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Identification of light and dark hypertrophic chondrocytes in mouse and rat chondrocyte pellet cultures

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ABSTRACT

Hypertrophic "light" and "dark" chondrocytes have been reported as morphologically distinct cell types in growth cartilage during endochondral ossification in many species, but functional differences between the two cell types have not been described. The aim of the current study was to develop a pellet culture system using chondrocytes isolated from epiphyseal cartilage of neonatal mice and rats, for the study of functional differences between these two cell types. Hypertrophic chondrocytes resembling those described *in vivo* were observed by light and electron microscopy in sections of pellets treated with triiodothyronine, 1% fetal calf or mouse serum, 10% fetal calf serum or 1.7 MPa centrifugal pressure at day 14, and in pellets cultured with insulin or 0.1% fetal calf or mouse serum at day 21. A mixed population of light and dark chondrocytes was found in all conditions leading to induction of chondrocyte hypertrophy. This rodent culture system allows the differentiation of light and dark chondrocytes under various conditions *in vitro* and will be useful for future studies on tissue engineering and mechanisms of chondrocyte hypertrophy.

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1. Introduction

In mammals, development of most bones is initiated by formation of a cartilage model, which is progressively replaced by bone tissue through the process of endochondral ossification (Mackie et al., 2008). During this process, chondrocytes in growth cartilage undergo programmed morphological and functional changes: proliferation, hypertrophy and death, which are regulated by various hormones and growth factors. Blood vessels then invade the growth cartilage, bringing osteogenic cells to deposit bone extracellular matrix on the remaining cartilage tissue.

Two types of hypertrophic chondrocytes, "light" and "dark", have been described with distinct ultrastructural morphology in several species (Anderson, 1964; Hwang, 1978; Wilsman et al., 1981; Erenpreisa and Roach, 1998; Roach et al., 1998; Roach and Clarke, 1999, 2000; Ahmed et al., 2007b). Light hypertrophic chondrocytes are typically rounded and are characterised by electron-lucent cytoplasm with sparse rough endoplasmic reticulum (RER); their nuclei contain loose chromatin (i.e. euchromatin). In contrast, dark hypertrophic chondrocytes are irregularly shaped with cytoplasmic processes, and contain electron-dense cytoplasm with abundant RER and Golgi apparatus; patches of condensed chromatin (heterochromatin) are contained in the nuclei. A recent *in vivo* and *in vitro* study with equine chondrocytes has provided evidence that light and dark chondrocytes constitute two different populations identifiable following proliferation (Ahmed et al., 2007b). It was demonstrated that equine light and dark chondrocytes undergo ultrastructural morphological changes suggestive of distinct, cell type-specific non-apoptotic modes of physiological cell death. Light chondrocytes appear to disintegrate gradually within their preserved cytoplasmic membrane and contain pale nuclei, while dark cells appear to undergo progressive extrusion of the cytoplasm into the extracellular space and contain condensed nuclei with patches of chromatin (Ahmed et al., 2007b). However, the roles of light and dark hypertrophic chondrocytes during endochondral ossification remain unclear.

Several chondrocyte culture methods and various culture conditions have been established to investigate chondrocyte proliferation and hypertrophic differentiation (Yasumoto et al., 1980; Kato et al., 1988; Pieper et al., 2002; Masuda et al., 2003; Malpeli et al., 2004). In monolayer cultures, chondrocytes lose their spherical phenotype and dedifferentiate into fibroblast-like cells; they express type I collagen instead of expressing cartilage-specific type II collagen (reviewed by Lin et al., 2006). Three-dimensional (3-D) chondrocyte culture systems, such as pellet culture (Kato et al., 1988), and culture in agarose, collagen or alginate gels (Böhme et al., 1992; Izumi et al., 2000; Kim et al., 2003) have been used to maintain chondrocyte phenotype. Among these 3-D cultures, chondrocyte pellet culture is the simplest method of obtaining cartilage-like tissue *in vitro*, and has been widely used for cartilage

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studies in different species (Kato et al., 1988; Ballock and Reddi, 1994; Okubo and Reddi, 2003; Malpeli et al., 2004; Ahmed et al., 2007b); the phenotype of chondrocytes and matrix composition in pellet cultures has been shown to resemble those in native cartilage (Zhang et al., 2004).

Insulin or insulin-like growth factor I or II (IGF-I and -II) has been reported to maintain chondrocyte phenotype and viability in 3-D cultures (Böhme et al., 1992; Ballock and Reddi, 1994). In addition, high concentrations of fetal calf serum (FCS; Bruckner et al., 1989) or thyroxine treatment (Ballock and Reddi, 1994; Okubo and Reddi, 2003) in pellet culture induce chondrocyte hypertrophy. Recently, light and dark hypertrophic chondrocytes have been observed in pellet culture of equine chondrocytes. In these cultures, the proportion of light and dark chondrocytes could be manipulated; light hypertrophic chondrocytes were mainly observed in triiodothyronine (T3)-treated pellets, while dark hypertrophic chondrocytes were prominent in pellets cultured in 10% FCS (Ahmed et al., 2007b).

The aim of the current study was to establish a chondrocyte pellet culture system using cells from mice and rats that could be used to study molecular and functional differences between the two cell types while taking advantage of the broad range of reagents and more detailed genetic information available for these species.

2. Materials and methods

2.1. Materials

Reagents were obtained from Sigma–Aldrich Pty Ltd. (St Louis, MO, USA) unless otherwise stated.

2.2. Chondrocyte isolation and pellet culture

Chondrocytes were isolated from the epiphyseal growth cartilages of the long bones before the formation of the secondary centre of ossification in 5-day-old mice or of the distal femurs and the proximal tibiae in 7-day-old rats. Animals were euthanased, then cartilages were carefully excised under a stereomicroscope (SZ 30, Olympus, City, Country). Chondroepiphyses were excised with an incision 1 mm away from the chondro-osseous junction in order to obtain resting chondrocytes without hypertrophic chondrocyte contamination. The cartilages were digested using 0.3% collagenase A (Roche, Basel, Switzerland) in Dulbeccos' Modified Eagle's Medium (DMEM; Gibco, Carlsbad, USA) at 37 °C until completely digested. The viability of isolated chondrocytes was determined using the trypan blue exclusion method; more than 95% of cells were viable in all isolates.

Fresh chondrocytes isolated from the growth cartilage were cultured in 15 ml polypropylene conical tubes (Becton Dickinson Labware, Franklin Lakes, USA). The method of 3-D pellet culture was modified from that of Kato et al. (1988). One ml aliquots of cell suspension at a density of 7×10^5 cells/ml were placed in 15 ml polypropylene tubes, and the cells were centrifuged at 699 rcf for 5 min at 20 °C to form an aggregate. The tubes were then incubated at 37 °C in an atmosphere of 5% CO_2. The caps of the tubes were loosened in the incubator for gas exchange. The isolated chondrocytes were cultured in DMEM containing gentamycin (50 mg/ml), L-glutamine (300 mg/ml), amphotericin B (2.5 mg/ml), ascorbic acid (50 μg/ml) and different concentrations of FCS (0.1%, 1% or 10%) or mouse serum (MS; 0.1% or 1%; School of Veterinary Science Animal House, The University of Melbourne). The medium was changed every other day. Disc-shaped, cartilage-like pellets were formed after 48 h of incubation (Fig. 1). The pellets were then generally collected at the 14th or the 21st day of incubation. In some pellet cultures using mouse chondrocytes, T3 (100 ng/ml) was added to the medium containing 0.1% FCS at the beginning of pellet culture, while T3 (100 ng/ml) was added at day 5 of rat pellet culture. In some experiments, insulin (5 μg/ml) or retinoic acid (1 μM) was supplied to the pellets cultured in 0.1% FCS on the fifth day.

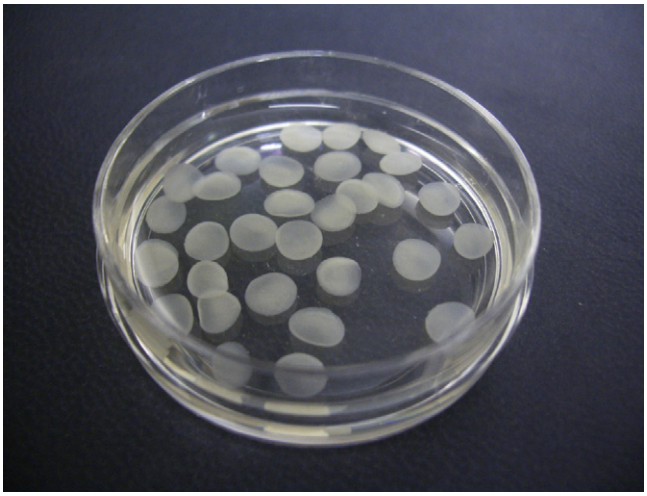

Fig. 1. Cartilage-like tissue formation from rat chondrocytes. Chondrocytes isolated from 7-day-old rat growth cartilage were cultured as pellets for 14 days; these pellets were pooled in a 35 mm Petri dish.

2.3. Specimen preparation for light and electron microscopy

Pellets were fixed with 2.5% glutaraldehyde (ProSciTech, Thuringowa, Australia) and 4% paraformaldehyde (pH 7.4; BDH, Poole, UK) in 0.1 M cacodylate buffer (0.1 M sodium cacodylate; pH 7.4) for 1 day at 4 °C. The pellets were post-fixed in 1% osmium tetroxide (ProSciTech, Thuringowa, Australia) and 1.5% potassium-ferrocyanide then embedded in Spurr's resin (ProSciTech, Thuringowa, Australia). Semi-thin sections (1 μm) were stained with 1% methylene blue and examined under light microscopy. Ultra-thin sections were stained with 5% uranyl acetate and Reynold's lead citrate and examined under a transmission electron microscope (Philips 300).

2.4. Cell morphology analysis and cell counts

The cell morphology in each pellet was examined in a semi-thin section taken transversely at one third of the pellet diameter. Counts of hypertrophic light and dark hypertrophic chondrocytes, non-hypertrophic and apoptotic chondrocytes were conducted on sections of pellets cultured under different conditions ($n = 3$ pellets/treatment) by an operator blinded to the treatments. Values for each of the cell types were expressed as a percentage of total cell number. Cell profiles that did not contain enough cytoplasm for assessment of cell type were not counted.

2.5. RNA extraction and polymerase chain reaction (PCR)

Total RNA was extracted from homogenized T3-treated pellets using TRI Reagent (Molecular Research Centre, Inc., Cincinnati, OH, USA), and was reverse transcribed using Superscript III (Invitrogen) according to the manufacturer's instructions. Primers for genes of interest (*Col10a1, Col2a1, Col1a1, Sox9, Runx2, Aggrecan*) were used as described (Chen et al., in press).

2.6. Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was performed using an MX3000P real-time PCR machine (Stratagene, La Jolla, USA). RP-S23 was used as a house-

keeping gene (Wang et al., 2004). Expression of genes of interest (normalised to RP-S23 expression) in T3-treated pellets was calculated relative to that of pellets without T3 treatment using the REST software tool (Pfaffl, 2001).

2.7. Statistical analysis

The proportion of different cell types in pellets were analysed using one-way ANOVA. RT-qPCR results were analysed for significant differences by a 2000 sample pairwise fixed reallocation randomisation test using REST-384 software (Pfaffl et al., 2002). Data are presented as the mean ± standard error. A P-value of less than 0.05 was considered to be significant for each comparison. Statistical significance is represented as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Induction of hypertrophic light and dark chondrocytes in rodent pellet culture

Chondrocytes isolated from 5-day-old mice or 7-day-old rats were initially cultured in 10% FCS or in the presence of T3 in 0.1% FCS in the hope that they would induce differentiation of dark and light chondrocytes, respectively, as described for equine chondrocytes (Ahmed et al., 2007b). Pellets cultured in 0.1% FCS were used as controls. Chondrocytes from mice or rats behaved identically in the pellets in response to the treatments. After 14 days, the morphology of the cells in pellets cultured in 0.1% FCS resembled that of resting or proliferative chondrocytes *in vivo* (Fig. 2A–D). Hypertrophic chondrocytes were observed in pellets cultured in 10% FCS (Fig. 2E–H) or T3 (100 ng/ml; Fig. 2I–L), in which there was increased apparently empty space surrounding chondrocytes in lacunae. A thick perichondrium-like layer was observed surrounding the pellets cultured in 10% FCS (Fig. 2E and G), but not other culture conditions.

Hypertrophic chondrocytes in 10% FCS- and T3-treated pellets could be identified as either light or dark under light microscopy as described (Ahmed et al., 2007b; Fig. 2F, H, J, L). Electron microscopy showed that dark chondrocytes contained electron-dense cytoplasm with abundant secretory vesicles and Golgi apparatus (Fig. 2R and T) and their nuclei were characterised by irregular patches of condensed heterochromatin (arrows in Fig. 2N and P). In contrast, light chondrocytes were characterised by electron-lucent cytoplasm with sparse rough endoplasmic reticulum (Fig. 2Q and S) and pale nuclei (Fig. 2M and O). Apoptotic chondrocytes were occasionally found in the pellet cultures with various treatments, and their

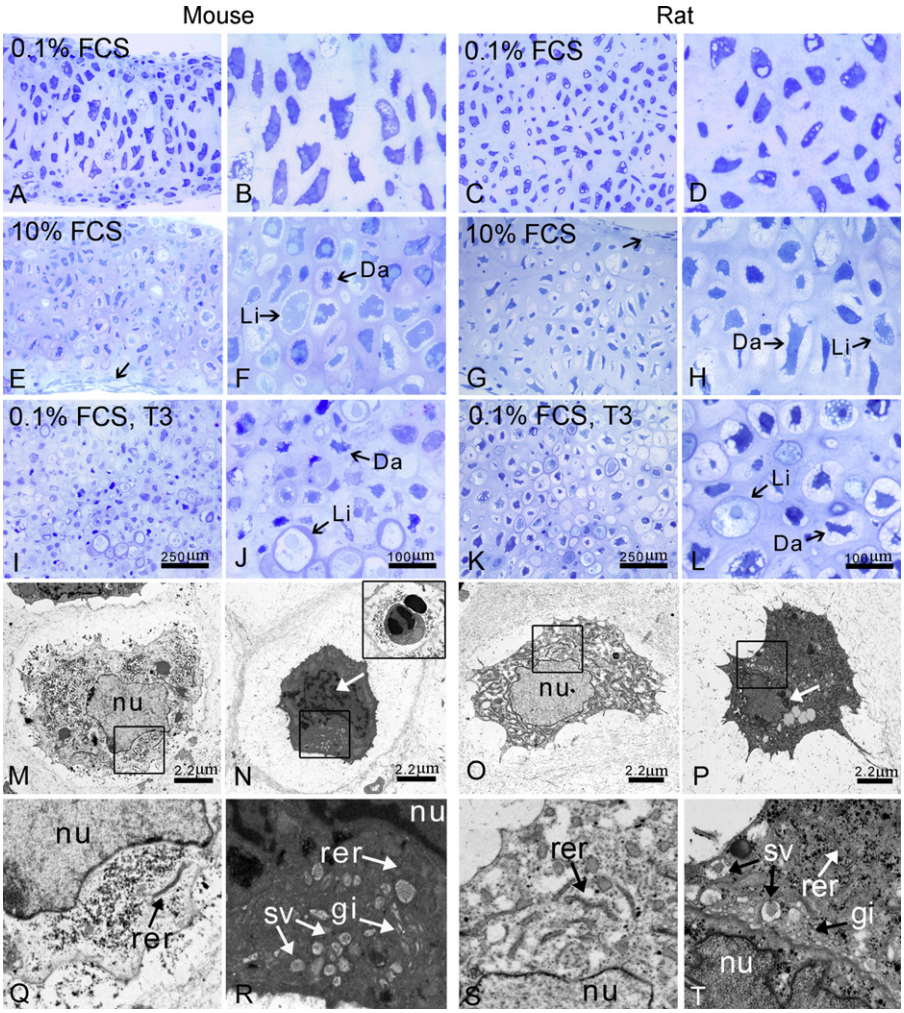

Fig. 2. Induction of chondrocyte hypertrophy in mouse and rat chondrocyte pellet culture *in vitro*. Light micrographs show non-hypertrophic chondrocytes in sections of a pellet cultured in 0.1% FCS (A–D), and of light and dark hypertrophic chondrocytes in sections of pellets cultured in 10% FCS (E–H) and T3-treated pellets (I–L) at day 14. Arrows in E, G indicate perichondrium-like layers in the pellets cultured in 10% FCS. Electron micrographs of light (M, O) and dark (N, P) hypertrophic chondrocytes in T3-treated pellets from mouse chondrocytes (M, N) and in 10% FCS-treated pellets from rat chondrocytes (O, P). Arrows in N and P show patchy nuclear condensation. Q, R, S, T are higher magnifications of M, N, O, P, respectively. Insert in N indicates an apoptotic cell. Li, Light cell; Da, dark cell; nu, nucleus; rer, rough endoplasmic reticulum; sv, secretory vesicle; gi, Golgi apparatus.

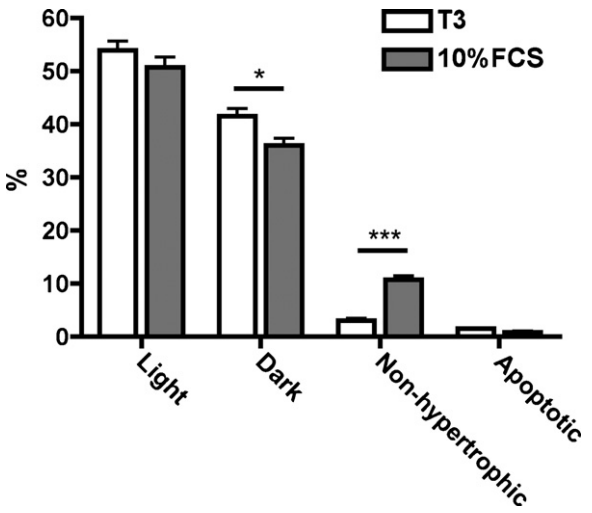

Fig. 3. The proportion of different types of chondrocytes in T3-treated pellets and in pellets cultured in 10% FCS. Light hypertrophic, dark hypertrophic, non-hypertrophic and apoptotic chondrocytes were counted as a percentage of total cells in specific fields of sections of rat chondrocyte pellets cultured with T3 in 0.1% FCS or in 10% FCS for 14 days. These results from one experiment are representative of results from 3 independent experiments. Results are presented as mean ± SEM. $n = 3$.

nuclei showed chromatin condensed into geometric masses (insert in Fig. 2N).

3.2. Proportions of light and dark chondrocytes in pellet cultures

Light and dark hypertrophic chondrocytes were observed in both 10% FCS and in T3-treated pellets, but both conditions appeared to contain a mixed population of light and dark chondrocytes. Cell counts were conducted to determine the proportions of light hypertrophic, dark hypertrophic, non-hypertrophic and apoptotic cells in rat chondrocyte pellets (Fig. 3). It was found that the proportion of light chondrocytes was higher than that of dark chondrocytes in both conditions; the ratio of light:dark chondrocytes in the pellets in 10% FCS and in the presence of T3 was 1.4 and 1.3, respectively, indicating no obvious differences in the ratio between treatments. Slightly more dark hypertrophic chondrocytes and fewer non-hypertrophic chondrocytes were observed in T3 treatment than in 10% FCS, and very few apoptotic chondrocytes were found with either treatment. However, these data indicated that in rodent (unlike equine) chondrocyte cultures, these treatment conditions could not be used to produce light or dark hypertrophic chondrocyte-enriched cultures.

3.3. Other culture conditions for light or dark chondrocyte differentiation

Chondrocytes isolated from 5-day-old mice were further cultured as pellets in 0.1% and 1% MS, and 1% FCS (Fig. 4). The purpose of using MS for the mouse pellet culture was to examine whether the species-matched serum was able to increase the proportion of either light or dark hypertrophic chondrocytes, since horse serum provides advantages over FCS in equine pellet culture with respect to increasing the proportion of dark chondrocytes (Ahmed et al., 2007a). At day 14, chondrocytes maintained their spherical morphology resembling resting chondrocytes *in vivo* in the pellets cultured in 0.1% MS (Fig. 4A and B), while both light and dark hypertrophic chondrocytes could be identified in the pellets cultured in 1% FCS and 1% MS (Fig. 4C–F). Chondrocytes in 1% MS appeared more hypertrophic than those in 1% FCS. However, there remained a mixed population of light and dark chondrocytes in these culture conditions, and neither light nor dark chondrocytes were enriched.

Chondrocytes were also cultured in the presence of insulin (5 μg/ml in 0.1% FCS), or in the presence of T3 in 0.1% MS (Fig. 5A–D). The morphology of chondrocytes treated with insulin resembled that of resting chondrocytes (Fig. 5A and B), which was similar to those chondrocytes without insulin treatment (Fig. 4A and B). Chondrocytes cultured in T3 in 0.1% MS were hypertrophic, and a mixed population of light and dark chondrocytes could be observed (Fig. 5C and D), resembling those treated with T3 in 0.1% FCS (Fig. 2I and J).

Centrifugal pressure (1.7 MPa) and retinoic acid (RA) were also applied to pellet cultures to examine whether they could change the proportion of light or dark hypertrophic chondrocytes in the pellets at day 14. Light and dark hypertrophic chondrocytes were observed in the pellets under centrifugal pressure (Fig. 5E and F). However, there were no obvious differences in the morphology or the proportion of light and dark chondrocytes compared with the chondrocyte pellets cultured without centrifugal pressure (Fig. 2G and H). Rat chondrocytes in the pellets treated with retinoic acid neither underwent hypertrophy nor showed the morphology of chondrocytes *in vivo* (Fig. 5G and H).

We also examined whether light or dark chondrocytes could be enriched in lower serum concentration with a prolonged culture period. At day 21, pellets cultured in 0.1% FCS, 0.1% MS or insulin in 0.1% FCS, that had contained resting or non-hypertrophic chondrocytes at day 14, contained some hypertrophic chondrocytes (Fig. 6). However, chondrocytes in those pellets without insulin treatment were at the early stage of hypertrophy and it was difficult to identify light and dark chondrocytes definitively under light microscopy (Fig. 6A–D). In contrast, chondrocytes treated with insulin were more hypertrophic than those without insulin treatment, and light and dark hypertrophic chondrocytes could be identified, but neither cell type was predominant (Fig. 6E and F).

3.4. Further characterization of T3-treated pellets

Markers for hypertrophic chondrocytes were investigated in 14-day rat chondrocyte pellets treated with T3 using RT-qPCR. The results of qPCR showed that mRNA expression of *Runx2* and *Col10a1*, markers for hypertrophic chondrocytes, were significantly up-regulated in pellets treated with T3 in 0.1% FCS, by comparison with pellets treated with 0.1% FCS alone (Fig. 7).

4. Discussion

The induction of chondrocyte hypertrophy was observed in mouse or rat chondrocytes initially cultured in 10% FCS or in the presence of T3 in 0.1% FCS. These results resembled those of previous studies of pellet culture of chondrocytes from rats, chickens and horses in high serum concentration or T3 (Bruckner et al., 1989; Böhme et al., 1992; Ballock and Reddi, 1994; Ahmed et al., 2007b). Under electron microscopy, the morphology of light and dark hypertrophic chondrocytes in these two conditions was similar to those in tissues and those described in horse pellet culture. However, the proportion of light or dark chondrocytes could not be manipulated, which was different from the observations made in equine chondrocyte pellet culture, where the proportion of dark chondrocytes increased with 10% FCS treatment and more than 70% of chondrocytes were dark. This is probably due to the differences between species in proportions of dark and light cells in growth cartilage *in vivo*. We have recently found that dark chondrocytes were the predominant cell type (75%) in the physeal growth cartilage in the horse, while light chondrocytes were the major cell population (90%) in rat growth cartilage (Chen et al., manuscript in preparation). In the current study, the proportion of light chondrocytes was higher than that of dark chondrocytes in both 10% FCS

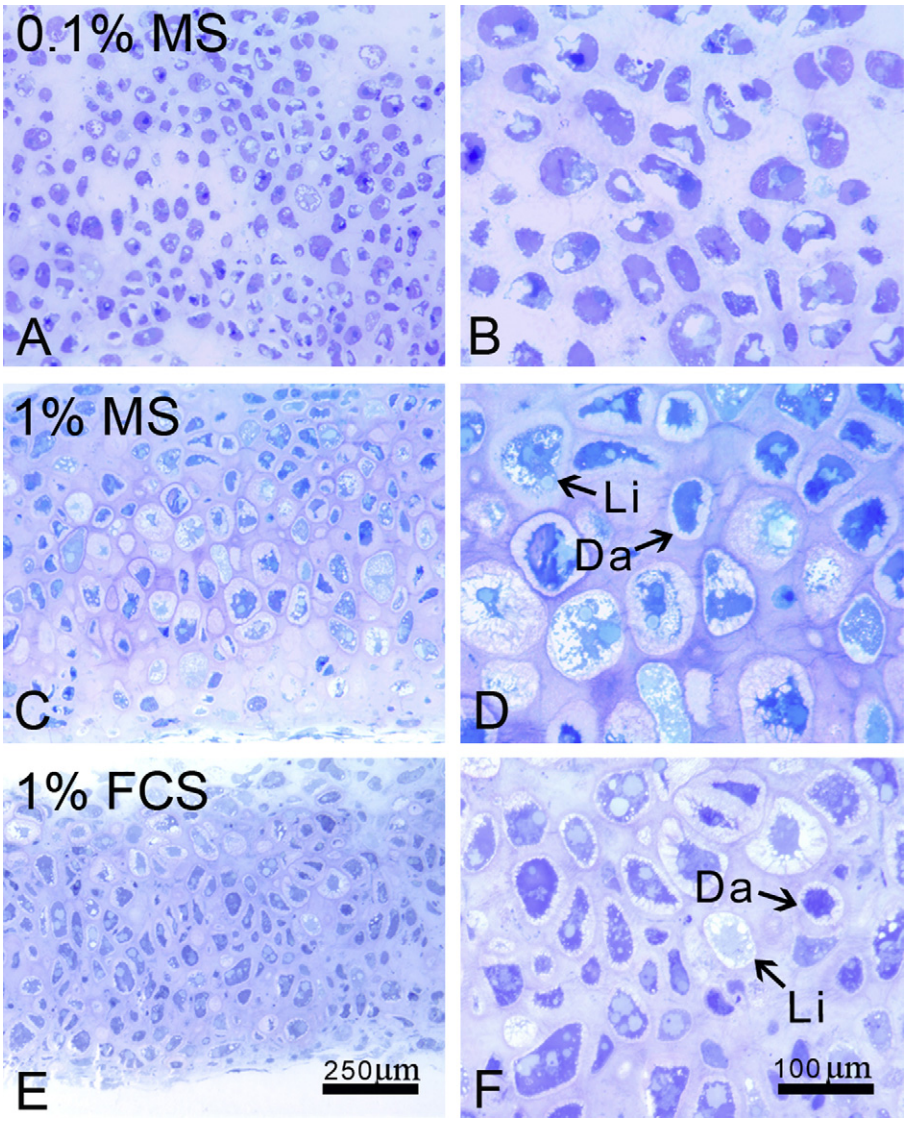

Fig. 4. Chondrocyte morphology in pellets cultured in MS and FCS at day 14. Light micrographs show non-hypertrophic chondrocytes from mouse pellets cultured in 0.1% MS (A and B). Hypertrophic chondrocytes can be seen in the pellets cultured in 1% MS (C and D) and 1% FCS (E and F), and light and dark hypertrophic chondrocytes can be identified. Li, Light chondrocyte; Da, dark chondrocyte.

and T3 treatment probably because most of the hypertrophic chondrocytes in rat growth cartilages are light chondrocytes. Therefore, other methods should be developed for the isolation of dark and light chondrocytes.

Compared with the morphology of hypertrophic chondrocytes in the pellets treated with 10% FCS, chondrocytes treated with T3 had more clearly delineated lacunae, suggesting that they had degraded more of the extracellular matrix immediately surrounding them, as observed in late hypertrophic chondrocytes *in vivo*. In addition, the proportion of non-hypertrophic chondrocytes was higher in pellets treated with 10% FCS than those treated with T3, suggesting that T3 induced more generalised chondrocyte hypertrophy in pellets than 10% FCS. Taken together, it can be concluded that T3 is superior to 10% FCS for the study of hypertrophy in pellet cultures of chondrocytes isolated from rats and mice.

A variety of culture conditions were examined that had not previously been used to assess the proportions of light and dark hypertrophic chondrocytes. In general, a mixed population of light and dark hypertrophic chondrocytes was observed in all the culture conditions that induced chondrocyte hypertrophy. Chondrocytes cultured in low serum concentration or in the presence of insulin resembled resting or proliferating chondrocytes *in vivo* at day 14 and started to undergo hypertrophy at day 21, as observed in a previous study using rat chondrocytes (Ballock and Reddi, 1994). Insulin was considered by others to maintain chondrocyte phenotype but not to induce hypertrophy (Ballock and Reddi, 1994; Böhme et al., 1992). However, light and dark chondrocytes could be identified in the pellets treated with insulin but not in those without insulin treatment, suggesting that insulin exerted a mild induction of hypertrophy. In greater concentrations of serum or in the presence of T3, chondrocyte hypertrophy was observed at day 14. In contrast to the effect of species-matched serum on equine chondrocyte pellets, MS could not enrich mouse pellet cultures in dark chondrocytes, but mouse chondrocytes treated with MS may undergo hypertrophy earlier than those cultured in sera from other species.

The proportion of dark chondrocytes was found to be lower in cultures of chondrocytes isolated from rats than in those isolated from horses. One possible explanation for this is the difference in

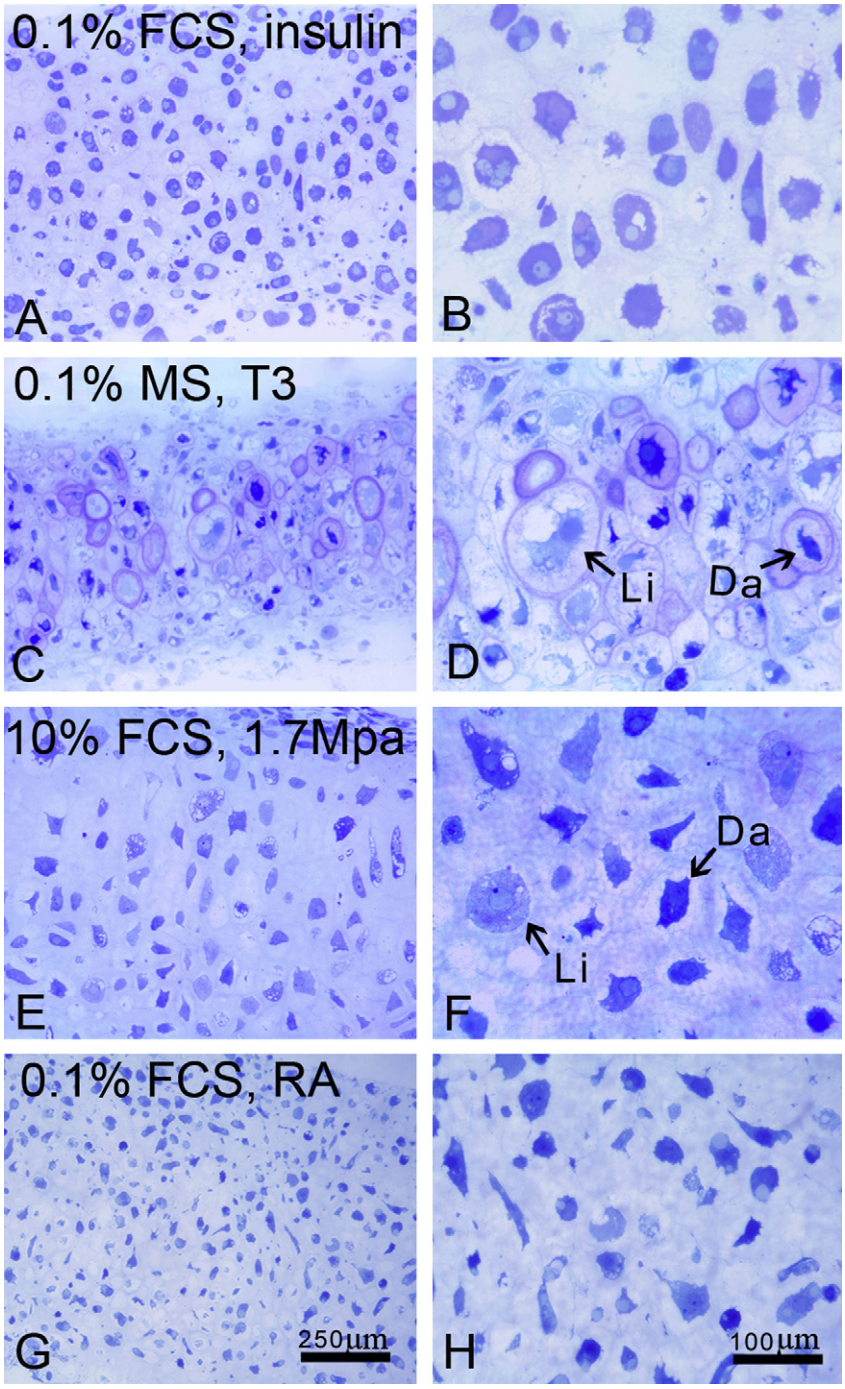

Fig. 5. Other conditions used for induction of light and dark hypertrophic chondrocytes at 14 days. Chondrocytes resemble resting chondrocytes in the pellets treated with insulin (A and B). Light and dark hypertrophic chondrocytes can be seen in the mouse pellets cultured in 0.1% MS with T3 (C and D) and in the rat pellets cultured in 10% FCS with 1.7 MPa centrifugal pressure (E and F). Loss of normal chondrocyte morphology can be observed in rat pellets cultured in 0.1% FCS in the presence of retinoic acid (RA; G and H). Li, Light chondrocyte; Da, dark chondrocyte.

mechanical loading of growth cartilage resulting from differences in body weight. To investigate this possibility, centrifugal pressure, which has been employed to study the changes in *IGF-I* mRNA expression and collagen fibre arrangement in chondrocyte pellet culture (Maeda et al., 2005), was applied to pellets cultured in 10% FCS. However, when centrifugal pressure of 1.7 MPa was applied to pellets for 20 min, twice daily for 14 days, no obvious differences in the proportion of dark chondrocytes were found compared to control.

Retinoic acid has been reported to stimulate expression of collagen type X (Col-X; a marker of chondrocyte hypertrophy) and morphological hypertrophy in some *in vitro* studies using chicken cells (Iwamoto et al., 1994; Koyama et al., 1999; Li et al., 2003). However, retinoic acid did not induce chondrocyte hypertrophy in our rodent system. Other studies have shown contradictory effects of retinoic acid on chondrocyte hypertrophy (Iwamoto et al., 1993; Ballock et al., 2001); the addition of retinoic acid to hypertrophic chondrocytes which express Col-X may inhibit *Col-X* gene expression instead of stimulating it. Thus, more investigation is required to determine the effects of altering the timing of addition of retinoic acid to rodent chondrocyte pellet cultures.

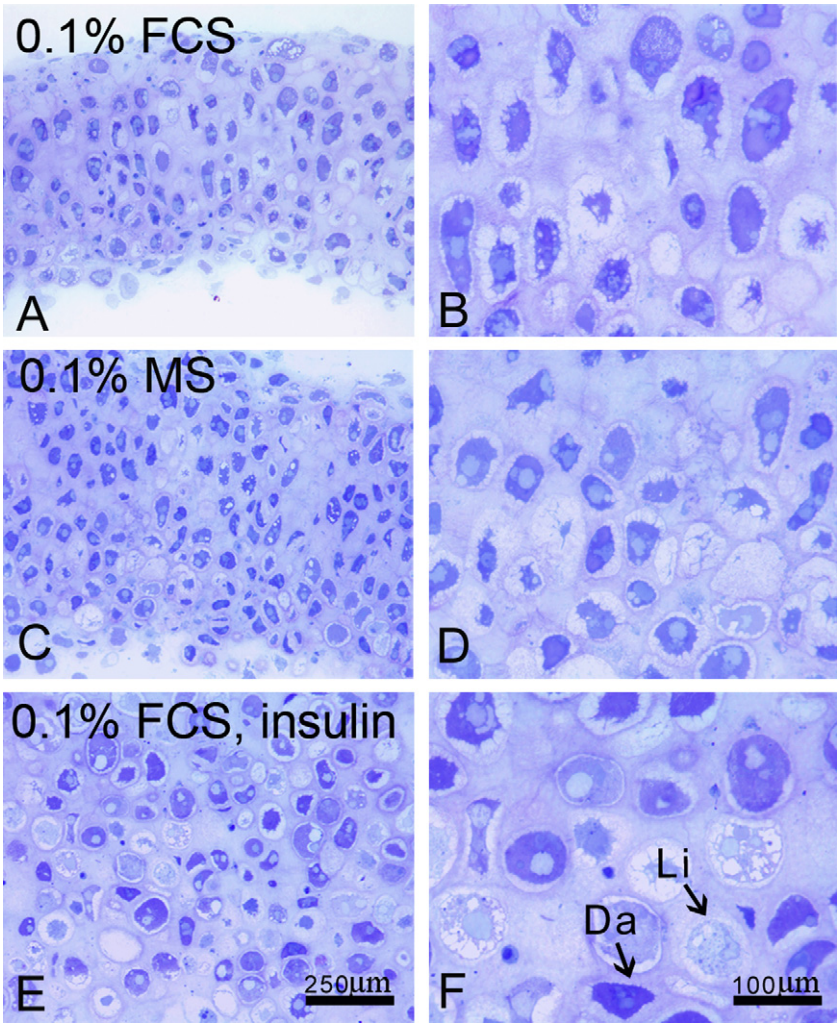

Fig. 6. Prolonged culture period for induction of chondrocyte hypertrophy and light or dark chondrocyte differentiation. Some early hypertrophic chondrocytes can be seen in mouse pellets cultured in 0.1% FCS (A and B) and MS (C and D) at day 21, while advanced hypertrophic chondrocytes can be seen in mouse pellets cultured in insulin in 0.1% FCS at day 21 (E and F); dark and light chondrocytes can be identified. Li, light; Da, dark.

Compared with the chondrocyte morphology in T3-treated pellets at day 14, at day 21 many dying chondrocytes (i.e. disintegrated light chondrocytes and condensed dark chondrocytes) and empty lacunae were observed, but the proportion of apoptotic chondrocytes did not increase (data not shown). This indicated that light and dark hypertrophic chondrocytes underwent non-apoptotic cell type-specific modes of cell death, which supports previous findings in horse chondrocytes (Ahmed et al., 2007b).

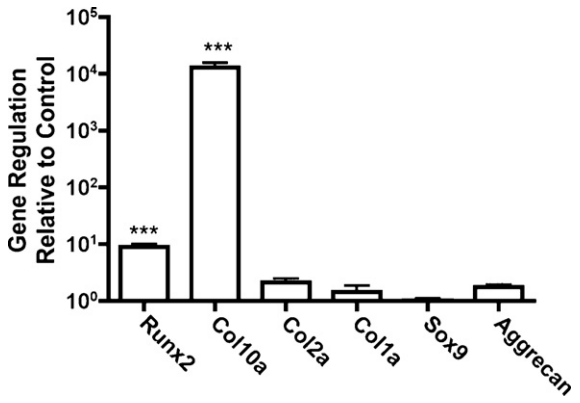

Fig. 7. The effect of T3 on gene expression in rat chondrocyte pellet culture. RT-qPCR analysis of gene of interest (normalised to RP-S23) of T3-treated pellets as compared with that of non-T3-treated pellets at day 14. These results are the combined results from three separate cell isolates prepared at different times. MNE ± SEM ($n = 3$); $^{***}P < 0.001$.

In this rodent system, we found that chondrocytes from mice or rats appeared to behave similarly in all the culture conditions. However, rats have advantages over mice for these cultures since 7-day-old rats are larger compared with 5-day-old mice, thus making it much easier to perform the dissection. In addition, more neonatal rats than mice are normally produced in one litter meaning that many more chondrocytes can be obtained, which facilitates multiple treatments for one single experiment. Thus we concluded that, of the conditions studied, T3 treatment of rat chondrocyte pellet cultures provides the best system for the study of light and dark hypertrophic chondrocytes. Analysis of gene expression provided molecular confirmation that chondrocytes in these cultures undergo hypertrophy.

In conclusion, a rodent chondrocyte pellet culture system in which chondrocytes can be induced to undergo differentiation into light and dark hypertrophic chondrocytes similar to those observed *in vivo* was successfully established in this study. We have documented the changes in chondrocyte morphology under various culture conditions using this system. These observations will lay the basis for future studies on chondrocyte hypertrophy and developmental orthopaedic diseases.

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