Genes & Diseases (2024) 11, 546–549

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RAPID COMMUNICATION

Isolation and culture of human primary osteoblasts: Comparing the effects of differences in method details on osteoblast characteristics

Osteoblasts are essential in the maintenance of human bone homeostasis.[1] The abnormal formation and impaired differentiation ability of osteoblasts are pivotal factors leading to bone-related diseases, suggesting that an in-depth study of osteoblasts can provide novel treatment strategies for these diseases.[2] Human primary osteoblasts are precious experimental models in bone-related research.[3] However, the isolation and culture of human primary osteoblasts still have some difficulties and limitations. Here, we refined a method to isolate and culture human primary osteoblasts by taking cancellous bone through a bone tissue sampler and pre-digesting it with pancreatin, which makes the process easier, cheaper, and more efficient. Furthermore, we compared the effects of the subtle changes in isolation and culture methods on the phenotype of human primary osteoblasts and examined the differences in morphology, proliferation, and differentiation between micro-explants cultured osteoblasts (MEC-OBs) and explants cultured osteoblasts (EC-OBs). Our results showed that although both MEC-OBs and EC-OBs have prominent osteoblastic phenotypes, they still have their own characteristics. Collectively, this improved method could reduce the difficulty and provide benefits for the research and application of human primary osteoblasts, such as human bone-related organoid culture and medical treatment of bone-related diseases.

In our study, we improved the isolation and culture method of human primary osteoblasts, the specific operation steps and advantages of the method were described in detail in the supplementary data, and we also provided the operation video of this method in the supplementary material. During the culture process, we continuously observed the morphological changes of human primary osteoblasts under the microscope at different culture times (Fig. 1A). Digested cells and micro-explants that can pass through the cell strainer with 70 μm pores were collected and cultured. When cultured for 4–6 days, several cells grew out and attached to the bottom of the culture dish. Subsequently, the cell processes gradually extended, and the cells became longer and larger, forming the spindle, triangle, or polygonal osteoblast morphology (Fig. 1A). As the cells continued to proliferate, about 30 days later, the cells fully converged. Then hematoxylin and eosin (HE) staining was performed on MEC-OBs and EC-OBs before and after induction to further observe their morphology. Before induction, MEC-OBs and EC-OBs were mainly triangular and polygonal, and the nuclei-stained purple by hematoxylin was round or oval (Fig. 1B). After induction, MEC-OBs and EC-OBs became long fusiform as the increase in growth density (Fig. 1B). Comparing these two types of cells, we found that MEC-OBs were more uniform in cell size and morphology, while EC-OBs showed polymorphism in cell size and morphology. This implies that the differentiation stage of MEC-OBs may be more synchronized and MEC-OBs are more homogeneous, while the differentiation stage between EC-OBs may have a larger span.

Furthermore, we examined the proliferation characteristics of MEC-OBs and EC-OBs. During 0–3 days, the proliferation of MEC-OBs and EC-OBs were basically the same (Fig. 1C). Subsequently, the proliferation rate of MEC-OBs slowed down and reached the plateau on day 6, while the proliferation rate of EC-OBs was higher than that of MEC-OBs and reached the plateau on day 7 (Fig. 1C). This indicates that compared with MEC-OBs, EC-OBs have stronger and more lasting proliferation activity.

We also explored the differences in the expression of human osteoblast markers in MEC-OBs, EC-OBs, and MG-

Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2023.03.024

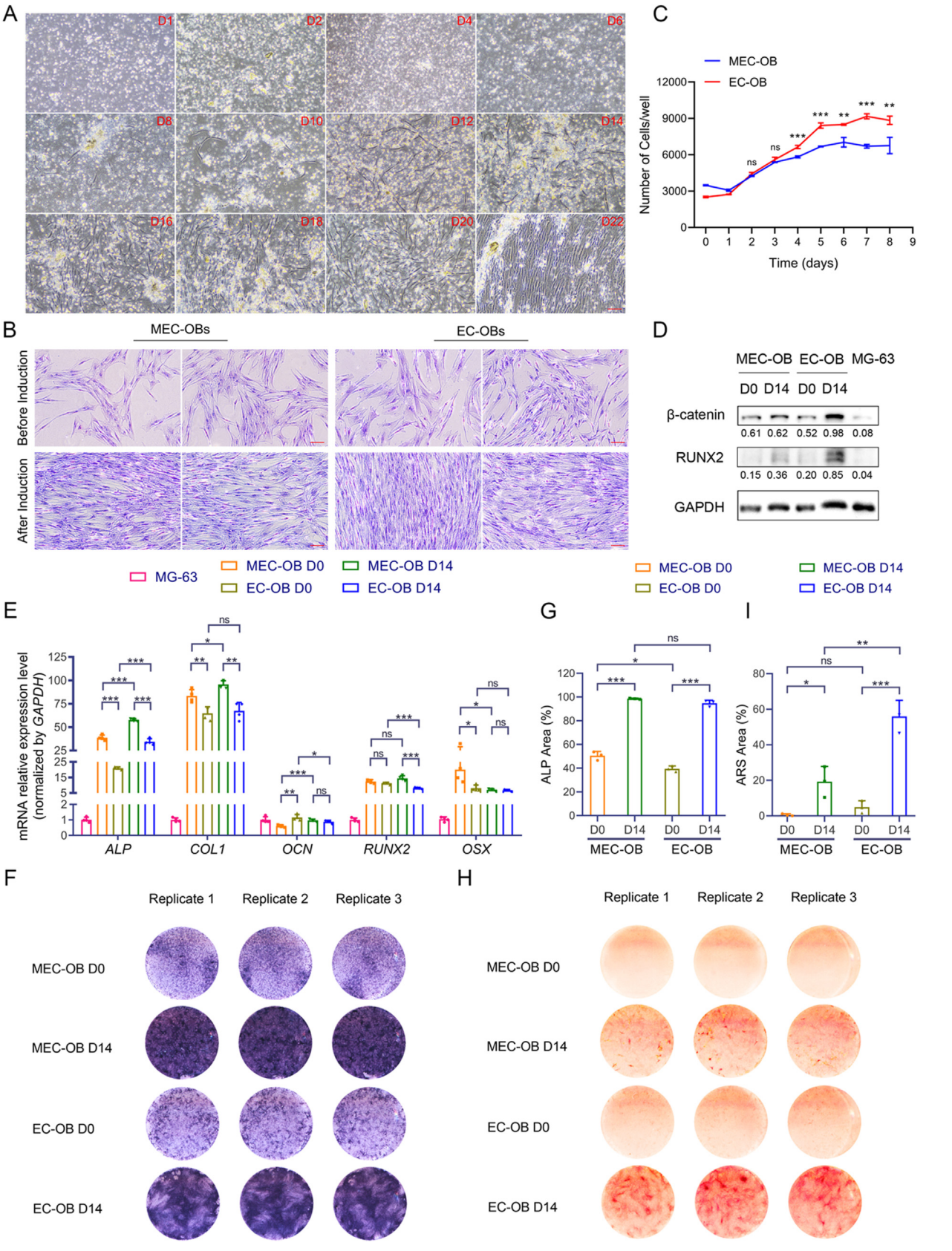

Figure 1 Characterization of MEC-OBs and EC-OBs in morphology, proliferation, osteogenic markers expression, differentiation, and mineralization. **(A)** Morphology of human primary osteoblasts during growth. The morphology of human primary osteoblasts was observed by phase contrast microscopy on days 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 of growth, respectively. Scale bar = 100 μm. **(B)** HE staining of MEC-OBs and EC-OBs before and after induction. Scale bar = 100 μm. **(C)** The proliferation curves

63 cells. Before and after induction of MEC-OBs and EC-OBs, the mRNA expressions of alkaline phosphatase (*ALP*), collagen type I (*COL1*), Runt-related transcription factor 2 (*RUNX2*), and Osterix (*OSX*) were significantly higher than that of MG-63 (*ALP*: ~20- to 60-fold, *COL1*: ~50- to 100-fold, *RUNX2*: ~10- to 15-fold, *OSX*: ~5- to 30-fold), indicating that human primary osteoblasts cannot be replaced by osteosarcoma cell lines in terms of osteoblast phenotype characterization (Fig. 1E). Before induction, the mRNA expression of early osteoblast differentiation markers (*ALP*, *COL1*) in MEC-OBs was significantly higher than that of EC-OBs (*ALP*: $P = 2.32 \times 10^{-5}$, *COL1*: $P = 0.00862$), while the mRNA expression of late osteoblast differentiation marker osteocalcin (*OCN*) was significantly lower than that of EC-OBs ($P = 0.00165$), suggesting that compared with MEC-OBs, EC-OBs contained more osteoblasts that were in the late stage of differentiation (Fig. 1E). Compared with before induction, the mRNA expression of *ALP*, *COL1*, and *OCN* in MEC-OBs increased significantly after induction, while only the mRNA expression of *ALP* in EC-OBs increased significantly after induction (Fig. 1E). This indicates that MEC-OBs are more sensitive to differentiation induction than EC-OBs. Before and after induction of MEC-OBs and EC-OBs, the protein expressions of RUNX2 and β-catenin were obviously higher than that of MG-63 (RUNX2: ~4- to 21-fold, β-catenin: ~7- to 13-fold), which further supports our conclusion at the transcriptional level that the phenotype of human primary osteoblasts is difficult to mimic by osteosarcoma cell lines (Fig. 1D). Compared with before induction, the protein expressions of RUNX2 and β-catenin in EC-OBs both increased significantly after induction, while only the protein expression of RUNX2 in MEC-OBs increased significantly after induction (Fig. 1D). This suggests that β-catenin-dependent Wnt signaling has an important role in promoting the differentiation of EC-OBs, whereas the differentiation of MEC-OBs is mainly promoted by other β-catenin-independent pathways.

To further investigate the differences in osteoblasts differentiation and mineralized nodule formation between MEC-OBs and EC-OBs, we performed ALP and alizarin red S (ARS) staining on MEC-OBs and EC-OBs before and after induction. The ALP activities of MEC-OBs and EC-OBs were significantly increased after induction (MEC-OBs: $P = 2.12 \times 10^{-5}$, EC-OBs: $P = 1.21 \times 10^{-5}$), indicating that both types of osteoblasts are sensitive to differentiation induction and have robust differentiation ability. Further observation of the staining results found that the ALP activity of MEC-OBs was significantly higher than that of EC-OBs before induction (Fig. 1F, G). In addition, in MEC-OBs, ALP activity was more even between cells, while in EC-OBs, there were clusters of cells with higher ALP activity, and this phenomenon is more obvious after induction (Fig. 1F). This further demonstrates that, compared with EC-OBs, MEC-OBs have more uniform expression profiles and MEC-OBs are more homogeneous. Compared with before induction, the calcium deposits of MEC-OBs and EC-OBs were significantly increased after induction (MEC-OBs: $P = 0.0201$, EC-OBs: $P = 0.000813$), indicating that both types of osteoblasts are sensitive to differentiation induction and have robust mineralization ability. Further observation of the staining results found that the calcium deposits of EC-OBs were significantly more than that of MEC-OBs after induction (Fig. 1H, I). This demonstrates that EC-OBs have stronger mineralization ability than MEC-OBs, which further implies that, compared with MEC-OBs, EC-OBs contained more osteoblasts that were in the late stage of differentiation.

In summary, with our improved method, we obtained two kinds of human primary osteoblasts, MEC-OBs and EC-OBs, both of which have excellent osteoblast characteristics. Furthermore, we compared the phenotypic differences between MEC-OBs and EC-OBs. Based on our results, we speculated that the differentiation stages of the cells in the MEC-OBs population were relatively synchronized, and most of the MEC-OBs were in the matrix maturation stage at the time of detection; while the differentiation stages of the cells in the EC-OBs population span a wide range, and EC-OBs may contain osteoblasts in various differentiation stages at the same time, and contain more osteoprogenitors and late osteoblasts. In addition to their use in basic research, our method to isolate and culture human primary osteoblasts could also contribute to clinical and preclinical personalized medicine and drug development if further combined with single-cell multi-omics technologies,[4] genome editing, and bone organoid culture.[5] In general, human primary osteoblasts still have an expansive application space to be developed.

Ethics declaration

All procedures performed in this study involving human samples were in accordance with the ethical standards of the Ethics Committee of Xi'an Jiaotong University.

Author contributions

YG, BY, TLY, and YZ conceived and designed the study. BY and BCL provided the human cancellous bone samples. FC, YZ, PP, and XTH performed the experiments. FC, YZ, and

of MEC-OBs and EC-OBs. The optical density (OD) values at different time points were measured by the CCK8 kit and the corresponding number of cells was calculated from the OD values. $n = 3$. **(D)** Real-time PCR detected mRNA expression of human osteoblast markers in MEC-OBs and EC-OBs before and after induction and in MG-63 cells. D0 means no induction and D14 means induction for 14 days. $n = 4$. **(E)** Western blot detected protein expression of human osteoblast markers in MEC-OBs and EC-OBs before and after induction and in MG-63 cells. The numbers below the bands represent the ratio of the gray value of the target protein to the gray value of GAPDH. **(F)** Scanned images of the ALP staining results of MEC-OBs and EC-OBs before and after induction. **(G)** Histogram of the ALP staining results of MEC-OBs and EC-OBs before and after induction. $n = 3$. **(H)** Scanned images of the ARS staining results of MEC-OBs and EC-OBs before and after induction. **(I)** Histogram of the ARS staining results of MEC-OBs and EC-OBs before and after induction. $n = 3$. Data were presented as mean ± standard deviation. ns, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

ZHQ wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors have declared that no conflict of interests exists.

Funding

This work was supported by the National Natural Science Foundation of China (No. 82170896, 31970569), Science Fund for Distinguished Young Scholars of Shaanxi Province, China (No. 2021JC-02), Innovation Capability Support Program of Shaanxi Province, China (No. 2022TD-44), China Postdoctoral Science Foundation (No. 2021M692582), Natural Science Basic Research Program of Shaanxi Province, China (No. 2022JQ-808, 2023-YBSF-180), and Fundamental Research Funds for the Central Universities (China) (No. xzy012022032).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.03.024.

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3 November 2022
Available online 28 April 2023

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