Advancing Bone Disease Research – Dissociated Rat Calvariae Osteoblasts Display Optimal Mineralization and Are Easily Transfected Using Nucleofection

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Abstract

High-quality, batch-tested dissociated rat calvariae osteoblasts, cryopreserved and ready-to-use, offer a standardized primary cell test bed approach for evaluating proliferation-mineralization *in vitro.* Combined with the Nucleofector™ System, which is an effective and time-saving transfection tool, this provides a potent *in vitro* system for investigating bone biology.

Introduction

The predominant research interest in bone biology has been osteoporosis, which is increasing in prevalence. Osteoporosis-related fractures are more common than the combined incidences of cardiac arrest, stroke and breast cancer. In North America, more than ten million people have this disease, and during their lifetime, approximately 30% of women and 20% of men will suffer an osteoporotic fracture.[1]

Toward more effective treatments, researchers are seeking to develop strategies for slowing down bone remodeling and thereby restore bone strength and integrity. Much of the osteoporosis research has been undertaken using primary bone cell cultures. Employing dissociated osteoblasts offers advantages for studying cell growth control mechanisms and mineralization, since osteoblasts are also responsible for the regulation of the differentiation and activity of osteoclasts. A key issue for studies employing primary cultures is having the dissociated bone cells retain the specific function of bone forming.[2]

The most extensively studied osteoblasts are those dissociated from calvariae and maintained in primary cultures.[3, 4, 5] These studies have now been made easier with the availability of batch-tested and hence "standardized" dissociated rat calvariae osteoblasts from Lonza, cryopreserved in their native state. This provides the researcher with the ideal tool for speeding the research workflow. The dissociated bone cells can be simply thawed and cultured and display excellent viability. We demonstrate herein the quality of the cryopreserved rat calvariae osteoblasts (developed by QBM Cell Science, Inc.) in long-term culture, and show the flexibility in their application for evaluating osteogenesis-mineralization, and the sensitivity of the cells to transfection employing the 4D-Nucleofector™ System. Nucleofection allows plasmids to be transfected directly into the nucleus[6, 7] with the advantage of better transfection rates, cell survival and quality of the cell culture post transfection, due to the specificity of the electroporation together with use of physiological buffers to optimize cell viability.[8, 9] This non-viral transfection system can be used to transfect cells and assist gene delivery to prescribed populations of cells.

Methods

Cells

Vials of frozen cells (Lonza Cat. No.: R-Ost-583) were thawed and prepared for cell culture studies and for transfection using the 4D-Nucleofector™ X Unit. Dissociated from the calvariae of Sprague Dawley rat embryos (E20,21), the osteoblasts are harvested and cryopreserved in the native state. For application in culture-based mineralization studies, the cells cannot be passaged since this affects the mineralization capacity of the cells. For proliferation studies, this is not a restrictive factor and the cells can be thawed and cultured through ten population doublings. Viability of the cryopreserved calvariae osteoblasts is guaranteed based upon batch sampling for morphology and mineralization assay using Alizarin Red, a biochemical marker to determine the presence of calcific deposition by cells, and staining for Alkaline Phosphatase (ALP), a biochemical and histochemical marker for primary osteoblasts in proliferative phase.

Morphological Assessment/Imaging

Fluorescent micrographs were used to assess viability, transfection efficiency, and morphology of the osteoblasts. Expression of GFP-fluorescent protein was examined together with immunohistochemical identification of the cell cultures directly in the wells using the commercially available antibody for vimentin (abcam Cat. No.: ab24525), α-actin (Millipore Cat. No.: CBL171) and phalloidin (Sigma Cat. No.: P1951). Cell nuclei were stained using Hoechst (Sigma Cat. No.: H6024). Expression of the T lymphocyte differentiation antigen, Thy-1, was examined using anti-Thy-1 (Chemicon MAB 1406).

All immuno- and bright field staining was viewed directly in the wells using a Zeiss inverted microscope, except for Thy-1 labeling where calvariae osteoblasts were cultured on cover slips for evaluation using a Zeiss transmission fluorescence microscope.

Mineralization Assays

Osteoblasts were prepared in 6-well plates and then cultured for up to 35 days. Rat osteoblast media for mineralization was prepared using 87 mL Dulbecco's modified Eagle Medium (DMEM) supplemented with 10 mL FBS (heat inactivated) and 1 mL penicillin/streptomycin. The medium was filtered and stored sterile. Prior to use, 50 µg/mL ascorbic acid (Sigma Cat. No.: A-4034), 10 mM s-glycerophosphate (Sigma Cat. No.: G-6376) and 0.1 µM dexamethasone were added. The mineralization cultures were fixed with 4% paraformaldehyde for 20 minutes at room temperature. After fixation, the cultures were washed with 10 nM PBS. The PBS was then removed and each well rinsed with 1–2 mL of Millipore H_2O. Cultures were then stained for ALP and for Alizarin Red S (Sigma Cat. No.: A3757).

For Alizarin Red staining, H_2O was removed and enough Alizarin Red stain was added to completely cover the cell layer, and the cultures were incubated at room temperature for 30 minutes. Wells were washed with Millipore H_2O to remove most of the dye, followed by another 2 – 3 washes at 5 – 10-minute intervals. Cultures were stored in 10 mM PBS + azide.

For the ALP assay, the PBS was removed from the culture well and BCIP/NBT Liquid Substrate (Sigma Cat. No.: B-1911) was added to completely cover the culture. The substrate stains cells blue-violet when ALP is present. The culture was incubated for 30 minutes at room temperature and then 10 mM PBS was added to the well to stop the reaction.

The cultures were then washed with 10 mM PBS.

Transfection

The cells were thawed and immediately used for Nucleofection. As the first step, optimal Nucleofection Conditions were determined in the 16-well 20 µL Nucleocuvette™ Strip following the recommendations of the Primary Cell Optimization Protocol using 4D-Nucleofector™ Solutions P1–P5 and 15 different programs. The optimal conditions were transferable to the 100 µL single Nucleocuvette™ Vessel. Per sample, 1×10^5 cells (in 20 µL strips) or 1×10^6 cells (in 100 µL cuvettes) were transfected with 2 µg/100 µL pmaxGFP™ Vector. After Nucleofection, prewarmed media was added and 100 µL of cells from each cuvette were transferred to a microtube containing 800 µL of warm media. Cells were plated in 96-well plates (0.8 or 1.6×10^5 cells/well), cultured 24 hours and then processed for morphological assessment.

Results and Discussion

Morphological Assessment of Osteoblast Cultures

Primary osteoblast cultures offer an excellent model system for the study of bone cell growth and differentiation related to a mineralizing matrix. Employing our optimized protocols, the cryopreserved dissociated calvariae osteoblasts were easy to culture and displayed normal morphology, growth and mineralization properties. The time course for morphological differentiation of the cryopreserved calvariae osteoblasts, following plating, was characterized for up to 21 days in culture. These can be compared with freshly prepared and cultured calvariae osteoblasts (Figure 1). All of the osteoblasts were positive for vimentin, a Type III intermediate filament (IF) protein. Osteoblasts showed a distinct morphology that could be easily distinguished from osteoclasts (which were rare) based upon size, shape and a single nucleus versus multiple nuclei (typical of osteoclasts).

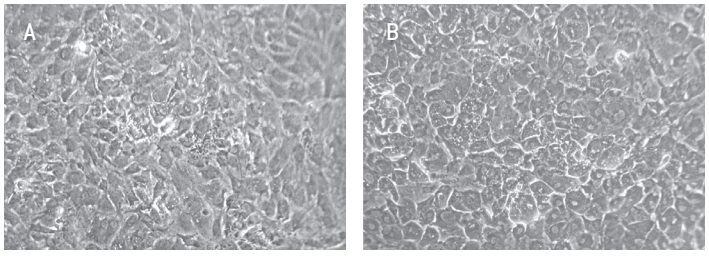

Figure 1
Comparison of cell morphology of freshly prepared versus cryopreserved calvariae osteoblasts in culture. The phase contrast images show osteoblasts dissociated and immediately cultured for 7 days in a 96-well plate **(A)**. Cryopreserved osteoblasts from the same dissociation batch were cryopreserved, then thawed and cultured for 7 days in a 96-well plate **(B)**.

The cytoskeleton filaments of osteoblasts are critical components for bone formation and normal signaling in response to mechanical stimulation. The actin filament cytoskeleton arrangement in the cultured cryopreserved calvariae osteoblasts can be seen in Figure 2. The calvariae osteoblast cultures displayed distinct populations of cells: those with predominant bundles of α-actin filament traversing the cytoplasm, or cells with a meshwork of thinner F-actin (phalloidin-positive) filaments. This is consistent with osteo-differentiation and maturation where there is remodeling of the actin cytoskeleton.

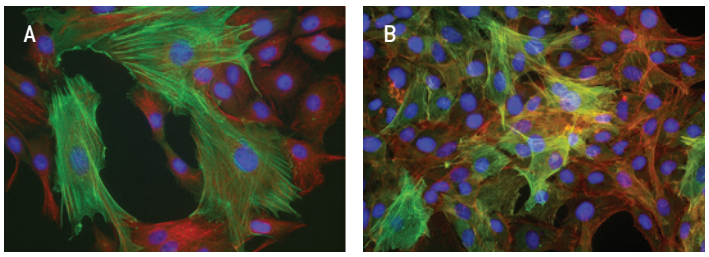

Figure 2
Fluorescence micrographs of cytoskeleton filaments. Cryopreserved calvariae osteoblasts thawed and cultured for 3 days were immunostained for **(A)** α-actin (green) and vimentin (red) or **(B)** α-actin (green) and phalloidin (red). Nuclei (blue) were stained with Hoechst.

Osteoblasts express Thy-1 antigen, and this expression has been proposed to be a useful differentiation marker for osteogenesis.[10] The cultures of cryopreserved calvariae osteoblasts displayed extensive Thy-1 immunoreactivity from Day 3 in culture (Figure 3). Almost all of the cells displayed some typical punctate Thy-1 labeling with many cells showing intense levels.

Furthermore, ALP (an enzyme that has a role in the mineralization of bone) activity is evident as early as Day 3 in our calvariae osteoblast cultures (Figure 4). Together, these are hallmarks of osteogenesis and the ability of these cells to mineralize is significant at 21 days in culture. By Day 35 there is profound mineralization and ALP activity (Figure 5).

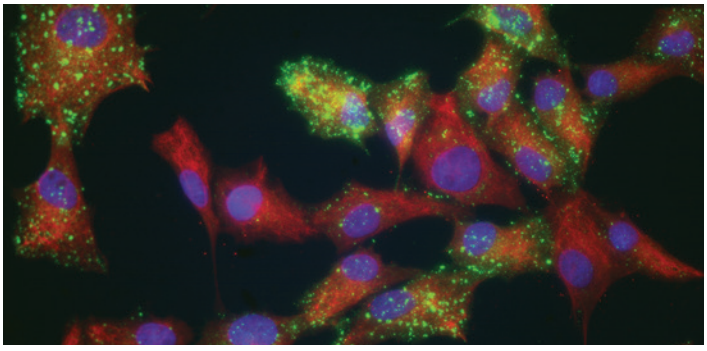

Figure 3
Fluorescence micrographs of immunohistological staining for Thy-1 antigen. Cryopreserved calvariae osteoblasts thawed and cultured for 3 days showed extensive staining for Thy-1 (green) which can be easily seen against vimentin (red) immunostaining. Blue = Hoechst-stained nuclei.

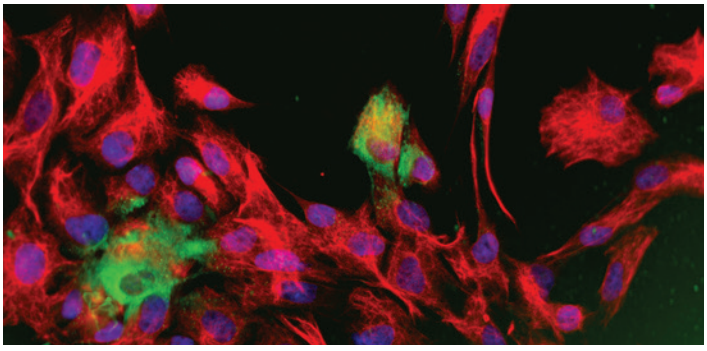

Figure 4
ALP localization in cryopreserved calvariae osteoblasts. Cells were thawed and cultured for 3 days. As shown by fluorescence microscopy, ALP activity (green) is evident in some osteoblasts easily identified in the culture stained with anti-vimentin.

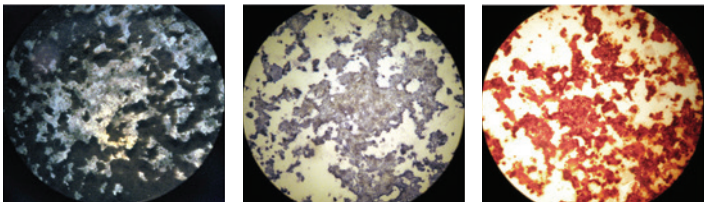

Figure 5
Mineralization of cryopreserved rat calvariae osteoblasts. Mineralization was examined using Alizarin Red-staining together with staining for ALP activity. Both were profound in cryopreserved calvariae osteoblasts thawed and cultured for 35 days.

Nucleofection of Rat Osteoblasts

Our results showed that high quality transfection of the cryopreserved dissociated osteoblasts could be obtained in suspension using the 4D-Nucleofector™ X Unit. Following the recommended optimization protocol for primary cells in the 20 µL Nucleocuvette™ Strips, we identified all of the 15 recommended programs to be effective for transfecting the osteoblasts. However, programs DS-150 and DS-137, employed together with P1 Primary Cell Solution, gave the optimal results. The results of the cuvette-based transfection under these conditions are shown in Figures 6 and 7. Following Nucleofection and plating, and culture in the 96-well format, the cells displayed typical osteoblast morphology. This was consistent across different plating densities (Figure 7). Based upon observer cell counts, Nucleofection using the 4D-Nucleofector™ X Unit gave a transfection rate of >50%.

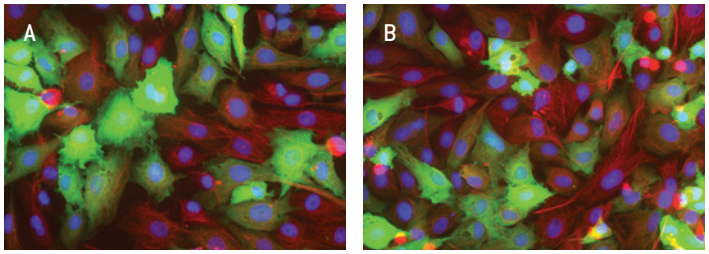

Figure 6
Transfection of cryopreserved calvariae osteoblasts using the 4D-Nucleofector™ X Unit. Cells were thawed and directly subjected to Nucleofection (1 × 10^6 cells/100 µL cuvette) using P1 Primary Cell Solution and program DS-150 **(A)** or DS-137 **(B)**. Post Nucleofection, cells were plated in 96-well plates with a plating density of 1.6 × 10^5 cells/well and fixed after 24 hours for fluorescence microscopy analysis. The cells were easily and efficiently transfected (green = maxGFP™ Protein) and show comparable morphology to the non-transfected cells (not shown here) which can be appreciated through immunostaining for vimentin (red).

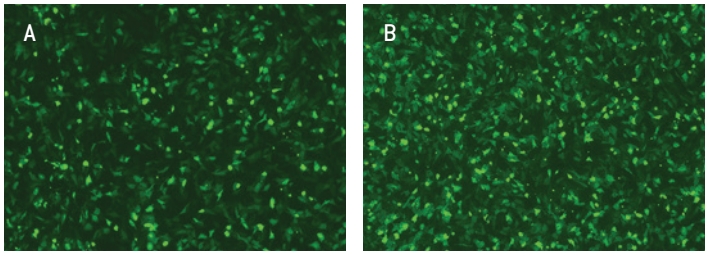

Figure 7
High quality transfection of rat calvariae osteoblasts using the 4D-Nucleofector™ X Unit. Cells were transfected using P1 Primary Cell Solution and program DS-150. Post Nucleofection, cells were plated at a density of 0.8 × 10^5 cells/well **(A)** or 1.6 × 10^5 cells/well **(B)** in 96-well plates. Expression of maxGFP™ Protein was analyzed after 24 hours and showed high quality transfection across cultures.

Conclusion

Together, these data show the quality and sensitivity of cryopreserved dissociated rat calvariae osteoblasts for *in vitro* studies. Furthermore, application of the 4D-Nucleofector™ System for efficient transfection of dissociated osteoblasts represents a significant advantage for investigating bone biology.

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