing study: **Equine osteochondrosis results from a failure of endochondral ossification during skeletal growth. Endochondral ossification involves chondrocyte proliferation, hypertrophy and death. Until recently no culture system was available to study these processes in equine chondrocytes.**

Objective: **To optimise an *in vitro* model in which equine chondrocytes can be induced to undergo hypertrophy and physiological death as seen *in vivo*.**

Methods: **Chondrocytes isolated from fetal or older (neonatal, growing and mature) horses were cultured as pellets in 10% fetal calf serum (FCS) or 10% horse serum (HS). The pellets were examined by light and electron microscopy. Total RNA was extracted from the pellets, and quantitative PCR carried out to investigate changes in expression of a number of genes regulating endochondral ossification.**

Results: **Chondrocytes from fetal foals, grown as pellets, underwent hypertrophy and died by a process morphologically similar to that seen *in vivo*. Chondrocytes from horses age >5 months did not undergo hypertrophy in pellet culture. They formed intramembranous inclusion bodies and the cultures included cells of osteoblastic appearance. Pellets from neonatal foals cultured in FCS resembled pellets from older horses, however pellets grown in HS underwent hypertrophy but contained inclusion bodies. Chondrocytes from fetal foals formed a typical cartilage-like tissue grossly and histologically, and expressed the cartilage markers collagen *type II* and aggrecan mRNA. Expression of Sox9, collagen *type II*, Runx2, matrix metalloproteinase-13 and connective tissue growth factor mRNA increased at different times in culture. Expression of fibroblast growth factor receptor-3 and vascular endothelial growth factor mRNA decreased with time in culture.**

Conclusions: **Freshly isolated cells from fetal growth cartilage cultured as pellets provide optimal conditions for studying hypertrophy and death of equine chondrocytes.**

Potential relevance: **This culture system should greatly assist laboratory studies aimed at elucidating the pathogenesis of osteochondrosis.**

Introduction

Osteochondrosis is a disease of growing horses that results from a disturbance in endochondral ossification, the process by which the cartilaginous skeleton of the fetus is replaced by bone during development and growth. The disease is initiated by formation of foci of retained cartilage in subchondral bone, which leads to the clinically important manifestations of osteochondrosis, including subchondral fractures and cysts, osteochondritis, cartilage flaps and synovitis.

During endochondral ossification, chondrocytes in growth cartilage undergo an organised programme of functional changes, starting with proliferation and progressing to hypertrophy then death, before the cartilage tissue is invaded by blood vessels and bone cells. Chondrocyte proliferation, differentiation and hypertrophy are regulated by a variety of growth factors, hormones and components of the cartilage matrix (van der Eerden *et al*. 2003; Nilsson *et al*. 2005; Provot and Schipani 2005). Many of these factors have been characterised, although it is probable that more remain to be identified. The process of physiological cell death (PCD) undergone by chondrocytes is less well characterised; until recently it was assumed to occur through apoptosis, but recent studies have indicated that hypertrophic chondrocytes undergo an unidentified nonapoptotic mode of death (Roach and Clarke 2000; Roach *et al*. 2004; Shapiro *et al*. 2005). Two populations of hypertrophic chondrocytes are detectable ultrastructurally ('light' and 'dark' chondrocytes; Hwang 1978; Wilsman *et al*. 1981; Erenpreisa and Roach 1998; Roach 2002), and each of these 2 cell types dies by a morphologically distinct nonapoptotic mechanism (Ahmed *et al*. 2007).

The sequence of events leading to the retention of growth cartilage in osteochondrosis is poorly understood, but it clearly involves a perturbation of the normal progression of chondrocytes from the resting state through to hypertrophy and death. In order to study the factors that influence this progression, it is important to have access to a culture system in which chondrocytes behave as they do *in vivo*.

Chondrocytes cultured in monolayer dedifferentiate, losing their characteristic spherical morphology and taking on a fibroblast-like morphology; they switch from expression of the cartilage-specific collagen *type II* to expression of the more widely expressed collagen *type I* (Stewart *et al*. 2000). A number

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of culture systems have been developed to maintain the differentiated state of chondrocytes. These systems include culturing chondrocytes in scaffolds such as agarose, collagen or alginate (Hunter *et al*. 1993; Chaipinyo *et al*. 2002; Masuda *et al*. 2003). Another method is to culture chondrocytes as pellets in tubes, a simple method that requires no specialised materials (Kato *et al*. 1988). We have recently observed that chondrocytes isolated from equine fetuses and grown in pellet culture in the presence of 10% FCS undergo hypertrophy and physiological death; and that such cells are morphologically indistinguishable from chondrocytes in growth cartilage *in vivo* (Ahmed *et al*. 2007). The aim of the current study was to optimise and fully characterise the pellet culture model for use with equine chondrocytes; such a model is necessary for investigation of mechanisms of chondrocyte hypertrophy and death, and of their perturbation in osteochondrosis.

Materials and methods

Sample collection

Metacarpophalangeal joints of neonatal (age 7 days), growing (age 6 and 12 months) and mature (age 17 years) horses were collected from a local abattoir or from animals that died from diseases unrelated to the skeleton at the University of Melbourne Veterinary Clinic and Hospital. Metacarpophalangeal joints of fetal foals were collected from the abattoir and the gestational stages (3, 6 and 9 months) were estimated on the basis of crown–rump length (Noden and de Lahunta 1985).

Chondrocyte isolation and culture

The joints were opened under aseptic conditions and cartilage excised from the articular surface, excluding any bone tissue. The excised cartilage was dissected into small pieces and chondrocytes isolated by overnight digestion in sterile Dulbecco's modified Eagle's medium (DMEM)[1] containing 0.5% collagenase A[2] and 0.1% FCS[1] at 37°C. The resulting chondrocytes were cultured as pellets using a modification of the method described by Kato *et al*. (1988). Briefly, 5 x 10^5 cells in 1 ml DMEM were placed in 15 ml polypropylene tubes. The DMEM contained gentamicin[1] (50 μg/ml), amphotericin B[1] (2.5 μg/ml), L-glutamine[1] (300 μg/ml), L-ascorbic acid[3] (50 μg/ml) and 10% FCS or 10% HS[1]. The cells were centrifuged for 5 min at 67 x ***g*** and incubated at 37°C with the caps of the tubes closed. After 48 h, the cultured cells formed white disc-shaped pellets that could be manipulated with forceps. Pellets were collected at 0, 7, 14, 21 and 28 days. Pellets were weighed before being fixed.

Histology

Some pellets were fixed in 2.5% gluteraldehyde/4% paraformaldehyde in 0.1 mol/l sodium cacodylate buffer at 4°C for 24 h. They were post fixed in 1% osmium tetroxide/1.5% potassium ferrocyanide and embedded in Spurr's resin. Semi-thin cross-sections were stained with methylene blue and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and Reynold's stain, and examined by transmission electron microscope (Philips 300).

Photomicrographs of semi-thin sections were captured with a digital camera and measurements of pellet thickness and cellular density were made using image analysis software (Image-Pro Plus)[4]. The thickness was measured at the centre of the pellet, and the cellular density defined as the number of cells/mm^2 in a specific region of each section. Counts of dark, light or resting chondrocytes as well as different forms of PCD were carried out manually as described (Ahmed *et al*. 2007), and expressed as a percentage of total cell number.

Some pellets were fixed in 4% paraformaldehyde for 30 min, embedded in OCT compound[5] and frozen in liquid nitrogen. Cryosections (5-10 μm) were cut using a cryostat, and alkaline phosphatase activity was detected using a kit[3] following the manufacturer's instructions. Mineralisation in cryosections was detected using Von Kossa's method. Sections were counterstained with Mayer's haematoxylin and mounted with aqueous medium.

Polymerase chain reaction (PCR)

Total RNA was extracted and purified from 3 pools of at least 10 pellets cultured in 10% FCS for various times using a combination of Tri Reagent[3] and SV RNA Isolation columns[6] according to the manufacturers' instructions. The first strand cDNA was synthesised using M-MLV reverse transcriptase RNase H Minus[6]. Primers[7] for collagen type II, aggrecan, Sox9, Runx2, vascular endothelial growth factor (VEGF), fibroblast growth factor receptor-3 (FGFR3), matrix metalloproteinase-13 (MMP-13), connective tissue growth factor (CTGF) and ribosomal protein S23 (RP-S23) were as described (Ahmed *et al*. 2007). Collagen *type X* primer sequences were as follows (numbers indicate exonic location of primer): 2-forward: CCCACTACCCAACACCAAGA, 3-reverse: TCCGTAGCCTGGTTTTCCT. Part of the equine collagen *type X* exonic sequence was identified and this novel sequence information used to design primers. All RT-PCR primers were designed as exonic primers to amplify products of 80-150 base pairs, with each primer set spanning an intron. Primers were designed using equine cDNA sequences where available. PCR products were separated by electrophoresis and visualised using a gel documentation system (Chemi-Smart 2000)[8]. Automated sequencing was carried out on all PCR products to confirm their identity (AGRF, Melbourne, Australia).

Quantitative PCR (qPCR) was carried out with Platinum SYBR Green Supermix-UDG[1] using a Stratagene MX3000 qPCR machine[9]. Results were analysed using the pair-wise fixed reallocation randomisation test from the Relative Expression Software Tool-384 (REST-384) (Pfaffl *et al*. 2002). The results for each gene of interest normalised to results for the housekeeping gene (RP-S23) are presented as level of expression at each time point relative to expression at Day 7. Efficiencies for primer products were very similar, with almost all lying within the range of 100% ± 10%, with a few exceptions. The REST-384 spreadsheet, however, allows different primer efficiencies to be taken into account in the calculation of relative expression.

Statistical analysis

Results are presented as mean ± s.e. ($n \geq 3$). For parameters other than qPCR, statistical differences between groups were evaluated using a one way ANOVA; P values <0.05 were considered significant.

Results

Pellet cultures: effect of age of horse and serum type

Experiments were undertaken to determine whether FCS or HS provides a better environment for equine chondrocytes in pellet culture and whether cells derived from neonatal, growing and mature horses could be used for such cultures. Pellet cultures from fetal foals, in either serum type, were similar to those described previously for 10% FCS (Ahmed *et al*. 2007). Electron microscopy revealed the presence of 2 types of hypertrophic chondrocytes, dark and light cells, evenly distributed throughout the pellets (Fig 1A, B). Dark chondrocytes had dark nuclei and cytoplasm containing well developed and often dilated rough endoplasmic reticulum (RER; Fig 1A). In contrast, light chondrocytes had pale nuclei and cytoplasm containing less developed RER (Fig 1B). Dark and light hypertrophic chondrocytes were observed to die by the same modes of PCD observed in equine growth cartilage *in vivo* (not shown; Ahmed

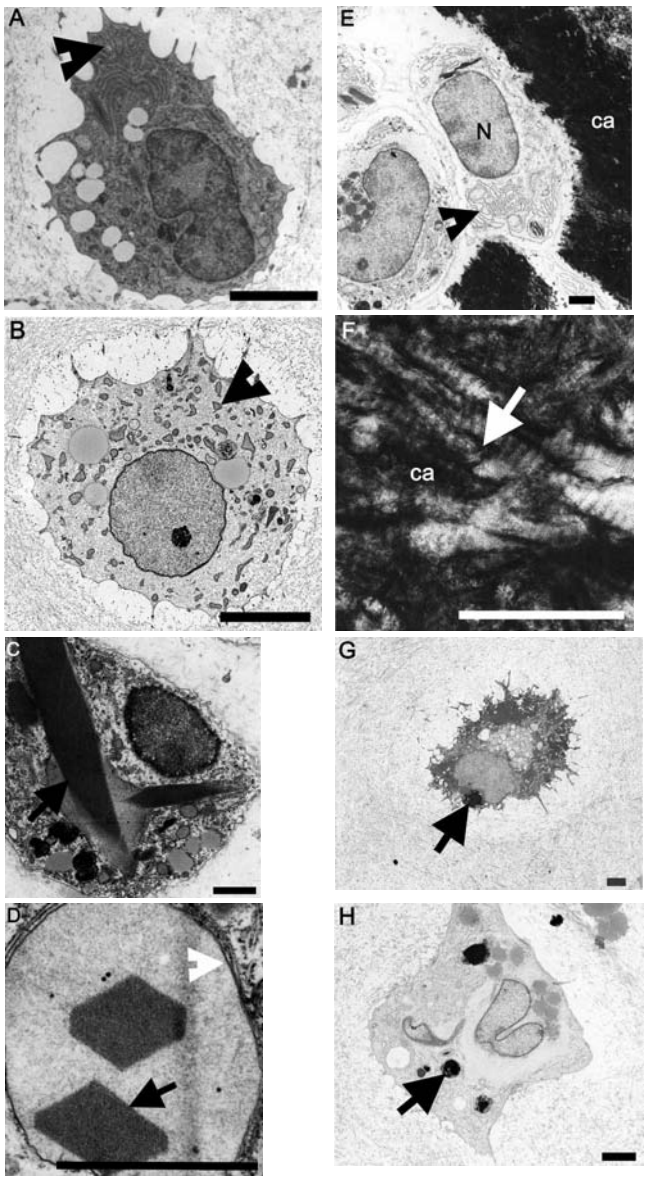

Fig 1: Morphology of chondrocyte pellet culture from 3 month fetal (A, B), 6 month post natal (C–F) and 7 day neonatal (G, H) horses. Electron micrographs from pellets cultured in FCS (A–F) or HS (G, H). A, B: Hypertrophic dark (A) and light (B) chondrocytes; arrowheads indicate RER. C, D: Cells containing inclusion bodies (arrows) in longitudinal (C) and transverse (D) sections; arrowhead in D indicates membranes surrounding inclusion bodies. E: Cells with osteoblastic appearance with large, pale nucleus (N) and RER (arrowhead), surrounded by calcified matrix (ca). F: collagen type I-like structures (arrow) in calcified matrix (ca). G, H: Hypertrophic dark (G) and light chondrocytes (H) containing inclusion bodies (arrows). All bars = 1 µm.

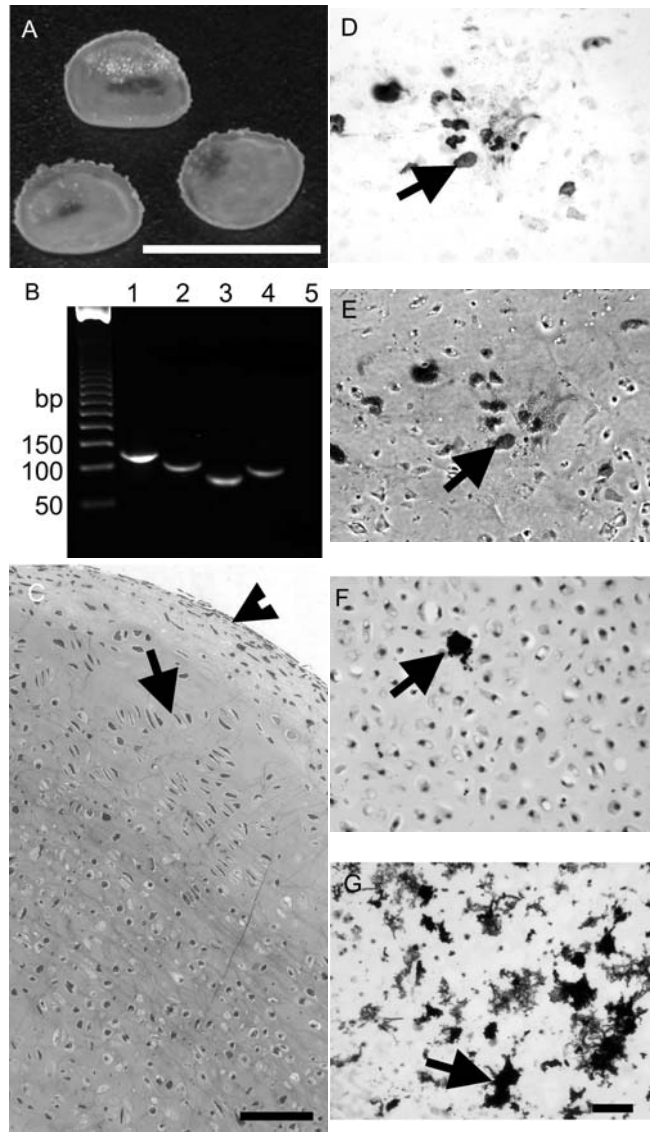

Fig 2: Cartilage-like tissue formation by fetal chondrocytes. Chondrocytes from equine fetal growth cartilage were cultured as pellets in FCS for up to 28 days. A: Photograph of gross pellet culture at 14 days. B: Agarose gel showing RP-S23 (1), collagen type II *(2), aggrecan (3), collagen* type X *(4) and negative control (5) PCR products derived from RNA extracted from pellets cultured for 21 days. C: Semi-thin section of a 21 day pellet showing hyaline cartilage-like tissue containing chondrocytes embedded in abundant ECM and organised in columns (arrow), and surrounded by a perichondrium-like layer (arrowhead). D, E: Cryosection of a pellet cultured for 21 days showing alkaline phosphatase activity (arrows) in brightfield (D) and phase contrast (E). F, G: Cryosections stained using Von Kossa's method showing increased ECM calcification (arrows) from Day 7 (F) to Day 28 (G). Bar = 10 mm in A and 25 µm in C. D–G have the same magnification; bar = 25 µm.*

et al. 2007). Dying dark chondrocytes showed extrusion of cytoplasm into the extracellular matrix (ECM) while dying light chondrocytes showed disintegration of cytoplasmic contents within the cell membrane. A small number of apoptotic chondrocytes were present at all stages of culture close to the perichondrium-like outer layer (not shown; Ahmed *et al*. 2007).

Chondrocytes from growing and mature horses, cultured in 10% FCS or 10% HS, formed pellets, but did not undergo hypertrophy. Accordingly, no light and dark cells could be distinguished. Many cells contained intramembranous inclusion bodies (Fig 1C, D). Some cells had an osteoblastic appearance; they had pale nuclei and few cytoplasmic organelles, mainly RER, and were embedded in a highly mineralised matrix (Fig 1E). In some areas, the ECM showed a structure similar in appearance and mineralisation to collagen *type I* (Bonucci and Motta 1990), the main collagen in bone tissue (Fig 1F).

Chondrocytes from neonatal foals, grown as pellets in FCS, were similar to those from older horses. Pellets cultured in HS contained chondrocytes undergoing dark (Fig 1G) and light (Fig 1H) hypertrophic differentiation but they also showed the inclusion bodies described in pellets from older horses (Fig 1G, H).

Characterisation of pellet culture from fetal horses

Chondrocyte pellet cultures from fetal foals were further characterised. After 14 days in culture, the chondrocytes formed a grossly (Fig 2A) and histologically (Fig 2C) hyaline cartilage-like tissue. The pellets contained chondrocytes in lacunae distributed uniformly throughout an abundant ECM; in some areas, they were arranged in a column-like structure similar to that seen in the proliferative zone in growth cartilage (Fig 2C). A perichondrium-like layer of flattened cells surrounded the pellet (Fig 2C). Chondrocytes expressed the cartilage marker genes, collagen *type II* and aggrecan (Fig 2B). Hypertrophic chondrocytes were first detectable morphologically at 14 days. After 21 days in culture, more than 80% of cells had differentiated into hypertrophic chondrocytes. Chondrocyte hypertrophy was further confirmed by expression of collagen *type X* (Fig 2B) and alkaline phosphatase activity at Day 21 (Fig 2D, E), and increased ECM mineralisation from Day 7 (Fig 2F) to Day 28 (Fig 2G).

Morphometrical studies revealed that the pellets were undergoing progressive changes during the culture period. Pellet weight (Fig 3A) and thickness (Fig 3B) increased, but cellular

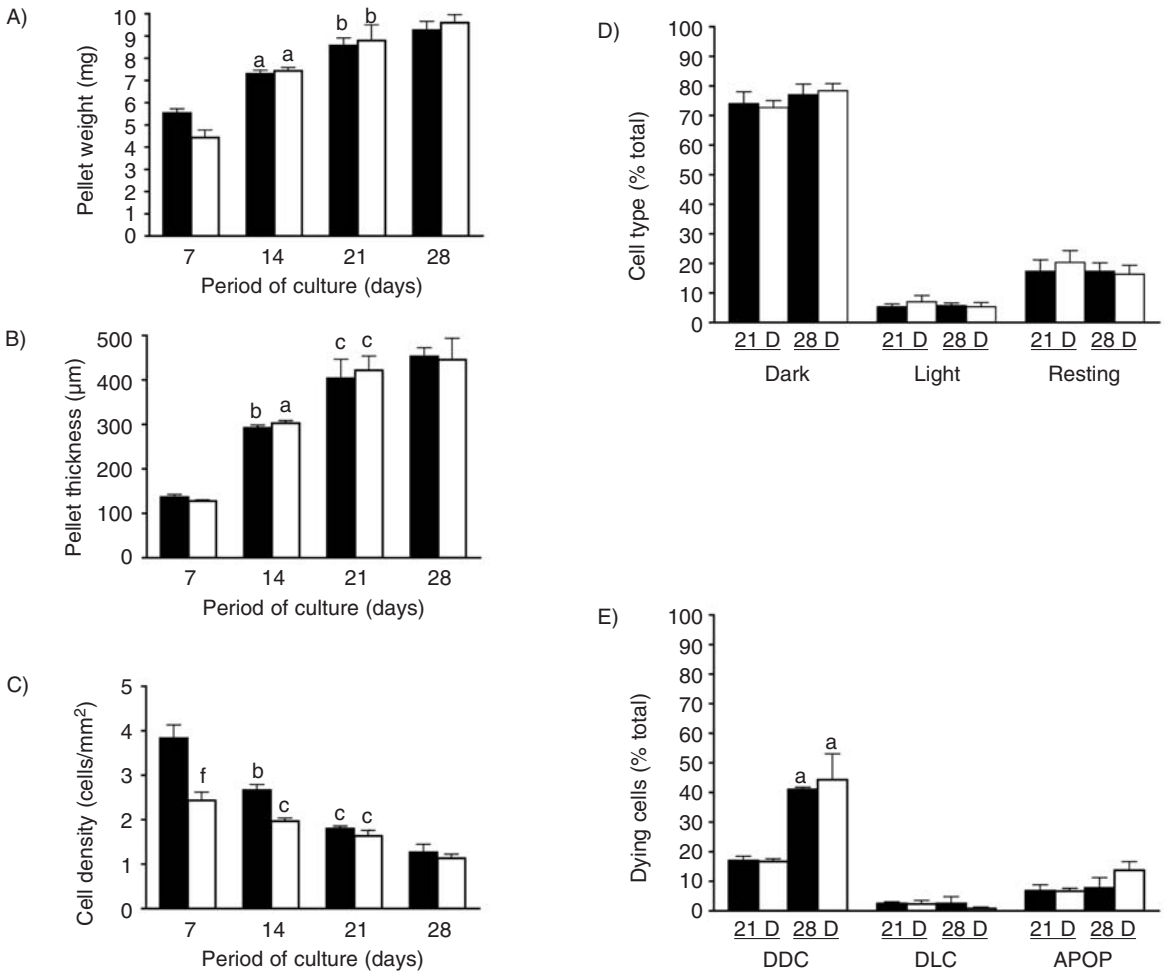

Fig 3: Characteristics of pellets cultured from equine fetal chondrocytes. Pellets cultured in FCS ■ and HS □ were collected at different time points, and pellet weight (A), thickness (B) and cellular density (C) were measured. D) The proportion of dark, light or resting chondrocytes expressed as a percentage of total cells. E) The proportion of dying dark cells (DDC), dying light cells (DLC) and cells undergoing apoptosis (APOP) at 21 (21D) or 28 (28D) days. Columns represent mean ± s.e.; n ≥3. Significant differences between values are expressed as: a, P<0.001, b, P<0.01, c, P<0.05 for comparison between each 2 successive time points; f, P<0.05 for comparison between treatments.

density (Fig 3C) decreased over time in culture. No significant differences were observed between pellets cultured in FCS or HS except at Day 7, when the pellets in HS showed a significantly lower cellular density than pellets in FCS.

Of the hypertrophic chondrocytes present at Day 21, dark chondrocytes were present in a higher proportion than light chondrocytes (Fig 3D). A high proportion of dark chondrocytes were dying by 28 days and no significant differences were observed between pellets cultured in FCS and HS (Fig 3E).

Quantitative PCR was carried out to compare changes in gene expression during the culture period relative to expression at Day 7 in pellets cultured in 10% FCS (Fig 4). Expression was significantly upregulated for Sox9 (1.7-fold), collagen *type II* (1.8-fold) and Runx2 (2-fold) after 14 days, and for MMP-13 (2.8-fold) and CTGF (1.5-fold) after 21 days. VEGF and FGFR3 were downregulated at the later time points. Aggrecan mRNA expression remained unchanged at Days 14 and 21. After 28 days, most of the genes examined were expressed at lower levels than at 7 days.

Discussion

The current study was undertaken in order to identify the optimal conditions for a system of culture in which isolated chondrocytes behaved in a similar way to cells in growth cartilage, differentiating into hypertrophic dark and light cells then dying by the same modes of PCD observed *in vivo*. Chondrocytes were isolated from growth cartilage of horses of a variety of ages, ranging from fetal to ageing mature individuals, and cultured as pellets in the presence of either FCS or HS. The morphology of the pellets revealed a considerable variability in relation to the age of the horses from which chondrocytes were isolated.

Chondrocytes isolated from fetal horses and cultured in HS differentiated into light and dark hypertrophic cells, and died by

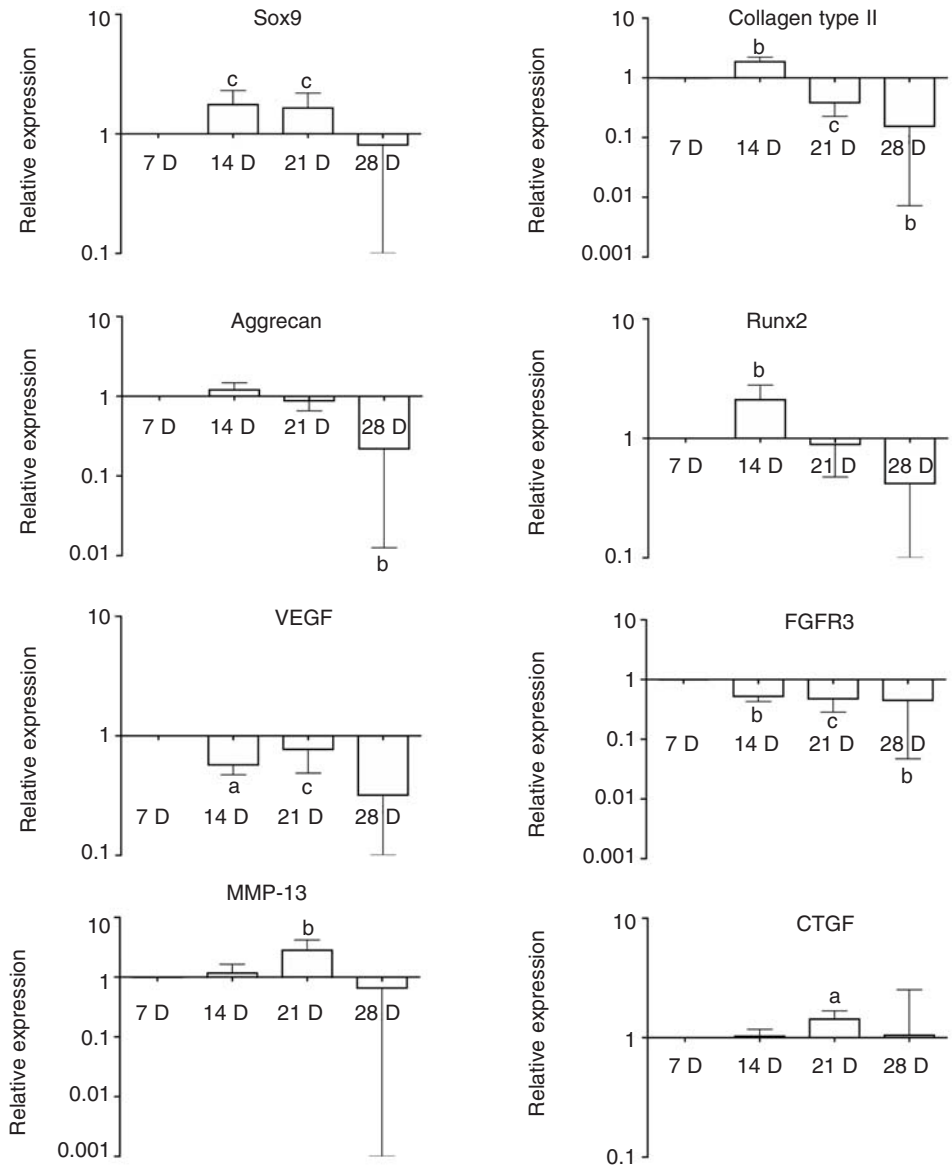

Fig 4: Quantitative PCR analysis of gene expression in chondrocyte pellet culture from fetal foals. Results for gene expression at 14 (14D), 21 (21D) and 28 (28D) days are expressed relative to expression at Day 7. Significant differences between values for 14D, 21D or 28D and 7 days are expressed as: a, P<0.001; b, P<0.01; c, P<0.05. Data represent mean ± s.e.; n = 3.

the same modes of PCD observed for these cells *in vivo*, as previously demonstrated for these cells when cultured in FCS (Ahmed *et al*. 2007). In addition, a few apoptotic cells were present at all stages of culture. The proportion of light cells was very low (less than 10%) in pellets cultured in 10% FCS or 10% HS, but it has previously been shown that the proportion of light cells can be increased by culture in 0.1% FCS, and further increased by the addition of triiodothyronine to 0.1% FCS (Ahmed *et al*. 2007).

Chondrocytes isolated from growing and mature horses formed inclusion bodies in pellet culture, which may be pro-collagen retained within the cells. Many cells had the appearance of osteoblasts, suggesting that chondrocytes from post natal horses were undergoing transformation into osteoblasts. Transformation of chondrocytes into osteoblasts has been reported in organ cultures (Thesingh *et al*. 1991; Roach 1992; Roach *et al*. 1995). Pellet cultures from neonatal foals showed different features when grown in different serum types. In FCS, the pellets were similar to pellet cultures from older horses. However, in HS their morphology was similar to that of pellet cultures from fetal foals, differentiating into dark and light hypertrophic chondrocytes, although some cells contained inclusion bodies. These observations indicate that as the age of the horse increases, chondrocytes lose their ability to undergo hypertrophy in culture.

Cultured chondrocytes from fetal foals formed a hyaline cartilage-like tissue as confirmed by morphology and cartilage-specific gene expression. Moreover, these cells underwent hypertrophy as indicated by their appearance, expression of collagen *type X* mRNA, alkaline phosphatase activity and increased ECM calcification. Previous studies have reported that chondrocytes from other species cultured in pellets undergo hypertrophy (Kato *et al*. 1988; Ballock and Reddi 1994; Okubo and Reddi 2003). In studies evaluating the growth characteristics of the pellet cultures, it was observed that the pellet weight and thickness significantly increased but the cellular density significantly decreased over the culture period, indicating that the growth was due more to ECM production than cellular proliferation. It has been reported that in rat chondrocyte pellet culture, cell proliferation occurs mostly during the first 8 days and sharply decreases later (Yasuda *et al*. 1995). Quantitative PCR studies showed that Sox9 and collagen *type II* were upregulated after 14 days, indicating progressive differentiation over this period; the transcription factor Sox9 is essential for maintaining the phenotype of human cultured chondrocytes, and enhances collagen *type II* expression (Tew *et al*. 2005). The transcription factor Runx2, which stimulates hypertrophy of mouse chondrocytes (Takeda *et al*. 2001), was upregulated after 14 days when hypertrophic chondrocytes were first detectable morphologically. MMP-13 and CTGF were upregulated after 21 days. CTGF has been shown to be expressed by hypertrophic chondrocytes (Fukunaga *et al*. 2003) and MMP-13 is essential for degradation of cartilage ECM during endochondral ossification (Inada *et al*. 2004). FGFR3 was downregulated at 14 days. Activation of FGFR3 inhibits chondrocyte hypertrophy (Ornitz and Marie 2002), therefore it is probable that downregulation of FGFR3 in pellet cultures allows cellular hypertrophy. VEGF expression was downregulated at all time points compared to 7 days. This observation is surprising since, in mice, VEGF expression is upregulated with chondrocyte hypertrophy and dependent on Runx2 expression (Zelzer *et al*. 2001). It is possible that there are species differences in the regulation of VEGF expression by hypertrophic chondrocytes.

In conclusion, the present findings demonstrate that chondrocytes freshly isolated from equine fetuses and grown as pellets in FCS or HS undergo the same modes of hypertrophy and PCD seen *in vivo*. Chondrocytes from older horses are not suitable for the study of chondrocyte hypertrophy in pellet culture. This culture system should greatly assist in further basic studies of equine developmental orthopaedic diseases, such as osteochondrosis.

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Manufacturers' addresses

[1]Invitrogen, Carlsbad, California, USA.
[2]Roche Diagnostics, Basel, Switzerland.
[3]Sigma-Aldrich, St. Louis, Missouri, USA.
[4]Media Cybernetics, Silver Spring, Maryland, USA.
[5]Leica, Wetzlar, Germany.
[6]Promega, Madison, Wisconsin, USA.
[7]Gene Works, Rundle Mall, SA, Australia.
[8]Vilber Lourmat, Marne-la-Vallée, France.
[9]Stratagene, La Jolla, California, USA.

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