Bone Reports 7 (2017) 98–107

Contents lists available at ScienceDirect

Bone Reports

journal homepage: www.elsevier.com/locate/bonr

The vacuolar H^+ ATPase V_0 subunit d_2 is associated with chondrocyte hypertrophy and supports chondrocyte differentiation

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ARTICLE INFO

Keywords:
Chondrocyte
Hypertrophy
Endochondral ossification
ATP6V0D2
Vacuolar H^+-ATPase

ABSTRACT

Chondrocyte hypertrophy makes important contributions to bone development and growth. We have investigated a number of novel cartilage genes identified in a recent transcriptomic study to determine whether they are differentially expressed between different zones of equine foetal growth cartilage. Twelve genes (*ATP6V0D2*, *BAK1*, *DDX5*, *GNB1*, *PIP4K2A*, *RAP1B*, *RPS7*, *SRSF3*, *SUB1*, *TMSB4*, *TPI1* and *WSB2*) were found to be more highly expressed in the zone of hypertrophic chondrocytes than in the reserve or proliferative zones, whereas *FOXA3* and *SERPINA1* were expressed at lower levels in the hypertrophic zone than in the reserve zone. *ATP6V0D2*, which encodes vacuolar H^+ ATPase (V-ATPase) V_0 subunit d_2 (ATP6V0D2), was selected for further study. Immunohistochemical analysis of ATP6V0D2 in growth cartilage showed stronger staining in hypertrophic than in reserve zone or proliferative chondrocytes. Expression of ATP6V0D2 mRNA and protein was upregulated in the mouse chondrocytic ATDC5 cell line by conditions inducing expression of hypertrophy-associated genes including *Col10a1* and *Mmp13* (differentiation medium). In ATDC5 cells cultured in control medium, knockdown of *Atp6v0d2* or inhibition of V-ATPase activity using bafilomycin A1 caused a decrease in *Col2a1* expression, and in cells cultured in differentiation medium the two treatments caused a decrease in nuclear area. Inhibition of V-ATPase, but not *Atp6v0d2* knockdown, prevented the upregulation of *Col10a1*, *Mmp13* and *Vegf* by differentiation medium, while *Atp6v0d2* knockdown, but not inhibition of V-ATPase, caused an increase in the number of ATDC5 cells cultured in differentiation medium. These observations identify ATP6V0D2 as a novel chondrocyte hypertrophy-associated gene. The results are consistent with roles for V-ATPase, both ATP6V0D2-dependent and -independent, in supporting chondrocyte differentiation and hypertrophy.

1. Introduction

Most bones develop through the process of endochondral ossification, which involves the initial formation of a cartilage anlage and its replacement by bone tissue (Mackie et al., 2011). As part of this process, chondrocytes proliferate, secrete cartilage matrix and undergo hypertrophy. Chondrocyte hypertrophy plays critical roles in bone elongation and in preparation of the cartilage matrix for invasion by the complex mixture of cells that comprise the ossification front.

The orderly progression of chondrocyte proliferation, matrix secretion and hypertrophy in growth cartilage is regulated by the coordinated actions of a variety of systemic and locally secreted factors, acting through stage-specific transcription factors. Thyroid hormones are important circulating inducers of hypertrophy, and their actions appear to be mediated by pathways including the WNT/β-catenin, insulin-like growth factor 1 and fibroblast growth factor receptor 3-mediated pathways (Mackie et al., 2011; Shao et al., 2006). The transcription factor RUNX2 induces expression of hypertrophy-associated genes including that encoding collagen type X (*COL10A1*), and stimulates hypertrophy (Lefebvre and Smits, 2005).

While much has been learnt in recent years about regulation of chondrocyte hypertrophy, there are still many aspects of this process that remain unclear. For example, while remodelling of the extracellular matrix is clearly required to accommodate the swelling of individual cells, no matrix-degrading enzyme has yet been identified as being critical for this process (see discussion in Mackie et al. (Mackie et al., 2011)).

Abbreviations: ABH, alcian blue/haematoxylin/eosin/acid fuchsin stain; ATP6V0D2, vacuolar H^+ ATPase V_0 subunit d_2; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; MMP-13, matrix metalloproteinase-13; MNE, mean normalised expression; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; V-ATPase, vacuolar H^+ ATPase

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http://dx.doi.org/10.1016/j.bonr.2017.08.002
Received 8 June 2017; Received in revised form 21 July 2017; Accepted 17 August 2017
Available online 18 August 2017

Chondrocytes undergoing hypertrophy not only express molecules that contribute to their own morphological and functional changes, but also secrete factors that regulate behaviour of cells in the invading ossification front. For example, hypertrophic chondrocytes promote vascular invasion of the growth plate through expression of vascular endothelial growth factor (Zelzer et al., 2001; Zelzer et al., 2004), and osteoclast differentiation through expression of receptor activator of NFκB ligand (Usui et al., 2008).

In a recent unbiased transcriptomic study of cartilage from lesions of equine osteochondrosis (a developmental orthopaedic disease), we identified a number of genes that had not previously been described in cartilage (Mirams et al., 2016). The current study was undertaken to determine whether expression of any of these genes is regulated in association with chondrocyte hypertrophy, in order to shed light on molecular mechanisms of this process. These studies made use of equine foetal growth cartilage and ATDC5 cells, a mouse chondrocyte line that can be induced to express hypertrophy-associated genes including those encoding collagen type X and matrix metalloproteinase-13 (MMP13) (Shukunami et al., 1997; Wang et al., 2004). Following the identification of several novel hypertrophy-associated genes, one of these, *ATP6V0D2*, was selected for further study.

2. Materials and methods

2.1. Tissue samples

Samples were collected from the distal end of the right third metatarsal bones of 22 foetal horses obtained from pregnant mares killed at a Melbourne knackery. Gestational age of foetuses was estimated using the crown-rump length method and ranged from 120 to 300 days. Foetuses with gross skeletal deformities were excluded. The specimens included some collected before the secondary ossification centre was formed in the distal epiphysis (designated as early samples; n = 8), and some collected after formation of the secondary ossification centre (late samples; n = 14). The distal end of the bone (4 cm in length) was excised and bisected longitudinally; one piece was used for histology and the other for gene expression studies. All samples included part of the primary ossification centre. The collection of these samples met the requirements of the University of Melbourne Animal Ethics Committee.

2.2. Histology

At the time of collection, samples were fixed in 4% w/v paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 at 4 °C overnight, then rinsed and demineralised using 0.33 M ethylenediaminetetraacetic acid, pH 7.4. Demineralised specimens were incubated in 25% sucrose in PBS overnight then trimmed to remove all except the growth plate and a small amount of primary ossification centre and secondary ossification centre, if present; for specimens lacking a secondary ossification centre, the region remaining after trimming extended from the primary ossification centre to the articular surface. Specimens were then embedded in Tissue-Tek® OCT compound (ThermoFisher Scientific, Waltham USA). Frozen sections (10 μm) were cut using a cryostat (Leica Microsystems, Germany). Some sections were stained with alcian blue and counterstained with haematoxylin, eosin and acid fuchsin (ABH) using standard procedures; other sections were stained by immunohistochemistry.

2.3. Immunohistochemistry

Sections to be stained for the presence of collagen type X were first treated with bovine testicular hyaluronidase (1000 U/ml in 0.15 M sodium chloride and 20 mM sodium acetate, pH 5.0). All sections were permeabilised using 0.1% v/v Triton-X100 in PBS before being blocked for one hour using either 10% v/v foetal calf serum (FCS; for anti-collagen type X) or 2.5% FCS (for anti-ATP6V0D2) in PBS. Sections were incubated overnight at 4 °C with rabbit anti-human collagen type X (1:100 in 5% FCS in PBS; Abcam, Cambridge, UK), rabbit anti-human ATP6V0D2 (0.02 μg/μl in PBS containing 0.5% Triton X100 and 1% w/v bovine serum albumin; Abcam) or normal rabbit serum (control for anti-collagen type X) or purified rabbit Ig (control for anti ATP6V0D2) diluted appropriately. Sections were rinsed with PBS prior to incubation with AlexaFluor 488-conjugated goat anti-rabbit immunoglobulin (1:300 in PBS; ThermoFisher Scientific). After being washed, sections were mounted in gelvatol containing 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml), then examined by standard fluorescence microscopy.

2.4. Microdissection of growth cartilage

Specimens harvested for analysis of gene expression were wrapped in aluminium foil, frozen in liquid nitrogen and stored at − 80 °C. Specimens were trimmed to remove any bone and embedded frozen in OCT compound; sections (15 μm) were collected on glass slides then held on dry ice until they were microdissected. The region dissected was the growth cartilage giving rise to the primary centre of ossification, whether or not a secondary ossification centre was present. Under an inverted phase contrast microscope, the reserve, proliferative and hypertrophic zones were identified by reference to morphological features observed in ABH-stained sections (Fig. 1A), and the hypertrophic zone in collagen type X-stained sections. For each section, tissue from each of the reserve, proliferative and hypertrophic zones was collected using a scalpel and placed in separate microcentrifuge tubes. For each sample, the tissue for each zone was pooled from 20 to 30 sections.

2.5. Cell culture and immunocytochemistry

Cells of the ATDC5 mouse chondrocyte cell line (kindly provided by Prof. A. Fosang, Murdoch Childrens Research Institute, Melbourne) were maintained in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 nutrient mix (1:1; DMEM/F12; 'control medium'). For induction of hypertrophy-associated gene expression, the medium of confluent cells was changed to differentiation medium consisting of α minimum essential medium containing insulin-transferrin-selenium (1% w/v), ascorbate-2-phosphate (100 μg/ml) and triiodothyronine (100 nM), in which cells were cultured for a further 4 days. This medium was selected as a combination of factors used in previous studies (Siebler et al., 2002; Temu et al., 2010), following optimisation experiments (data not shown). Some cultures (in control medium or in differentiation medium) were treated with bafilomycin A1 (1 nM; Sigma Aldrich, St Louis, USA) over this 4-day period.

For immunocytochemistry, cells on glass coverslips were fixed with 4% w/v paraformaldehyde in PBS for 5 min, then rinsed, blocked, stained with anti-collagen type X or anti-ATP6V0D2, and mounted in gelvatol containing DAPI as described above for tissue sections. In some cases, cells on glass coverslips were incubated with Lysotracker® Red DND-99 (1:20,000; ThermoFisher Scientific) or wheat germ agglutinin, AlexaFluor® 488 conjugate (1:2000; ThermoFisher Scientific) for 30 min before fixation (as described above) or incubated with AlexaFluor® 488/568 phalloidin (1:300; ThermoFisher Scientific; 30 min) after fixation. For cell counting and morphometry, coverslips were examined by standard fluorescence microscopy. Images were taken of 8 fields per coverslip, and cell number, nuclear area and cell area were determined using Image Pro software (Media Cybernetics, Rockville, USA); nuclear area was assessed as the area of DAPI staining, and to measure cell area phalloidin-stained cells were outlined manually. Mitotic figures (visualised with DAPI staining) were counted and expressed as a percentage of total nuclei. Some coverslips were examined by confocal microscopy using a Zeiss LSM 510 Meta microscope (Carl Zeiss, Germany).