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Isolation and gene expression of haematopoietic-cell-free preparations of highly purified murine osteocytes

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ABSTRACT

To define their gene expression and function, osteocytes are commonly isolated and purified by fluorescence-activated cell sorting (FACS) from mice expressing GFP directed by the dentin matrix protein 1 (*Dmp1*) promoter (DMP1-GFP). These cells express mRNA for osteocyte genes, including sclerostin (*Sost*) and *Dmp1*, and genes associated with the osteoclast phenotype: *Dcstamp*, *Oscar*, Cathepsin K (*Ctsk*), tartrate resistant acid phosphatase (TRAP/*Acp5*) and calcitonin receptor (*Calcr*). This suggests either that osteoclasts and osteocytes share genes and functions or that DMP1-GFP$^+$ preparations contain haematopoietic osteoclasts.

To resolve this we stained DMP1-GFP cells for haematopoietic lineage (Lin) surface markers (CD2, CD3e, CD4, CD45, CD5, CD8, CD11b, B220, Gr1, Ter119) and CD31. Lin$^-$CD31$^-$ (Lin$^-$) and Lin$^+$CD31$^+$ (Lin$^+$) populations were analysed for GFP, and the four resulting populations assessed by quantitative real-time PCR.

Lin$^-$GFP$^+$ cells expressed mRNAs for *Sost*, *Dmp1*, and *Mepe*, confirming their osteocyte identity. *Dcstamp* and *Oscar* mRNAs were restricted to haematopoietic (Lin$^+$) cells, but *Calcr*, *Ctsk* and *Acp5* were readily detected in purified osteocytes (Lin$^-$GFP$^+$). The capacity of these purified osteocytes to support osteoclastogenesis was assessed: no TRAP+ cells with >2 nuclei were formed when purified osteocytes were cultured with bone marrow macrophages and stimulated with 1,25-dihydroxyvitamin-D_3/prostaglandin E_2. Lin$^-$GFP$^+$ osteocytes also expressed lower levels of *Tnfsf11* (RANKL) mRNA than the osteoblast-enriched population (Lin$^-$GFP$^-$).

This demonstrates the importance of haematopoietic depletion in generating highly purified osteocytes and shows that osteocytes express *Acp5*, *Ctsk* and *Calcr*, but not other osteoclast markers, and do not fully support osteoclast formation in vitro.

Introduction

Osteocytes are terminally differentiated osteoblasts and constitute more than 95% of all bone cells in the adult skeleton [1]. They reside in lacunae, small cavities within the mineralised bone matrix, and possess long, dendrite-like processes. These extend through small tunnels in the bone matrix (canaliculi) thereby connecting osteocytes with each other and with cells on the bone surface, including osteoblasts and bone lining cells [1,2]. This forms a well-organised network suitable for integration of local and systemic signals. In recent years, osteocytes have been reported to play significant paracrine and endocrine roles that regulate bone formation [3,4], phosphate metabolism [5–7], and osteoclast differentiation [8–12]. Despite their abundance, these cells remain poorly understood, mainly due to their position, deeply embedded in mineralised bone, and the difficulty of isolating highly purified primary cells.

Several methods to isolate a highly purified osteocyte population have been described. Initial efforts using cells digested from embryonic chick calvaria purified them using a monoclonal antibody to the osteocytic protein, phosphate regulating endopeptidase homolog, X-linked (*Phex*) [13,14]. Osteocytes were also collected from serial digestion of newborn rat calvariae [15] or outgrowth of cells from previously digested bone [16]. Both methods generated a population of osteocytes that showed typical stellate shape in culture, that produced low levels of alkaline phosphatase [15,17] and high levels of extracellular matrix proteins including osteocalcin [15,18], osteonectin [18] and osteopontin [18]. However, these cultures were impure and contaminating cells, including fibroblasts, proliferated and overgrew the post mitotic osteocytes [15,17].

The more recent identification of several osteocytic gene markers such as *Dmp1*, *Mepe*, *Phex*, podoplanin (*Pdpn*/E11) and *Sost* indicated that late digest fractions contain higher levels of osteocyte genes including *Dmp1* and *Sost* compared to earlier osteoblast-enriched fractions [19], allowing isolation of cells including a high proportion of osteocytes. Stern et al. [20] also successfully isolated primary cells displaying several characteristics of osteocytes from mice by combining serial

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digestions with tissue homogenisation. In their studies, the late digest fraction expressed several osteocytic genes, including *Pdpn*, *Sost*, *Mepe*, *Dmp1* and *Phex* [20]. The possibility of contaminating proliferating cells such as fibroblasts in the cell preparation remained an open question.

Based on the restricted expression of *Dmp1* in osteocytes [21,22], Kalajzic et al. [22] used 8 kb of the *Dmp1* promoter region to generate a transgenic mouse line with GFP expression directed to osteocytes in osteoid and bone [22]. Subsequently, cells sorted for GFP from calvarial bones of neonatal DMP1-GFP mice have been used to examine osteocytic gene expression compared to osteoblasts [19,23]. An alternative approach to isolate osteocytes from neonatal calvariae used fluorescent labelling directed by a 10 kb-promoter region driven DMP1-Cre transgene [8]. However, expression of GFP driven by the 10 kb DMP1-Cre transgene also occurs in osteoblasts [12,24], perhaps providing an explanation for recent contradicting data obtained when RANKL levels are compared between osteocytes and osteoblasts prepared by this method [8] vs a comparison of early and late digest fractions [25].

We used the 8 kb DMP1-GFP transgenic mice to generate osteocytes. By FACS sorting for GFP only, we observed the presence of osteoclast-specific markers *Dcstamp* and *Oscar* mRNA in the GFP positive population, consistent with previous reports of common genes expressed by osteocytes and haematopoietic osteoclasts including *Ctsk* [26], *Acp5* [26], *Oscar*, *Dcstamp* [23], *Calcr* [23,27,28] and *Sost* [29]. We developed a method to further purify this osteocyte population, utilising standard haematopoietic-lineage and endothelial cell depletion methods [30–33] in order to determine the extent to which osteocyte gene expression overlaps with that of osteoclasts.

Materials and methods

Animal experiments

DMP1-GFP mice, that express green fluorescent protein (GFP) under the control of 8 kb of the DMP1 promoter region plus 4439 bp of the first exon, first intron and part of the second exon [22], were obtained from Dr Ivo Kalajzic, University of Connecticut Health Science Center, via the colony of Dr Hong Zhou, ANZAC Research Institute, Sydney. All animal experiments were approved by the St. Vincent's Melbourne Health Animal Ethics Committee.

Osteocyte isolation

To allow comparison with previously published work [8,19,23], calvariae were dissected from 4 to 9 day old DMP1-GFP transgenic mice and subjected to 7 sequential 15-minute digestions in a 2 ml mixture containing 4 mg dispase (Gibco, NY, USA) and 2 mg collagenase type 2 (Worthington, Australia) as previously described [27]. Cell fractions 1–7 were collected, pooled, and resuspended in alpha modified Eagle's medium (αMEM, Gibco) containing 10% FBS, and centrifuged. Pellets were resuspended in erythroid cell lysis buffer (150 mM NH_4CI, 10 mM $CHKO_3$, 0.1 mM Na_2EDTA, pH 7.2) before cell sorting.

Fluorescence activated cell sorting (FACS) of green-fluorescent protein (GFP) positive osteocytes

Isolated cells were subjected to FACS for GFP expression alone or lineage depletion plus GFP as follows (Fig. 1). Prior to sorting, dead cells and debris were removed based on side scatter (SSC) area and forward scatter (FSC) area, and doublets were excluded based both on SSC width (W) vs SSC height (H) and on FSC-W vs FSC-H. Cells were sorted with excitation 488 nm and 530/30 or 530/40-emission filter for GFP on a BD FACSAria cell sorter (BD Bioscience) or a BD FACS Influx cell sorter (BD Bioscience), respectively. For each preparation, calvarial digests from C57BL/6 mice were used to determine the GFP negative gate.

For haematopoietic lineage and endothelial cell depletion, cells were stained for 30 min with an antibody cocktail comprised of biotin-labelled antibodies against mouse CD2, CD3e, CD4, CD45, CD5, CD8, CD11b, B220, Gr1, Ter119 (all eBioscience) to identify the haematopoietic lineage population [30–33] and CD31 (eBioscience) to identify endothelial cells [34]. None of these markers have been detected by microarray in any mesenchymal cell (BioGPS: http://biogps.org; accessed 14 October 2014) or in neonatal osteocytes by in situ hybridization (Gene eXpression Database: http://www.informatics.jax.org/mgihome/homepages/expression.shtml; accessed 14 October 2014). In the text that follows, the Lin^+ population includes $CD31^+$ cells, and the Lin^- population is also $CD31^-$. Biotinylated antibodies were detected with Qdot 605 streptavidin conjugate (Invitrogen) as previously described [35]. Cells were then subjected to FACS with excitation at 405 nm and 610/20 emission filter for Qdot 605, and GFP as described above.

The following cell populations were isolated: Lin^+GFP^+ (fluorescent haematopoietic cells), Lin^+GFP^- (haematopoietic lineage cells), Lin^-GFP^+ (highly purified osteocytes) and Lin^-GFP^- (mesenchymal lineage cells, including osteoblasts) (Fig. 1).

cDNA preparation and PCR

cDNA was prepared from RNA using random hexamers (Promega, Australia) and Superscript III (Invitrogen, Australia) according to the manufacturer's protocol. Real time quantitative RT-PCR (qPCR) was performed using SYBR green detection on a Stratagene Mx3000P (Invitrogen, Australia) as previously described [27]. Cycling conditions were (95 °C for 10′), (95 °C for 15″, 60 °C for 1′) × 40 cycles. Samples were analysed using Stratagene software MxPro and reported using linear ΔCT values normalised to the geometric average of *Bms1* and *Pgk1*. Primers for genes of interest are as previously published: *Calcr*, *Dmp1*, *Mepe*, *Sost* and *Bms1* [27,28]; *Alpl*, *Pthr1* and *Tnfsf11* [36]; *Dcstamp* and *Ctsk* [37]; *Tnfrsf11b* [38]; and *Bglap* [39]; novel primers are listed in Table 1. *Calcr* transcript was confirmed by sequencing as follows: PCR products were cloned into pGEMT™T-A cloning vector (Promega) and transformed into Stable 3 bacterial cells. Plasmid DNA was prepared using Qiagen mini-prep kit (Qiagen) and cycle sequencing was performed by Applied Genetic Diagnostics (Department of Pathology, The University of Melbourne).

In vitro osteoclastogenesis

To generate bone marrow macrophages, mouse bone marrow was isolated from C57BL/6 mice and cultured in L-cell conditioned media for 2 days. Non-adherent cells (bone marrow macrophages) (250,000 cells/cm^2) were used as osteoclast precursor cells, which were cocultured with osteoblasts (50,000 cells/cm^2) or osteocytes (50,000 cells/cm^2) in the presence of 10^{-7} M 1,25-dihydroxyvitamin D_3 and 10^{-7} M prostaglandin E_2 for 6–7 days as previously described [8]. Cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min on ice and stained for tartrate-resistant acid phosphatase (TRAP) enzyme activity [40].

Results

GFP^+ population generated by FACS for GFP without haematopoietic depletion contains osteoclasts

DMP1-GFP^+ calvarial cells obtained by FACS for GFP alone from 4 to 9 day old mice expressed the osteocyte marker, *Sost* at a three-fold higher level than GFP^- calvarial cells, confirming enrichment of osteocytes in this population (Fig. 2). Consistent with our previous finding, *Calcr* was also detected in this population [28] (Fig. 2). Testing for the presence of other osteoclast markers, such as dendritic cell-specific transmembrane protein (*Dcstamp*) and osteoclast-associated immunoglobulin-like receptor (*Oscar*), revealed their presence in both GFP^+ and GFP^- populations (Fig. 2). This indicated either that both osteocytes and osteoclasts express