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Periostin expression distinguishes between light and dark hypertrophic chondrocytes

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ABSTRACT

Hypertrophic chondrocytes exist in two forms detectable by electron microscopy, light and dark chondrocytes; the functional implications of the heterogeneous morphology are unknown. The aims of the study were to establish a method for separating light from dark hypertrophic chondrocytes and to identify genes differentially expressed between the two populations. Three-dimensional pellet cultures of chondrocytes from cartilage of neonatal rats were induced to undergo hypertrophy by treatment with triiodothyronine. Cultures were dissociated and subjected to density gradient centrifugation. The cell fraction with the lowest density comprised predominantly light hypertrophic chondrocytes, and the fraction with the highest density comprised predominantly dark hypertrophic chondrocytes. An Affymetrix GeneChip® rat expression array was used to compare expression between dark cell-containing pellets and the light cell-enriched fraction. Genes identified on the array as putative dark cell-selective genes included genes encoding extracellular matrix proteins and enzymic modulators thereof. Expression of a subset of genes (*Col1a1, periostin, osteoglycin, tPA/Plat*, and *Chst11*) was confirmed as dark cell-selective using quantitative reverse transcriptase polymerase chain reaction. The most highly differentially expressed dark cell-selective gene was *periostin*. In immunocytochemical studies of light and dark cell-enriched fractions, periostin staining was detectable in dark, but not light hypertrophic chondrocytes. The results provide insight into molecular differences between light and dark hypertrophic chondrocytes.

1. Introduction

Most of the bones in the mammalian skeleton develop through the process of endochondral ossification, whereby a cartilage model of the future bone is formed then progressively replaced by bone tissue (Mackie et al., 2008). The longitudinal expansion of the bone results from proliferation of chondrocytes within the growth plate, a cartilaginous structure that persists until growth has ceased. Following proliferation, growth plate chondrocytes undergo hypertrophy, dramatically expanding their volume while partially degrading the extracellular matrix (ECM) surrounding them. Hypertrophic chondrocytes then die, and blood vessels and osteogenic cells invade the remaining cartilage tissue, depositing bone ECM on the cartilage remnants.

Hypertrophic chondrocytes are generally considered to comprise a single population that undergoes progressive morphological and molecular changes during the process of terminal differentiation and death. Ultrastructural studies have, however, described two morphologically distinct types of chondrocytes, 'light' and 'dark', in the growth plate of several species (Ahmed et al., 2007; Anderson, 1964; Erenpreisa and Roach, 1998; Hwang, 1978; Roach and Clarke, 2000; Wilsman et al., 1981). Dark hypertrophic chondrocytes are irregularly shaped with cytoplasmic processes; they contain electron-dense cytoplasm, abundant rough endoplasmic reticulum (RER) and Golgi apparatus and their nucleus contains patches of condensed chromatin (heterochromatin). Light hypertrophic chondrocytes, in contrast, are typically round; the cytoplasm is electron-lucent with sparse RER, and the nuclear chromatin is not condensed (i.e. euchromatin). We have recently provided strong evidence that, in the horse, light and dark hypertrophic chondrocytes constitute two distinct post-proliferative populations, rather than different stages of differentiation of a single population (Ahmed et al., 2007). Dark and light chondrocytes can be observed at all levels of the growth plate, from the zone of proliferative chondrocytes to the ossification front, each undergoing distinct cell type-specific morphological changes during hypertrophy and death. In three-dimensional chondrocyte pellet culture, the ratio of light: dark cells can be manipulated using different treatments, but once the cells have started to undergo hypertrophy and can be distinguished as light or dark, the ratio

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Table 1
Primer sequences for genes analysed by RT-qPCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
RP-S23	*CCTGAAGGCCAATCCGTTT*	*GGCTTCAACCCCTACTTTTTCC*
Col1a1	*ACATGCCGTGACCTCAAGAT*	*CTGACCTGTCTCCATGTTGC*
Col2a1	*AGAACTGGTGGAGCAGCAAG*	*CGGAGGAAAGTCATCTGGAC*
Col10a1	*TTCCCTGGATCTAAGGGTGA*	*CTCTGTCCGCTCTTTGTGAA*
Sox9	*AGGAGCTGGCAGACCAGTA*	*TCCACGAAGGGTCTCTTCTC*
Runx2	*CCCTGAACTCAGCACCAAGT*	*GGTGGGGAGGATTGTGTCT*
Aggrecan	*CCTTCGCTCCAATGACTCTG*	*GTCCAGTGTGTAGCGTGTGG*
Periostin	*AACCAAGGACCTGAAACACG*	*CAACACCATTTGTGGCAATC*
Osteoglycin	*TGGAATCTGTGCCTCCTAAT*	*CTCCCGAATGTACCGAGTGT*
tPA	*GGTGCTGATCAGTTCCTGCT*	*ATGTCTGCTCCTCCTCTCCA*
Chst11	*CCTTTGGCGTGGACATCT*	*AGGATGGCAGTGTTGGAGAG*
Arpc1b	*GCTTCCAGAACCTCGACAAG*	*GCTGAGCACCGAGATTTGA*

does not change, that is, cells do not transform from light to dark or vice-versa with time in culture.

Hypertrophic chondrocytes play a fundamental role in skeletal development and growth, and perturbation of their function leads to skeletal dysplasia, thus it is important to understand all aspects of their behaviour. Because it has only been possible to distinguish between light and dark chondrocytes using transmission electron microscopy (TEM) or light microscopy of semi-thin (1 μm) sections of heavily fixed tissues prepared for TEM, it has not been possible to investigate functional differences between these cell types. As a first step in this direction, we used quantitative reverse transcription polymerase chain reaction (RT-qPCR) to investigate patterns of expression of a small number of chondrocyte-associated genes in equine chondrocyte pellet cultures enriched in light cells or dark cells, and identified differences between the two (Ahmed et al., 2007).

The aim of the current study was to use a genome-wide array to investigate differences in expression of a much larger number of genes between light and dark hypertrophic chondrocytes, so as to maximise the possibility of identifying differentially expressed genes. Achievement of this aim was considered to be useful for two reasons: first, the identification of light or dark cell-specific genes would allow the identification of these cells using immunochemistry in combination with a variety of cell biological methods and thus assist in the study of their different functions; second, the nature of the differentially expressed genes is likely to provide insights into the different functions of the two cell types. In order to obtain access to the maximum availability of genetic information and research reagents, we chose to change species from horse to rat. Before undertaking the array study, it was necessary to develop a method for separating light from dark hypertrophic chondrocytes. Here we describe development of such a method, its use to prepare material for an array study, and fur-

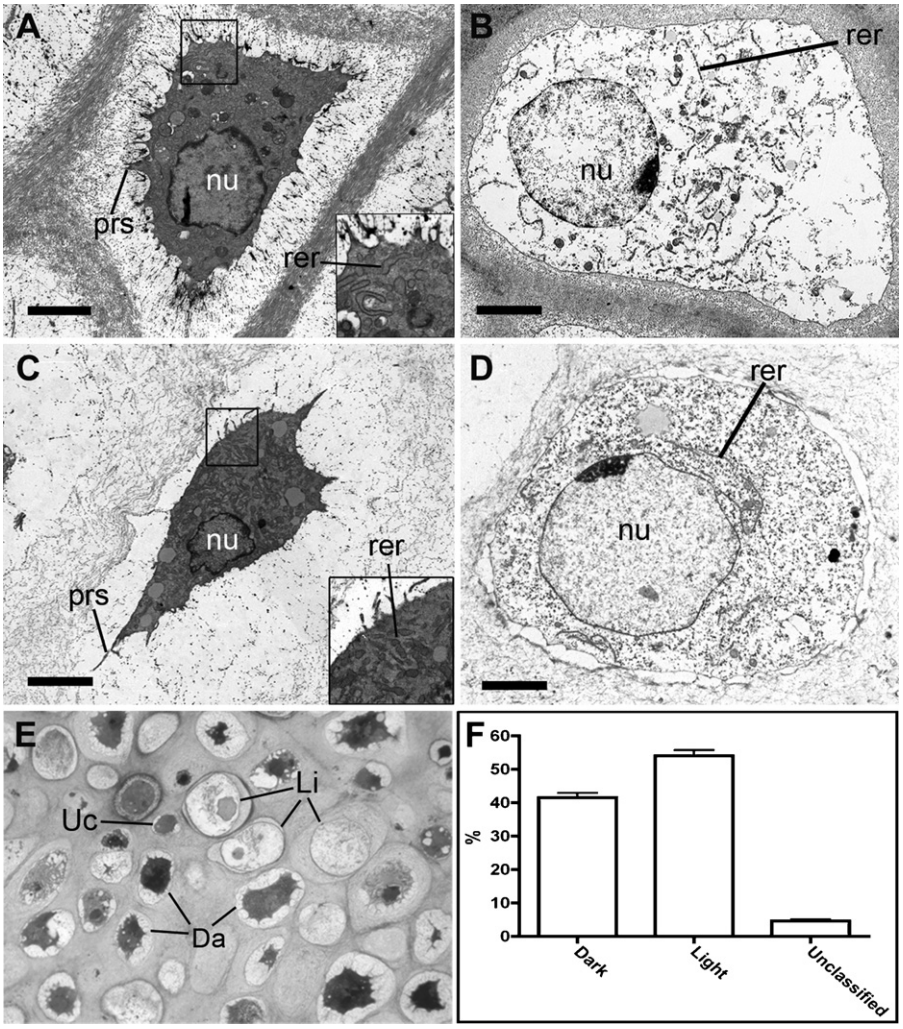

Fig. 1. Morphology of dark and light chondrocytes *in vivo* and *in vitro*. A–D: Electron micrographs of dark (A, C) and light (B, D) hypertrophic chondrocytes in sections of distal femoral growth plate of one-month-old rat (A, B) or T3-treated rat chondrocyte pellet cultured for 14 days (C, D). nu—nucleus; prs—process; rer—rough endoplasmic reticulum. E: Light micrograph of section of pellet. Li—light cell; Da—dark cell; Uc—unclassified cell. F: Percentages of different cell types (dark hypertrophic, light hypertrophic or unclassified) in sections of pellets. Mean ± SEM (n = 3 pellets). Bar = 2.2 μm.

ther studies of selected genes identified as being differentially expressed.

2. Materials and methods

2.1. Reagents

Reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. Dulbeccos' Modified Eagle's Medium (DMEM), L-glutamine, amphotericin B and gentamycin were purchased from Invitrogen (Carlsbad USA). Oligonucleotide primers for each target gene, designed to span at least one intron, were synthesized by Geneworks (Hindmarsh, SA, Australia). Ficoll-Paque™ Plus (Ficoll) was purchased from GE Healthcare BioSciences (Uppsala Sweden).

2.2. Chondrocyte pellet culture

Primary chondrocytes were isolated by enzymatic digestion of the femoral and tibial epiphyseal cartilages of seven-day-old PVG/C rats. The cartilages were digested in DMEM containing 0.3% collagenase A (Roche, Basel, Switzerland) at 37 °C until a single chondrocyte suspension was obtained; chondrocytes were cultured as pellets (7×10^5 cells/pellet) as described[3]. Triiodothyronine (T3, 100 ng/ml), which stimulates hypertrophy in chondrocyte pellet culture (Ahmed et al., 2007), was added to the cultures at day 5 and pellets were harvested at day 14.

2.3. Separation of light and dark chondrocytes

A Ficoll concentration gradient (100%, 70%, 60% and 50%) was prepared in a 15 ml polypropylene tube. Chondrocytes isolated by collagenase digestion from T3-treated pellets were suspended in DMEM, loaded on the Ficoll gradient and centrifuged in a swinging bucket at 4100 *g* for 20 min at 18 °C. Cell fractions at the interface between different Ficoll concentrations were removed carefully and centrifuged to form aggregates to be processed for microscopy or RNA extraction.

2.4. Light and electron microscopy

Chondrocytes in tissue specimens of growth cartilage from 1- and 4-week-old rats, cultured pellets and cell fractions obtained by Ficoll separation were examined under light and electron

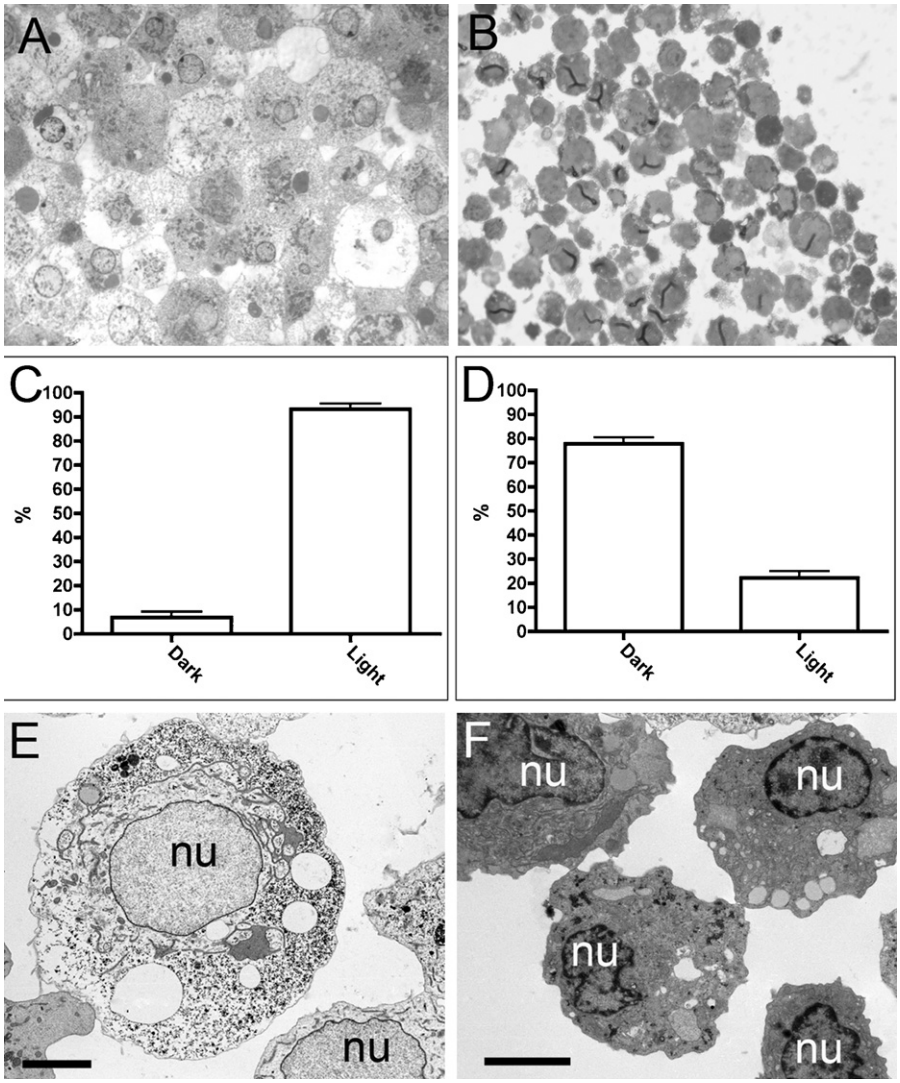

Fig. 2. Ficoll separation of light from dark hypertrophic chondrocytes. A, B: Light micrographs of sections of light (A) and dark (B) cell-enriched fractions. C, D: Percentages of dark and light cells in the light cell-enriched (C) and dark cell-enriched (D) fractions. Data represent the combined results from three separate cell isolates prepared at different times. Mean ± SEM ($n = 3$). E, F: Electron micrographs of sections of light (E) and dark (F) cell-enriched fractions. nu—nucleus. Bar = 3.2 μm.

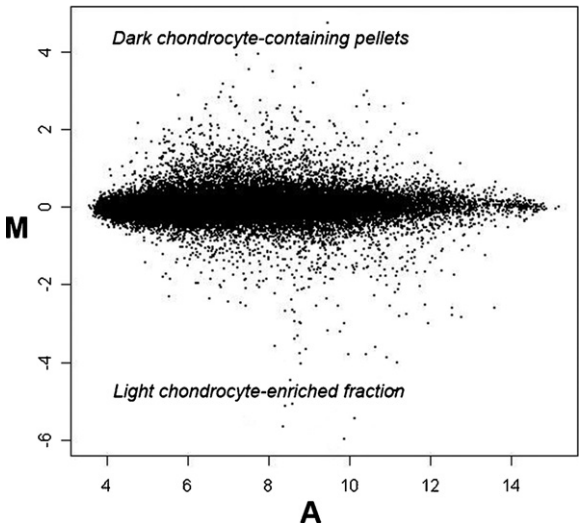

Fig. 3. Gene expression profile of dark chondrocyte-containing pellets as compared with the light cell-enriched fraction. MA scatter plot of expression array data. Each dot represents a single gene. *M* indicates $\log_2$ ratio and A represents $\log_2$ intensity. Genes with positive *M* values are more highly expressed in pellets and those with a negative *M* value are more highly expressed in the light chondrocyte-enriched fraction.

microscopy. These specimens were fixed in 2.5% glutaraldehyde/4% paraformaldehyde, and processed as described (Ahmed et al., 2007). One semi-thin section from each of three pellets was collected for counts of light and dark hypertrophic chondrocytes and unclassified chondrocytes. Six microscopic fields including two areas from the centre and four areas approximately one quarter of the cross-sectional diameter from the centre were selected for the counts. Values for each cell type were expressed as percentage of total cell number. If cell profiles did not contain enough cytoplasm for assessment of cell type, they were not counted. The numbers of light and dark hypertrophic chondrocytes in semi-thin sections of aggregates of cell fractions were also counted under light microscopy. The proportions of light and dark hypertrophic chondrocytes in tissue were counted under light microscopy. All cells located between the ossification front and a line through the mid-point of the hypertrophic zone were counted. Values for each cell type were expressed as a percentage of total cells. Counts were undertaken on specimens from three different animals at each age.

2.5. RNA extraction and cDNA microarray analysis

Total RNA was extracted from homogenized T3-treated pellets using TRI Reagent (Molecular Research Centre, Inc., Cincinnati, OH, USA), and from cell fractions using RNeasy columns (Promega, Madison, WI, USA) according to the manufacturers' instructions. Total RNA (15 μg) from T3-treated pellets and from the fraction enriched in hypertrophic light chondrocytes was sent to Australian Genome Research Facility (AGRF) Melbourne, Australia for microarray analysis. The Affymetrix GeneChip® rat expression set 230 was used to examine differences in the levels of gene expression between samples. Results of the array analysis were used to plot $\log_2$ expression ratios (*M*) against normalised $\log_2$ array intensity (*A*) as an MA scatter plot. Each dot represents a gene, and those with a positive *M* value are those identified on the array as being more highly expressed in dark chondrocyte-containing pellets as compared with the light cell-enriched fraction, and those with a negative *M* value were identified as more highly expressed in the light cell-enriched fraction. Arbitrary *M* value cut-offs of 2 and -2 were used to select putative dark chondrocyte-selective and light chondrocyte-selective genes, respectively. Literature searching was undertaken to organise the genes into functional groupings on the basis of known function; note was taken of whether the genes were known to be expressed by chondrocytes.

2.6. Polymerase chain reaction (PCR)

Samples of RNA from T3-treated pellets and from fractions enriched in light or dark chondrocytes were reverse transcribed using Superscript III (Invitrogen) according to the manufacturer's instructions. Primers for genes of interest selected from the microarray data were designed for PCR (Table 1). The primers for ribosomal protein S23 (RP-S23) were used as described (Wang et al., 2004). All PCR products were sequenced to confirm their identity (AGRF).

2.7. 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 housekeeping gene because of its consistent expression in proliferative and hypertrophic chondrocytes (Wang et al., 2004). Mean normalised expression (MNE) and standard error of the mean (SEM) of genes of interest were calculated using the Q-gene software application (Muller et al., 2002). Expression of genes of interest (normalised to RP-S23 expression) in one cell population was calculated relative to that of another population using the REST software tool (Pfaffl, 2001).

2.8. Immunocytochemistry

Cytospot preparations of all Ficoll-separated cell fractions were prepared on 3-aminopropyltriethoxysilane (TESPA)-coated glass slides. Non-specific staining was blocked with 10% FBS for 1 h, then slides were incubated either with rabbit anti-murine periostin (8 μg/ml, Abcam, Cambridge, UK) or with normal rabbit immunoglobulin (Ig; 8 μg/ml) overnight at 4 °C. Sections were washed and incubated with swine anti-rabbit immunoglobulin conjugated with Tetramethylrhodamineisothiocyanate (TRITC; 1:250; DakoCytomation, Glostrup Denmark) for 30 min. Cells were mounted in aqueous mountant containing 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml) and observed using fluorescence microscopy.

2.9. Immunohistochemistry

Distal femoral growth cartilages of 1- and 4-week-old rats were excised, fixed and processed as described (Mackie and Ramsey, 1996). Cryosections were pre-treated with bovine testicular hyaluronidase (500 units/ml) for 1 h then stained for periostin as described above for cell fractions, except that Alexa Fluor® 488 phalloidin (1 unit/ml; Invitrogen) was included in the secondary antibody incubation mixture.

2.10. Statistical analysis

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). All numerical data are presented as mean ± SEM. $P \leq 0.05$ was considered significant.

3. Results

3.1. Morphology of light and dark chondrocytes in vitro is similar to that in vivo

The initial experiments in the current study were undertaken to optimise conditions for inducing hypertrophy in pellet cultures

Table 2
Genes highly expressed in dark chondrocyte-containing pellets (*M*: Log_2 expression ratio; *A*: Log_2 signal intensity).

Gene ID	Gene Symbol/Gene Name	*M*	*A*
ECM and ECM modification			
1388116_at/	**Col1a1/collagen, type 1, alpha 1**	4.72	9.44
1370864_at		3.19	9.10
1387854_at	**Col1a2/procollagen, type I, alpha 2**	2.13	11.12
1395280_at	**Col2a1/procollagen, type II, alpha 1**	2.63	10.48
1370959_at	**Col3a1/collagen, type III, alpha 1**	2.98	10.43
1376105_at	**Col14a1/collagen, type XIV, alpha 1 (undulin)**	2.11	6.72
1373911_at	**Postn/periostin, osteoblast specific factor**	3.90	7.20
1376749_at/	**Ogn/osteoglycin**	2.74	7.78
1385248_a_at/		2.53	6.43
1390450_a_at		2.23	6.08
1367700_at	**Fmod/fibromodulin**	2.59	10.17
1387197_at	**Omd/osteomodulin**	2.17	10.43
1370301_at	**Mmp2/matrix metallopeptidase 2**	2.05	9.12
1368657_at	**Mmp3/matrix metallopeptidase 3**	2.59	10.88
1367800_at	**Plat/plasminogen activator, tissue**	2.08	9.62
1396659_at/	**Chst11/carbohydrate sulfotransferase 11**	2.31	8.63
1397045_at		2.18	8.06
1380726_at	**Aspn/asporin**	2.15	4.76
Regulation of cytoskeleton and cell adhesion			
1386925_at	Arpc1b/actin related protein 2/3 complex, subunit 1B	2.65	11.33
1397449_at/	Enah/enabled homolog (Drosophila)	2.17	8.93
1391373_at		2.02	8.46
1388936_at	**Cdh11/cadherin 11**	2.43	7.78
1392311_at	Epb4.1/erythrocyte membrane protein band 4.1	2.24	8.66
1368887_at	Cdh22/cadherin 22	2.11	9.59
1391171_at	**Itm2b/integral membrane protein 2B**	2.17	9.00
MAPK pathway			
1393520_at	Stk39/serine/threonine kinase 39, STE20/SPS1 homolog (yeast)	3.16	6.87
PTHrP/Ihh pathway			
1376614_at	**Runx2/runt related transcription factor 2**	3.09	8.64
1396494_at	**Ptch1/patched homolog 1 (Drosophila)**	2.59	8.27
1382982_at	Gnb1/guanine nucleotide binding protein, beta 1	2.34	6.55
Tyrosine kinase signaling pathway			
1382810_at	**Epha4/Eph receptor A4**	2.80	7.03
Wnt signaling pathway			
1397286_at	Tcf4/transcription factor 4	2.01	7.63
1392650_at	**Csnk2a1/casein kinase II, alpha 1 polypeptide**	2.13	7.87
Jak-Stat signaling pathway			
1392017_at	Stam2/signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	2.11	6.32
Regulation of apoptosis			
1374786_at	Tia1/cytotoxic granule-associated RNA binding protein 1	3.08	7.12
Others			
1380908_at	Mox2/antigen identified by monoclonal antibody MRC OX-2	3.93	7.72
1368271_a_at	Fabp4/fatty acid binding protein 4, adipocyte	3.56	8.77
1367973_at	Ccl2/chemokine (C-C motif) ligand 2	3.48	8.09
1388985_at	LOC310926/hypothetical protein LOC310926	2.87	10.34
1393766_at	RGD1311849/similar to Mkiaa1797 protein	2.87	5.74
1386965_at	Lpl/lipoprotein lipase	2.81	6.76
1382810_at	**Epha4/Eph receptor A4**	2.80	7.03
1377086_at	**C1qtnf3/C1q and tumor necrosis factor related protein 3**	2.62	6.45
1394756_a	RGD1308297/similar to CG10084-PA	2.61	7.18
1378831_at	Srgap2/SLIT-ROBO Rho GTPase activating protein 2	2.57	7.17
1367628_at	Lgals1/lectin, galactose binding, soluble 1	2.53	8.20
1397676_at	RGD1563148/similar to osteoclast inhibitory lectin	2.43	8.78
1375669_at	Fkbp2/FK506 binding protein	2.43	8.58
1397259_at	Fam126b/Family with sequence similarity 126 member B	2.25	5.79
1382673_at	Nrd1/N-arginine dibasic convertase 1	2.25	8.65
1396947_at	Gpr48/G protein-coupled receptor 48	2.24	7.92
1388340_at	Ns5atp9/NS5A (hepatitis C virus) transactivated protein 9	2.20	7.42
1384146_at	Clec2l/C-type lectin domain family member L	2.11	7.83
1373250_at	RGD1562446/similar to 60S ribosomal protein L7a	2.07	6.57

Gene symbol/name in bold indicates that the gene is known to be associated with cartilage biology.

of rat chondrocytes. Of a number of different culture conditions, T3 was found to provide the most reproducible induction of hypertrophy, based on both morphological observation and RT-qPCR assessment of induction of the hypertrophy-associated genes *Runx2* and *Col10a1* (data not shown). Light and electron microscopic studies confirmed that most of the cells in pellets cultured under these conditions could be identified as dark or light hypertrophic chondrocytes with the typical features of these cells in tissue specimens (Fig. 1A–D). In pellets, hypertrophic chondrocytes with electron-dense cytoplasm (i.e. dark cells) contained

Table 3
Genes highly expressed in light chondrocyte-enriched fraction (*M*: Log_2 expression ratio; *A*: Log_2 signal intensity).

Gene ID	Gene Symbol/Gene Name	*M*	*A*
ECM modification			
1387269_s_at	**Plaur/urokinase-type plasminogen activator receptor**	2.70	8.64
Regulation of cytoskeleton and cell adhesion			
1369928_at	**Acta1/actin, alpha 1, skeletal muscle**	4.45	8.53
1369313_at/	Fhl2/four and a half LIM domains 2	2.54	7.42
1371951_at		2.39	8.54
1382710_at	Enc1/ectodermal-neural cortex 1	2.36	6.52
1368860_at	Phlda1/pleckstrin homology-like domain, family A, member1	2.07	6.80
MAPK pathway			
1368124_at	Dusp5/dual specificity phosphatase 5	3.76	8.76
1368147_at/	Dusp1/dual specificity phosphatase 1	3.20	9.75
1368146_at		2.58	12.52
1369268_at	**Atf3/activating transcription factor 3**	3.61	10.62
1375043_at	**Fos/FBJ murine osteosarcoma viral oncogene homolog**	2.78	12.54
1387788_at	**Junb/Jun-B oncogene**	2.75	11.41
1369788_s_at/	**Jun/Jun oncogene**	2.55	9.17
1389528_s_at		2.01	7.70
1368489_at	**Fosl1/fos-like antigen 1**	2.13	8.70
FGF signaling pathway			
1374864_at	**Spry2/sprouty homolog 2 (Drosophila)**	3.00	9.86
Wnt signaling pathway			
1375604_at	Grb10/growth factor receptor bound protein 10	5.12	8.40
VEGF signaling pathway			
1368254_a_at	Sphk1/sphingosine kinase 1	2.93	8.63
1371840_at	Edg1/endothelial differentiation sphingolipid G-protein-coupled receptor 1	2.67	8.40
TGF-β signaling pathway			
1368896_at	**Madh7/MAD homolog 7 (Drosophila)**	2.36	9.42
Cell cycle			
1368596_at	Snf1lk/SNF1-like kinase	3.40	8.64
1386994_at/	Btg2/B-cell translocation gene 2, anti-proliferative	2.99	11.94
1386995_at		2.83	12.77
1372389_at	Ier2/immediate early response 2	2.20	11.12
1389355_at	Ier5/immediate early response 5	2.00	10.91
Regulation of calcium release			
1370997_at/	Homer1/homer homolog 1 (Drosophila)	5.66	8.35
1370454_at		2.05	7.75
1387074_at/	**Rgs2/regulator of G-protein signaling 2**	3.88	10.93
1368144_at		3.78	10.40
Regulation of apoptosis and cell proliferation			
1369067_at/	Nr4a3/nuclear receptor subfamily 4, group A, member 3	5.43	10.16
1393389_at/		4.03	8.79
1369217_at		2.64	8.57
1387410_at/	**Nr4a2/nuclear receptor subfamily 4, group A, member 2**	5.03	8.57
1369007_at		4.89	9.89
1386935_at	Nr4a1/nuclear receptor subfamily 4, group A, member 1	4.71	11.11
1367802_at	Sgk/serum/glucocorticoid regulated kinase	2.52	11.86
Other			
1387306_a_at	**Egr2/early growth response 2**	4.00	11.17
1376828_at	Rai3/retinoic acid induced 3	3.32	8.71
1373093_at	**Errfi/ERBB receptor feedback inhibitor**	2.80	11.22
1387870_at	Zfp36/zinc finger protein 36	2.55	11.00
1368488_at	Nfil3/nuclear factor, interleukin 3 regulated	2.54	9.71
1381070_at	Wdr36/WD repeat domain 36	2.47	7.30
1381810_at	Aff1/AF4/FMR2 family member 1	2.45	7.00
1394555_at	Smek1/SMEK homolog 1, suppressor of mek1 (Dictyostelium)	2.31	5.53
1373767_at	Zfand2a/zinc finger AN1-type domain 2A	2.29	10.61
1370174_at	Myd116/myeloid differentiation primary response gene 116	2.12	11.20
1387087_at	Cebpb/CCAAT/enhancer binding protein (C/EBP), beta	2.03	11.73

Gene symbol/name in bold indicates that the gene is known to be associated with cartilage biology.

numerous cytoplasmic processes and abundant RER; their nuclei contained heterochromatin (Fig. 1C). Hypertrophic chondrocytes with electron-lucent cytoplasm (i.e. light cells), in contrast, had smooth rounded profiles and contained sparse RER; their nuclei contained euchromatin (Fig. 1D). Most of the cells in pellets cultured under these conditions could be classified as either dark or light hypertrophic chondrocytes (with slightly more light than dark); the small 'unclassified' remainder had the appearance of non-hypertrophic chondrocytes (Fig. 1E, F).

3.2. Separation of light and dark hypertrophic chondrocytes

Density gradient centrifugation of T3-treated pellets yielded four cell fractions, of which the top fraction contained predomi-

nantly light hypertrophic chondrocytes (Fig. 2A, C) and the bottom fraction predominantly dark hypertrophic chondrocytes (Fig. 2B, D), as confirmed by electron microscopic examination (Fig. 2E, F). These two fractions are referred to here as 'light cell-enriched' and 'dark cell-enriched' fractions. The two middle fractions contained mixtures of light and dark cells, and were not used for further studies. Multiple cell isolates were used in the studies described below, and for each isolate part of each of the light and dark cell-enriched fractions were examined histologically to confirm that they comprised the appropriate cell type.

3.3. Expression array studies

RNA was extracted from the two cell fractions, with the aim of using the Affymetrix array to compare patterns of gene expression between light and dark hypertrophic chondrocytes. However, the dark cell-enriched fraction of each cell isolate (derived from an entire litter of rat pups) only contained about 1×10^5 cells, and yielded 2 μg RNA, while the array required 15 μg per sample. Thus, gene expression was compared between the entire pellet (comprised of a mixture of light and dark cells) and the almost pure light cell fraction (which contained about 5×10^6 cells/isolate).

A number of genes were differentially expressed between the two samples, as demonstrated by the MA scatter plot (Fig. 3). About one third of the putative dark chondrocyte-selective genes were found to be genes encoding ECM proteins or ECM-modulating enzymes; another substantial grouping comprised genes involved in regulation of the cytoskeleton and cell adhesion (Table 2). Many of the remainder were as yet unidentified genes. Most of the putative light chondrocyte-selective genes were components of growth factor signalling pathways, including a number of transcription factors (Table 3).

3.4. Dark chondrocyte-selective genes

From the list of genes presented in Table 2, eight putative dark chondrocyte-selective genes were selected for confirmation by RT-qPCR. In the selection of these candidates for further study, preference was given to genes for which antibodies or inhibitors for further investigation were available, as well as those showing higher levels of differential gene expression. It was also considered appropriate to include at least one gene that had not previously been shown to be expressed by chondrocytes. The genes subjected to further study were *collagen type I (Col1a1), collagen type II (Col2a1), periostin (Postn), osteoglycin, tissue plasminogen activator (tPA/Plat), carbohydrate sulfotransferase (Chst11), actin related protein 2/3 complex subunit 1B (Arpc1b),* and *Runx2*. For the RT-qPCR studies, much less material was required than for the array, thus RNA extracted from the dark chondrocyte-enriched fraction was used in a comparison with RNA from the light chondrocyte-enriched fraction. In these studies, selective expression by dark chondrocytes of *Col1a1, Postn, osteoglycin, tPA/Plat,* and *Chst11* was confirmed (Fig. 4, Table 4). Contrary to the array result, RT-qPCR showed that *Col2a1* and *Arpc1b* were not differentially expressed between light and dark cells, and *Runx2* was more highly expressed by light than dark cells. *Aggrecan* and *Sox9* (expressed by all chondrocytes) and *Col10a1* (expressed by hypertrophic chondrocytes) were not identified from the array as being differentially expressed, but were included in the analysis. There was no difference in the level of expression of *Sox9* between light and dark cells, but *aggrecan* and *Col10a1* were found to be significantly more highly expressed by light cells than by dark cells.

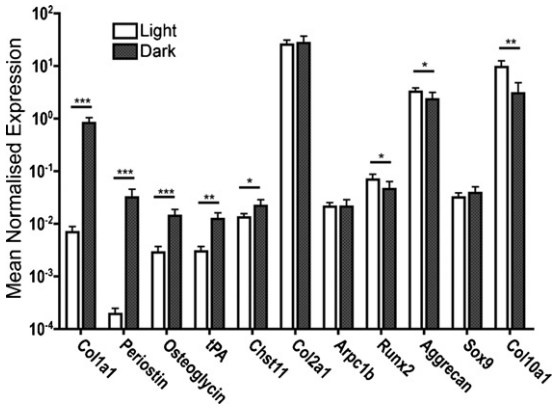

Fig. 4. Analysis of expression of selected genes in light hypertrophic as compared with dark hypertrophic chondrocytes. RT-qPCR analysis of Ficoll-separated light hypertrophic cell-enriched as compared with dark hypertrophic cell-enriched fractions. These results are the combined results from four separate cell isolates prepared at different times. MNE ± SEM ($n = 4$); $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

Table 4
Expression of candidate genes in dark cell-enriched fractions relative to light cell-enriched fractions.

Gene	*Expression relative to light cells*[#]	*P-value*[##]
Col1a1	118.2	0.001
Periostin	164.5	0.001
Osteoglycin	5.0	0.001
tPA	4.2	0.004
Chst11	1.7	0.023
Col2a1	1.1	0.758
Arpc1b	1.0	0.978
Runx2	0.6	0.036
Aggrecan	0.7	0.025
Sox9	1.2	0.536
Col10a1	0.3	0.008

[#] Expression of dark chondrocytes was calculated relative to that of light chondrocytes using the Relative Expression Software Tool.
[##] Pairwise randomisation reallocation test; $P < 0.05$ was considered significant.

3.5. Characterization of periostin as a dark cell-selective gene product

Periostin was selected for further investigation not only because it was found to be more highly differentially expressed (164-fold; Table 4) than any of the other dark chondrocyte-selective genes, but also because its MNE value suggested that its expression level in light chondrocytes may be substantially lower than that of any of the other genes (Fig. 4). Expression of periostin was examined in Ficoll-separated cell fractions using immunocytochemistry. Specific staining for periostin was detected in most cells in the dark chondrocyte-enriched fraction, but in fewer than 5% of cells in the light cell-enriched fraction (Fig. 5).

Expression of periostin was also examined by immunohistochemistry in cryosections of growth plates from young growing rats at different ages. Before undertaking the immunohistochemistry, an analysis of the proportions of dark and light hypertrophic chondrocytes in the growth plate at different ages was undertaken. At one week of age, 7.6% (±1.74%) of cells within the zone of hypertrophic chondrocytes were dark (with the remainder light), and at one month of age, 11.7% (±1.14%) were dark. Extracellular staining for periostin was observed in the perichondrium (Fig. 6A, B). Staining for periostin was also observed within the cytoplasm of growth plate chondrocytes. The staining was weak in the zone of resting chondrocytes and stronger throughout the zone of proliferative chondrocytes at both ages examined (Fig. 6). Periostin staining was observed in a small proportion of cells within the zone of hypertrophic chondrocytes in specimens from one-week old rats (approximately 6.3%), and in a higher proportion of cells in specimens from one month-old rats

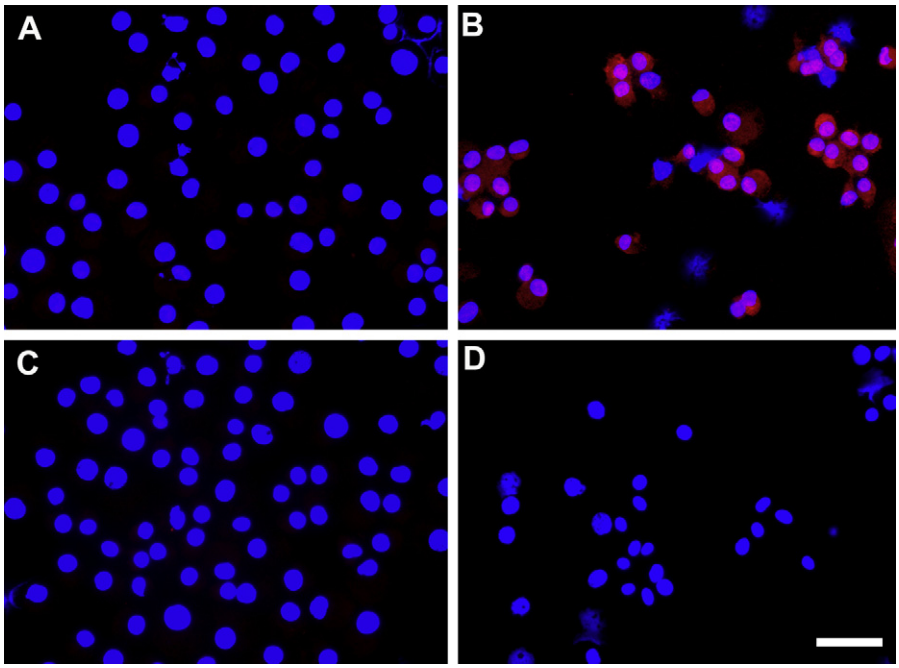

Fig. 5. Immunofluorescence analysis of periostin expression in cells separated by Ficoll density gradient. The light (A, C) and dark (B, D) hypertrophic chondrocyte-enriched fractions were stained with anti-periostin (A, B; red) or normal rabbit Ig (C, D) and nuclei were counterstained with DAPI (blue). Bar = 125 μm.

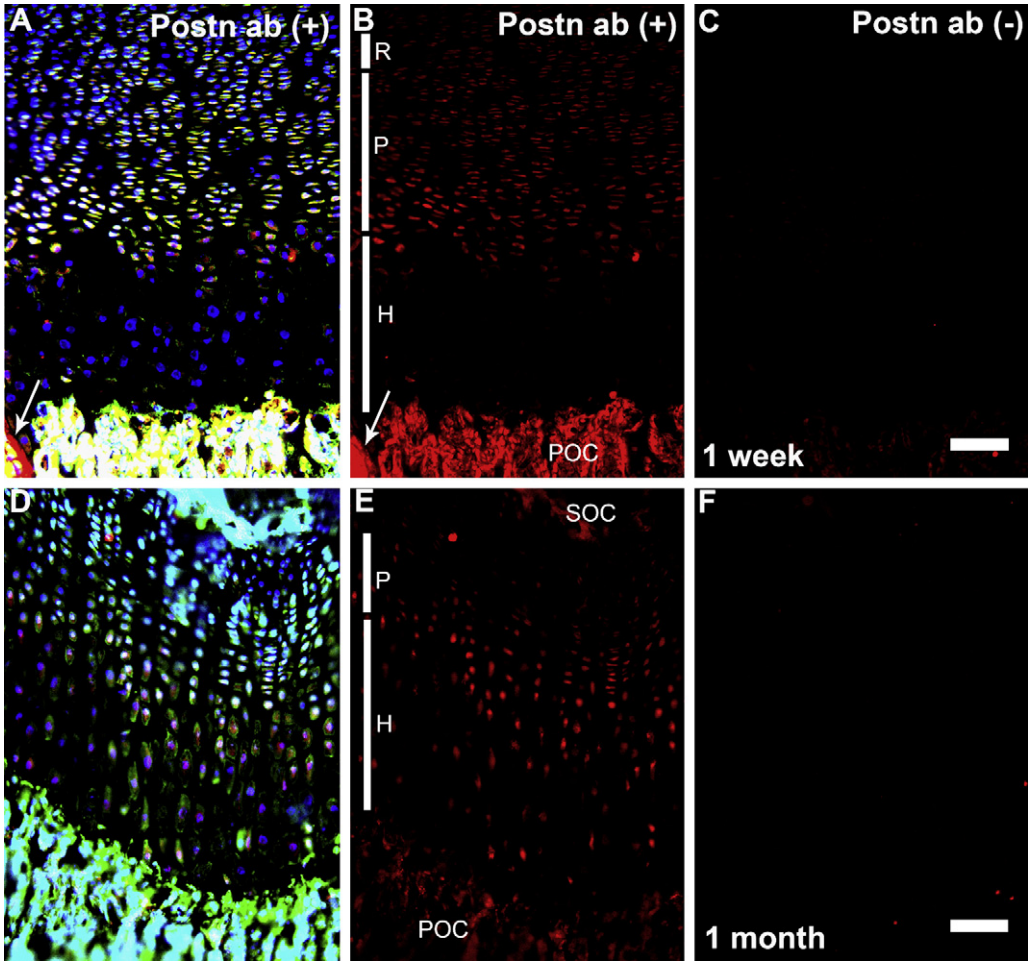

Fig. 6. Immunofluorescence analysis of periostin expression in the growth plate of growing rats. Cryosections of distal femoral growth plate of one-week-old (A–C) and one-month-old (D–F) rats stained with anti-periostin (A, B, D, E; Postn, red) or normal rabbit Ig (C, F), and counterstained with Alexa Fluor 488 phalloidin (green) and DAPI (blue), shown in A, D. Arrows indicate periosteum. The magnification for A, B is the same as that for C (Bar = 250 μm). The magnification for D, E is the same as that for F (Bar = 125 μm).

(approximately 14.1%). The specimens presented in Fig. 6 were treated with hyaluronidase to enhance antibody penetration; parallel specimens without hyaluronidase pre-treatment showed a similar pattern of staining, although slightly weaker (data not shown).

4. Discussion

Light and dark chondrocytes have been identified using ultrastructural methods as morphologically distinct populations of hypertrophic chondrocytes in a number of species (Ahmed et al., 2007; Anderson, 1964; Erenpreisa and Roach, 1998; Hwang, 1978; Roach and Clarke, 2000; Wilsman et al., 1981), but functional differences between these two cell types have not yet been identified. Until recently, the only available methodology for studying these cells was TEM of tissue specimens. We have recently demonstrated not only that it is possible to identify distinctive light and dark cells amongst chondrocytes induced to undergo hypertrophy in pellet culture, but also that it is possible to identify the two cell types by light microscopic analysis of specimens fixed and embedded as for TEM (Ahmed et al., 2007). Here we present the next stage of our development of methods for the study of functional differences between the two populations, together with the results of experiments using these methods.

The results presented here demonstrate that it is possible to separate light from dark hypertrophic chondrocytes for the purpose of analysing gene expression. Ficoll gradients have previously been used to separate chondrocytes of different sizes from articular cartilage specimens, with the larger cells being found in the lower fractions (Scheinberg et al., 1982). When rat chondrocytes expanded and induced to undergo hypertrophy in pellet culture were subjected to this method, it was found that dark chondrocytes had a higher density than light chondrocytes (although tending to be smaller), and were found in the lower fractions.

Our approach was to use cells separated by this method to obtain an overview of patterns of differential gene expression in light and dark hypertrophic chondrocytes. The array yielded a large number of putative dark or light cell-selective genes. It has not so far been possible logistically to confirm the differential expression of most of these genes, but for eight putative dark chondrocyte-selective genes this has been undertaken using RT-qPCR, and most of them were confirmed as being dark cell-selective. Three were shown not to be dark cell-selective in the more definitive qRT-PCR studies: *Col2a1* and *Arpc1b*, which were shown not to be differentially expressed, and *Runx2,* which was shown to be light cell-selective. For the first two, the reason for the discrepancy is likely to be that the array was undertaken with entire pellets, that is, a mixed cell population rather than the dark cell-enriched fraction used for the RT-qPCR studies. For *Runx2*, RT-qPCR using the same material as the array contradicted the array data and provided the same result as the RT-qPCR with the dark cell-enriched fraction (data not shown), which suggests that there may be a lack of homology between *Runx2* expressed in rat chondrocytes and the sequence on the array.

On the basis of the array study, we have observed that a substantial proportion of dark cell-selective genes are genes encoding ECM proteins or enzymes that modify ECM proteins, including matrix-degrading proteases. The most important function for chondrocytes is synthesis and secretion of the mixture of cartilage-specific ECM components, which confer on cartilage its special structural properties. The two most abundant ECM proteins in cartilage are the fibrillar collagen, type II, and the major cartilage proteoglycan, aggrecan, however the RT-qPCR studies demonstrated that neither of the genes encoding these proteins is differentially expressed between the two cell types. Hypertrophic chondrocytes have the additional function of modifying the cartilage matrix surrounding themselves so as to allow their own expansion as well as ultimately the invasion of the growth plate by the cells responsible for replacing it with bone tissue. The selective expression by dark hypertrophic chondrocytes of a number of minor ECM proteins and ECM-modifying enzymes (some putative, some confirmed by RT-qPCR), suggests that these cells may play a greater role in preparing the growth plate ECM for invasion by bone tissue than do light hypertrophic chondrocytes. After *Postn* (discussed further below), the most highly dark cell-selective gene was *Col1a1*. Cells of mesenchymal origin express decreasing levels of collagen type I with increasing chondrocytic differentiation, and thus expression of this collagen by chondrocytes is often considered to be a marker of de-differentiation. Sandell et al. (1994) and Wang et al. (2004) have, however, described the expression of collagen type I by hypertrophic chondrocytes. This protein has been found to promote angiogenesis in many tissues during embryonic development (Twardowski et al., 2007), thus it is possible that dark hypertrophic chondrocytes assist in the vascular invasion of the growth plate through expression of collagen type I.

Genes identified as putative light chondrocyte-selective genes on the array largely fell under groupings associated with growth factor signalling and regulation of cell cycle, proliferation and apoptosis. Confirmation of the differential expression of these genes using RT-qPCR will be required before the implications for light hypertrophic chondrocyte function can be considered. Contrary to the array, RT-qPCR data showed *Runx2* and *Col10a1* to be significantly more highly expressed by light than by dark hypertrophic chondrocytes. Both of these genes are up-regulated with chondrocyte hypertrophy; our data indicate that light hypertrophic chondrocytes contribute more to this up-regulation than do dark hypertrophic chondrocytes.

A grouping of genes that was well represented in lists of both putative dark chondrocyte-selective and putative light chondrocyte-selective genes is that of genes involved in regulation of the cytoskeleton and cell adhesion. It is not surprising that there are differences in expression of such genes between light and dark hypertrophic chondrocytes, since the shape of the two cell types differs substantially. Differences in the cytoskeleton (and thus cell shape) are of some interest, because our earlier observations indicate that the different cell shapes are functionally associated with different modes of secretion and physiological death in the two cell types (Ahmed et al., 2007).

Postn was confirmed in RT-qPCR studies to be the most highly dark cell-selective gene investigated, so further studies of periostin were undertaken. Immunocytochemical studies of Ficoll-separated hypertrophic chondrocytes demonstrated that periostin is detectable in dark but not light cells. This observation provides a means of distinguishing between light and dark cells for future use in a much wider variety of cell biological research methods than previously possible.

Expression of periostin in growth plate of postnatal rats has not previously been investigated. In such specimens, periostin staining was found to be present in the zone of proliferative chondrocytes, but absent from all but a small proportion of cells in the zone of hypertrophic chondrocytes; there was an increase in the proportion of periostin-positive hypertrophic chondrocytes with age, in keeping with the increase in the proportion of dark hypertrophic chondrocytes observed in parallel specimens. Thus, it appears that periostin is uniformly expressed by proliferative chondrocytes in the growth plate, and down-regulated in light but not dark cells with hypertrophy.

Periostin is considered to be an ECM protein, and it is certainly present in the ECM of periosteum, from which it takes its name, as described by others (Blumer et al., 2006; Horiuchi et al., 1999) and observed in the immunohistochemical studies presented here. In cartilage, however, periostin is known to be located in the chondrocyte cytoplasm rather than the ECM (Blumer et al., 2006; Zhu et al.,

2008), as observed in the current study. Yoshioka et al. (2002) have provided evidence that periostin located intracellularly suppresses anchorage-independent growth of a cancer cell line. Thus it is possible that cytoplasmic periostin may play a functional role in dark hypertrophic chondrocytes, but what this role may be is not clear. Embryonic bone development in periostin-null mice is normal, but growth of these mice is slower than in wildtype mice 3–4 weeks after birth (Rios et al., 2005). In the current study, the increase in periostin-positive hypertrophic chondrocytes (and concomitant increase in dark hypertrophic chondrocytes) was also observed at 4 weeks, perhaps indicating that the growth retardation results from a defect in the function of dark chondrocytes in the absence of periostin.

In conclusion, light and dark hypertrophic chondrocytes can be separated by density gradient centrifugation and subjected to investigations of gene and protein expression. A variety of putative and/or confirmed light and dark cell-selective genes have been identified. The results of the expression array will be extremely useful in the future analysis of the differential functions of light and dark hypertrophic chondrocytes. Moreover, expression in chondrocytes of a number of genes identified in the array study has not previously been described, thus the results will be helpful in the understanding of hypertrophic chondrocyte biology in general. Periostin has been identified as a dark cell-specific gene product, at least as far as hypertrophic chondrocytes are concerned; this observation will provide enormous benefits to the future study of hypertrophic chondrocyte populations.

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