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Single cell gene expression profiling of cortical osteoblast lineage cells

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ABSTRACT

In tissues with complex architectures such as bone, it is often difficult to purify and characterize specific cell types via molecular profiling. Single cell gene expression profiling is an emerging technology useful for characterizing transcriptional profiles of individual cells isolated from heterogeneous populations. In this study we describe a novel procedure for the isolation and characterization of gene expression profiles of single osteoblast lineage cells derived from cortical bone. Mixed populations of different cell types were isolated from adult long bones of C57BL/6J mice by enzymatic digestion, and subsequently subjected to FACS to purify and characterize osteoblast lineage cells via a selection strategy using antibodies against CD31, CD45, and alkaline phosphatase (AP), specific for mature osteoblasts. The purified individual osteoblast lineage cells were then profiled at the single cell level via nanofluidic PCR. This method permits robust gene expression profiling on single osteoblast lineage cells derived from mature bone, potentially from anatomically distinct sites. In conjunction with this technique, we have also shown that it is possible to carry out single cell profiling on cells purified from fixed and frozen bone samples without compromising the gene expression signal. The latter finding means the technique can be extended to biopsies of bone from diseased individuals. Our approach for single cell expression profiling provides a new dimension to the transcriptional profile of the primary osteoblast lineage population *in vivo*, and has the capacity to greatly expand our understanding of how these cells may function *in vivo* under normal and diseased states.

Introduction

Background

The skeleton is a complex organ system containing a number of tissues comprised of unique cell populations involved in maintaining structure and function. Within the long bones of the appendicular skeleton, calcified bone can be divided into the trabecular and cortical tissues. Recently, there has been tremendous progress in understanding and treating age-related disorders to prevent the loss of cortical bone tissue [1]. However, there have been few studies that examine primary bone cell populations derived from *in vivo* sources. One potential reason for this is the inherent difficulty in studying the cell types involved in maintaining bone, as these cells are typically encased within an ossified matrix. Consequently, characterization of these cell types has usually been performed upon differentiated osteoblast culture models [2–4], often after multiple passages *in vitro*. Thus, there is always the uncertainty of how faithfully the cells cultivated *in vitro* mirror the behavior of osteoblasts functioning *in vivo*. It is also not well understood how osteoblasts transcriptionally vary from one type of bone to another [5], nor even within the same bone. For example, it has been demonstrated that the gene expression profiles from calvarial bone are quite different than those derived from long bones [6]. There are also questions about potential differences in osteoblast function *in vivo* as a consequence of specific location or physiological state; periosteal versus endosteal for the former, and degree of mechanical loading or physiological age for the latter.

The need for single cell methods to study cells involved in bone formation

There have been a number of recent advances in nucleic acid manipulation and amplification technology that allow for the quantitative assessment of multiple genes using high throughput PCR platforms [7]. Similarly, there have been recent advances in the analysis of single cell data in specific cell populations to elucidate subtle expression differences between cell types in developmental or pathological processes such as tumor progression [8,9]. These approaches have not yet been widely used in studying age-related changes, and are particularly underutilized in the context of bone tissue [10,11]. This may partially be due to a lack of robust procedures to obtain specific cell types from bone for gene expression profiling. To begin to address these issues, we sought to develop methods to isolate and purify cells from the cortical bone matrix, using mouse long bones (femurs), with the eventual goal of developing methods to transcriptionally profile dozens of genes of interest, or potentially carry out whole genome profiling at the single cell level, similar to procedures we recently reported for the cardiomyocyte [12]. Briefly, the method isolates cells from the cortical

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bone, sorts them based upon canonical markers for osteoblast lineage, pre-amplifies the message using a targeted amplification, and finally analyzes the expression profiles of scores of cells simultaneously using nanofluidic qPCR. Subsequent analysis of single cell data provides data about the variance and co-expression of transcripts that are not observable in bulk tissue preparations.

Materials and methods

Animals

Five-month-old female C57BL/6J mice were used in this study. The mice were sacrificed via CO_2 overdose and cervical dislocation. Femurs were then immediately isolated. The bones were stripped of muscle and immediately placed in ice cold PBS (pH 7.4). The tissue was then prepared for immediate tissue digestion or prepared for long term preservation as described in Materials and Methods. All animal procedures were carried out under approved IACUC protocols of the Buck Institute for Research on Aging.

Cortical bone isolation and preservation

The collected bone samples were maintained in PBS on ice after their removal from the animal. The samples were submerged in a petri dish of PBS where any remaining soft tissue was stripped from the bone. The ends of the femur were then cut from the shaft of the bone using small scissors or a scalpel. The shaft of the femur was then thoroughly flushed using a 21-gauge needle and syringe of PBS to remove as much marrow material as possible. Note the difference in the appearance before and after (Fig. S1, top) cleaning the bone sample (Fig. S1, bottom). Once the bone samples are prepared it is possible to proceed directly to the digestion phase of the cortical bone cell isolation or preservation procedure for later analysis.

To preserve the bone for later analysis a method was modified from a procedure by Oh et al. [13] where the bone was quickly prepared for cryopreservation. Briefly, after the femurs were flushed with PBS to remove marrow they were placed into 2 ml cryovials containing a solution of M199 media containing 10% DMSO and 10% FBS. These samples were then placed into a 4 °C refrigerator to equilibrate for 30 min. The sample tubes were then transferred to a −80 °C freezer for storage for a minimum of 48 hours. Upon removal from the freezer the tubes were immediately thawed in a 37 °C water bath. The sample preserving solution was replaced with stepwise 5 min washes of M199 media containing 10% FBS supplemented with sucrose (0.5 M) to wash out any remaining DMSO from the tissue sample. This was followed by two more washes in media with 0.25 M sucrose and finally M199 media alone. From this point, the tissue sample was immediately processed using the digestion procedure described in Enzymatic removal of bone matrix.

Enzymatic removal of bone matrix

After preparing the bone sample as described above, the tissue is digested to yield a cell suspension. Two femur shafts are placed into one 1.6 ml conical tube that is then filled with collagenase digestion solution consisting of 0.2% collagenase in PBS (Worthington Biochemical, Type 2). This tube is then placed into a 37 °C shaking incubator for 30 min. After this incubation, the supernatant is removed and discarded. The bone is then pulverized using small scissors or an appropriate sized pestle. The bone was then further broken into smaller pieces leaving no large fragments. The tube is then filled with collagenase digestion solution and incubated for another hour at 37 °C with gentle shaking. After one hour of digestion, the tubes are removed from the incubator and the solution is gently triturated with a pipette. The solution should then appear opaque with digested material. The supernatant is carefully removed and transferred to a clean sterile 1.6 ml conical tube. The supernatant containing the cell population is then spun at 750 rpm for 8 min to pellet the cells. The supernatant is then removed taking care not to disturb the cell pellet. The cells were then resuspended with M199 media supplemented with 10% FBS, pH 7.4 to neutralize the collagenase. From this point, the bone-derived cells were prepared for either FACS analysis or immunocytochemistry staining.

Immunohistochemistry and immunocytochemistry

To identify bone specific markers within the bone cells, we prepared cell smears of the total population isolated from bone, or tissue sections of cortical bone. First, a concentrated cell suspension was placed onto a glass superfrost® plus charged slide and allowed to dry at room temperature. Once completely dry, the slide was rinsed with PBS and then incubated with 4% paraformaldehyde solution overnight. The next day the slides were processed for the presence of the osteoblast marker, alkaline phosphatase (AP) according to an immunohistochemistry protocol (see supplement for detailed protocol). The optimal concentration for the primary AP antibody was a 1:100 dilution (Sigma Anti-mouse IgG (whole molecule), Cat #A4312). The biotinylated secondary antibody was used at a concentration of 1:200, and visualized with VectaStain Elite DAB kit. Once stained, cells were mounted with Permount® and allowed to dry overnight. The following day the cells were imaged on a bright field microscope at 20× and 60× magnification.

For fluorescence immunocytochemistry of cortical bone sections we obtained paraffin embedded sections of a three month old C57Bl6 mouse from Zyagen laboratories. These sections were stained using the same directly conjugated Alkaline phosphatase antibody used in the FACS experiments (1:100; R & D systems, Cat #FAB1448P) and an antibody against Osteocalcin (1:100; Thermo Scientific, Cat #PA1-85754) with an Alexa Fluor 647 nm secondary antibody (1:1000; Life Technologies, Cat #A21447) using standard staining procedures. After staining, the sections were mounted with Prolong Gold Antifade reagent (Invitrogen), and dried for 48 hours prior to imaging. These sections were subsequently imaged using a Zeiss Confocal LSM 780 under 20× objective.

Cell staining and FACS analysis

The flow cytometry procedure was developed to determine the optimal treatment of cells to capture the osteoblast lineage cell population while maintaining the RNA quality so that it was suitable for nanofluidic PCR. The cells isolated from long bones were first incubated on ice for 20 min in purified rat anti-mouse CD16/CD32 Fc Block (BD Pharmingen, Cat #553142) diluted 1:100 in media. After incubation the cells were spun down at 250 ×*g* for 7 min. The supernatant was removed and the cells were resuspended with PBS. All wash steps were carried out in a similar manner. We chose CD31 and CD45 as cell surface markers to select against cells derived from the hematopoietic lineage, to enrich for osteoblasts in the resultant population. For the cell surface marker labeling of CD31 (BD Pharmingen PE-Cy7 rat anti-mouse CD31, Cat #561410) and CD45 (BD Pharmingen FITC rat anti-mouse CD45, Cat #553080), cells were incubated on ice with directly conjugated antibody for 30 min at a 1:100 dilution followed by washing. Labeling with the third antibody for alkaline phosphatase to define the osteoblast lineage cells requires a gentle fixation and permeabilization treatment to achieve sufficient labeling of this intracellular marker and to preserve the mRNA quality. This was performed using the BD Cytofix/Cytoperm Kit (Cat #554714) after staining for CD31 and CD45. Cells were incubated in the Fix/Perm solution for 20 min on ice, spun down, and stained with anti-human/mouse/rat alkaline phosphatase-phycoerythrin (R & D systems, Cat #FAB1448P) diluted 1:100 in PBS. NB: This step was omitted in some experiments to examine the qPCR signal from unfixed cells. After incubation, the cells were washed once