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Association of Fpr1 Gene Expression with Osteogenesis and Adipogenesis of Adipose Derived Stem Cells

Wan'an Xiao[1,2], **Quang Le**[1], **Di Zhu**[1], **Abhijit Dighe**[1], **Quanjun Cui**[1], **Xinlin Yang**[1,*]

[1]Department of Orthopaedic Surgery, University of Virginia, Charlottesville, VA, USA, 450 Ray C. Hunt Drive, Room 2323, Charlottesville, Virginia 22908

[2]Department of Orthopaedic Surgery, Shengjing Hospital of China Medical University, Shenyang, China

Abstract

Formyl peptide receptors (Fprs) play fundamental roles in multiple cell functions including promotion of osteogenesis and bone fracture healing. In this study, the role of Fpr1 gene in osteogenic and adipogenic differentiation of adipose derived stem cells (ADSCs) was investigated. Primary ADSCs (mADSCs) from either Fpr1 knockout (KO) or wild type (WT) mice and human ADSCs (hADSCs) were treated by osteogenic (OM) or adipogenic (AM) medium, with basal medium as control. Osteogenesis and adipogenesis were measured by histological and biochemical methods. In both hADSCs and mADSCs, Fpr1 gene expression, osteogenic gene expression, as well as mineralization were increased after osteogenic induction. The osteogenic capacity of KO ADSCs was remarkably reduced compared to WT ADSCs, with decreased levels of expression of osteogenic markers, alkaline phosphatase activity, and mineralization. In contrast, the adipogenesis of KO ADSCs was remarkably enhanced compared with WT ADSCs, forming more lipid droplets, and increasing expression of adipogenic markers PPARγ and aP2. Expression of the nuclear transcription factor Forkhead box protein O1 (FoxO1) was decreased in KO ADSCs, while OM and AM caused increase and decrease in FoxO1 expression, respectively. The current study revealed a correlation of Fpr1 gene expression with osteogenesis and adipogenesis of mADSCs, underlying a mechanism involving FoxO1. Our present research suggests that targeting Fpr1 might be a novel strategy to enhance osteogenesis of ADSCs.

Keywords

Adipose-derived Stem Cells; Formyl Peptide Receptors; Osteogenesis; Adipogenesis; Forkhead box protein O1; Bone Regeneration

[*]Corresponding Author: Xinlin Yang, Department of Orthopaedic Surgery, University of Virginia School of Medicine, 450 Ray C. Hunt Drive, Room 2323, Charlottesville, Virginia 22908, xy3c@virginia.edu.

There is no conflict of interest for each author.

INTRODUCTION:

Adult stem cells, or mesenchymal stem cells, is a population of cells in adult tissue possessing regenerative capacity recently recognized as a promising research direction in tissue engineering. Adipose-derived stem cells (ADSCs), specifically, is a subset of adult stem cells obtained from the processing of adipose tissue, which gives it many desirable clinical qualities. Most notably, ADSC can be obtained in large volume, through a simple procedure, and is associated with little donor site morbidity. In addition, ADSCs has been reported to have better proliferation and longer multipotency compared to bone marrow-derived stem cells[1]. In recent years, extensive research has been done to further elucidate the possible therapeutic use of ADSCs in a variety of conditions including osteoarthritis, diabetes mellitus, soft tissue reconstruction, and heart disease[2]. Within orthopedics, ADSCs has been noted for its ability to treat critical size bone defects *in vivo*[3–5]. ADSCs has also been found to be safe and to have some benefit for osteoarthritis patients in early clinical trials [6–12].

Formyl peptide receptor 1 (Fpr1) is a known chemoattractant of neutrophils belonging to the family of G-protein coupled receptors[13]. Its ligands are N-formyl peptides, which could originate from bacteria or cellular death/dysfunction[14,15]. As such, Fpr1 has been noted for its role in modulation of the immune system through recruitment of myeloid cells in response to external and internal stimuli[13]. Of interest, Fpr1 has been found to be expressed on the surface of human bone marrow-derived mesenchymal stem cells (BMMSC) and influence the migration of these cells[16]. Additionally, it has also been reported that Fpr1 promotes the differentiation of human BMMSCs into osteoblasts[17]. Given its major role in migration and differentiation of BMMSCs, we are interested in Fpr1's analogous function in the regulation of ADSCs. First, Fpr1 gene expression were compared between osteogenically induced human ADSCs and basal media (BM) control to examine Fpr1 expression changes in response to osteogenic media (OM). Second, Fpr1 knockout (KO) mice ADSCs in different growth media were compared to wild type (WT) to evaluate Fpr1's impact on osteogenesis and adipogenesis. Additionally, we characterized the Forkhead box protein O1 (FOXO1) metabolic pathway's interaction with Fpr1. FOXO1 is a gene previously noted for its role regulation of the cell cycle and adipogenesis[18].

MATERIALS AND METHODS:

Osteogenic induction of human ADSCs

Human ADSCs were obtained from Lonza (Basel, Switzerland) and cultured in Dulbecco's Modified Eagle's Medium (Gibco BRL, Gaithersburg, MD, USA) with 50 μg/ml sodium ascorbate, 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah, USA), 100 IU/ml penicillin G, and 100 μg/ml streptomycin of culture media in a humidified atmosphere of 5% carbon dioxide at 37°C. This culture medium is designated the basal medium (BM). The osteogenic medium (OM) is the basal medium with the addition of 10 mM β-glycerophosphate and 10^{-7} M dexamethasone. Experiments were performed using cells at passage 8 with starting density of 5,000 cells/cm^2.

Osteogenic and adipogenic induction of mouse ADSCs

Use of animal follows the protocol approved by the Institutional Animal Care and Use Committee at University of Virginia in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. Wild-type C57BL/6 mice (male, 8 weeks old) were purchased from Charles River (Boston, MA) while the Fpr1 knockout animals (male, 8 weeks old) were graciously supplied by Dr. Olefsky at UC San Diego. Mouse ADSCs were obtained from mouse inguinal fat pad and processed using the standard collagenase digestion method[19]. Cells were subsequently cultured in basal and osteogenic media similar to hADSCs as described above. Cells were also induced by the adipogenic medium (AM) which is the basal medium supplemented with 1 μM dexamethasone, 10 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.2 mM indomethacin.

Gene Expression Evaluation using Quantitative RT-PCR

We used the RNeasy® Mini kit (Qiagen, Valencia, VA, USA) to isolate total ribonucleic acid (RNA), and used the Reverse Transcription System kit (Promega, Madison, WI, USA) to obtain the complementary DNA (cDNA) according to the manufacturers' instructions. The QuantiTect® SYBR Green PCR master mix (Qiagen, Valencia, CA, USA) was utilized to perform Real-time PCR. Amplification plots were calculated based on the threshold cycle value. We analyzed the data based on the 2-ΔΔCT method with 18s ribosomal RNA (rRNA) serving as the reference[20]. We normalized all gene expression to the control group's expression in each experiment and represented experimental values as folds of change of WT BM values. The targeted genes and their primer sequences are as follows: runt-related transcription factor 2 (Runx2) (forward: 5′-AGATGATGACACTGCCACC TCTG-3′, reverse: 5′-GGATGAAATGCTTGGGAACTGC-3′), peroxisome proliferator-activated gamma (PPARg) (forward: 5′-CCTGAAACTTCAAGAGTACCA-3′, reverse: 5′-TCATGAGGCTTATTGTAGAGC-3′), adipocyte-specific fatty acid binding protein (aP2) (forward: 5′-AGTGGGAGTGGGCTTTGC-3′, reverse: 5′-CCTGTCGTCTGCGGTGAT-3′), human formyl peptide receptor 1 (hFpr1) (forward: 5'-CCACATGTGGAGCAGACAAG-3', reverse: 5'-CCGAGGACAAAGGTGACTG-3'), mouse formyl peptide receptor 1 (mFpr1) (forward: 5'-CCACATGTGGAGCAGACAAG-3', reverse: 5'-CCGAGGACAAAGGTGACTG-3'), mouse FoxO1 (forward: 5'-ACGGGCTGTCTGTCTGTC-3', reverse: 5'-TAAGTGAAGTTTCTCTGTGGTTTC-3'), mouse alkaline phosphatase (ALP) (forward: 5′-ACGAGATGCCACCAGAGG-3′, reverse: 5′-AGTTCAGTGCGGTTCCAG-3′), mouse collagen type 1, alpha 1 (mCOL1A1) (forward: 5′-CCCACCCCAGCCGCAAAGAG-3′, reverse: 5′-GCCATGCGTCAGGAGGGCAG-3′).

Alkaline Phosphatase Activity

In order to measure the activity of alkaline phosphatase in mADSCs, we utilized the Alkaline Phosphatase, Diethanolamine Detection Kit (Sigma-Aldrich Company, St. Louis, MO). Different groups of mADSCs were rinsed twice with Hanks' balanced salt solution and then added to a mixture of 0.67 M p-nitrophenyl phosphate in 1.0 M Diethanolamine and 0.50 mM MgCl2, pH 9.8, at 37 °C. Inversion mixing was immediately performed.

Absorbance of p-nitrophenol produced by ALP activity was monitored at 405 nm using a microplate spectrophotometer over 5 minutes.

Histological Analysis

Alizarin Red staining was utilized to quantify mineralization by ADSCs using our laboratory's standardized procedure[19]. Cells were stained with Alizarin Red S (Sigma-Aldrich Company) for 20 minutes at 40 mM concentration (pH = 4.0). The sample was washed with water to remove excess dye subsequently. A picture was then taken of the plate by a digital camera. Afterwards, the sample was washed with 10% (vol/vol) acetic acid to extract the dye that was bound to deposited minerals. The solution obtained from acetic acid washing was then neutralized using 10% (volume/volume) NH_4OH before transfer to a 96-well plate. Optical density was measured at 405 nm using a microplate reader (Eppendorf, Hamburg, Germany).

Oil Red O staining was utilized to quantify adipogenesis via lipid droplets assessment. PBS was first used to wash the cells twice before the sample was fixed with 10% formaldehyde at room temperature for 30 minutes. Afterwards, the samples were washed twice with distilled water and with 70% isopropanol once. The samples were then stained using filtered Oil Red O (Sigma-Aldrich Company) at room temperature for 1 hour. The filtered Oil Red O solution was a mixture of 60% stock Oil Red O (0.5% wt/vol in isopropanol) and 40% distilled water. The stained samples were washed with water and PBS, each twice, before being examined with a light microscope. Optical Density was measured at 490 nm on a microplate reader (Eppendorf, Hamburg, Germany).

Statistical Analysis

All data were reported as mean ± SD. A two-tailed Student's t-test was used to determine statistically significant differences between two groups, which was defined as results with $p<0.05$.

RESULTS:

Increased Fpr1 expression is associated with osteogenic induction in hADSC

hADSCs grown in OM expressed an increased level Fpr1 than in BM ($p<0.05$) (Figure 1A). At the same time, hADSCs after induction also expressed significantly more RUNX2 compared to BM ($p<0.05$), pointing to the association between increased Fpr1 and improved osteogenesis (Figure 1A). Superior mineralization in OM group was also confirmed by Alizarin Red staining to visualize calcium deposits (Fig 1B). OM group exhibited significantly higher OD value than found in BM group ($p<0.05$).

Increased Fpr1 expression is associated with osteogenic induction and improved bone formation in mADSC

Similar to the results found in experiment with hADSCs, wild type (WT) mADSCs cultured in OM expressed over twice the level of Fpr1 (day 2: 2.51±0.56, day 7: 2.35±0.54) found in BM control (day 2: 1.02±0.23, day 7: 1.02±0.22) ($p<0.05$) (Figure 2A). In terms of osteogenic markers, WT ADSCs yielded significantly higher expression of RUNX2

following osteogenic induction via OM (2.31±0.18) compared to BM control (1.05±0.24) ($p<0.05$) (Figure 2B). Similarly, WT OM ADSCs' expressions of COL1A1 (1.80±0.32) and ALP (3.19±0.53) were both higher than WT BM control's level of COL1A1 (1.02±0.29) and ALP (1.01±0.20) ($p<0.05$) (Figure 2D and 2E). A similar trend was found in evaluation of OD value following Alizarin Red staining of OM and BM WT groups ($p<0.05$) (Figure 2F). Visually, the WT OM culture had a much more robust staining compared to WT BM.

Fpr1 KO mADSCs is associated with weakened osteogenic capacity

In Figure 2B, KO OM mADSCs was shown to express only around half of the amount of RUNX2 (1.21±0.15) found in WT OM population (2.31±0.18) ($p<0.05$) (Figure 2B). Similarly, KO OM mADSCs expressed much weaker COL1A1 (0.75±0.26) and ALP (0.89±0.11) than the WT OM culture (COL1A1: 1.80±0.32, ALP: 3.19±0.53) ($p<0.05$) (Figure 2C and 2D). Additionally, the expressions of the three aforementioned markers were largely similar between Fpr1 KO mADSCs grown in BM or OM. Alkaline phosphatase assay also demonstrated lower level of activity in KO OM mADSCs compared to WT OM ($p<0.05$) (Figure 2E). Finally, reduced mineralization in KO OM mADSCs following osteogenic induction was illustrated by less robust Alizarin Red staining, confirmed by significantly lower OD value than found in WT OM culture (Figure 2F).

Fpr1 KO mADSCs is associated with improved adipogenic capacity

Successful induction of adipogenesis using AM was confirmed by significantly higher levels of PPARγ, aP2, and more robust Oil Red staining in AM groups compared to BM groups regardless of whether the cells were Fpr1 WT or KO ($p<0.05$) (Figure 3). Adipogenic induction was associated with significantly lower expression of Fpr1 in WT AM group (0.36±0.06) compared to BM control (1.05±0.40) ($p<0.05$) (Figure 3A). Using Oil Red O staining, Fpr1 KO AM mADSCs yielded significantly higher OD value (0.131±0.010) than found in WT AM (0.116±0.003) ($p<0.05$) (Figure 3C). Histologically, more lipid droplets were found on the surface of KO AM populations (Figure 3B). The difference was even more pronounced using adipogenic markers. Fpr1 KO AM mADSCs expressed over 3 times more aP2 (21.74±1.47) and over 15 times more PPARγ (16.96±2.47) than WT AM mADSCs did (aP2: 6.03±1.07, PPARγ: 0.98±0.12) ($p<0.05$) (Figure 3D, 3E).

Fpr1 KO is associated with decreased expression of FoxO1

FoxO1 is a protein previously understood to be involved in the commitment of preadipocyte[21]. We found that regardless of medium, Fpr1 KO mADSCs expressed significantly reduced levels of FoxO1 compared to WT ($p<0.05$) (Figure 4). In WT mADSCs, osteogenic induction is associated with a significantly higher level of FoxO1 expression (1.72±0.24) compared to BM (1.03±0.29) ($p<0.05$) (Figure 4A). On the other hand, KO OM mADSCs shows a mild non-significant increase of FoxO1 over KO BM (0.98±0.23 vs 0.81±0.12). Following adipogenic induction, both WT (0.56±0.17) and KO (0.34±0.07) mADSCs exhibited significantly decreased FoxO1 expression compared to BM WT (1.02±0.14) and BM KO (0.48±0.06) ($p<0.05$) (Figure 4B). Additionally, KO AM mADSCs expressed significantly lower levels of FoxO1 compared to WT AM ($p<0.05$). A possible interaction between Fpr1, FoxO1 and mADSC differentiation was illustrated in Figure 4C.

DISCUSSION:

From the results of the study, it is sufficient to establish a correlation between expression of the Fpr1 and the differentiation of ADSCs in favor of the osteoblast and against the adipocyte lineage. Fpr1 has traditionally been thought of as a chemoattractant, supporting migration of phagocytic myeloid cells such as neutrophils or monocytes in the context of acute immune reaction[13]. Kim et al and Shin et al also further investigated the role of Fpr1 and its ligand in the recruitment and lineage regulation of human BMMSCs [16,17]. Nonetheless, Fpr1's activity in the population of adipose-derived stem cells has not been studied. In this study, we demonstrated Fpr1's novel likely role as a factor which commits ADSCs to the osteoblast pathway instead of adipocytes.

As shown in Figure 1, human ADSCs cultured in osteogenic media is associated with a significantly higher level of Fpr1 expression as well as other markers of osteogenesis such as Runx2 and mineralization. Mice ADSCs grown in OM also showed similar characteristics as Fpr1 expression is associated with higher level of osteogenic markers, alkaline phosphatase activity and mineralization. Notably, as shown in Figure 2, when Fpr1 is knockout, ADSCs no longer exhibit robust osteogenesis when cultured in OM. This lack of response to OM indicates the pivotal role played by Fpr1 in lineage differentiation and its upstream position in the metabolic pathway compared to Runx2, COL1A1 and alkaline phosphatase.

Figure 3 provides several strong suggestions about the role of Fpr1 in mediating adipogenesis in mouse ADSCs. First, adipogenesis in mADSCs is associated with decreased expression of Fpr1. Moreover, based on figures 3A and 3B, staining by Oil Red O indicates significantly more adipogenesis in Fpr1 knockout cells compared to wild type when grown in AM. This is corroborated by figures 3D and 3E showing dramatic increase in adipogenic markers PPARγ and aP2 in KO AM ADSCs. As the result, it is likely that Fpr1 act as a suppressor of adipogenesis. Fpr1's qualities as a potential inducer of osteogenesis and suppressor of adipogenesis in ADSCs fit with current understanding of these two processes, which are often a reciprocal balance act. Interaction of Fpr1 with both processes points to its importance in the metabolic regulation of ADSC differentiation.

We attempt to further elucidate the mechanism of action of Fpr1 by examining its interaction with FOXO1, which has been recognized for its crucial role in regulated cell death, the cell cycle, and most notably, adipogenesis[18]. FOXO1 has been previously described to be an activator of cycle inhibitors p21 and p27, leading to cell cycle arrest, and an inhibitor of PPARγ, leading to suppression of adipogenesis[18]. As demonstrated in Figure 4, Fpr1 KO mADSCs in both OM and AM showed significantly lower levels of FOXO1 gene expression compared to Fpr1 WT in correlation with lower levels of Fpr1. This likely means that Fpr1 is upstream compared to FOXO1 in the metabolic pathway regulating ADSCs differentiation and acts as an activator of FOXO1 transcription (Figure 4C). Moreover, the lack of significant difference in FoxO1 levels in KO BM and OM mADSCs may indicate a more sensitive interaction between Fpr1 and FoxO1 in adipogenic differentiation compared to in osteogenesis. The result of this experiment further supports Fpr1's central role.

As noted earlier, ADSCs hold many clinical advantages and is regarded by many to be the future of regenerative medicine. Nonetheless, there are still quite a few concerns about the inherent osteogenicity of ADSCs under current definitions. Several groups have encountered disappointing results on use of ADSCs to enhance bone healing in different defect models[22–25]. Implantation of human ADSCs were also found to be incapable of inducing *de novo* ectopic osteogenesis in immunodeficient animals[26,27]. As such, we believe that development of strategies to further activate osteogenesis and suppress adipogenesis of ADSCs is the natural next step for researchers in the field. To that end, Fpr1 manipulation is likely to hold a pivotal role. We have established Fpr1 expression's correlation with improved osteogenesis. In our opinion, future studies should build upon several directions. First, it is necessary to investigate available Fpr1 ligands and agonists to evaluate their effects on osteogenesis. Moreover, researchers could consider genetic manipulation of Fpr1 to evaluate Fpr1-overexpression ADSCs as a possible therapeutic tool. Further considerations should also be focused on the combination of Fpr1 targeting with other known pathways of osteogenesis induction in ADSCs, for example using bone morphogenetic proteins. Synergistic effects may be found, which would reduce the necessary dose of BMP, which is known to have many side effects[28]. Finally, it is imperative that the discussed ADSC constructs be tested on actual animal disease model. Due to its favorable clinical properties, ADSCs in general, and Fpr1-manipulated ADSCs specifically, promises great clinical benefits to patients suffering from many different bone and joint conditions, such as osteoarthritis, nonunion, or fracture.

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Highlights:

- Formyl peptide receptor-1 expression is abundant in adipose-derived stem cells and is associated with improved osteogenesis.
- Formyl peptide receptor-1 knock-out in adipose-derived stem cells leads to worsened osteogenesis but improved adipogenesis.
- Formyl peptide receptor-1's signaling mechanism on osteogenic and adipogenic differentiation of adipose-derived stem cells possibly involves the nuclear transcription factor Forkhead box protein O1.

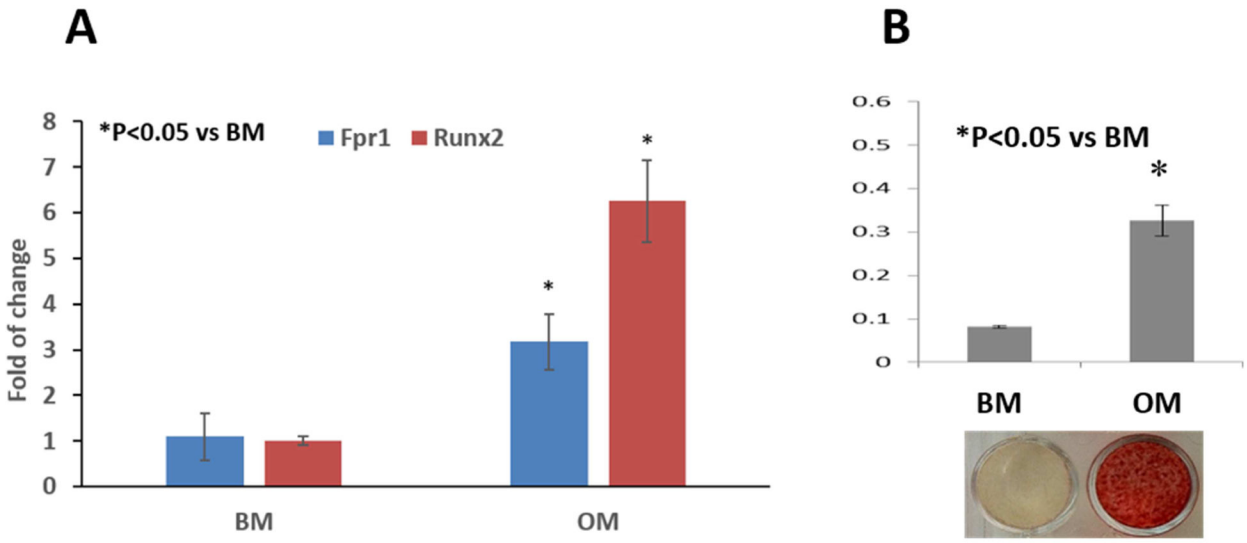

Figure 1. Increased Fpr1 expression and osteogenic capacity found in hADSCs following osteogenic induction at day 11.

A. Expression of Fpr1 and Runx2 in hADSCs cultures represented as folds of change of BM value. B. Top panel: OD value at 405 nm of hADSCs cultured in either basal or osteogenic media following Alizarin Red S staining, Bottom panel: Visual representation of Alizaren Red S staining. There were 4 replicates in each group (n=4).

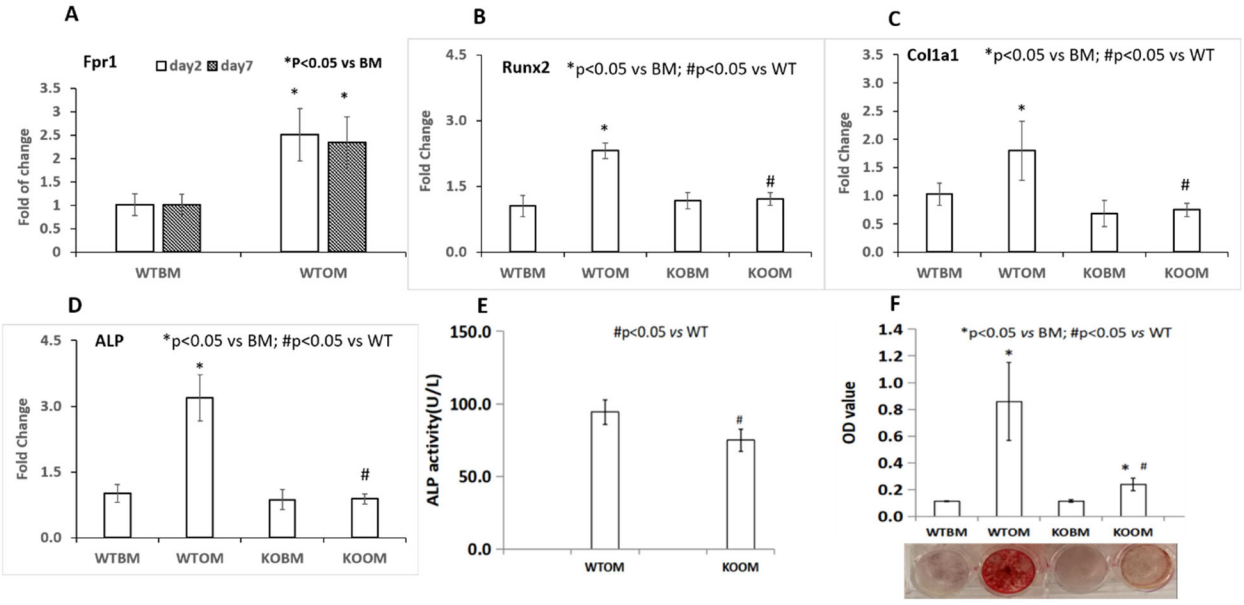

Figure 2. Increased Fpr1 associated with improved osteogenic capacity in WT mADSCs while Fpr1 KO mADSCs showed decreased osteogenicity at day 7.

A. Comparison of Fpr1 expresion between mADSCs grown in BM and OM at day 2 and day 7; B. Runx2 expression in WT or KO mADSCs grown in either BM or OM; C. COL1A1 expression in WT or KO mADSCs grown in either BM or OM; D. ALP expression in WT or KO mADSCs grown in either BM or OM; E. ALP activity measured in WT OM and KO OM cultures; F. Top panel: OD value at 405 nm of WT or KO mADSCs cultured in either BM or OM following Alizarin Red S staining, Bottom panel: Visual representation of Alizaren Red S staining. There were 4 replicates in each group (n=4).

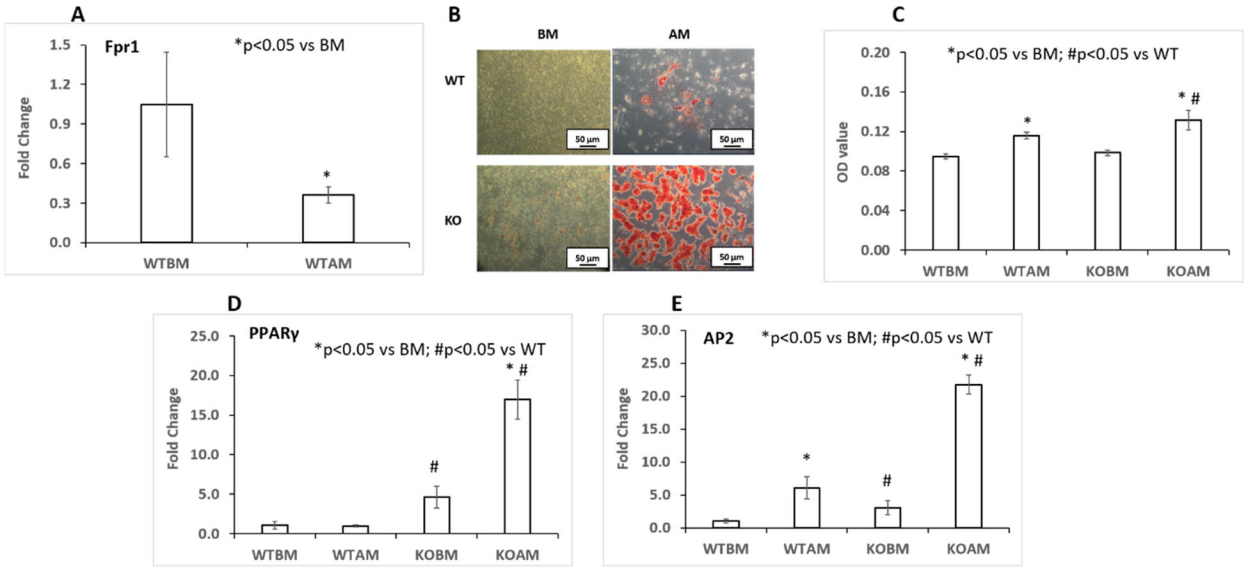

Figure 3. Fpr1 KO mADSCs demonstrates increased adipogenesis capacity compared to WT at day 9.

A. Fpr1 expression in WT mADSCs following adipogenic induction compared to BM control. B. Visual representation of WT or KO mADSCs following Oil Red O staining. Left column is from cultures grown in BM, right column is from cultures grown in AM. Upper row is from WT cultures, lower row is from KO cultures; C. OD value at 490 nm of WT or KO mADSCs cultured in either BM or AM following Oil Red O staining; D. PPARγ expression in WT or KO mADSCs grown in either BM or AM; E. aP2 expression in WT or KO mADSCs grown in either BM or AM. There were 4 replicates in each group (n=4).

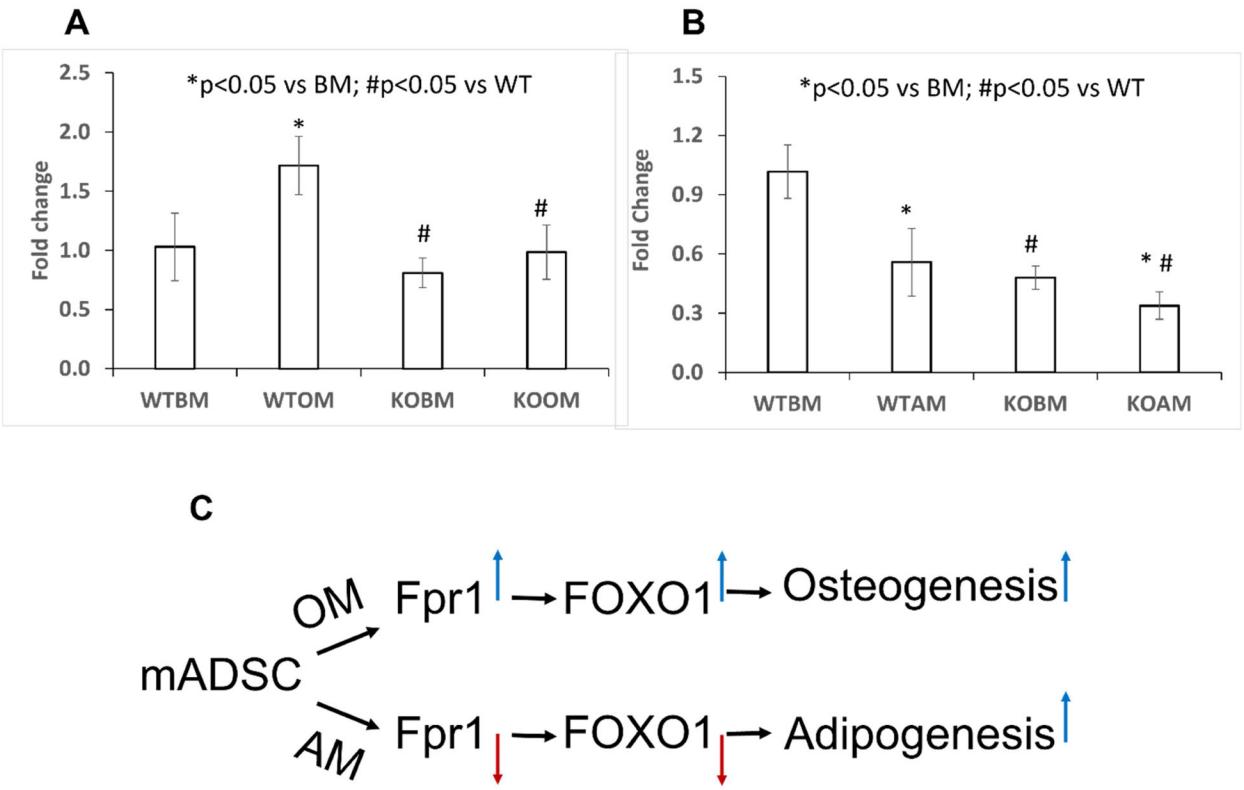

Figure 4. FoxO1 expression in WT or Fpr1 KO mADSCs following osteogenic and adipogenic induction.

A. FoxO1 expression in WT or KO mADSCs grown in either BM or OM for 7 days.

B. FoxO1 expression in WT or KO mADSCs grown in either BM or AM for 7 days.

C. Basic diagram representing the possible interaction between Fpr1, FoxO1 and mADSC differentiation. In A and B, there were 4 replicates in each group (n=4).