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Murine calvaria-derived progenitor cells express high levels of osterix and lose their adipogenic capacity

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ABSTRACT

Though the mouse is the most widely used biomedical animal model, it is difficult to isolate murine mesenchymal stem cells (MSCs) from the bone marrow because of contamination by hematopoietic cells. The murine compact bone tissue of long bones is considered a novel and reliable source of MSCs with low hematopoietic cell contamination. We investigated whether the murine compact bone of the calvaria would be a promising source of MSCs due to its low bone marrow content. We isolated cells from both long bones and the calvaria using the same method. Although they shared morphological features and surface antigens similar to those of long bone-derived MSCs, the calvaria-derived cells highly expressed the osteogenic transcription factor osterix, lost their adipogenic capacity and gained a higher osteogenic capacity. These findings suggest that the cells that migrated from the calvaria were progenitor cells rather than MSCs and that the differentiation fate of mesenchymal stem/progenitor cells existing in different murine compact bone deposits is already committed.

1. Introduction

Mesoderm-derived mesenchymal stem cells (MSCs) are present in many tissues postnatally and can differentiate into osteocytes, adipocytes and chondrocytes under appropriate conditions [1,2]. Aside from their multiple differentiation capacity, MSCs also play important roles in supporting hematopoiesis and in suppressing the immunoresponse of T lymphocytes. Because of these characteristics, the application of MSCs in cytotherapy is promising, such as their use in tissue repair and in reducing immunological rejection [3,4].

MSC culture was first developed by Friedenstein et al. from bone marrow, and since then, bone marrow has become the common source for isolating MSCs [5]. Thus far, MSCs have been isolated from the bone marrow of many species, including humans, rats and pigs. In contrast to other species, murine MSCs cannot be easily harvested from the bone marrow due to contamination by hematopoietic cells, and this contamination is difficult to eliminate using the characteristic plastic adherence of MSCs. Recent studies showed that the compact bone tissue of long bones is a novel source of MSCs for both humans and mice [6–8]. These studies developed a novel method to obtain high-purity murine MSCs by culturing collagenase-digested compact bone fragments. Some procedures in this method help avoid hematopoietic cell contamination, such as by removing bone marrow before collagenase digestion and discarding the released cells after collagenase digestion. Furthermore, subsequent experiments showed that compact bone is a richer source of MSCs than the marrow plug within it [9,10]. Based on these findings, we postulated that murine MSCs could be isolated from the compact bone of the calvaria, which has a relatively low bone marrow content. In this study, we found that cells migrating from the calvaria possess morphological characteristics and surface antigen profiles similar to those of MSCs derived from long bones. However, these calvaria-derived cells highly expressed the osteogenic transcription factor osterix. The calvaria-derived cells lost their adipogenic capacity but gained a higher osteogenic capacity. These results suggest that not all types of murine compact bones in the body are sources of MSCs and that the differentiation fate of mesenchymal stem/progenitor cells in different types of compact bones is already committed. The cells that migrate from the calvaria should be considered progenitor cells rather than MSCs.

2. Materials and methods

2.1. Animal

Wild-type inbred C57BL/6 mice (~1.5 weeks old) were purchased from the Experimental Animal Center of the Chinese Academy of Military Medicine Science. All experimental procedures

Abbreviations: CEBP, CCAAT/enhancer-binding protein; HPRT, hypoxanthine phosphoribosyltransferase; PPAR, peroxisome proliferator-activated receptor; Runx2, Runt-related transcription factor 2.

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fully complied with the relevant laboratory animal regulations of the Chinese Academy of Military Medicine Science.

2.2. Isolation and culture of compact bone-derived MSCs/progenitor cells from murine long bones and calvariae

To isolate long bone-derived MSCs, we followed previously described methods [9,10]. To isolate calvaria-derived cells, a similar approach was employed, but the bone marrow was not flushed. The two sources of cells were cultured in α-modified minimum essential medium (α-MEM, HyClone) containing 10% MSC-qualified fetal bovine serum (Gibco) at 37 °C in 5% CO_2.

2.3. Flow cytometry analysis

The two sources of third-passage cells were collected by trypsinization and incubated with phycoerythrin-conjugated monoclonal antibodies against mouse stem cell antigen-1 (Sca-1), CD29, CD11b, CD44, CD31, CD34, CD45 and their isotype IgG (eBioscience) for 30 min in the dark at room temperature. After three washes, the cells were analyzed in a FACSCalibur flow cytometer (Becton Dickinson).

2.4. Adipogenic and osteogenic differentiations assays

Adipogenic induction medium consisted of α-MEM supplemented with 10% fetal bovine serum, 10^{-6} M dexamethasone, 500 μM 3-isobutyl-1-methylxanthine and 10 μg/ml insulin (Sigma–Aldrich). Osteogenic induction medium consisted of α-MEM supplemented with 10% fetal bovine serum, 10^{-8} M dexamethasone, 100 μg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma–Aldrich). For alizarin red staining, cells exposed to osteogenic induction medium for 21 days were fixed with 4% formaldehyde for 10 min and then stained with alizarin red solution (1% alizarin red and 2% ethanol in distilled water) for 15 min at room temperature. Excess stain was removed by washing with distilled water several times prior to observation. Oil red-O staining and von Kossa staining were performed according to the instructions provided in a previous study [11].

2.5. Real-time quantitative PCR analysis

The total RNA was extracted using the GeneJET RNA purification kit (Fermentas). The subsequent reverse transcription reactions were performed with a ReverTra Ace Kit (TOYOBO). Real-time quantitative PCR was performed with a SYBR PCR Master Mix Kit (TOYOBO) and 0.3 μM specific primers in a 25 μl volume. The data were acquired using an iQ5 system (Bio-Rad). Specific primers to the following genes were used:

HPRT: 5′-GTGATTAGCGATGATGAACC-3′(forward); 5′-AAGTCT TTCAGTCCTGTCCA-3′ (reverse). PPARγ: 5′-GTCATCACAGACACCC TCTC-3′ (forward); 5′-CATATTCGACACTCGATGTTCAG-3′(reverse). CEBPα: 5′-CAAGAACAGC- AACGAGTACCG-3′ (forward); 5′-GTCACT GGTCAACTCCAGCAC-3′ (reverse). aP-2: 5′-GTGAAGAGCATCATAA CCCT-3′(forward); 5′-TTTCATAACACATTC- CACCACC-3′ (reverse). Runx2: 5′-CTAGTTTGTTCTCTGATCGCC-3′ (forward); 5′-GCCTGGG ATCTGTAATCTG-3′ (reverse). osterix: 5′-ATGGCGTCCTCTCT- GCT TG-3′ (forward); 5′-TGAAAGGTCAGCGTATGGCTT-3′ (reverse). osteo- calcin: 5′-CTGACAAAGCCTTCATGTCC-3′ (forward); 5′-CTAC CACTATGGA- AGGCTAAGG-3′ (reverse).

2.6. Statistical analysis

The data were analyzed with the paired Student's *t* test. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Cells that migrate from the calvaria have morphological features and surface antigens similar to those of long bone-derived MSCs

Historically, isolating murine MSCs from the bone marrow has been difficult due to contamination caused by hematopoietic cells. In the past few years, several research groups have obtained high-purity murine MSCs by cultivating collagenase-digested long bone fragments after discarding bone marrow. Prompted by these findings, we hypothesized that the compact bone from the calvaria would be a better source of MSCs due to its lower bone marrow content and the simpler procedures involved in isolating MSCs from the calvaria. To test this hypothesis, we cultivated collagenase-digested bone fragments from calvariae using long bone-derived bone fragments as a positive control. After two days in culture, similar to their counterparts from long bone, fibroblastic cells were observed migrating from the bone fragments in the calvaria-derived culture (Fig. 1A). In the next few days in both cultures, a steady stream of cells migrated from the bone fragments, adhered to the plastic flask and proliferated (Fig. 1A). There was no visible difference in the morphologic features of calvaria-derived cells compared to MSCs that migrated from the long bone.

Next, we analyzed the surface antigens of the cells from both sources. According to previous studies, murine long bone-derived MSCs do not display purity until the third passage [9,10]. In our study, based on this knowledge, both the third-passage long bone-derived MSCs and calvaria-derived cells were analyzed by flow cytometry to determine their surface antigens. As shown in Fig. 1B, approximately 95% of the cells in both cohorts expressed surface antigens indicative of an MSC phenotype, including Sca-1 (murine pan-stem/progenitor cell marker), CD29 (receptor for extracellular matrix components) and CD44 (receptor for osteopontin and hyaluronate). Meanwhile, the two cohorts of cells were homogeneously negative for CD45 (hematopoietic maker), CD11b (monocyte marker) and CD31 (endothelial marker). These results indicate that calvaria-derived cells display cell surface antigens similar to those of long bone-derived MSCs.

3.2. Calvaria-derived cells display extremely high osterix expression

Multiple differentiation capacity is a vital characteristic of MSCs. The differentiation program of MSCs is driven by lineage-specific transcription factors, which respond to stimulation signals and activate the transcription of various of cell type-specific genes. Though the cells derived from long bones and calvariae shared similar morphologic and immuno-phenotypic features, we further investigated the expression of some lineage differentiation markers to test whether calvaria-derived cells are identical to long bone-derived MSCs. After passage to the third generation in growth medium, we employed real-time quantitative PCR to measure the basal levels of several adipogenic and osteogenic marker mRNAs in these two groups of cells. The adipogenic markers were PPARγ, CEBPα and adipocyte protein 2 (aP-2), a fatty acid-binding protein. Compared to the long bone-derived MSCs, the calvaria-derived cells demonstrated 2- to 3-fold decreases in CEBPα and aP-2 expression. By contrast, PPARγ expression was comparable between both groups of cells (Fig. 2A).

The osteogenic transcription factors Runx2 and osterix and a bone-specific protein, osteocalcin (OC), were measured by real-time quantitative PCR as osteogenesis indicators. As shown in Fig. 2B, the calvaria-derived cells expressed 20 times more osterix than MSCs derived from the long bone, but no significant differences were observed in Runx2 or OC transcription.

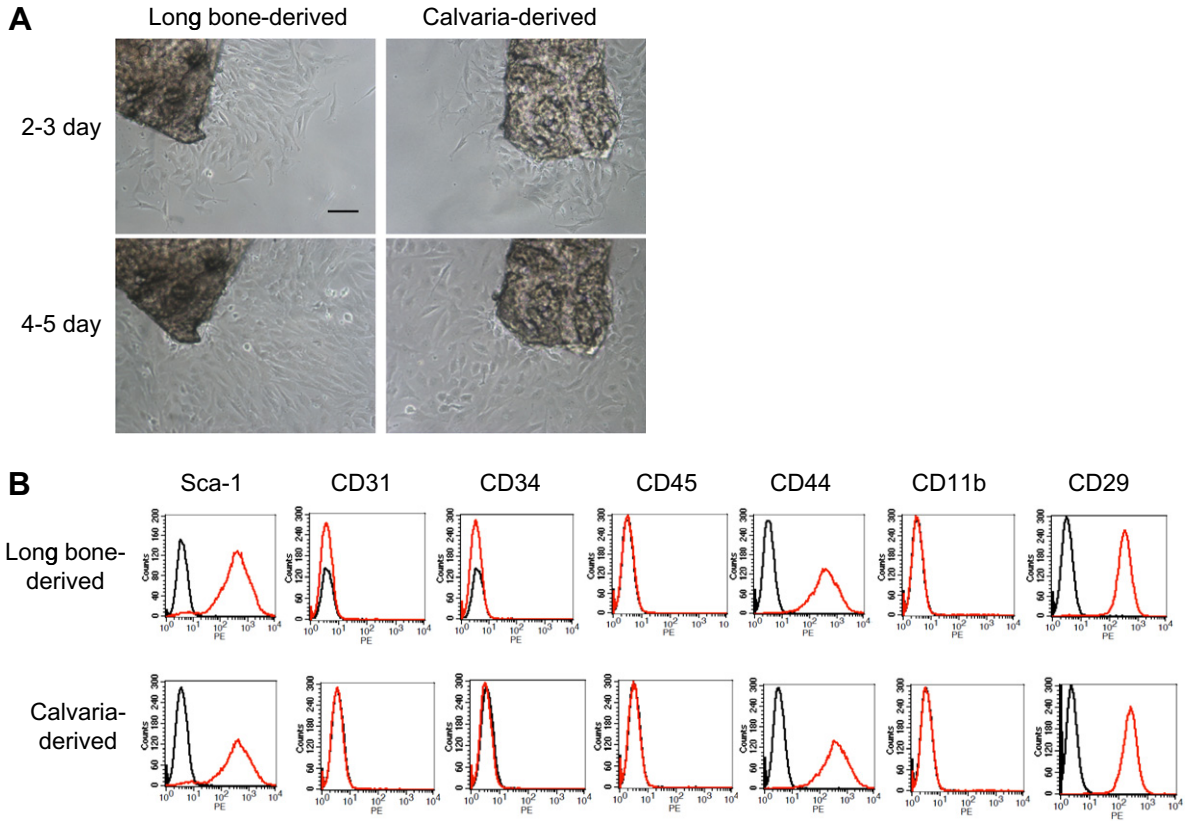

Fig. 1. There are no significant differences in morphological features or surface antigens between calvaria-derived cells and long bone-derived MSCs. (A) The cells that migrated from long bone chips and from calvaria chips possessed similar morphological features. The scale bar represents 100 μm. (B) Cells from the two sources also showed similar surface antigens. Red line, antibody; black line, the corresponding IgG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Calvaria-derived cells lack adipogenic capacity

On the basis of the above observations, we investigated whether the differences in the expression of these differentiation markers between the two cohorts of cells would be amplified during differentiation induction and would therefore change the cells' differentiation capacity. We first compared their adipogenic differentiation capacity. The third-passage cells of both sources were collected by trypsinization and then reseeded at the same cell density. At confluence, they were cultured in normal control or adipogenic induction medium. After six days, lipid droplet-containing cells became evident in long bone-derived MSC culture exposed to adipogenic stimuli, but no cells with similar characteristics were found in their counterparts derived from calvariae (Fig. 3A). In accordance with changes in morphology, real-time quantitative PCR showed that the transcript levels of adipogenic marker genes in the calvaria-derived cells were dramatically lower than those of long bone-derived MSCs when cultured in adipogenic induction medium (Fig. 3B). Moreover, significant differences were observed in normal growth medium after six days of culture (Fig. 3B). When long bone-derived MSCs were exposed to adipogenic stimuli for 14 days, the previously observed small cytosolic lipid droplets converged into a large lipid droplet, and the cells acquired a mature adipocyte phenotype. In contrast, no lipid droplet-containing cells formed in the calvaria-derived culture by day fourteen (Fig. 3A).

3.4. Calvaria-derived cells display enhanced osteogenic capacity

We next investigated whether the higher osterix expression accompanied higher osteogenic capacity in the calvaria-derived cells. Trypsinized third-passage cells of both cohorts were reseeded at the same cell density and cultured in normal control or osteogenic induction medium. After eight days of induction, real-time quantitative PCR was utilized to analyze relevant osteogenic marker genes. In both control and osteogenic induction medium, the calvaria-derived cells displayed significantly higher transcription levels of Runx2, osterix and OC than the MSCs derived from long bone (Fig. 4A). During differentiation, osteoblasts secrete large amounts of matrix (composed mainly of type I collagen), mineralize the matrix and become mature osteocytes. After 21 days of induction, the two groups of cells were subjected to von Kossa staining (brown) and alizarin red staining (red) to evaluate extracellular mineralization deposits. In accord with the previous molecular analysis, the calvaria-derived cells showed more enhanced and extensive mineralization than long bone-derived MSCs (Fig. 4B).

4. Discussion

In recent years, a novel method of isolating MSCs from compact bone was developed successfully in both humans and mice. Moreover, compact bone-derived MSCs display higher purity and yield than their counterparts derived from bone marrow [6–10]. However, for mice, this method requires flushing the bone marrow and washing the cavity thoroughly to avoid possible contamination by hematopoietic cells [9,10]. In this study, to simplify the procedure as well as to avoid contamination, we isolated MSCs by culturing collagenase-digested bone chips of murine calvariae. We chose the calvaria because it has a low bone marrow content. The calvaria-derived cells shared morphologic features and surface antigens similar to those of MSCs cultured from the long bone. Unlike MSCs, the adipogenic capacity of the calvaria-derived cells

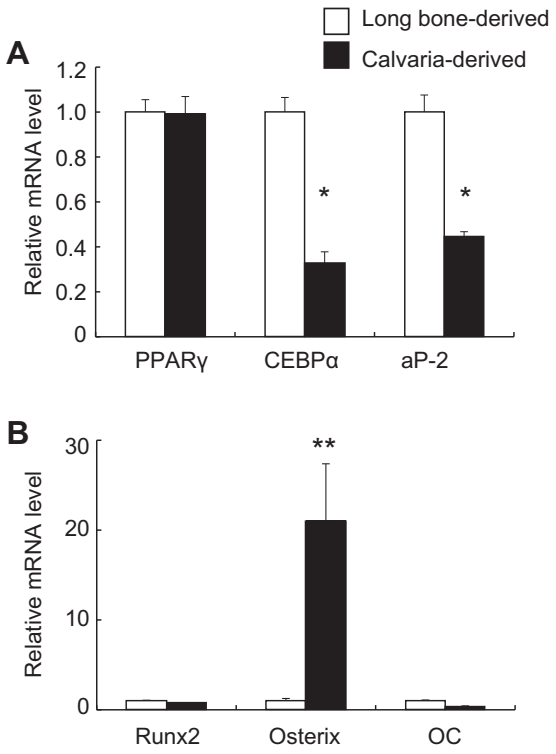

Fig. 2. Real-time quantitative PCR analysis of marker genes in both groups of cells in basal growth medium. The mRNA levels of adipogenesis marker genes (A) and osteogenesis marker genes (B) in cells from the two sources were analyzed with real-time quantitative PCR in the basal (non-differentiating) state. The data are representative of three independent experiments after normalization to HPRT and are presented as the mean ± standard deviation (SD). $^{*}P<0.05$ and $^{**}P<0.01$.

was deficient, but their osteogenic capacity was enhanced. These findings suggest that cells that reside in murine calvariae are progenitor cells rather than MSCs. These results also indicate that not every compact bone deposit in the murine body is a source of MSCs and that the fate of mesenchymal stem/progenitor cells in compact bone deposits is already committed. Because the osteogenesis mechanism is relatively conserved in mammalian species, similar situations might also exist in humans and other species.

Because no specific surface antigens for MSCs have been defined, various surface antigens were analyzed to identify MSCs collectively. The selection of surface antigens was imperfect because the calvaria-derived cells shared these surface antigens with MSCs derived from long bones. For this reason, it is necessary to identify more specific surface antigens for the identification of MSCs.

In addition, long bones and calvariae are two typical types of compact bone. Endochondral ossification and intramembranous ossification are two major mechanisms of developing bone tissue. Long bone formation and development are considered endochondral ossification, while calvariae are formed by intramembranous ossification [12–14]. According to our results, the calvaria-derived cells displayed different committed fates compared with long bone-derived MSCs. Further investigation is needed to determine whether these differences are related to the two different ossification pathways.

Osterix, the crucial osteogenic transcription factor, was expressed more highly in the calvaria-derived cells than in MSCs derived from long bones in this study, whereas Runx2, another crucial osteogenic transcription factor, showed no significant difference in expression between the two cell types. Osterix and Runx2 are essential osteogenic transcription factors, and a deficiency in either leads to a lack of bone formation in mouse models [15–16]. Osterix is a downstream target gene of Runx2. According to our findings, the calvaria-derived progenitor cells expressing

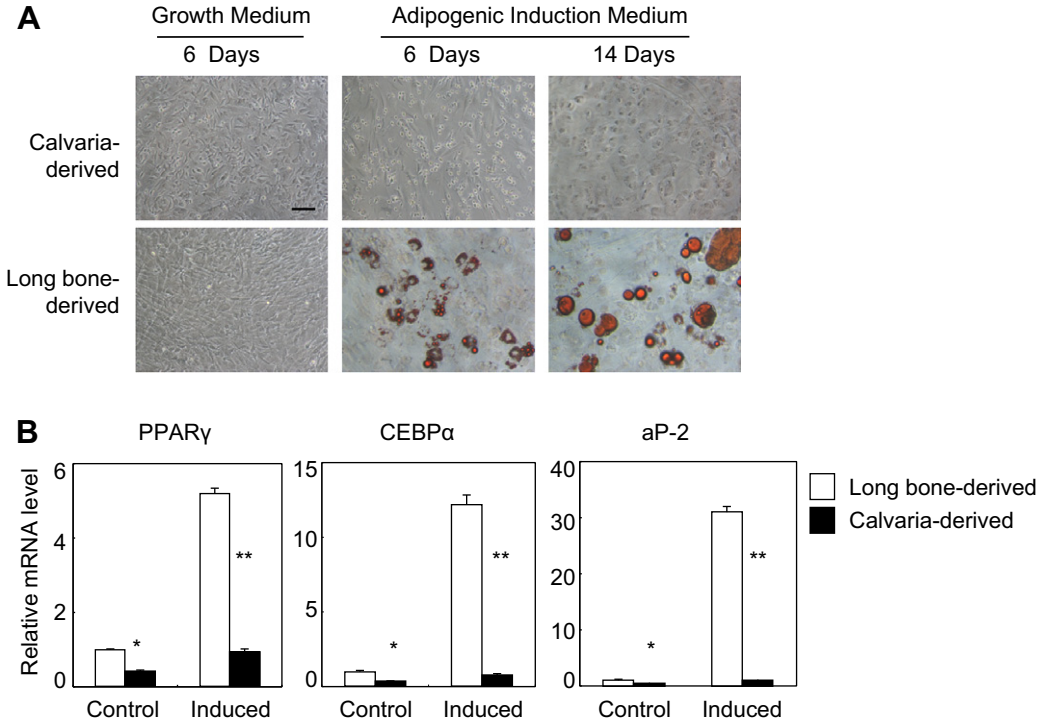

Fig. 3. The calvaria-derived cells have deficient adipogenic capacity. (A) Adipogenic capacity was evaluated by oil red-O staining in both groups of cells at the indicated time points and conditions. The scale bar represents 100 μm. (B) Adipogenic marker genes were measured by real-time quantitative PCR in both groups of cells exposed to adipogenic stimulation or normal (control) medium for six days. The data are representative of three independent experiments after normalization to HPRT and are presented as the mean ± SD. $^{*}P<0.05$ and $^{**}P<0.01$.

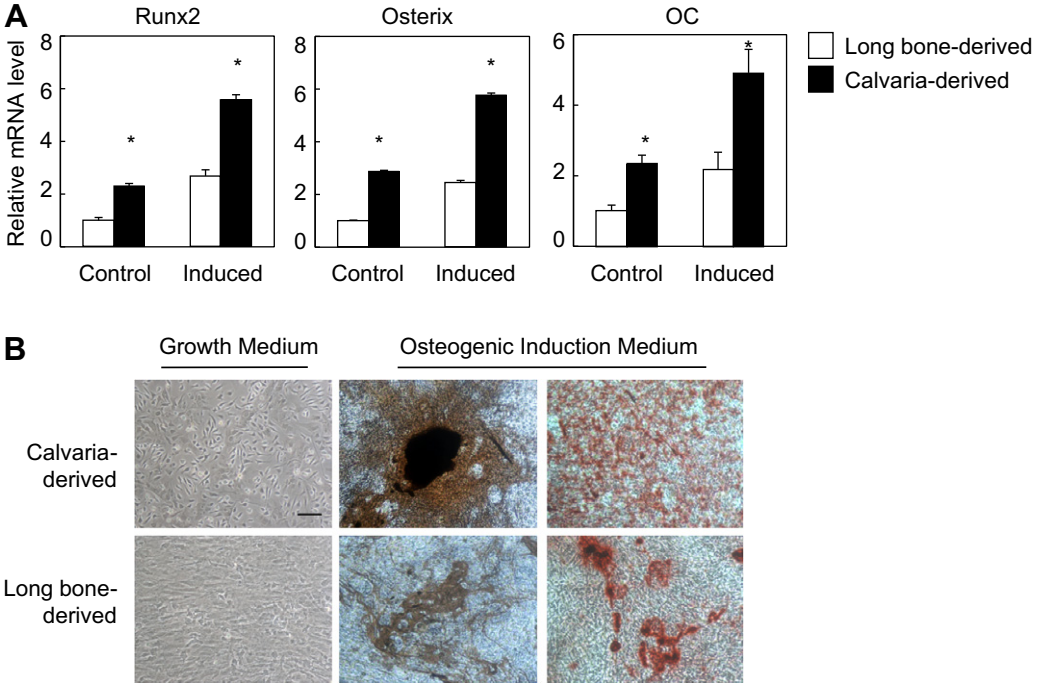

Fig. 4. The calvaria-derived cells display enhanced osteogenic capacity compared to long bone-derived MSCs. (A) Osteogenic marker genes were measured by real-time quantitative PCR in both groups of cells exposed to osteogenic stimulation or normal (control) medium for eight days. The data are representative of three independent experiments after normalization to HPRT and are presented as the mean ± SD. *$P<0.05$. (B) Extracellular mineralization deposits were detected with von Kossa staining (brown) and alizarin red staining (red) in both groups of cells after 21 days of osteogenic induction. The scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

high levels of osterix demonstrated deficient adipogenesis and enhanced osteogenesis. Whether osterix rather than Runx2 regulates the differentiation fate decisions of mesenchymal stem/progenitor cells needs further investigation.

Acknowledgments

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