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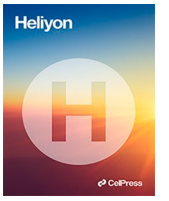

Research article

FGF2/HGF priming facilitates adipose-derived stem cell-mediated bone formation in osteoporotic defects

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ABSTRACT

Aims: The activity of adipose-derived stem cells (ADSCs) is susceptible to the physiological conditions of the donor. Therefore, employing ADSCs from donors of advanced age or with diseases for cell therapy necessitates a strategy to enhance therapeutic efficacy before transplantation. This study aims to investigate the impact of supplementing Fibroblast Growth Factor 2 (FGF2) and Hepatocyte Growth Factor (HGF) on ADSC-mediated osteogenesis under osteoporotic conditions and to explore the underlying mechanisms of action.

Main methods: Adipose-derived stem cells (ADSCs) obtained from ovariectomized (OVX) rats were cultured ex vivo. These cells were cultured in an osteogenic medium supplemented with FGF2 and HGF and subsequently autologously transplanted into osteoporotic femur defects using Hydroxyapatite-Tricalcium Phosphate. The assessment of bone formation was conducted four weeks post-transplantation.

Key findings: Osteoporosis detrimentally affects the viability and osteogenic differentiation potential of ADSCs, often accompanied by a deficiency in FGF2 and HGF signaling. However, priming with FGF2 and HGF facilitated the formation of immature osteoblasts from OVX ADSCs *in vitro*, promoting the expression of osteoblastogenic proteins, including Runx-2, osterix, and ALP, during the early phase of osteogenesis. Furthermore, FGF2/HGF priming augmented the levels of VEGF and SDF-1α in the microenvironment of OVX ADSCs under osteogenic induction. Importantly, transplantation of OVX ADSCs primed with FGF2/HGF for 6 days significantly enhanced bone formation compared to non-primed cells. The success of bone regeneration was confirmed by the expression of type-1 collagen and osteocalcin in the bone tissue of the deficient area.

Significance: Our findings corroborate that priming with FGF2/HGF can improve the differentiation potential of ADSCs. This could be applied in autologous stem cell therapy for skeletal disease in the geriatric population.

1. Introduction

Osteoporosis is a metabolic skeletal disease characterized by the deterioration of bone microstructure, leading to bone fragility. This condition is prevalent among the geriatric population and poses a significant challenge in modern societies [1,2]. Fractures in

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osteoporotic conditions present difficulties in performing adequate surgery and prolong the healing time due to low bone density. The delayed healing of fractures in elderly patients may lead to disability and increased morbidity [3,4]. Furthermore, osteoporotic fractures have been associated with an elevated risk of subsequent fractures [5]. Current treatments for osteoporotic fractures encompass the administration of pain relievers, anti-osteoporotic drugs, external bracing, vertebroplasty, and surgery [6–9]. However, these interventions offer temporary relief, emphasizing the need for a fundamental and more enduring solution.

Stem cell transplantation has emerged as a novel regenerative medical treatment. Stem cells exhibit self-renewal properties, paracrine potential, pluripotency, and immune-modulatory functions [10–12]. While bone marrow-derived stem cells (BMSCs) have been widely employed in stem cell therapy, their utility is constrained by a low survival rate and diminished differentiation potential, especially in the presence of background factors such as age and disease [13]. Additionally, acquiring bone marrow aspirates from patients with osteoporosis poses considerable challenges. Adipose-derived stem cells (ADSCs) have proven to be a valuable alternative to BMSCs in clinical applications. ADSCs are easily obtainable, entail low morbidity at the donor site, and are devoid of ethical controversies [14–17]. Furthermore, ADSCs are well-suited for cell therapy due to their rapid ex vivo repopulation, differentiation potential, and immune modulation through paracrine action. Nevertheless, because ADSCs are committed to adipose tissue, their differentiation potential may be less robust than that of BMSCs, and their osteogenic activity may be suboptimal [18].

ADSC therapy has demonstrated efficacy and holds significant promise in regenerative medicine [19–23]. The clinical determination of differences in efficacy between autologous and allogeneic ADSCs has not been established conclusively. Nevertheless, some non-clinical reports have suggested that autologous ADSCs may be superior to allogeneic ADSCs in terms of tissue repair [24,25]. As clinical outcomes of stem cell therapy can be influenced by environmental stimuli, there should not be an inhibition of allogeneic stem cell therapy. However, research findings should be taken into consideration when contemplating the use of allogeneic ADSC therapy.

While the activity of ADSCs is influenced by the donor's background, including underlying diseases and aging, it is generally not as pronounced as in the case of BMSCs [26,27]. This suggests that ADSCs with compromised cellular activity may not yield satisfactory efficacy in vivo. Therefore, the development of novel strategies to enhance the therapeutic efficacy of ADSCs is imperative before their autologous transplantation in geriatric patients or those with underlying diseases.

Fibroblast growth factor 2 (FGF2) is a versatile growth factor that regulates cell proliferation, angiogenesis, tissue repair, and osteogenesis [28–31]. Hepatocyte growth factor (HGF) is a pleiotropic growth factor involved in organ regeneration, myogenesis, wound healing, angiogenesis, and osteogenesis via activation of the intrinsic receptor c-Met [32–35]. Our previous study provided evidence that aging inhibits FGF2/HGF signaling in ADSCs, resulting in a diminished osteogenic capacity. Notably, FGF2/HGF priming was shown to significantly restore the osteogenic potential of ADSCs derived from the geriatric population [36].

Significant osteogenic potential has been documented in immature osteoblasts and osteoblasts derived from bone marrow or adipose tissue [37–39]. Immature osteoblasts exhibit the ability to proliferate before reaching maturity, suggesting their potential to populate fracture sites through proliferation and subsequent terminal differentiation after transplantation in vivo. The osteogenic induction of aged ADSCs in the presence of FGF2/HGF induces the expression of immature osteoblast markers and the secretion of angiogenic factors, suggestive of the formation of immature osteoblasts. In this study, we posited that FGF2/HGF priming expedites the transition of ADSCs into immature osteoblasts, potentially contributing to enhanced osteogenesis under osteoporotic conditions.

2. Materials and methods

2.1. Materials

Alizarin Red S powder, formaldehyde solution, antibiotic-antimycotic solution, decalcifying solution, and cetylpyridinium chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). α-MEM medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Rat osteoblast differentiation medium was provided by Cell Applications (San Diego, CA, USA). Phosphate-buffered saline (PBS) and 0.25 % trypsin-EDTA solution were purchased from Welgene (Daegu, Korea). Primary antibodies against osterix, alkaline phosphatase (ALP), and GAPDH were purchased from Abcam (Cambridge, UK). Type-1 collagen and FGF2 were obtained from Novus Biologicals (Centennial, CO, USA). Antibodies to P-Met, c-Met, and Runx-2 were provided by Cell Signaling Technology (Danvers, MA, USA). The osteocalcin antibody was obtained from Bioss (Woburn, MA, USA). Hydroxyapatite/beta-tricalcium phosphate (HA/β-TCP) ceramic powder was purchased from Biomatlant (Vigneux, France). Rat HGF ELISA Kit, Rat FGF2 ELISA Kit and recombinant FGF2 protein were obtained from R&D Systems (Minneapolis, MN, USA). The rat VEGF enzyme-linked immunosorbent assay (ELISA) kit was purchased from Abclonal (Woburn, MA, USA). Rat SDF-1α ELISA Kit was provided by Biorbyt (Cambridge, UK). Recombinant rat HGF protein was purchased from LifeSpan BioSciences (Seattle, WA, USA).

2.2. Animals

Five-week-old Sprague Dawley (SD) rats (110–120 g, 21 females) were purchased from Daehan Bio Link (Seoul, Korea). All animals were housed under a 12-h light/dark cycle in an animal room and allowed to acclimatize to a new environment for 7 d before the experiments. All animals received a standard chow diet and water *ad libitum*. This study was approved by the Ethical Committee for Experimental Animals of Kyung Hee University Hospital (approval number: KHMC-IACUC 22-011).

2.3. Osteoporosis induction

To induce osteoporosis, female SD rats were anesthetized during the operation using intraperitoneal injections of ketamine (75 mg/

kg; Yuhan, Seoul, Korea) and Rompun (1.2 mg/kg; Bayer Healthcare, Kyunggi-do, Korea). An incision is made through the dorsal abdominal skin and muscles to reach the abdominal cavity. The fat-surrounding ovary was gently grasped using forceps and exposed. The bilateral ovaries and ovarian arteries were ligated with 5-0 silk and removed. Rats were randomly divided into two groups: (1) Surgery-operated control (sham) group (n = 7), (2) OVX group (n = 14).

2.4. ADSCs isolation

Ten weeks after ovariectomy, all rats (sham = 7 and OVX = 14) were anesthetized and shaved, approximately 1–1.5 cm-long skins were excised, and visceral adipose tissues were harvested. To compare non-osteogenic ADSC's efficacy, adipose tissue of sham group was also isolated and cultured to get ADSCs, following the same procedure used for osteogenic ADSC. Adipose tissues were immediately washed with PBS containing 5 % Antibiotic-Antimycotic solution and chopped into a semi-liquid. Minced tissues were enzymatically digested using 1 % collagenase I for 40 min at 37 °C. FBS was added to prevent excessive digestion, and digested tissues were filtered through a 70 μm cell strainer to elevate cellular purity. After washing in DPBS, pelleted cells were seeded in culture plates in α-MEM supplemented with 10 % FBS and 1 % Antibiotic-Antimycotic solution. ADSCs were cultured at 37 °C in a 5 % CO_2 incubator and culture media were changed every other day. 2 weeks later, ADSCs were autologously transplanted into the defect area.

2.5. WST-1 assay

3×10^4 ADSCs were seeded in each well of 96-well plates (4 wells per rat) and incubated for 24 h. Water-soluble tetrazolium salts-1 (WST-1) solution was added to each 96 well, equaling 10 % of the total volume of the medium, and the plate was incubated for 90 min at 37 °C in a 5 % CO_2 incubator. After incubation, the optical density of each well was measured at 450 nm using a Versa Max Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The data were obtained by averaging the optical density from 28 wells (per group) and this process was repeated three times.

2.6. Osteogenic differentiation assay and growth factor treatment

ADSCs were incubated in 6-well plates (1×10^5 cells/well) for two days in α-MEM (3 wells per rat). Then, the medium was replaced with rat osteoblast differentiation medium with 5 ng/mL FGF2 and 50 ng/mL HGF. FGF2/HGF treatment was sustained for 6 days, followed by osteogenic induction without growth factors (FGF2/HGF) for 12 d. The medium was changed every 3 d. On day 18 post-osteogenic induction, the cells were fixed using 3.7 % formaldehyde and stained with Alizarin Red S solution (2 %, w/w) for 10 min to visualize calcium deposition. Stained Alizarin Red S was eluted using cetylpyridinium chloride (10 %, w/w), and calcium deposition was determined by measuring the absorbance at 560 nm (Molecular Devices, Sunnyvale, CA, USA). To include the relation between the difference in the repopulation potency of ADSCs from sham and OVX and the extent of osteogenesis, same experiment was setting in parallel to get the final cell number. ADSCs were plated in 6-well plates at a density of 1×10^5 cells per well, with six wells per condition, and cultured according to the osteogenic induction protocol for 18 days. To determine the total cell count, cells were trypsinized and stained with trypan blue, and the average cell number per well was calculated. Osteogenesis quantification was assessed by measuring optical density (Alizarin Red S) relative to total cell number (average cell number/well). This was repeatedly four times.

2.7. Cytokine measurement

To obtain the conditioned medium of ADSCs, ADSCs were cultured in 6-well plates (3 wells per rat) and the conditioned medium was obtained at 48 h after the media change. The supernatants of each well were collected by centrifugation at 12,000 rpm for 20 min at 4 °C. HGF, SDF-1α, and VEGF in the conditioned medium of ADSCs (before osteogenic induction) were quantified using ELISA kits, according to the manufacturer's instructions. HGF/VEGF/SDF-1 concentration under osteogenic indiction was determined by collecting supernatant of ADSCs at day 3 and 6 after initiation of osteogenic stimulus.

Absorbance was measured at 450 nm using an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA) and it was quantified by standard value.

2.8. Sample preparation and Western blot analysis

ADSC before osteogenic induction (day 0) and ADSC after osteogenic media treatment (day 3 and day 6) were lysed to obtain cell lysates. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL, USA). Protein samples were denatured, electrophoresed by SDS-PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5 % skim milk or 5 % BSA, the membranes were incubated with primary antibodies for c-Met, P-Met, FGF2, Runx-2, osterix, ALP, and GAPDH, followed by anti-immunoglobulin G horseradish peroxidase-conjugated secondary antibody. Blots were developed and visualized using WESTAR ECL Substrates (Cyanagen, Bologna, Italy) and analyzed by chemiluminescence using Fusion Solo X (Vilber Lourmat, Marne-La-Vallée, France). The expression levels were quantified using ImageJ software.

2.9. Femur defect and cell transplantation

Experimental groups are consists of 1) Sham + autologous ADSCs 2) OVX group + autologous ADSCs 3) OVX group + autologous ADSCs with FGF2/HGF priming. All ADSCs were cultured in osteogenic medium for 6 days with or without FGF2/HGF. To transplant autologous ADSCs, 2×10^6 ADSCs were added to 20 mg of hydroxyapatite/beta-tricalcium phosphate (HA/β-TCP) ceramic powder (Biomatlante, Vigneux-de-Bretagne, France) in 1.5 mL vial and gently shook it without inverting. This vial was incubated at 37 °C for 2 h. Tapping is required to ensure equal distribution. SD rats were anesthetized, and a lateral longitudinal incision was made in the left femur skin. The femoral condyle is exposed after blunt muscle dissection. To create a bone defect, a hole with a diameter of 4 mm and depth of 3 mm was created on the surface of the bone using a dental drill (Saeshin Precision, Daegu, Korea). After washing the bone cavity with normal saline to remove debris, the defects were filled with a complex of ADSCs and HA/β-TCP, and the fascia and skin were closed. (n = 7/group).

2.10. Histological analysis

Rat femurs were harvested 4 weeks post-transplantation and fixed in 3.7 % formaldehyde. After fixation, femurs were decalcified in a decalcifying solution for 12 h and embedded in paraffin. Paraffin-embedded samples were stained with hematoxylin and eosin (H&E) and Masson's trichrome staining (MTC). For immunohistochemical (IHC) staining of newly formed bone, deparaffinized samples were treated with Type-1 collagen (1:100) or osteocalcin (1:300) and incubated with biotin-conjugated rabbit secondary antibody. After washing, the ABC reagent solution was added to amplify the reaction. The samples were treated with Nova RED (Vector Laboratories) to visualize the bone-forming area in the tissue. Stained area was quantified by analyzing 8 images per a sample, resulting in a total of 56 images (per group) being assessed.

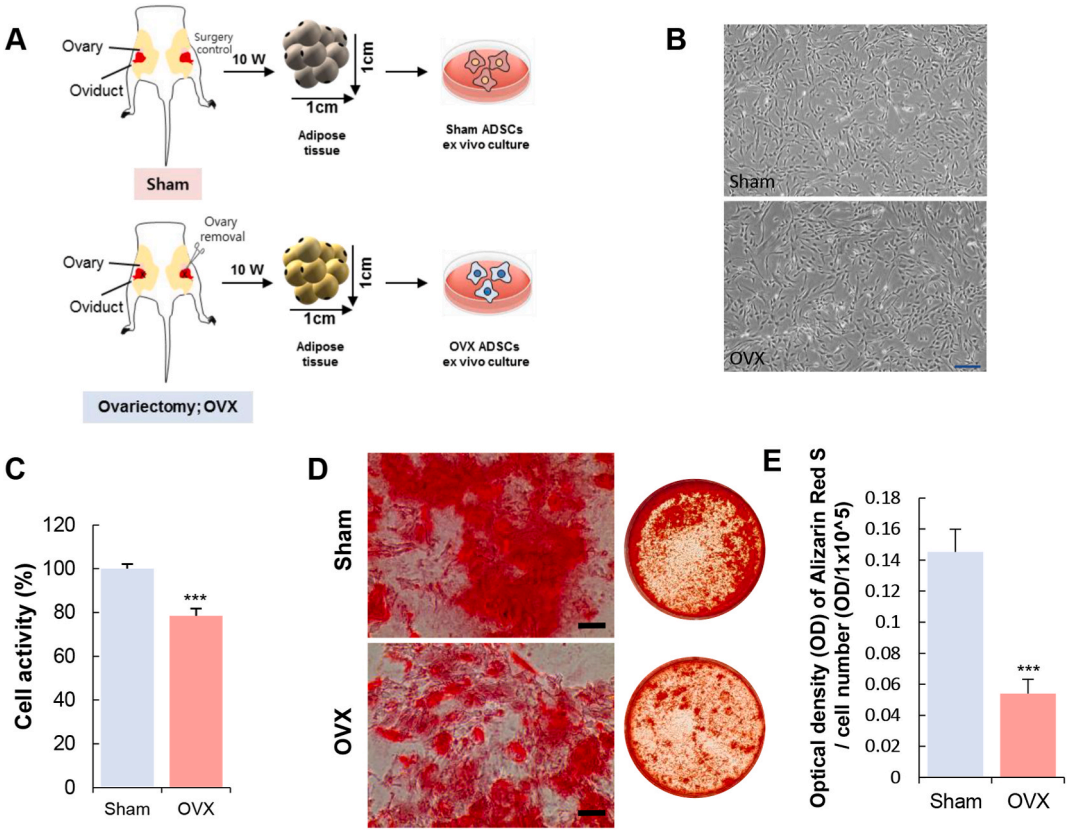


Fig. 1. Osteoporotic environments decrease the cellular activity of ADSCs. (A) Experimental scheme for culture of ADSCs from the sham and ovariectomized (OVX) rats. N = 7/group (B) Cellular morphology of sham ADSC and OVX ADSC was comparatively observed (C) WST-1 assay to check the cellular activity of ADSCs. Results are presented as the mean ± SD of at least three replicates for each group (D–E) Osteogenic induction was carried out for 18 d, and calcium deposit was determined by Alizarin Red S staining. Staining intensity was represented as optical density (OD) relative to average of total cell number/well. Scale bar, 100 μm. Results are presented as the mean ± SD of at least four replicates for each group. ***$P < 0.001$.

2.11. Statistical analysis

All data are presented as the mean ± standard deviation. Unpaired two-tailed Student's t-test analyses were performed using GraphPad Prism (GraphPad Software, version 5.01, San Diego, CA, USA). The p values < 0.05, 0.01, and 0.001 were considered statistically significant.

3. Results

3.1. Osteoporotic environments reduce cellular activity and osteogenic potential in ADSCs

To assess the impact of osteoporotic environments on the cellular activity of ADSCs, osteoporosis was induced in SD rats, and weight gain was monitored post-induction (Fig. S1). Following a notable reduction in bone density observed 8 weeks after OVX induction [26], adipose tissue was isolated for ADSC culture 10 weeks after the OVX induction (Fig. 1A). No differences in the morphology of ADSCs were observed between the surgery-operated control (sham) and OVX rats (Fig. 1B). Nevertheless, OVX ADSCs exhibited a lower optical density compared to sham ADSCs (Fig. 1C). The WST-1 assay involves measuring the mitochondrial activity of total cells. Consequently, the observed difference in cellular activity is anticipated to be attributable to variations in the proliferation rate. To assess the impact of osteoporotic status on the osteogenic potential of ADSCs, both sham and OVX ADSCs were cultured in osteogenic medium, and the calcium deposition rate was determined using Alizarin Red S staining. OVX ADSCs exhibited significantly lower osteogenic potential when compared to sham ADSCs (Fig. 1D). The Alizarin Red S-positive area was determined based on the cell number (Fig. 1E). This indicates that osteoporosis not only diminishes cellular activity but also impairs the osteogenic differentiation of ADSCs.

3.2. Osteoporosis interrupts production of FGF2/HGF and angiogenic factor in ADSCs

FGF2 and HGF signaling play pivotal roles in the osteogenesis of ADSCs through the activation of osteogenesis-related factors [40–43]. Subsequently, we explored the impact of the osteoporotic environment on FGF2 and HGF signaling in ADSCs (Fig. 2). OVX ADSCs exhibited hypophosphorylation and diminished expression of c-Met, the HGF receptor, coupled with reduced HGF secretion, in comparison to sham ADSCs (Fig. 2A-D). FGF2, a key growth factor favoring osteogenesis, exhibited insufficient expression in OVX ADSCs as compared to sham ADSCs (Fig. 2A and E). Angiogenesis plays a pivotal role in bone development by facilitating the transport of minerals and growth factors through blood vessels. Consequently, heightened angiogenic potential may enhance bone formation [44,45]. VEGF and SDF-1α, recognized as representative angiogenic factors, exhibited a significant reduction in secretion by ADSCs in an osteoporotic environment, as observed in Fig. 2F and G, in comparison to sham ADSCs.

These findings indicate a detrimental effect of osteoporotic conditions on FGF2/HGF signaling and the synthesis of angiogenic

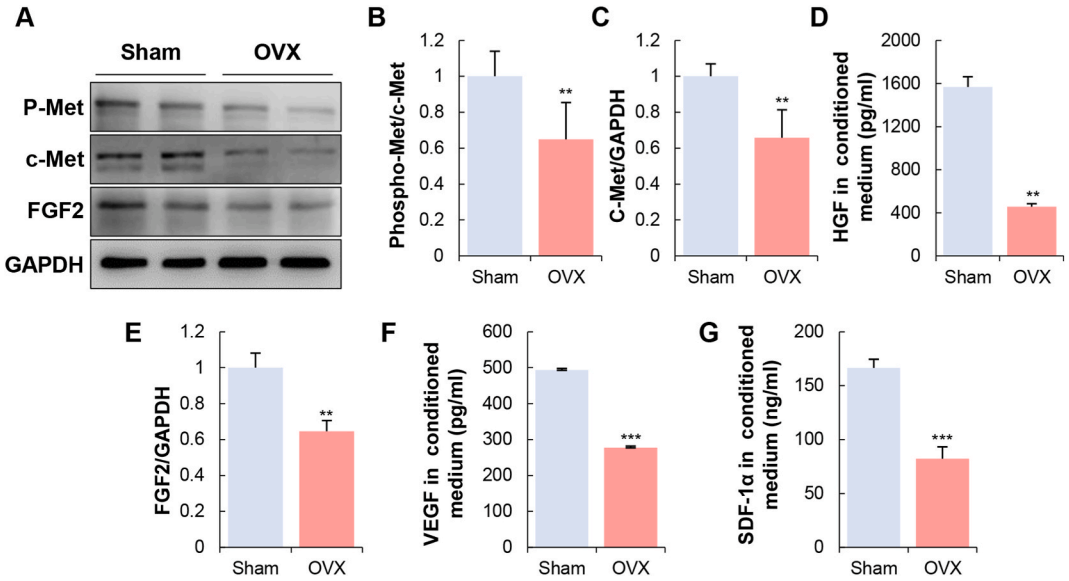


Fig. 2. Osteoporosis decreases FGF2/HGF levels and angiogenic factors in ADSC. ADSCs from both of sham and OVX rat were cultured *in vitro* and then, passage 2 ADSC was used for analysis. (A) Expression levels of P-Met, c-Met, and FGF2 in ADSCs was determined by Western blot (B–C, E) Expression levels quantified relative to c-Met or GAPDH. Original blot image is shown in Supplementary material. Levels of HGF (D), VEGF (F), and SDF-1α (G) in a conditioned medium was examined by ELISA. Results are presented as the mean ± SD of at least three replicates for each group. ***P < 0.01 and ***P < 0.001.*

factors in ADSCs.

3.3. OVX ADSCs fails to activate FGF2/HGF signaling during osteogenic induction

FGF2 and HGF signaling exhibited a decrease in ADSCs from OVX rats compared to sham-operated ADSCs, as depicted in Fig. 2. If the attenuation of FGF2 and HGF signaling persists in ADSCs from OVX rats even after osteogenic stimulation, it may lead to a loss of osteogenic potential. Therefore, the assessment of FGF2/HGF signaling at an early stage after osteogenic induction could serve as a predictive measure for osteogenic capacity. Subsequently, the levels of HGF/c-Met and FGF2 in both sham and OVX ADSCs were investigated following treatment with osteogenic medium (Fig. 3A). The osteoporotic environment maintained reduced levels of phosphorylated c-Met in ADSCs during osteogenic stimulus, as depicted in Fig. 3B and C. Quantitative analysis of HGF demonstrated that OVX ADSCs failed to secrete HGF in response to osteogenic induction, while sham ADSCs exhibited a rapid and substantial production of HGF within 3 days (Fig. 3D). The expression of FGF2 was also reduced in OVX ADSCs under osteoinductive conditions compared to sham ADSCs (Fig. 3B and E). Thus, OVX ADSCs respond to osteogenic stimuli with insufficient FGF2/HGF activity.

Runx-2 serves as the primary initiator of osteogenesis and an indicative marker of osteogenic progression. FGF2/HGF signaling has been closely associated with Runx-2 expression in various cell types [46,47]. The suppression of FGF2/HGF signaling in OVX ADSCs during the early osteogenic phase led to a reduced expression of Runx-2 on days 3 and 6 of the osteogenic process, in comparison to sham ADSCs (Fig. 3B and F). This observation suggests that sham ADSCs enter the osteogenic phase earlier than OVX ADSCs. Furthermore, VEGF secretion by sham ADSCs increased as differentiation progressed, while OVX ADSCs exhibited low secretion during the early stages of differentiation (Fig. 3G).

Considering these findings, the diminished osteogenic potential observed in OVX ADSCs is likely attributed to both inadequate FGF2/HGF signaling and a deficiency of angiogenic factors during the early phase of osteogenesis.

3.4. FGF2/HGF priming mitigates loss of osteogenic differentiation of ADSCs due to osteoporosis

The deficiencies in FGF2, HGF, and VEGF in OVX ADSCs are considered causal factors for their diminished osteogenic capacity.

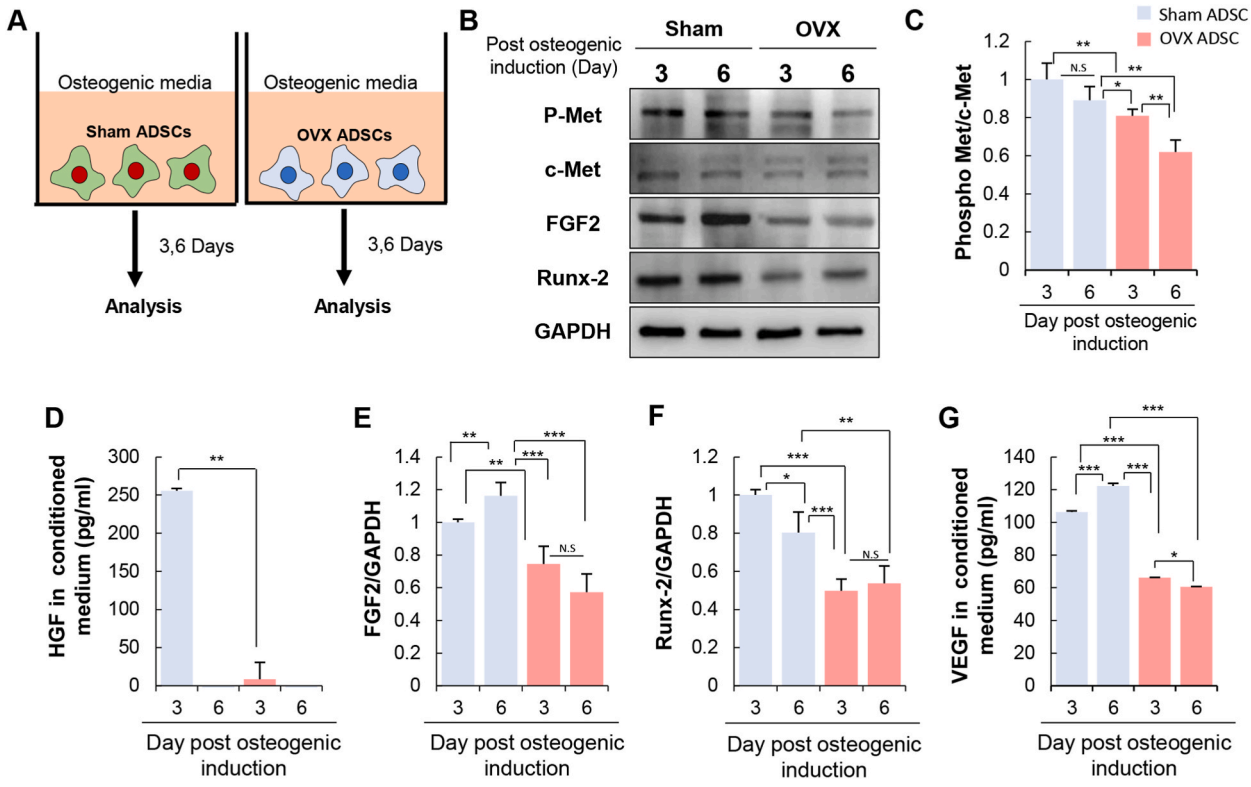


Fig. 3. Upon osteogenic stimulus, OVX ADSCs respond with insufficient FGF2/HGF, leading to low levels of Runx-2 and VEGF (A) Experimental scheme for analyzing the effect of osteoporotic environments on ADSCs from sham and OVX rat. ADSCs were exposed to osteogenic medium for either 3 or 6 days, and at each time point, cell lysates were prepared. (B) Expression levels of P-Met, c-Met, FGF2, and Runx-2 in ADSCs on days 3 and 6 after the osteogenic medium treatment were determined by Western blot. (C, E-F) Expression level was quantified relative to c-Met or GAPDH. Original blot image is shown in Supplementary material. (D) HGF and (G) VEGF were quantified using ELISA. Results are presented as the mean ± SD of at least three replicates for each group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. N.S: Non significant.

Previous studies have reported that FGF2 and HGF can enhance the expression of angiogenic factors in various cell types, providing further support for the association between these signaling factors and osteogenesis. It suggests that priming with FGF2/HGF is expected to enhance osteogenic differentiation in OVX ADSCs by addressing the deficiency of FGF2/HGF and creating a favorable environment for angiogenesis.

The study evaluated whether FGF2/HGF priming during the early osteogenic phase could enhance the osteogenic potential of OVX ADSCs. OVX ADSCs were primed with FGF2 and HGF for 6 days under osteogenic induction conditions and subsequently cultured in osteogenic induction medium without FGF2 and HGF for 12 days. The assessment of calcium deposition on day 18 was conducted using Alizarin Red S staining (Fig. 4A). The consistent loss of osteogenic capacity in OVX ADSCs was observed. However, when FGF2/HGF priming was applied for 6 days, it resulted in an approximately 51 % increase in calcium deposition in OVX ADSCs compared to non-primed OVX ADSCs (Fig. 4B and C). Additionally, FGF2/HGF were not detected in the osteogenic medium itself (Fig. S2). Therefore, it is inferred that the exogenously applied FGF2/HGF could enhance osteogenic capacity. This suggests that the application of FGF2/HGF priming during the initial osteogenic phase has the potential to restore the impaired osteogenic differentiation capacity of OVX ADSCs.

3.5. *FGF2/HGF priming facilities the entry of OVX ADSCs into immature osteoblasts by modulating expression of early osteogenic factors*

FGF2/HGF priming for 6 days resulted in a remarkable improvement in the osteogenic differentiation of OVX ADSCs *in vitro* (Fig. 4), indicating that osteogenic potential can be restored by osteogenic stimulation for 6 days. Next, the effect of FGF2/HGF priming on the expression of osteogenesis-related factors in OVX ADSCs was assessed at 3 and 6 days after the induction of osteogenesis (Fig. 5A). Compared to untreated OVX ADSCs, FGF2/HGF-primed OVX ADSCs showed increased levels of P-Met on day 6 (Fig. 5A and B). Additionally, FGF2 levels were higher in FGF2/HGF-primed OVX ADSCs than in untreated OVX ADSCs on days 3 and 6 (Fig. 5A and C). FGF2/HGF-priming also elevated the concentration of HGF in OVX ADSCs (Fig. 5D). Considering the short half-life of HGF, it is likely that the increased concentration is generated from ADSCs themselves rather than from exogenous treatment. FGF2/HGF-priming shifted OVX ADSCs to a cellular condition with a high potential for FGF2/HGF signaling.

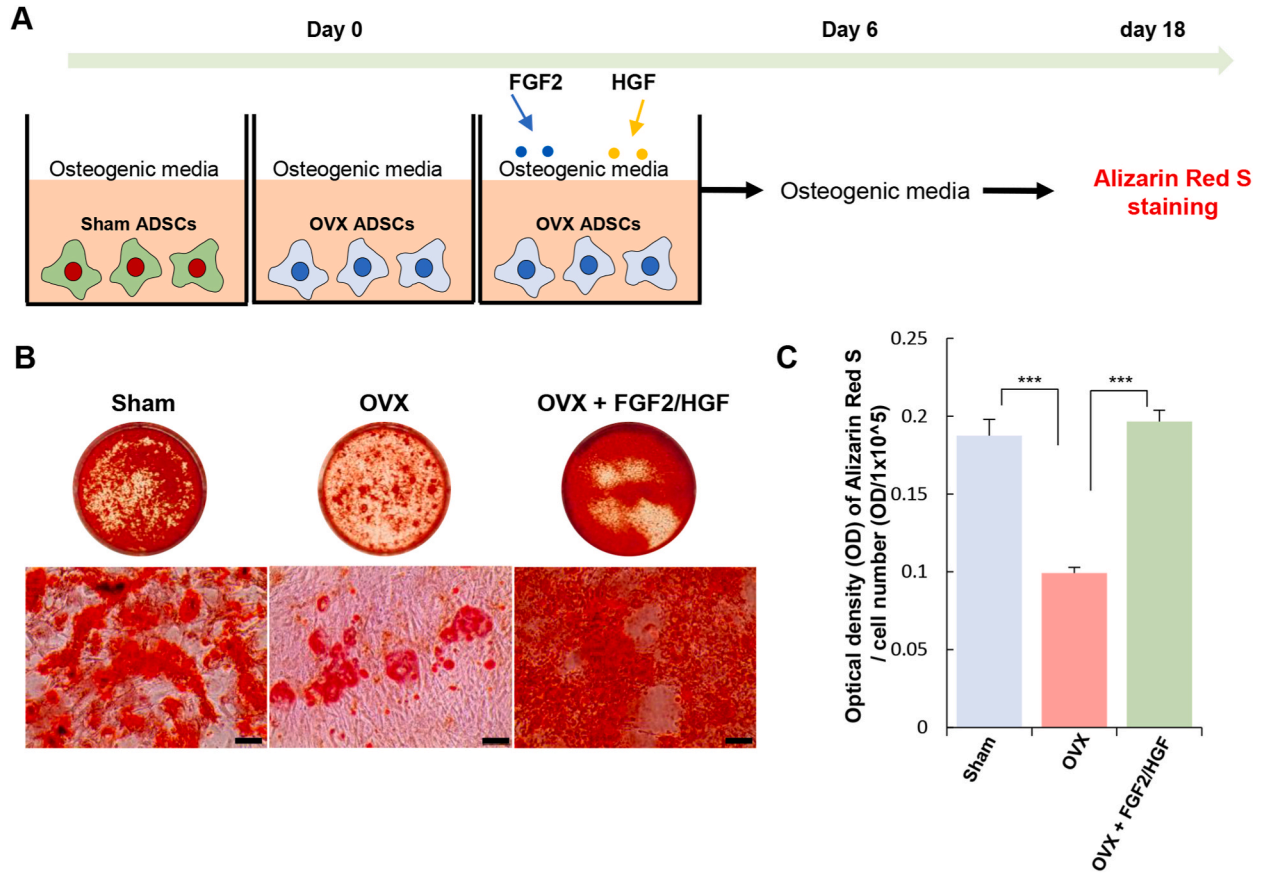


Fig. 4. FGF2/HGF treatment restores osteogenic differentiation of OVX ADSCs *in vitro*. (A) Experimental schedule to treat FGF2 and HGF to OVX ADSCs. FGF2 and HGF were added to ADSCs in the presence of osteogenic medium. Six days later, ADSCs were treated with osteogenic medium alone. (B) ADSCs were fixed with formalin and stained with alizarin red after osteogenic induction for 18 days. Representative images of Alizarin Red S staining of ADSCs. (C) Quantification of calcium deposition. Scale bar, 100 μm. Results are shown as the mean ± SD of at least four replicates for each group. ***$P < 0.001$.

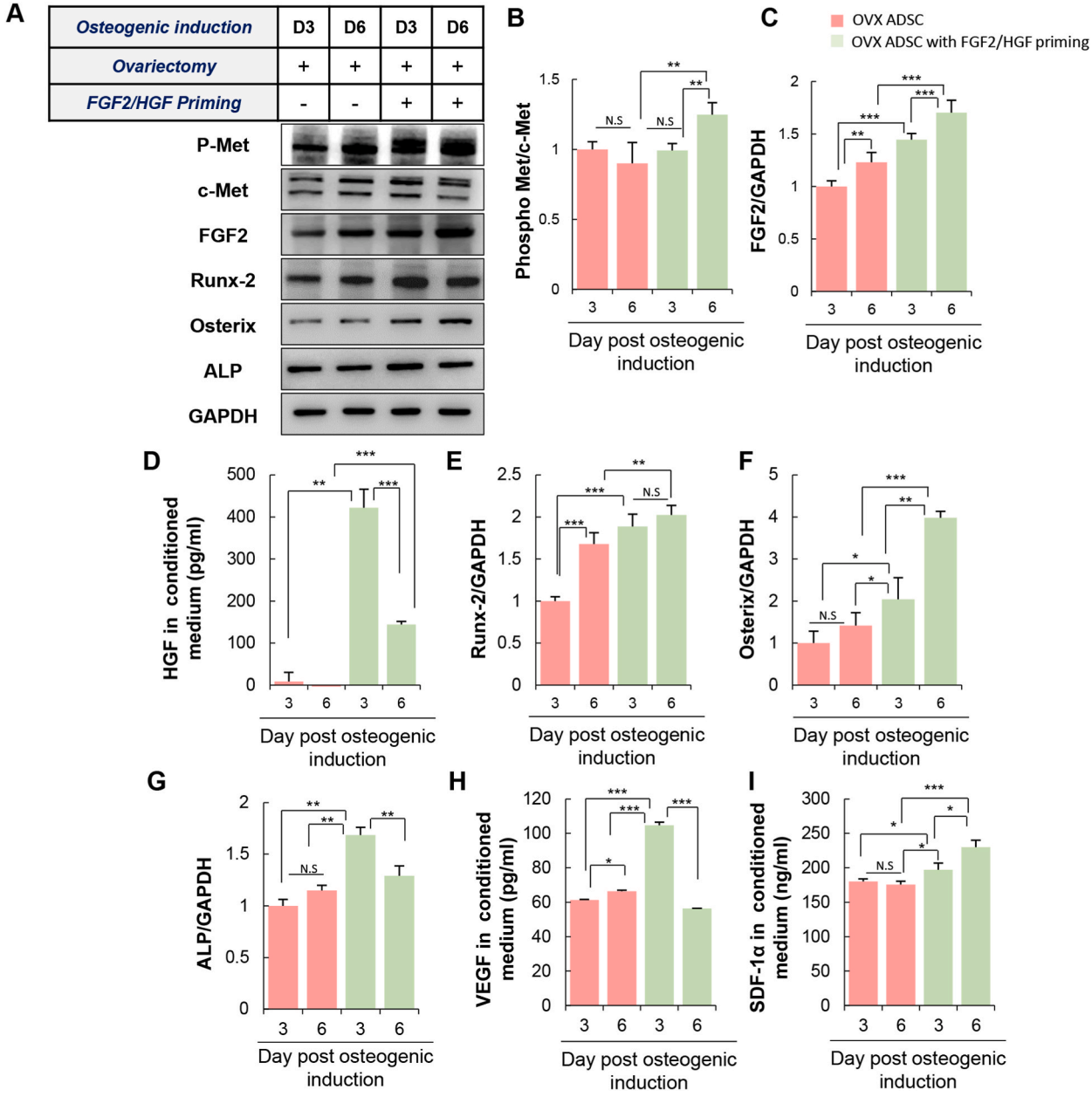
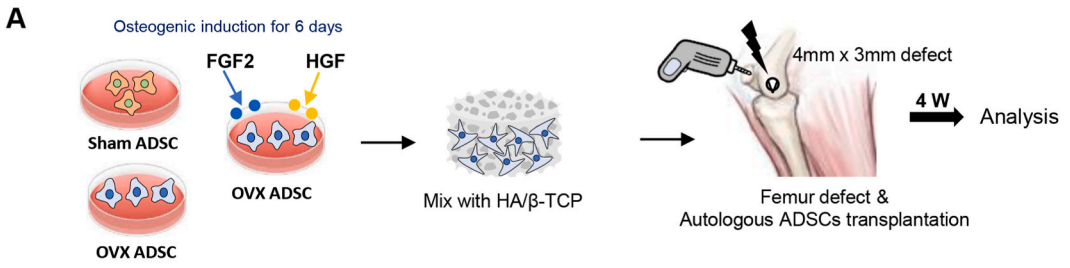
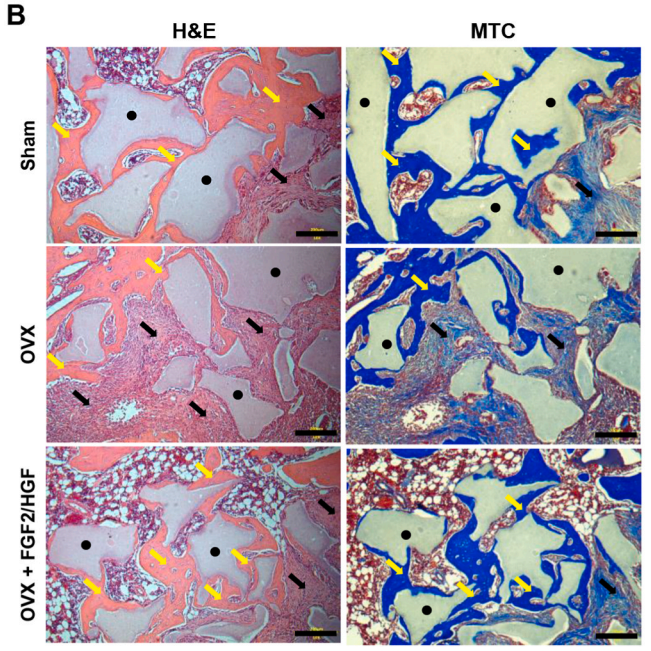
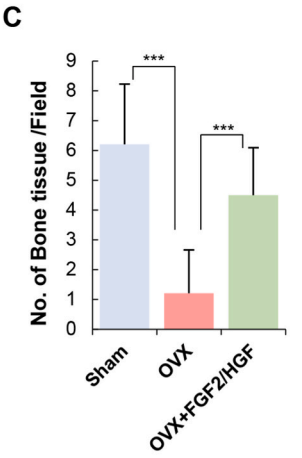


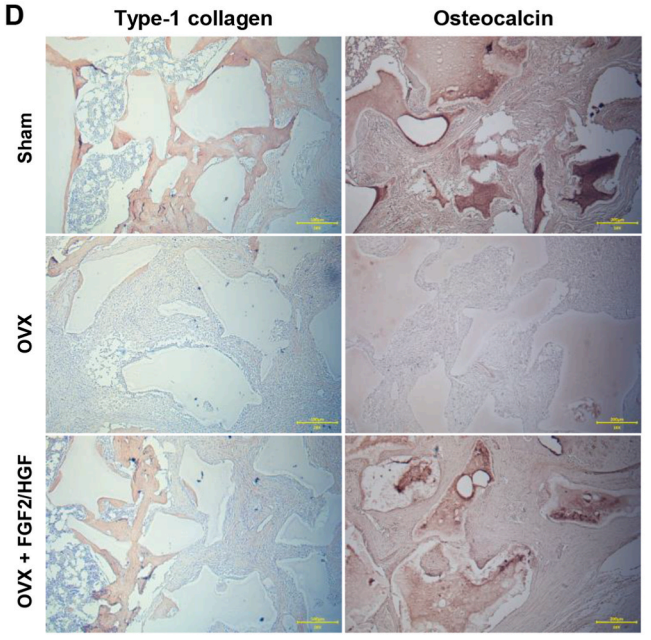
Fig. 5. FGF2/HGF treatment facilitates osteogenic marker expression and enriches angiogenic factors in OVX ADSCs. OVX ADSCs were treated with osteogenic medium, with or without the addition of FGF2/HGF. (A) At 3 and 6 day post osteogenic induction, expression levels of P-Met, c-Met, FGF2, Runx-2, osterix, and ALP on OVX ADSCs were examined by Western blot. Original blot image is shown in Supplementary material. (B–C) P-Met and FGF2 expression (D) HGF measured by ELISA (E–G) Runx-2, Osterix, and ALP expression (H–I) VEGF and SDF-1α examined by ELISA. Results are shown as the mean ± SD of at least three replicates for each group. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. N.S: Non significant.

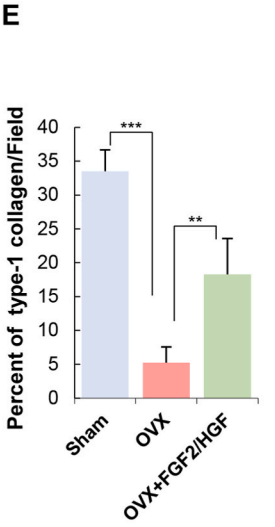
To determine whether FGF2/HGF-priming contributes to the elevation of early osteogenic markers in OVX ADSCs, the levels of Runx-2, osterix, ALP, VEGF, and SDF-1α were examined. Runx-2 expression was increased by FGF2/HGF-priming on days 3 and 6, with a notable difference observed on day 3 (Fig. 5A and E). Osterix is a crucial osteoblast-determining factor downstream of Runx-2. FGF2/HGF-primed OVX ADSCs exhibited higher osterix expression compared to untreated OVX ADSCs (Fig. 5A and F). ALP, an early bone formation marker secreted by osteoblasts, exhibited an increase on day 3 after FGF2/HGF priming (Fig. 5A and G). Additionally, VEGF secretion by OVX ADSCs was significantly enhanced by FGF2/HGF priming on day 3 of osteogenic differentiation. (Fig. 5H). SDF-1α has been reported to be highly expressed in immature osteoblasts in the endosteal niche and promote the homing of stem cells to the site of bone defects [48]. FGF2/HGF-primed OVX ADSCs also showed increased SDF-1α secretion on days 3 and 6 of osteogenic differentiation (Fig. 5I). The change in growth factor and osteogenesis markers implies the progression of osteogenesis.











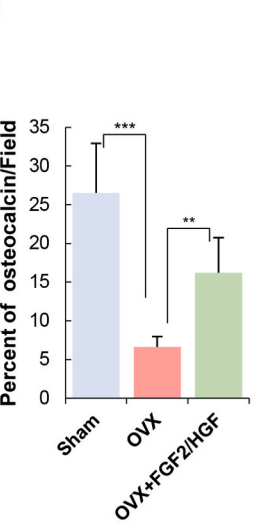


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Fig. 6. FGF2/HGF priming improves OVX ADSCs-mediated bone regeneration capacity *in vivo*. (A) Sham and OVX ADSC were cultured for 2 weeks for autologous transplantation. Schematic representation of autologous ADSCs transplantation in surgically induced femoral defect. (B) H&E and MTC staining were performed for the complex of transplanted cells and bone particles. Scale bar, 200 μm. (C) Number of regenerated bones in each group. (D) Representative images of immunohistochemical staining for type-1 collagen and osteocalcin in rat femurs implanted with ADSCs-scaffold complexes. Positive area: dark brown stained (E and F) Type-1 collagen and osteocalcin-positive area quantified by Image J. Scale bar, 200 μm. Results are displayed as the mean ± SD of 56 images for each group.$^{**}P < 0.01$ *and* $^{***}P < 0.001$. Black dots: HA/β-TCP particles. Black arrows: Fibrotic tissues. Yellow arrows: Regenerated bone. (n = 7/group).

Collectively, FGF2/HGF priming modulates the expression of osteoblastogenic proteins while enriching angiogenic factors in the cellular environment. This suggests that OVXADSCs may transition into immature osteoblasts during FGF2/HGF priming, potentially accelerating their differentiation into mature osteoblasts.

3.6. FGF2/HGF priming enhances ADSCs-mediated bone regeneration in vivo

To assess the bone-forming capacity of FGF2/HGF-primed OVX ADSCs *in vivo*, sham and OVX ADSCs were cultured in an osteogenic induction medium with or without FGF2 and HGF for 6 days (Fig. 6A). Following FGF2/HGF priming, ADSCs were combined with HA/β-TCP (6:4) and then transplanted into femoral defect sites. Four weeks post-transplantation, the defective femurs were harvested for subsequent analysis. (Fig. 6A and Fig. S3).

Histological analysis of the transplanted area was conducted using hematoxylin and eosin (H&E) as well as Masson's trichrome staining (MTC). As illustrated in Fig. 6B, the transplantation of sham ADSCs prompted the formation of well-organized bone tissue, while OVX ADSCs exhibited limited bone tissue formation at the defect site. Contrastingly, the grafts of FGF2/HGF-primed OVX ADSCs induced substantial bone formation *in vivo* (Fig. 6A and B). The extent of bone formation was quantified in each group (Fig. 6C). Immunohistochemistry using Nova Red was conducted, leading to the detection of a type-1 collagen-positive area (dark brown area) in the newly generated bone adjacent to the HA/β-TCP scaffold at the defect site. The expression of type-1 collagen in OVX ADSC grafts was rarely observed compared to the site where sham ADSCs were transplanted. However, the level of type-1 collagen significantly increased in the defective area of FGF2/HGF-primed OVX ADSCs (Fig. 6D and E). Osteocalcin is primarily secreted by osteoblasts and serves as a representative late marker of osteogenesis. It is noteworthy that osteocalcin has a strong affinity for hydroxyapatite (HA) and plays a crucial role in bone mineralization [49]. As expected, osteocalcin-positive areas were observed in both the HA/β-TCP scaffolds and newly formed bone tissue in the defect site. The staining intensity of osteocalcin was weak in the area transplanted with OVX ADSCs, but FGF2/HGF priming led to the recovery of osteocalcin levels at the transplanted site (Fig. 6F).

The results demonstrate that FGF2/HGF priming during the early osteogenesis phase in *ex vivo* cultures can enhance the bone regenerative potential of OVX ADSCs. This enhancement is likely attributed to the ability of ADSCs to enter the immature osteoblast phase before transplantation.

4. Discussion

The seriousness of bone fractures has emerged as a notable societal issue, emphasizing the need for further research progress in this field. In clinical practice, a range of techniques is employed for bone regeneration, including bone grafting and guided bone regeneration [50–52]. With an improved understanding of bone tissue biology and recent advancements in tissue engineering, mesenchymal stem cell (MSC) therapy has gained significant importance in improving bone tissue reconstruction [53]. The primary source of MSCs is the bone marrow; however, they can also be isolated and identified from adipose tissue, peripheral blood, placenta, and various other tissues. MSCs possess the capacity for direct differentiation into osteoblasts, particularly in the formation of bones such as the skull, facial bones, and pelvis. This process occurs through intramembranous ossification, bypassing the need for a cartilaginous template [54].

Autologous BMSC therapy is constrained by the donor's individual characteristics. MSCs originating from inflammatory microenvironments have shown a reduction in bone regenerative potential, which underscores the role of autologous MSCs in impaired bone healing [55,56]. MSCs from estrogen-deficient osteoporotic rats exhibited delayed healing of bone fractures and reduced osteogenic differentiation potential compared to BMSCs from healthy, normal rats [57,58]. In the context of estrogen deficiency, BMSCs displayed a compromised ability to induce osteoclast apoptosis, contributing to elevated bone resorption rates and subsequent bone loss [59,60].

ADSC transplantation can be applied through autologous or allogeneic approaches. Several completed clinical trials on allogeneic ADSC cell therapy have demonstrated therapeutic efficacy, significantly contributing to establishing ADSCs as a viable option in regenerative medicine and a potent tool in cell-based therapy for restoring damaged tissues [19–23]. However, a limited number of reports based on non-clinical experiments proved the superiority of autologous ADSC when compared to allogeneic ADSC therapy [24,25].

Concerning BMSCs, which share similarities with ADSCs, it has been reported that allogeneic BMSC transplantation can trigger an adaptive immune response, a distinction not observed with autologous BMSCs [61]. Within 14 days of transplantation, the allogeneic BMSCs at the injured site exhibited an initiation of elevated expression levels of MHC-Ia and MHC-II, transitioning from an initially immunoprivileged state to an immunogenic state [62]. This underscores a critical consideration for the clinical application of allogeneic BMSC therapy, emphasizing the potential need for additional immunosuppressive measures. Despite the advantages, such as immediate availability and donor selection, the observed shift to an immunogenic state suggests the importance of careful management to optimize therapeutic outcomes [63].

ADSCs are well-suited for autologous transplantation due to their accessibility for tissue retrieval and robust cellular activity, even in older or diseased individuals. However, it's important to note that ADSC activity may be influenced by donor age and underlying disease [64]. In our previous study, we found a strong correlation between FGF2 and HGF levels and the osteogenic capacity of ADSCs. FGF2/HGF priming was shown to enhance the osteogenic differentiation potential of elderly human ADSCs with low viability and self-renewal ability.

This study focused on stem cell therapy in osteoporotic conditions. A comparison between OVX ADSCs and sham ADSCs revealed that OVX ADSCs exhibited weaker repopulation and osteogenic potential compared to sham ADSCs. Furthermore, OVX ADSCs displayed significantly reduced expression levels of FGF2 and HGF compared to sham OVX ADSCs. This suggests that ADSCs within an osteoporotic environment may exhibit insufficient FGF2/HGF signaling activity.

Considering that OVX ADSCs have low levels of FGF2/HGF and that FGF2/HGF promotes Runx-2 expression, the poor FGF2/HGF signaling in OVX ADSCs is inferred to be the cause of the lack of osteogenic potential. The osteoporotic environment reduced the secretion of VEGF. The observed deficient osteogenic activity in OVX ADSCs may be attributed to the deficiency of Runx-2 and angiogenic factors, which are linked to the inadequate levels of FGF2 and HGF. FGF2/HGF priming improved the osteogenic potential of OVX ADSCs to a level similar to that of sham ADSCs. This underscores the significance of early control of osteogenic factors through FGF2/HGF priming, facilitating the transition of ADSCs to the immature osteoblast stage and ultimately restoring their osteogenic potential. FGF2/HGF priming had a positive effect on bone regeneration in an *in vivo* bone defect model. While OVX ADSCs alone had limited effectiveness in inducing bone regeneration, the group transplanted with FGF2/HGF-primed OVX ADSCs exhibited bone structure adjacent to the bone particles. Employing immature osteoblasts through FGF2/HGF priming combines the advantages of both stem cells and osteoblasts, given their self-renewal capacity and high osteogenic potential. This study confirmed the necessity of FGF2/HGF priming to enhance the osteogenic potential of ADSCs within the context of diseases, as demonstrated using an animal model.

Animal models enable the control of various variables, such as body condition and age, thereby ensuring consistent results following cell therapy. However, clinical application may present more complex challenges not encountered in the animal study, which is a thoughtful consideration. It's important to note that osteoporotic patients may also contend with age-related diseases, potentially giving rise to severe complications. This intricate physiological environment can significantly impact stem cell activity, potentially leading to a marked reduction in the regenerative capacity of stem cells. This aspect should be taken into account when considering autologous stem cell therapy.

As a next step, research involving human ADSCs from individuals with osteoporosis should be pursued. In this endeavor, it's essential to explore cellular impairments from various perspectives.

5. Conclusion

This study highlights the importance of FGF2/HGF priming for the use of ADSCs with low differentiation activity in skeletal diseases. Priming with FGF2/HGF plays a vital role in establishing a more conducive environment for bone regeneration, primarily through the stimulation of immature osteoblast formation. This approach holds the potential to significantly improve the success of bone regeneration procedures.

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Institutional review board statement

The protocol and procedures employed were ethically reviewed and approved by the Institutional Animal Care and Use Committee of Kyung Hee Medical Center (KHMC-IACUC 22-011).

Data availability statement

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Jeong Seop Park: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Do Young Kim:** Methodology, Investigation. **Hyun Sook Hong:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24554.

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