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### Authors

Yang, Haili  
Sun, Lei  
Cai, Wenqian  
[et al.](#)

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## DDR2, a discoidin domain receptor, is a marker of periosteal osteoblast and osteoblast progenitors

Haili Yang<sup>1</sup>, Lei Sun<sup>1</sup>, Wenqian Cai<sup>1</sup>, Jingkai Gu<sup>1</sup>, Dacai Xu<sup>1,2</sup>, Arjun Deb<sup>3</sup>, Jinzhu Duan<sup>1</sup>

<sup>1</sup>Heart Center and Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, No. 9 JinSui Road, Guangzhou 510120, Guangdong, China

<sup>2</sup>Institute Pasteur of Shanghai, Chinese Academy of Science, Shanghai 200031, China

<sup>3</sup>Division of Cardiology, Department of Medicine, Department of Molecular Cell and Developmental Biology, UCLA Cardiovascular Medicine Research Theme, Eli and Edythe Broad Institute of Regenerative Medicine and Stem Cell Research, David Geffen School of Medicine, University of California, Los Angeles, 675 Charles E Young Drive S, MRL 3609, Los Angeles, CA 90095, USA

### Abstract

**Introduction**—The periosteum has a bilayered structure that surrounds cortical bone. The outer layer is rich in connective tissue and fibroblasts, while the inner layer in contact with the cortical surface of the bone predominantly consists of osteoblasts and osteoblast progenitors. The identification of cell-specific surface markers of the bilayered structure of the periosteum is important for the purpose of tissue regeneration.

**Materials and methods**—We investigated the expression of the discoidin domain tyrosine kinase receptor DDR2, fibroblast specific protein-1 (FSP-1) and alkaline phosphatase (ALP) in the periosteum of cortical bone by immunohistochemistry. Osteogenic differentiation was compared between DDR2- and FSP-1-expressing cells flow-sorted from the periosteum.

**Results**—We showed that DDR2 predominantly labeled osteogenic cells residing in the inner layer of the periosteum and that Pearson's coefficient of colocalization indicated a significant correlation with the expression of ALP. The mineralization of DDR2-expressing osteogenic cells isolated from the periosteum was significantly induced. In contrast, FSP-1 predominantly labeled the outer layer of periosteal fibroblasts, and Pearson's coefficient of colocalization indicated that FSP-1 was poorly correlated with the expression of DDR2 and ALP. FSP-1-expressing periosteal fibroblasts did not exhibit osteogenic differentiation for the induction of bone mineralization.

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Arjun Deb, adeb@mednet.ucla.edu. Jinzhu Duan, duanjinzhu@163.com.

**Author contributions** HY performed most of the experiments and data analysis with LS, JG and DC, WQ, AD and JD designed the study and wrote the manuscript.

**Conflict of interest** The authors report no conflict of interest.

**Ethical approval** All animal studies were approved by the Institutional Animal Care and Use Committee of the Guangzhou Medical University (the acceptance number:2016-025) and all animal procedures conform to the National Institutes of Health (NIH) guidelines. This article does not contain any studies with human participants performed by any of the authors.

**Informed consent** No informed consent needed.

**Conclusion**—DDR2 is a novel potential cell surface marker for identifying and isolating osteoblasts and osteoblast progenitors within the periosteum that can be used for musculoskeletal regenerative therapies.

### Keywords

Osteoblasts; DDR2; FSP-1; Periosteum; Alkaline phosphatase

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### Introduction

The periosteum is a dense bilayered connective tissue that surrounds cortical bone [1]. The inner layer of the periosteum, also known as the cambial layer, is rich in osteoblasts and osteoblast progenitors, while the outer layer of the periosteum is rich in fibroblasts [2]. The periosteum plays a critical role in de novo bone formation and fracture repair [2]. Following a bone fracture, the periosteum overlying the injured site expands, and mesenchymal progenitors and osteoblasts are activated to invade the fracture site and form a callus that later ossifies to form new bone [3–5]. The periosteum is also widely recognized as a target for anabolic agents such as sex steroids and intermittently administered parathyroid hormone [6]. The impressive ability of the periosteum to regenerate new bone coupled with its reservoir of progenitor cells and osteoblasts has generated immense enthusiasm regarding the periosteum as a cellular source for tissue engineering and musculoskeletal regeneration [7–9]. There are a number of advantages of using periosteal cells for the purposes of tissue engineering and regeneration. First, the periosteum covers the surface of long bones except for where tendons and muscles attach and can be easily excised using minimal surgical exploration from a readily accessible anatomic site [10]. Second, periosteal osteogenic cells exhibit higher proliferation rates than those of osteoblasts or progenitor cells harvested from cortical or trabecular bone and thus are readily amenable for ex vivo expansion prior to cell therapy or tissue engineering [11]. Finally, the multipotent characteristics of isolated periosteal osteoprogenitors are equivalent to those of bone marrow-derived stromal cells [12]. Despite the scientific rationale and enthusiasm for using periosteal osteoblasts for tissue engineering and regeneration [2, 10], no specific markers exist for identifying and isolating periosteal osteogenic cells.

Discoidin domain tyrosine kinase receptor 2 (DDR2), a membrane-bound tyrosine kinase receptor, is activated by fibrillar collagens and stimulates osteoblast differentiation and bone formation through both ERK1/2 and p38 MAP kinase signaling [13–16]. Human DDR2 mutations cause spondylo-meta-epiphyseal dysplasia (SMED) [17], and mice harboring deletions in the DDR2 locus have a SMED-like phenotype characterized by dwarfism and a reduction in total bone mineral density [18]. DDR2<sup>slie/slie</sup> cells exhibit defective osteoblast differentiation and accelerated adipogenesis [19]. Furthermore, it was shown that DDR2 controls the expression of bone markers and osteogenic differentiation and chondrocyte maturation via modulation of Runx2, master transcription factor activation [20]. Recently, research showed that DDR2 functions as an inhibitor of osteoclastogenesis and is a potential therapeutic target in osteoporosis [21]. However, whether DDR2 itself can be served as a marker for periosteal osteoblasts and osteoblast progenitor has not been reported.

Here, we report that unexpectedly, the bilayered structure of the bone periosteum can be predominately labeled by two cell surface markers, namely, discoidin domain tyrosine kinase receptor 2 (DDR2) and fibroblast specific protein-1 (FSP-1). These newly identified cell surface markers can facilitate the isolation of different cellular components of the periosteum for tissue engineering or regenerative purposes.

## Materials and methods

### Reagents

Goat anti-DDR2 antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), rabbit anti-DDR2 antibody (Genetex, Inc. CA, USA), ALP (alkaline phosphatase) antibody (R&D systems, Inc., Minneapolis, MN) and FSP-1 (R&D systems, Inc., Minneapolis, MN) were purchased from respective vendors. ALP activity staining kit was purchased from Sigma.

### Immunohistochemistry and immunofluorescence staining

All animal studies were approved by the Institutional Animal Care and Use Committee of the Guangzhou Medical University (the acceptance number: 2016-025) and all animal procedures conform to the National Institutes of Health (NIH) guidelines. Ribs and femurs were harvested from C57bl/6 mice and frozen in OCT. Cryosections (7- $\mu$ m) were fixed in acetone for 10 min at  $-20^{\circ}\text{C}$  followed by staining for DDR2, FSP-1 and ALP according to the manufacturer's instructions (Vector Labs ABC kit, Burlingame, CA, USA) or standard immunofluorescence staining. A Leica SP2 AOBS upright laser scanning confocal microscope was used to obtain images. Then, co-localization analysis of confocal images was performed by the plugin called Just Another Colocalization Plugin or JACoP of the Image J software (NIH) according to the instructions of the software.

### Isolation of periosteal osteoblasts from the periosteum

We chose ribs as an example of cortical bone because ribs can be easily cut for cryosections. Periosteal osteoblasts and osteogenic cells were isolated adapting techniques described by Arnsdorf et al. [10]. Ribs were removed from the chest wall and the overlying muscle gently removed by blunt dissection. The ribs were cut into 1 mm<sup>3</sup> pieces, gently treated with Collagenase II and washed to remove bone marrow and subsequently plated in T25 tissue culture flasks with DMEM supplemented by 10% fetal bovine serum [22]. Following 7 days of culture, sub-confluent cells were observed to migrate from the leading edges of the cut bone pieces. The bone pieces were discarded and the cells were subsequently used for further experiments without further passaging.

### Flow cytometry

Indirect flow cytometry was done to sorting DDR2- and FSP-1-expressing cells isolated from the periosteum. Cells were incubated with the primary antibody DDR2 and FSP-1 for 30 min at  $4^{\circ}\text{C}$ , followed by staining with fluorescent conjugated secondary antibodies Anti-Rabbit IgG Alexa Fluor 647 (Jackson, Philadelphia, PA, USA) and anti-Rat IgG DyLight 488 (Invitrogen, USA). The cells subjected to flow cytometry without the addition of a primary antibody or secondary antibody served as controls.

## Bone mineralization differentiation and alizarin red staining

After flow cytometry sorting, DDR2-expressing cells and FSP-1-expressing cells were plated at a density of  $2.5 \times 10^4$  cells/cm<sup>2</sup> and osteogenesis was induced using differentiation medium (a-MEM supplemented with 10% FBS, 0.1  $\mu$ M dexamethasone, 10 mM  $\beta$ -glycerol phosphate and 50  $\mu$ M L-ascorbic acid) [23]. Differentiation was visualized after 21 days by staining with alizarin red staining. In brief, 2% paraformaldehyde-fixed cells were incubated with alizarin red solutions for 15 min at room temperature. After staining, the cells were washed thrice with 1 $\times$  wash buffer and the images were taken in a Leica microscope. The percentage of alizarin red staining area to high power field (HPF) were analyzed with Image pro plus 6.0 software (Media Cybernetics, USA).

## Statistical analysis

Statistical analysis was performed using the GraphPad 6.0 software (GraphPad software, Inc, La Jolla, CA, USA) according to the instructions of software. For paired comparison, we used the Student's *t* test (two-tailed). For multiple comparison, one-way analysis of variance (ANOVA) with Bonferroni post-hoc test analysis was applied. A probability value of  $p = 0.05$  was considered statistically significant. All results in the text are shown as mean  $\pm$  SEM (standard error of the mean).

## Results

### Expression of DDR2 and FSP-1 in the bilayered structure of the periosteum

The periosteum covers the external surface of cortical bones and consists of two histologically distinct layers. The outer fibrous layer comprises fibroblasts and the extracellular matrix, while the inner layer (in contact with the cortical bone surface), also known as the cambial layer, is highly cellular and predominantly consists of osteoblasts and osteoblast progenitors [2]. DDR2 is a receptor tyrosine kinase that is activated following collagen binding and plays an important role in the remodeling of extracellular matrix [13]. FSP-1 is expressed in fibroblasts in several organs and has been implicated in cell mobility and migration [24]. Periosteal osteoblasts, such as osteoblasts located elsewhere in the skeleton, express ALP (alkaline phosphatase). By performing immunohistochemistry on 7- $\mu$ m parallel frozen sections of bone, we demonstrated that the inner cambial layer of the periosteum abundantly expressed ALP (Fig. 1a) and was labeled by DDR2 (Fig. 1b, c). In contrast, the outer fibrous layer of the periosteum was labeled by FSP-1 (Fig. 1d). By performing double immunofluorescence staining on frozen sections of bone, we demonstrated that  $62.35 \pm 1.81\%$  (mean  $\pm$  SEM) of periosteal osteoblasts that expressed DDR2 also expressed ALP (Fig. 1e, h) and that only  $9.30 \pm 0.60\%$  of the FSP-1-positive cells located in the outer layer of the periosteum expressed ALP (Fig. 1f, h). Coexpression analysis of both FSP-1- and DDR2-expressing periosteal cells showed that  $10.96 \pm 1.96\%$  of DDR2-expressing cells also expressed FSP-1 and that  $13.59 \pm 1.57\%$  of FSP-1-expressing cells also expressed DDR2 (Fig. 1g, i). The Pearson's coefficient indicated that DDR2 was significantly correlated with ALP ( $0.74 \pm 0.02$ ), while FSP-1 was poorly correlated with DDR2 ( $0.20 \pm 0.20$ ) and ALP ( $0.31 \pm 0.05$ ) (Fig. 1e–g, j).

It is well recognized that periosteal anatomy as well as its regulation differ throughout the skeleton in a site-specific manner and that the periosteum around the femoral diaphysis is particularly thin [25]. To determine whether DDR2 and FSP-1 also label osteoblasts and fibroblasts in the periosteum over the femoral diaphysis, we analyzed the periosteum over the diaphysis region at the upper end of the femur. Immunohistochemistry demonstrated that the inner osteoblastic layer of the periosteum over the femoral diaphysis expressed ALP (Fig. 2a, b) and was intensely labeled by DDR2 (Fig. 2c, d), while the outer layer of periosteal fibroblasts expressed FSP-1 and was ALP-negative (Fig. 2a, b, e, f). These findings suggest that DDR2 and FSP-1 are specific markers of periosteal osteoblasts and fibroblasts covering cortical bone.

### **Mineralization of DDR2-expressing periosteal osteogenic cells can be significantly induced**

To investigate the difference in osteogenic differentiation between DDR2- expressing and FSP-1-expressing periosteal osteogenic cells in vitro, we isolated periosteal osteogenic cells from the cortical bone (ribs), taking care not to expose the endosteum so that the isolation of periosteal osteogenic cells was enriched [10]. We first performed immunofluorescence staining of isolated periosteal osteogenic cells. Consistent with the immunohistochemistry results, we observed that  $61.56 \pm 4.88\%$  of isolated osteogenic cells that expressed DDR2 also expressed ALP, while only  $33.38 \pm 4.18\%$  of FSP-1-positive cells expressed ALP (Fig. 3a, b). Pearson's coefficient of colocalization demonstrated a high correlation between DDR2 and ALP ( $0.62 \pm 0.02$ ) but a poor correlation between FSP-1 and ALP ( $0.19 \pm 0.02$ ) (Fig. 3c).

We then isolated the periosteal osteogenic cells and used flow cytometry to sort DDR2- and FSP-1-expressing periosteal osteogenic cells for bone mineralization differentiation (Fig. 3d). After 21 days, we observed a significant difference in bone mineralization differentiation between DDR2-expressing cells and FSP-1-expressing cells (Fig. 3e). Clearly, almost all DDR2-expressing cells were differentiated into mineralized cells; however, few FSP-1-expressing cells were alizarin red-positive (Fig. 3e). Based on the percentage of alizarin red-positive area relative to the HPF (high power field), we analyzed and compared the degree of osteogenic differentiation ability between DDR2- and FSP-1-expressing cells. The results showed that the mineralization of  $47.3 \pm 1.90\%$  of DDR2-expressing cells per HPF was induced, while the mineralization of only  $0.82 \pm 0.10\%$  of FSP-1-expressing cells per HPF was induced (Fig. 3e, f). These results suggest that compared to FSP-1-expressing cells, DDR2-expressing cells have a significantly greater potential to differentiate into bone and may play a more important role in bone repair. DDR2 is a potential cell marker of periosteal osteogenic progenitors.

## **Discussion**

The periosteum is recognized to have a population of osteoblasts and osteoblast progenitors that have regenerative potential and play a critical role in bone repair. In fact, lineage-tracing studies with bone grafts have suggested that cells of periosteal origin, rather than endosteal osteoblasts, which are in contact with the bone marrow, contribute to callus formation and

fracture healing [3–5]. Despite the obvious potential of the periosteum for the purposes of cell therapy in musculoskeletal regenerative medicine, there is a lack of cell surface markers that can be used for the isolation of osteogenic cells, including periosteal osteoblasts or osteoblast progenitors, and to prevent contamination with periosteal fibroblasts. In this report, we observed in situ that the expression of ALP is significantly correlated with the expression of DDR2, but not FSP-1. The mineralization of almost 100% of isolated DDR2-expressing cells from the periosteum can be induced, while few FSP-1-expressing cells exhibit osteogenic differentiation in vitro. More Interestingly, the fluorescence signal intensity of ALP increase from the outer layer to the inner layer, and DDR2-positive cells that express ALP are also increased from the outer layer to the inner layer (Fig. 1e), which indicates that DDR2-positive cells may migrate to the cortical bone surface and the expression of ALP may represent different maturation stages of DDR2-expressing cells.

DDR2 has been reported widely regarding the function and mechanism of DDR2 in osteogenic and chondrogenic differentiation [20]. In recent years, great attention has been paid to the function and signaling of DDR2 in bone-related disease. However, DDR2 itself has not been recognized as a specific marker of periosteal osteoblasts and osteoblast progenitors. Considering our observation were firstly got by using directly frozen ribs, the possible reason may be due to both the antibodies of DDR2 and FSP-1 currently available are more sensitive to fixation and decalcification, which may cause failed Immunohistochemistry staining for the bilayer structure of bone tissue. The phenotype that DDR2 is primarily expressed in periosteal osteoblasts and osteoblast progenitor is consistent with the functional importance of DDR2 in osteogenic and chondrogenic differentiation.

Intriguingly, DDR2 is enriched in cardiac fibroblasts and serves as one of the useful markers for identifying cardiac fibroblasts [26]. Our report raises an interesting question whether DDR2<sup>+</sup> cardiac fibroblast cells mainly contribute to the heart and valve calcification.

As this report demonstrates, periosteal fibroblasts rarely express DDR2; however, those in close proximity to the cortical bone express DDR2. Our work suggests that, in addition to its key function in bone-related diseases, DDR2 is also a marker of periosteal osteoblasts and progenitors, which can be used for the isolation and/or targeting of accurate regulation during bone formation and healing. Revealing the exact regulatory mechanism of DDR2 signaling in DDR2-expressing periosteal osteoblasts may be beneficial for effective musculoskeletal regenerative therapies.

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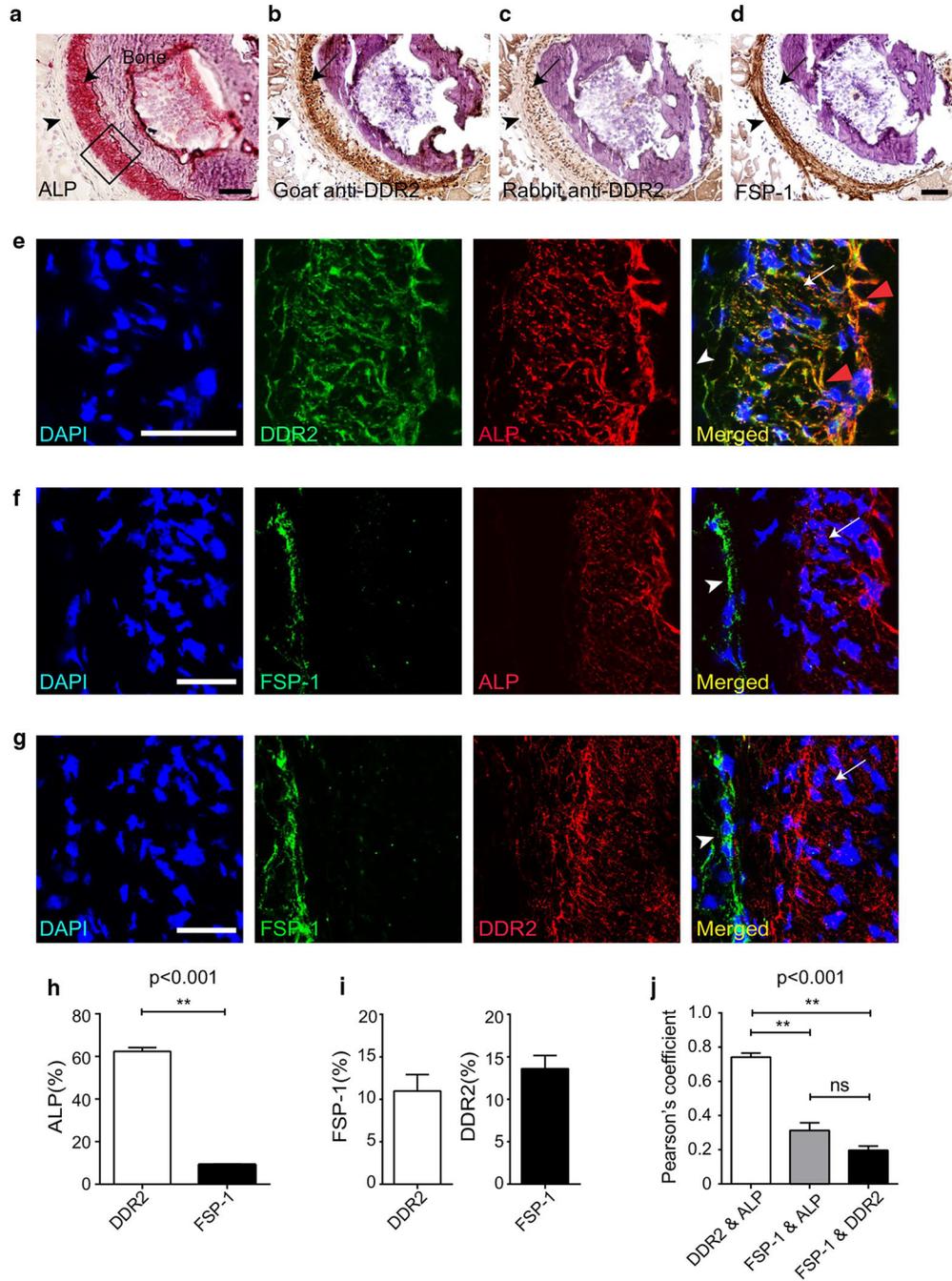
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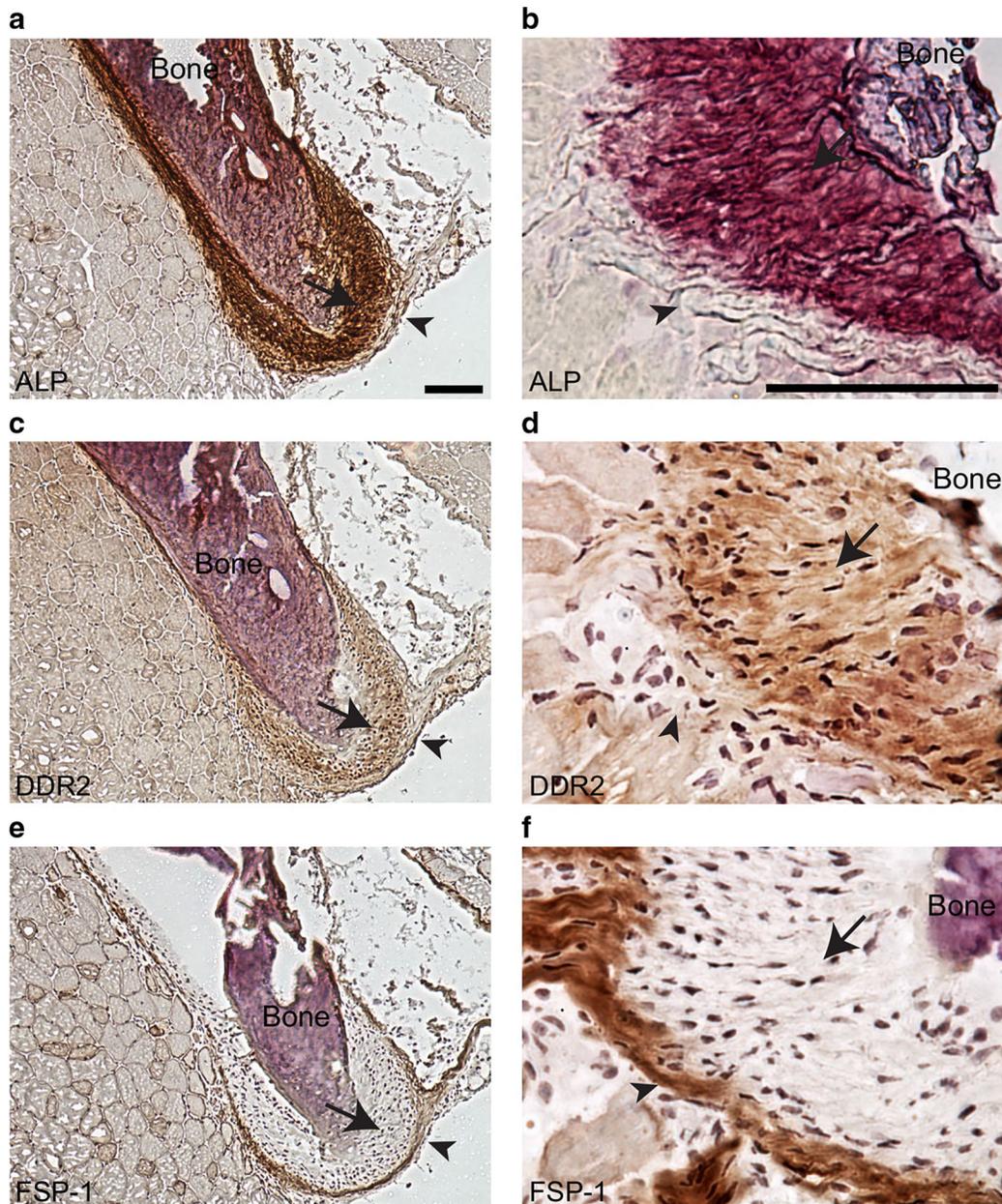
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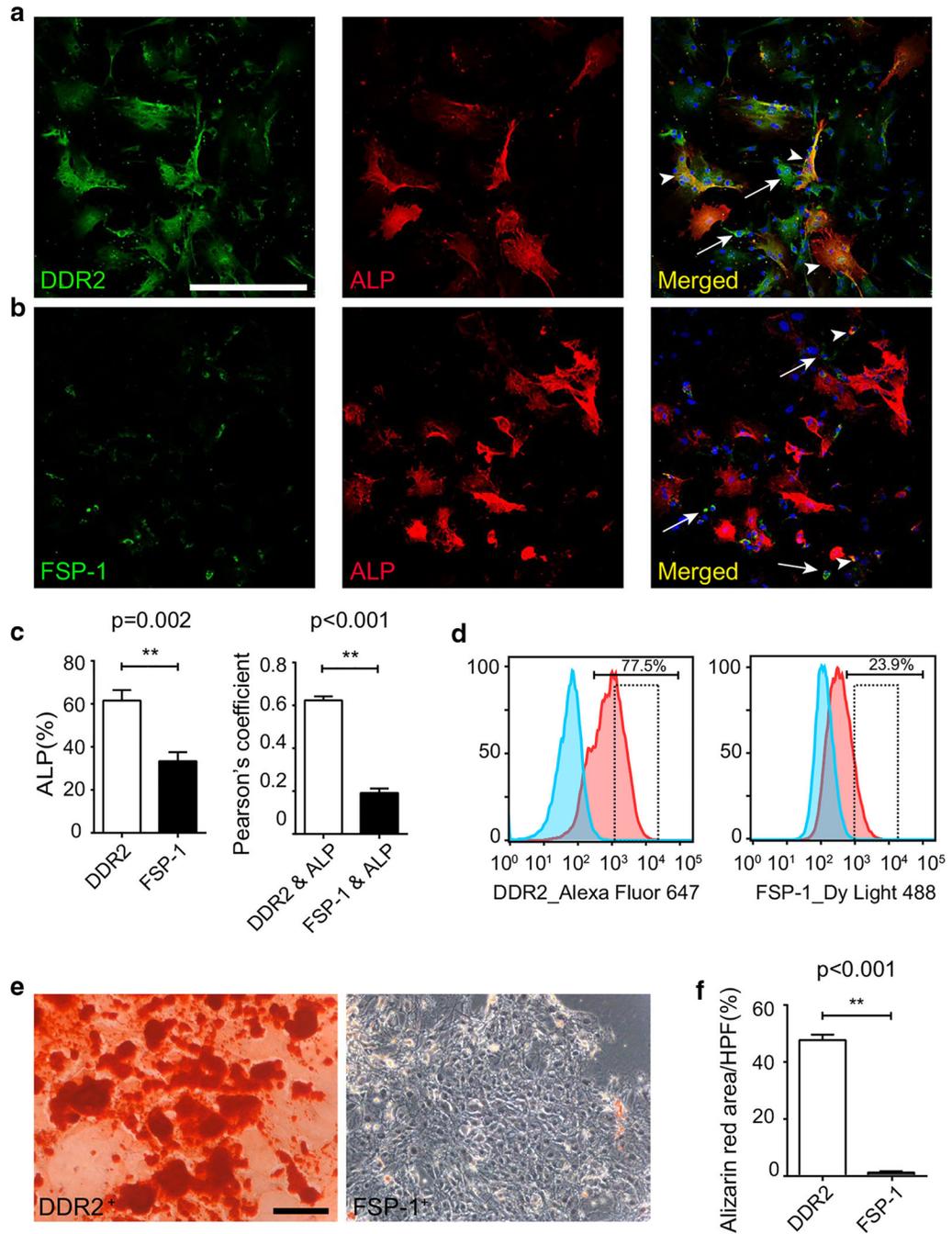


**Fig. 1.** Expression of ALP (alkaline phosphatase), DDR2 and FSP-1 in periosteum surrounding cortical bone (ribs). Outer layer of periosteum (white and black arrowhead); the inner layer of periosteum (arrow). **a** ALP expression determined by ALP activity in periosteum surrounding rib. Immunohistochemistry for **b** DDR2 (goat anti-DDR2 antibody), **c** DDR2 (rabbit anti-DDR2 antibody) and **d** FSP-1. **e–g** Immunofluorescence staining of the square area highlighted in **a**. **e** DDR2 and ALP staining with merged image demonstrating colocalization of fluorophores (red arrowhead). **f** Immunofluorescence staining of the outer

layer of periosteum for FSP-1 and ALP demonstrating lack of colocalization. **g** Immunofluorescence staining of the junction of outer and inner layers of periosteum for FSP-1 and DDR2 demonstrating lack of colocalization of fluorophores. **h** The percentage of DDR2-or FSP-1-expressing cells expressed ALP in **e, f, i** The colocalization between DDR2- and FSP-1-positive cells in **g, j** Pearson's coefficient of colocalization of ALP, DDR2 and FSP-1 in **e-g**. (All graphs were analyzed by image J and shown as mean  $\pm$  SEM,  $n = 3$  mice,  $**p < 0.01$ ,  $ns$  not significant, using ANOVA with post hoc tests. Scale bar: 25  $\mu\text{m}$ )



**Fig. 2.** Similar distribution of ALP, DDR2 and FSP-1 expression in the periosteum surrounding femoral diaphysis. **a** Immunohistochemistry staining for ALP and **b** ALP activity staining on bilayered-periosteum of femoral diaphysis. **c–f** Immunohistochemistry of DDR2 (**c–d**) and FSP-1 (**e–f**) in the similar region indicating no overlap staining between DDR2 and FSP-1. (Arrows point to the inner layer of periosteum, arrowheads point to the outer layer of periosteum, Scale bar: 100  $\mu$ m). The magnification in figures **a**, **c** and **e** is  $\times 100$ , magnification in figures **b**, **d** and **f** is  $\times 400$



**Fig. 3.** Bone mineralization differentiation of DDR2- and FSP-1-expressing Periosteal osteogenic cells. **a–c** double immunofluorescence staining of osteogenic cells isolated from the periosteum of ribs **a** DDR2 and ALP **b** FSP-1 and ALP **c** Quantitation of the colocalization in **a**, **b** (colocalization of fluorophores indicated by arrowhead; arrow points to the DDR2- or FSP-1-positive alone cells). **d** Flow sorting of DDR2-expressing cells and FSP-1-expressing cells from isolated periosteal osteogenic cells (dotted box showed the collected cells). **e** Alizarin red staining after 21 days bone differentiation of DDR2-expressing and FSP-1-

expressing periosteal osteogenic cells from **d**, and **f** The percentage of alizarin red-positive area relative to the HPF (high power field) in **e**. (results shown as mean  $\pm$  SEM;  $n = 7$ ,  $**p < 0.01$ , using an unpaired  $t$  test. Scale bar: 200  $\mu\text{m}$ )