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PGE2 Receptor Subtype 1 (EP1) Regulates Mesenchymal Stromal Cell Osteogenic Differentiation by Modulating Cellular Energy Metabolism

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Abstract

Mesenchymal stromal cells (MSCs) are multipotent progenitors capable of differentiation into osteoblasts and can potentially serve as a source for cell-based therapies for bone repair. Many factors have been shown to regulate MSC differentiation into the osteogenic lineage such as the Cyclooxygenase-2 (COX2)/Prostaglandin E2 (PGE2) signaling pathway that is critical for bone repair. PGE2 binds four different receptors EP1-4. While most studies focus on the role PGE2 receptors EP2 and EP4 in MSC differentiation, our study focuses on the less studied, receptor subtype 1 (EP1) in MSC function. Recent work from our laboratory showed that $EP1^{-/-}$ mice have enhanced fracture healing, stronger cortical bones, higher trabecular bone volume and increased *in vivo* bone formation, suggesting that EP1 is a negative regulator of bone formation. In this study, the regulation of MSC osteogenic differentiation by EP1 receptor was investigated using EP1 genetic deletion in $EP1^{-/-}$ mice. The data suggest that EP1 receptor functions to maintain MSCs in an undifferentiated state. Loss of the EP1 receptor changes MSC characteristics and permits stem cells to undergo more rapid osteogenic differentiation. Notably, our studies suggest that EP1 receptor regulates MSC differentiation by modulating MSC bioenergetics, preventing the shift to mitochondrial oxidative phosphorylation by maintaining high Hif1α activity. Loss of EP1 results in inactivation of Hif1α, increased oxygen consumption rate and thus increased osteoblast differentiation.

Keywords

Mesenchymal Stromal Cell; PGE2; EP1; Oxidative Phosphorylation

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Introduction

Mesenchymal stromal cells, also known as mesenchymal stem cells (MSCs) are adult multipotent progenitor cells that have the ability to differentiate into osteoblasts, chondrocytes and adipocytes and play a significant role in bone homeostasis and repair (2,3). Changes in MSC ability to differentiate into osteoblasts can result in either a reduction or enhancement in bone formation and fracture repair potential (2,3). Prostaglandin E2 (PGE2) is a known modulator of bone formation (4–6). PGE2 is a lipid compound synthesized from arachidonic acid by cyclooxygenases 1 or 2 (COX1 or COX2, respectively). PGE2 plays an important role in bone homeostasis and repair by its ability to regulate both bone formation and resorption (7,8). PGE2 is the most abundant prostaglandin in the body and is the main prostaglandin to be studied in bone tissue (9,10). Prostaglandin E2 was shown to increase bone marrow mineralized nodule formation when administrated systemically, suggesting that the number of committed progenitors is increased and that PGE2 has a role in bone homeostasis (11). PGE2 exerts its effect by binding to four receptors: EP1-EP4. Several studies demonstrated the bone anabolic effect of PGE2 by activating EP4 or EP2 receptors (12,13).

Recent studies suggest that prostaglandin E2 plays a role in stem cell biology. In hematopoietic cells PGE2 has been shown to maintain the primitive stem cell state (14). PGE2 was also shown to maintain long-term repopulating cells in culture by stabilizing the β-catenin pathway (15). PGE2 can prevent hematopoietic stem cell (HSC) death and promote recovery after radiation injury (14). *In vivo*, pretreatment of bone marrow cells with PGE2 before transplantation improves HSC engraftment (16). The effect of PGE2 on stem cell biology has been primarily attributed to signaling via the EP2 and EP4 receptors (15–17). However, limited investigations regarding the role of EP1 receptor as a mediator of PGE2 responses in stem cells have been performed. Our recent studies in $EP1^{-/-}$ mice show that EP1 is a negative regulator of bone formation (18,19). We demonstrated that $EP1^{-/-}$ mice exhibit enhanced fracture repair and an increased bone formation rate (20). Additionally, the mice exhibited increased bone formation *in vivo* leading to stronger bones that also protects from bone loss during ageing as well as following ovariectomy (21). Given the role of progenitor cells in the maintenance of bone mass and in injury and repair responses, these findings raised the possibility that the EP1 receptor was involved in the regulation of the bone progenitor cell population.

Recently, the importance of cellular bioenergetics in stem cell biology was demonstrated in several systems. First, it was shown that embryonic stem differentiation requires mitochondrial maturation and activation of oxidative phosphorylation (OxPhos) (22,23). In addition, maturation and differentiation of adult neuronal and hematopoietic stem cells also requires the switch in energy production to OxPphos (24). Changes in cellular bioenergetics in MSC differentiation was demonstrated by several laboratories. Chen and colleagues, showed that during MSC differentiation into the osteoblastic lineage, there is an increase in OxPhos with maturation of mitochondria, and that blocking mitochondrial activity inhibited osteoblast differentiation. Shum., L. *et al* (2016) showed that during osteogenic differentiation OxPhos is up regulated by down regulation of the Hif1α signaling pathway (25). In contrast, to create inducible pluripotent stem cells (iPSCs), it is necessary to reduce

OxPhos and increase glycolysis (26). In bone marrow, long repopulating HSCs were shown to have reduced mitochondrial potential and increased glycolysis (27,28).

As bone formation is dependent on MSC number and function, and given the potent regulatory role of EP1 in fracture healing, a series of experiments were performed to test the hypothesis that activation of the EP1 inhibits MSC differentiation. We found that $EP1^{-/-}$ bone marrow consists of a higher percentage of committed progenitors with higher potential to differentiate into the osteoblastic lineage. Additionally, our studies suggest that PGE2, through the EP1 receptor, regulates MSC fate through the modulation of Hif1α signaling, resulting in increased mitochondrial bioenergetics. To our knowledge this is the first study to demonstrate that PGE2 plays a role in cellular bioenergetics, thus affecting BMSC's differentiation potential.

Methods

Animals studies

The breeding colonies of C57BL/6 mice were purchased from Jackson Laboratory and expanded in the University of Rochester facility and used as Wild Type (WT) controls. $EP1^{-/-}$ mice were generously provided by Matthew Breyer (Vanderbilt University) (1). $EP1^{-/-}$ mice were created by introduction of a stop codon in exon 2 of EP1 gene. All animal breeding and procedures were approved by University Committee of Animal Resources (UCAR) at the University of Rochester.

Mesenchymal stromal cell isolation and culture

Bone marrow cells were isolated from 10–14 week-old $EP1^{-/-}$ or C57BL6/J mice. Mice were sacrificed and femuri and tibiae were removed and bone marrow was flushed with PBS supplemented with 3% FBS. The cells were strained through a 70mm mesh and collected by centrifugation at 1000 RPM for 5 minutes. The collected cells were resuspended in MesenCult Proliferation Kit with Stem Cell Stimulatory Supplements (Stem Cell Technologies # 05512, Vancouver, Canada) and 1% Streptomycin and Penicillin were used for further experiments. For Colony Forming Unit (CFU) assays, freshly isolated cells were plated at 20000 cells/per well of a six-well plate and cultured for 10 days. The cells were then fixed and stained with 0.5% crystal violet in methanol for CFU-F and with ALP substrate NBT/BCIP reagent (Thermo Scientific Pierce #34042 Grand Island, NY) for CFU-O. A colony was considered a cluster of more than 50 cells.

Flow cytometry

Expression of cell surface markers was performed by staining freshly isolated bone marrow cells that were resuspended in 100μl PBS with 3% FBS and stained with 1μl mouse BD Fc blocker (anti CD16/CD32, BD pharmingen # 553141, San Jose, CA) prior to staining with antibodies: CD45-PerCP, CD31-PE-Cy7, CD105-PE (BD pharmingen, # 561047, 561410, and 562759, respectively, San Jose, CA) and Sca1-APC (Thermo Scientific # 17–5981 Grand Island, NY). The cells were incubated with the antibodies for 30–45 minutes in the dark. The cells were analyzed using AC-CANTO (Becton Dickson, Franklin Lanes NJ) flow cytometer. The data was analyzed using FlowJo software (TreeStar, Ashland OR). For cell

sorting, freshly isolated cells were stained with cell surface fluorescent antibodies as described above and sorted based on expression of $Sca1^+$ and $CD105^+$ cells or $Sca1^+CD105^-$ cells. Sorting was performed using an 85micron nozzle FACSAria cell sorter (eBiosciences, San Diego CA) in the URMC Flow Cytometry Core. The cells were collected into warm MesenCult medium with 20% serum and supplements (Stem Cell Technologies # 05512, Vancouver, Canada). The collected cells were either seeded into 12-well plates for differentiation assays or washed once with PBS and RNA was isolated using RNEasy kit as described below.

Osteoblastic differentiation

Freshly isolated bone marrow cells were grown to confluence and media was changed to medium supplemented with 10mM β-glycerol phosphate (Sigma Aldrich #G9422, St.Louis, MI) and 50μg/ml Ascorbic Acid (Sigma Aldrich #A0278, St.Louis, MI). The medium was changed every 2 days and cells were harvested at designated time points.

Cell proliferation

Bromodeoxyuridine (BrdU-10mg/kg) (Invitrogen # 0103, UK) was injected 12 hours before sacrificing the mice. The cells were isolated as described above followed by fixation and permeabilization using a Perm and Fix kit (Thermo Fisher # GAS003, Grand Island, NY). After the fixation and permeabilization the cells were stained with cell surface antibodies as described before and with BrdU-FITC conjugated antibody (BDbiosciences # 347583, Canada). Flow cytomtery analysis was performed as described above, using a blue laser 454nm excitation and 520nm emission to detect BrdU positive cells.

Bioenergetic profiling

Passage one bone marrow cells (p1) were plated into XF24 Analyzer plates (Seahorse Bioscience, #100850, MA) at 10000 cells per well and allowed to adhere for 48 hours. Immediately before performing the assay the medium was changed to a Seahorse assay medium supplemented with 1 mM glutamine, 5mM glucose with no pyruvate, pH adjusted to 7.4. Oxygen consumption rate (OCR) and Extracellular Acidification Rate (ECAR) were measured following treatments with the following inhibitors, injected by the analyzer: oligomycin (1 μM final concentration), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP: 0.5 μM final concentration), and antimycin A (1 μM final concentration). The measurements of OCR and ECAR were taken at three time points during basal respiration and after each injection. After analysis, cells were trypsinized and counted. The OxPhos parameters were calculated as follows: Basal respiration = $OCR_{pre\ oligo}$ - $OCR_{post\ AntaA}$. ATP-linked respiration = $OCR_{pre\ oligo}$ - $OCR_{post\ oligo}$. ATP level was assessed using CellTiter-Glo Luminescence assay (Promega #G7570, Sweden).

Gene expression analysis

RNA was isolated using the RNeasy Mini Kit (Qiagen #74104, Germany) and cDNA made using the iSCRIPT cDNA Synthesis Kit (BioRad #1708890, Hercules CA). Real-Time quantitative (qRT-PCR) was performed using SYBR Green Mix (Quanta Biosciences # 9554-500, Gaithersburg, MD). Primers used were: *β actin* 5'-

AGATGTGGATCAGCAAGCAG-3’and 5’-GCGCAAGTTAGGTTTTGTCA-3’, Runx2 5’-TGATGACACTGCCACCTCTGACTT-3’ and 5’- ATGAAATGCTTGGGAACTGCCTGG-*3’, Alpl* 5’-TGACCTTCTCTCCTCCATCC-3’ and 5’-CTTCCTGGGAGTCTCATCCT-3’, Col1a1 5’-GCCAAGGCAACAGTCGCT −3’ and 5’-CTTGGTGGTTTTGTATTCGATGAC −3’, Sp7 5’-GGAAAGGAGGCACAAAGAAGCCAT −3’ and 5’-AGTCCATTGGTGCTTGAGAAGGGA −3’, Sox2 5’-TACCTCTTCCTCCCACTCCA-3’ and 5’-CCCTCCCAATTCCCTTGTAT-3’, Nanog 5’-CCAAAGGATGAAGTGCAAGC-3’ and 5’-TTGGTCCAGGTCTGGTTGTT-3’.

Western blotting

Proteins were isolated using Radioimmunoassay (RIPA buffer-Sigma-Aldrich # R0278, St. Louis, MO) following sonication for 12 pulses 15 seconds each and centrifugation for 30 minutes at 13000 RPM at 4°C. Proteins (20–50μg) were separated on 8% SDS-PAGE followed by a transfer to a polyvinylidene membrane. The membrane was incubated with primary rabbit anti-mouse HIF1α antibody (Novus Biologicals # NB100–449) at 1μg/ml overnight at 4°C. The membrane was then washed in TBS-T and incubated with secondary goat anti-rabbit HRP-conjugated antibody (Bio-Rad #1721019, CA) for 1 hour at room temperature followed by 3 washes with TBS-T, 15 minutes each. Immunostaining was detected using a Western Lightning Plus ECL (Perkin Elmer #NEL104001EA, Waltham, MA) and imaged using Kodak film (Hif1α protein) or a BioRad XP Chemidoc System (BioRad. Hercules CA) (β-actin).

Luciferase reporter assay

Hypoxia Response Element (HRE) transcriptional activity was measured using the Dual-Reporter Assay (Promega, Maddison WI). Bone marrow cells were grown until 75–80% and transfected with a construct in which a Hypoxia Response Element is upstream of the firefly luciferase on the pGL4 vector. Concomitantly, 5 ng of pRL vector (Promega #E1960, Sweden) carrying the Renilla uniformis gene was co-transfected to serve as an internal control. HRE luciferase activity was measured 24 hours following transfection.

Cellular viability

Calcein-AM (ThermoFisher # C3100, Grand Island, NY) was added to growth medium and incubated for 30 minutes. The cells were then washed three times with PBS and fluorescence was measured using green laser (480/520nm excitation/emission).

Statistical analysis

Results are presented as the average value plus or minus standard error of the mean. Statistical significance was measured using Student’s t-test or two-way ANOVA followed by Bonferroni test, unless otherwise specified. P values less than 0.05 were considered significant.

Results

EP1$^{-/-}$ bone marrow has more osteogenic progenitors

Since previous observations showed increased bone formation *in vivo* in EP1$^{-/-}$ mice, initial experiments were performed to analyze the colony forming abilities of bone marrow stromal cells isolated from the EP1$^{-/-}$ mice (29). Here, we confirmed that the bone marrow cells of EP1$^{-/-}$ mice form more osteoblastic colonies. Bone marrow MSCs (BMSCs) harvested from EP1$^{-/-}$ mice formed more fibroblastic colonies, CFU-F (WT 8 ± 2, EP1$^{-/-}$ 29 ± 2.6, $p < 0.01$) and more osteoblastic colonies, CFU-O (WT 2.2 ± 0.6, EP1$^{-/-}$ 17 ± 4.85, $p<0.05$) (Figure 1A–B). These data indicate that BMSCs from EP1$^{-/-}$ mice are both more clonogenic and osteogenic.

EP1$^{-/-}$ bone marrow cells exhibit increased osteoblastic differentiation

Given the increased amount of CFU-O of the bone marrow in EP1$^{-/-}$ mice we decided to test the osteoblastic differentiation of EP1$^{-/-}$ BMSCs. EP1$^{-/-}$ BMSCs showed increased staining of alkaline phosphatase and increased mineralization as shown by Alizarin Red staining after 14 days in osteogenic induction medium. Additionally, gene expression of *Alpl* (WT 0.99 ± 0.1, EP1$^{-/-}$ 1.75 ± 0.2, $p<0.05$), *Col1a1* (WT 1 ± 0.01, EP1$^{-/-}$1.56 ± 0.2, $p=0.05$) and *Sp7* (WT 1 ± 0.01, EP1$^{-/-}$ 1.7 ± 0.2 $p<0.05$) was higher in EP1$^{-/-}$ cultures compared to WT control cultures (Figure 1C–D). These results suggest that EP1$^{-/-}$ BMSCs have increased potential for osteogenic differentiation.

Cell surface markers have been used to characterize MSCs (3,30). Fluorescent activated cells sorting (FACS) was used to assess the presence of several well-known MSC surface markers on BMSCs from EP1$^{-/-}$ mice compared to WT mice. We excluded hematopoietic and endothelial cells using CD45 and CD31 markers, respectively. The CD105 and Sca1 markers were used to characterize the MSC population in cells harvested from bone marrow. While we did not observe significant changes in Sca1^{+}CD105^{+} cell populations (WT 0.013 ± 0.008, EP1$^{-/-}$ 0.027 ± 0.014, $p = 0.4$), freshly isolated bone marrow cells from EP1$^{-/-}$ mice had more Sca1^{+}CD105^{-} cells (WT 0.01 ± 0.002, EP1$^{-/-}$ 0.1 ± 0.02, $p<0.01$) compared to the BMSC population harvested from WT mice (Figure 2A–B).

The Sca1^{+}CD105^{-} cell population is a more differentiated osteogenic precursor

We separated the Sca1^{+}CD105^{+} and Sca1^{+}CD105^{-} cell populations obtained from WT mice and performed qRT-PCR to assess the relative gene expression of progenitor cell markers and markers of more differentiated cells. We found that Sca1^{+}CD105^{-} cells express increased *Runx2* levels (Sca1^{+}CD105^{+} 1 ± 0.09, Sca1^{+}CD105^{-} 1.3 ± 0.07, $p < 0.05$). In contrast, the relative levels of pluripotency genes *Sox2* and *Nanog* were higher in Sca1^{+}CD105^{+} cells compared to Sca1^{+}CD105^{-} cells (*Sox2*: Sca1^{+}CD105^{+} 1 ± 0.1, Sca1^{+}CD105^{-} 0.6 ± 0.1, $p < 0.05$. *Nanog*: Sca1^{+}CD105^{+} 1 ± 0.08, Sca1^{+}CD105^{-} 0.54 ± 0.043, $p < 0.01$) (Figure 2C).

We also measured proliferation rate of BMSCs obtained from WT and EP1$^{-/-}$ mice using BrdU labeling. Both WT and EP1$^{-/-}$ mice had a higher number of BrdU^{+} cells in the Sca1^{+}CD105^{-} population compared to the Sca1^{+}CD105^{+} population (WT Sca1^{+}CD105^{+} 7.8

± 3.7, $Sca1^{+}CD105^{-}$ 15.7 ± 7.9 $p < 0.05$, $EP1^{-/-}$ $Sca1^{+}CD105^{+}$ 7 ± 2.9, $EP1^{-/-}$ $Sca1^{+}CD105^{-}$ 17.1 ± 1.4, $p < 0.01$). Taken together, these results show that i) that $EP1^{-/-}$ bone marrow has a higher population of $Sca1^{+}CD105^{-}$ cells compared to WT mice; and ii) osteogenic differentiation potential is greater in the $Sca1^{+}CD105^{-}$ cell population.

$EP1^{-/-}$ BMSCs exhibit increased mitochondrial activity

Based on the increased differentiation of $EP1^{-/-}$ BMSCs we decided to test whether $EP1^{-/-}$ BMSCs have differences in their mitochondrial activity that reflects their differentiation potential. OxPhos of BMSCs was measured using the Seahorse XF24 analyzer. We observed that $EP1^{-/-}$ BMSCs have higher basal oxygen consumption rate (OCR), a measure of OxPhos (WT 1 ± 0.1, $EP1^{-/-}$ 2.26 ± 0.37, $p < 0.01$) and ATP-linked respiration (WT 1.1 ± 0.11, $EP1^{-/-}$ 2.46 ± 0.5, $p < 0.05$) (Fig 3A–C). Additionally, we found that ATP production was higher in $EP1^{-/-}$ BMSCs compared to WT BMCs (WT 1 ± 0.09, $EP1^{-/-}$ 1.28 ± 0.034, $p < 0.05$) (Figure 3D).

$EP1^{-/-}$ BMSCs enhanced osteogenic differentiation depends on OxPhos

Next, to test whether differentiation of BMSCs in $EP1^{-/-}$ mice is due to enhanced mitochondrial activity, we inhibited mitochondrial activity and measured $EP1^{-/-}$ BMSCs differentiation using Antimycin A an electron transfer blocker. We treated the cells with Antimycin A (100nM) and stained for ALP and performed gene expression analysis. Antimycin treatment reduced the basal respiration rate of $EP1^{-/-}$ BMSCs (Vehicle 1 ± 0.1, Antimycin 0.4 ± 0.2, $p < 0.05$) (Figure 4A). Antimycin A treatment also reduced ALP staining and the expression of *Alpl* gene in $EP1^{-/-}$ cells, but not in WT cells (WT Vehicle 1 ± 0.23, Antimycin 1 ± 0.68, $p > 0.05$, $EP1^{-/-}$ vehicle 2.23 ± 1.2, $EP1^{-/-}$ Antimycin 0.83 ± 0.35, $p < 0.01$) (Figure 4B–C). The treatment with Antimycin A did not reduce cell viability in either WT or $EP1^{-/-}$ cells, as measured by calcein incorporation (WT 1.09 ± 0.13, $EP1^{-/-}$ 0.95 ± 0.02, $p > 0.05$) (Figure 4D).

$EP1^{-/-}$ BMSCs exhibit reduced *Hif1α* activity

As Hif1α is a known negative regulator of mitochondrial activity in various cells, including BMSCs (31), we tested whether there was a change in Hif1α protein levels in $EP1^{-/-}$ BMSCs. The literature suggests that EP1 is a positive regulator of Hif1α activity (32). We, therefore, hypothesized that the observed increase in OxPhos in $EP1^{-/-}$ cells is due to lower Hif1α activity. Using Western blot analysis and densitometry we observed that $EP1^{-/-}$ BMSCs have lower levels of Hif1α protein compared to WT BMSCs (WT lanes 1–3 0.97 ± 0.3, $EP1^{-/-}$ lanes 4–6, 0.55 ± 0.17 $p < 0.05$) (Figure 5A–B). Similarly, Hif1α transcriptional activity as measured by hypoxia responsive luciferase reporter activity was reduced in $EP1^{-/-}$ BMSCs (WT 1.22 ± 0.14, $EP1^{-/-}$ 0.45 ± 0.066, $p < 0.05$) (Figure 5C).

We next tested whether re-activation of Hif1α signaling reduces the enhanced $EP1^{-/-}$ BMSC differentiation. We treated cells with Hif1α stabilizer, DMOG, and analyzed osteoblastic gene expression. DMOG treatment increased Hif1α protein levels both in WT and $EP1^{-/-}$ BMSCs (Figure 6A). DMOG treatment reduced the enhanced differentiation of $EP1^{-/-}$ BMSCs as evidenced by the decreased Alizarin Red staining and decreased expression of

Alpl (Figure 6B–C) (Vehicle 1.9 ± 0.5, EP1$^{-/-}$ 0.65 ± 0.097, $p \leq 0.05$) (Figure 6D). Cell viability was not changed upon treatment with DMOG (Figure 6E).

Taken together the data show that BMSCs from EP1$^{-/-}$ mice exhibit lower levels of Hif1α activity which results in increased mitochondrial respiration (OxPhos) which in turn contributes to enhanced osteoblastic differentiation of EP1$^{-/-}$ MSCs.

Discussion

The objective of this study was to characterize the BMSC of EP1$^{-/-}$ mice and to elucidate the mechanism by which the EP1 receptor regulates stromal cell differentiation. Here we report that EP1$^{-/-}$ bone marrow has more BMSCs with increased osteoblastic differentiation potential. Additionally, we observed that EP1$^{-/-}$ BMSCs exhibit enhanced mitochondrial activity which correlated with more differentiated cells. Moreover, EP1 down regulation was associated with reduced Hif1α activity. Thus, we identified a potential role for EP1 receptor in regulating cellular metabolism and stem cell fate determination, which has not been previously reported.

MSCs are essential components of bone homeostasis and repair as well as part of the bone marrow niche (33). The regulation of MSC differentiation in a controlled manner is essential to renewal the MSC pool and long term maintenance of this cell population (34). Our data suggest that down regulation of EP1 receptor results in increased differentiation of MSCs into the osteoblastic lineage, and correlatively, that activation of the EP1 receptor maintains MSCs in a more progenitor state and acts as a “brake” on MSC differentiation. Previous *in vitro* and *in vivo* studies showed that EP2 and EP4 agonist administration results in increased bone formation (5,35,36). Our laboratory showed that EP1$^{-/-}$ mice exhibit increased bone formation rate as well as reduced bone loss with ageing (29). Furthermore, our findings suggest that the EP1 receptor regulates bone formation through the regulation of metabolic activity in MSCs.

While characterizing the EP1$^{-/-}$ BMSC population, we observed a significant increase in the percentage of Sca1^{+}CD105^{-} cells in bone marrow compared to the number of Sca1^{+}CD105^{-} cells in WT bone marrow. This was accompanied by an increased ratio of CFU-Os in bone marrow from EP1$^{-/-}$ mice compared to WT mice that formed under non-stimulated conditions. Previously it has been shown in various cell populations, including umbilical cord cells, adipose tissue and bone marrow cells, that the population of progenitor cells expressing Sca1 but lacking CD105 marker expression have higher osteoblastic potential (37–39). More importantly, these cells have been shown to have higher regeneration potential in the critical-size mouse calvarial defect model (38). In our study, we showed that EP1$^{-/-}$ bone marrow cells have more osteoblastic cells, more committed progenitors and higher osteoblastic differentiation potential. In addition, Sca1^{+}CD105^{-} cells exhibited increased expression of RUNX2 transcription factor that drives MSC osteogenic differentiation. In support of these results we also observed increased differentiation into the osteoblastic lineage. All of this supports the hypothesis that EP1$^{-/-}$ bone marrow consists of a MSC population with increased differentiation potential.

Next, we wanted to begin defining the mechanisms through which EP1 receptor regulates MSC differentiation and focused our experiments on mitochondrial energy metabolism. A role of metabolic bioenergetics in regulating stem cell self-renewal or differentiation has been suggested in several recent studies (27,40,41). These studies show that the intracellular metabolic state is a characteristic of specific cell stage, and in addition that intracellular metabolism has a mechanistic role in the differentiation process and cell fate determination (39,42–45). Multiple studies using MSCs show that changes in mitochondrial structure and function occur with differentiation (28,46). Specifically, osteoblastic differentiation was accompanied with increased OCR, mitochondrial gene expression and oxidative stress (46). In the current experiments we similarly showed that the increased osteoblastic differentiation observed in $EP1^{-/-}$ BMSCs was paralleled by changes in mitochondrial metabolism. We observed an increase in basal levels of respiration, ATP production, and maximal respiratory capacity in $EP1^{-/-}$ BMSCs. More importantly, the increased osteoblastic differentiation in BMSCs from $EP1^{-/-}$ mice was blocked when oxidative phosphorylation was inhibited. Interestingly, inhibition of OxPhos did not cause changes in WT BMSC differentiation. These results support the notion in the $EP1^{-/-}$ MSCs have increased oxidative phosphorylation that results in increased osteoblastic differentiation. Consistently with this suggestion, Chen *et al* (2008) showed that human MSCs differentiated into the osteoblastic lineage are more sensitive to inhibition of OxPhos compared to undifferentiated MSCs (46). Conversely, Esen *et. al.*(47) showed that a high level of glycolysis is required for osteoblastic differentiation to take place. One possible explanation is that during differentiation, while elevated metabolic requirements result in increased OxPhos however, there is still a need for higher glycolytic activity to supply the intermediates for OxPhos.

Hif1α protein level was measured as a possible regulator of mitochondrial bioenergetics changes observed in $EP1^{-/-}$ BMSCs. Previously, it was shown that EP1 upregulated Hif1α translation (32). Additionally, it has been shown that in osteoblasts, hypoxia can upregulate EP1 expression, suggesting that there is an interplay and possible co-regulation of EP1 and Hif1α. As Hif1α is considered to be a master regulator of mitochondrial activity and has an important role in stem cell differentiation and reprogramming, we tested whether there were changes in Hif1α levels in $EP1^{-/-}$ BMSCs. Both protein levels and transcriptional activity were decreased in BMSCs from $EP1^{-/-}$ mice. Importantly, the treatment of BMSCs cells with the Hif1α stabilizer (DMOG) blocked the increased osteoblast differentiation observed in $EP1^{-/-}$ BMSCs. Others have similarly shown that Hif1***signaling regulates MSC differentiation into the osteoblastic lineage. These data support our hypothesis that down regulation of EP1 reduces Hif1*** activity leading to activation of OxPhos and, thus to a BMSC cell population with greater osteoblastic differentiation potential (Figure 6).

As the role of PGE2 in stem cell biology has been implicated previously, our work presents novel observations regarding PGE2 signaling and MSCs, and proposes an important regulatory role for EP1 in MSC differentiation. However, more studies are required to understand the exact mechanism of EP1 regulation of MSCs. One question is how EP1 regulates Hif1*** activity. It has been suggested that in a hepatic cell line, PGE2 activates Hif1α through the regulation of mTOR signaling and increased Hif1*** translation. mTOR signaling in general has been associated with an important role in stem cell biology, partly

by regulating Hif1α activity. It will be important to test whether EP1 activity has similar effects in BMSCs.

In summary, the studies presented here provide novel findings regarding the function of the PGE2 receptor, EP1, in stem cell biology. The data suggest that the EP1 receptor functions to maintain the MSCs in an undifferentiated state, indicating that EP1 is a negative regulator of osteoblastic differentiation and bone formation. The loss of the EP1 receptor changes MSC characteristics, results in an increase in the $CD105^{-}$ population and OxPhos, and promotes cell differentiation along the osteoblast lineage. This is the first time that PGE2 is shown to have a role in cellular bioenergetics. The research presented here introduces the EP1 receptor as being a novel target to enhance fracture healing in addition to being a potential therapeutic target.

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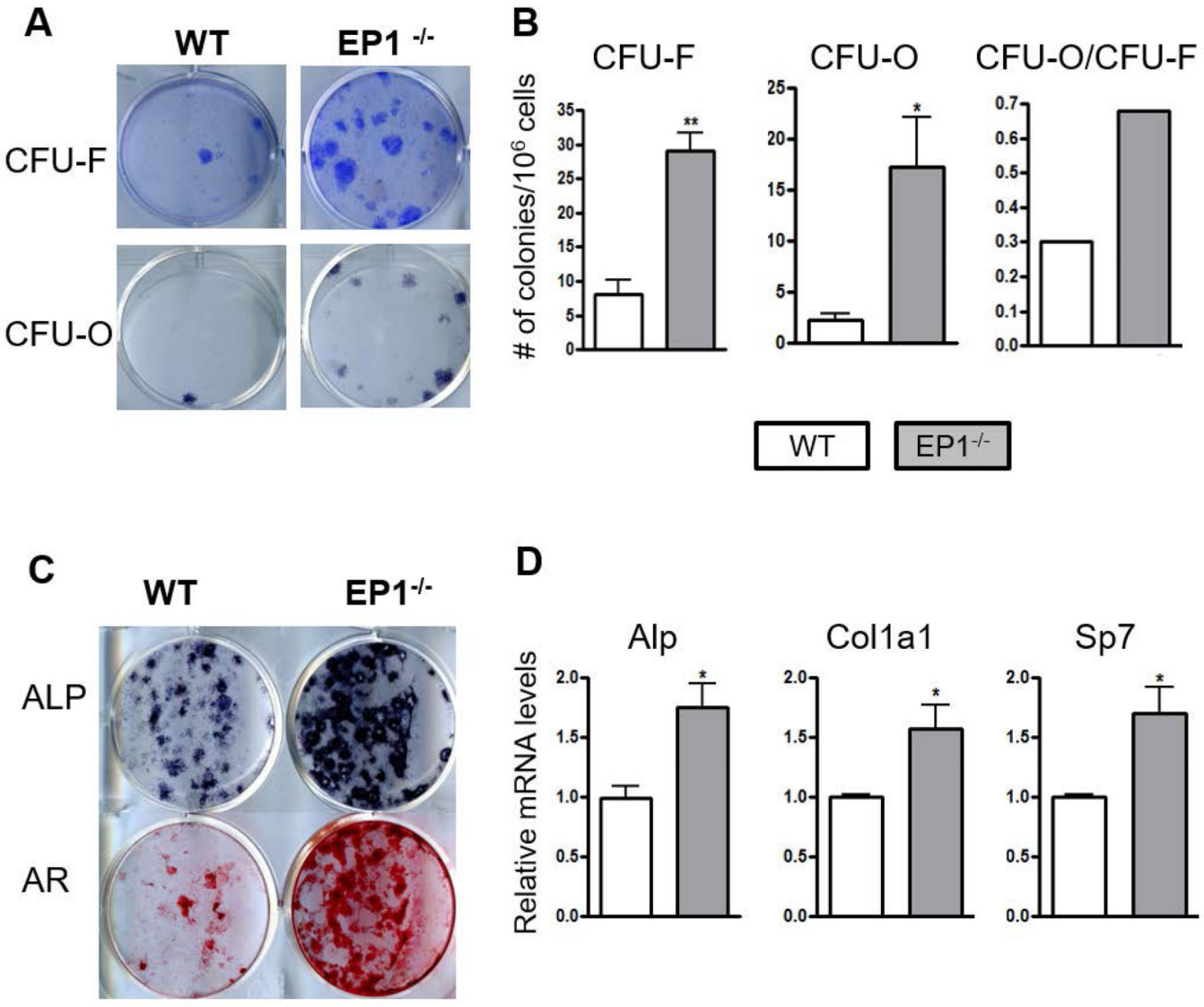

Figure 1. EP1$^{-/-}$ BMSCs exhibit higher osteoblastic differentiation

Bone marrow cells from WT and EP1$^{-/-}$ mice were isolated and cultured *in vitro*. (A) Representative images and (B) quantifications of CFUs from freshly isolated bone marrow of WT and EP1$^{-/-}$ mice assessed after 10 days in regular (CFU-F) or osteogenic (CFU-O) medium. Representative images of AR and ALP staining and (C) osteoblastic gene expression (D) after incubation in osteogenic medium for 14 days (N=5 per group). Data are means ± SEM. Statistical analysis was performed using paired student t-test. (*) = $p<0.05$ (**) = $p<0.01$ compared to age-matched WT. White bars represent samples of WT mice and grey bars represent samples from EP1$^{-/-}$ mice.

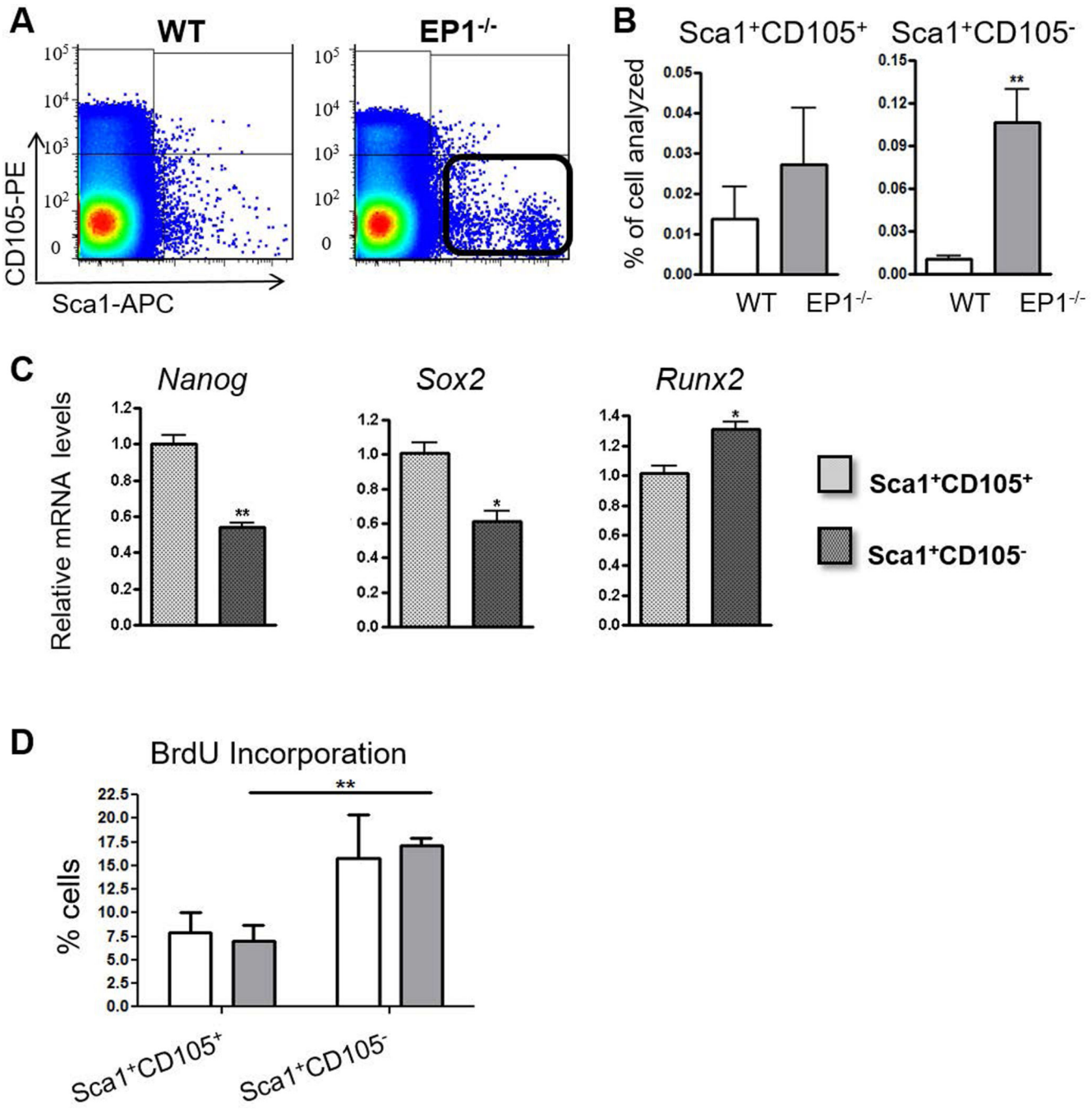

Figure 2. EP1$^{-/-}$ BMSCs have more osteogenic progenitors

Freshly isolated bone marrow cells were stained with antibodies against CD45, CD31, CD105 and Sca1 markers. CD45^{+} and CD31^{+} cells were excluded from the analysis. (A) Representative scatter plots and (B) quantification of Sca1^{+} and CD105 cells. (N=9) Sca1^{+}CD105^{+} cells and Sca1^{+}CD105 cells were sorted into separate tubes and (C) mRNA was isolated and qPCR was performed. (N=3). (D) Mice were injected with BrdU 12 hours before being sacrifice. The cells were stained with antibodies against cell surface markers and BrdU and flow cytometry performed. (N=3)

Results are shown as mean ± SEM. Statistical analysis was performed using Student t- test and Two-way ANOVA with multiple comparison analysis performed for panel D. (*) = $p<0.05$ (**) = $p<0.01$versus age-matched vehicle treated animals.

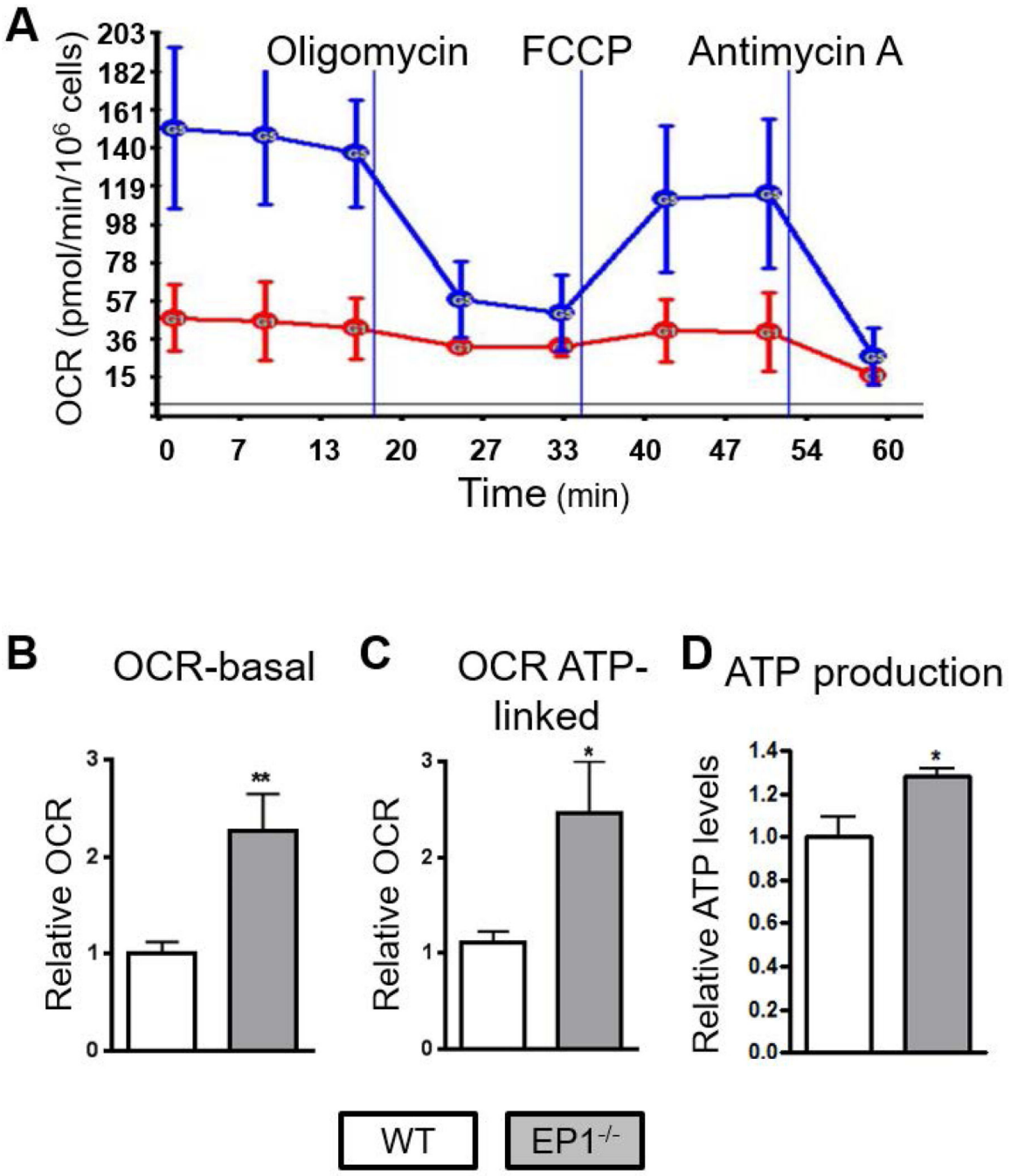

Figure 3. EP1$^{-/-}$ BMSCs exhibit increased mitochondrial activity

First passage BMSCs cultured with maintenance medium were plated on a XF24 plate and a mitochondrial stress test was performed. (A) Representative graph of mitochondrial stress test and (B) quantified basal oxygen consumption rate (OCR) and (C) ATP-linked-OCR (N=6). (D) Bone marrow cells were expanded in maintenance medium and intracellular ATP levels were measured (N=3).

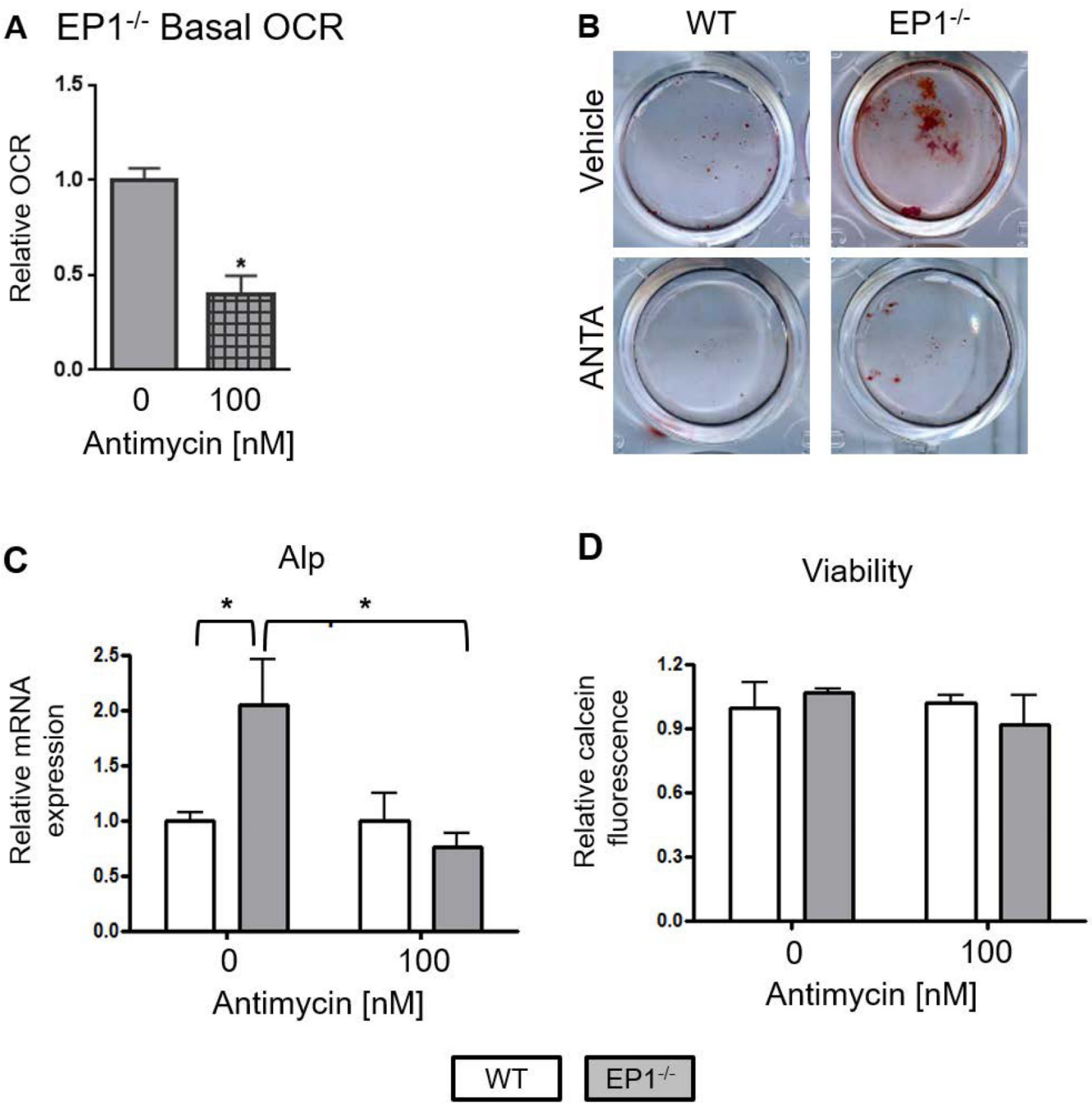

Figure 4. EP1$^{-/-}$ BMSCs enhanced osteogenic differentiation depends on OxPhos

Freshly isolated bone marrow cells were cultured in osteogenic medium and supplemented with vehicle or antimycin A. (A) OCR was measured using Seahorse XF24. (B) Representative images of Alizarin Red (AR) staining. (C) qRT-PCR of *Alpl* expression in cells cultured in maintenance medium supplemented with or without 100 nM antimycin A. (D) Cell viability was measured using calcein fluorescence in cells cultured with or without antimycin A. Two-way ANOVA with multiple comparison analysis performed for statistical analysis . (*) = $p<0.05$.

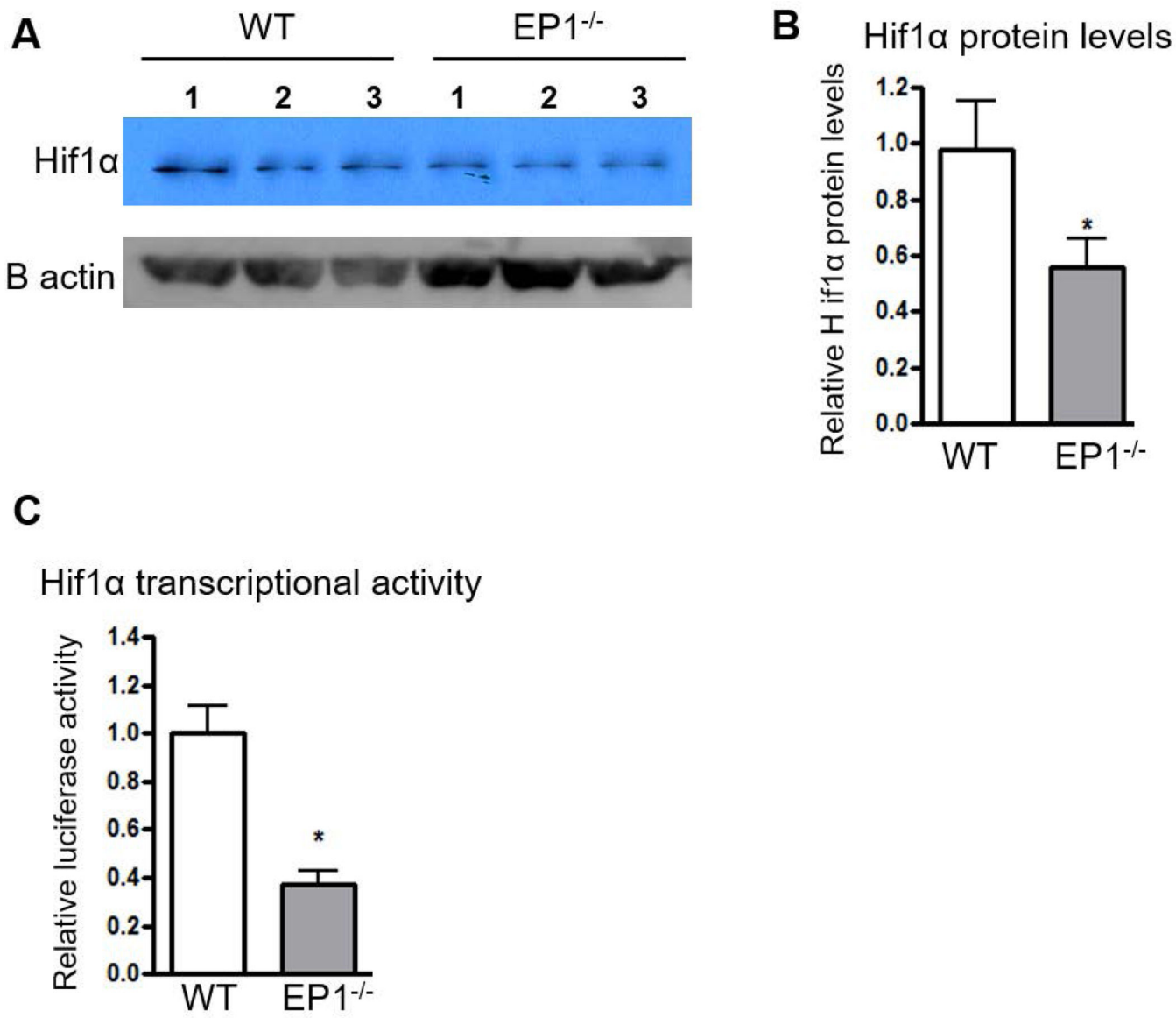

Figure 5. EP1$^{-/-}$ BMSCs have reduced HIF1α activity

(A) Representative Western blot analysis of Hif1α protein levels of cultured WT or EP1BMSCs. (B) Densitometry of Hif1α signal normalized to b-actin signal. C) Hif transcriptional activity was measured using hypoxia response element (HRE) luciferase construct and luciferase assay in WT or EP1$^{-/-}$ cultured BMSCs (N=5). Results are shown as mean ± SEM. Statistical analysis was performed using Student t- test. *, $p<0.05$ versus age-matched WT animals.

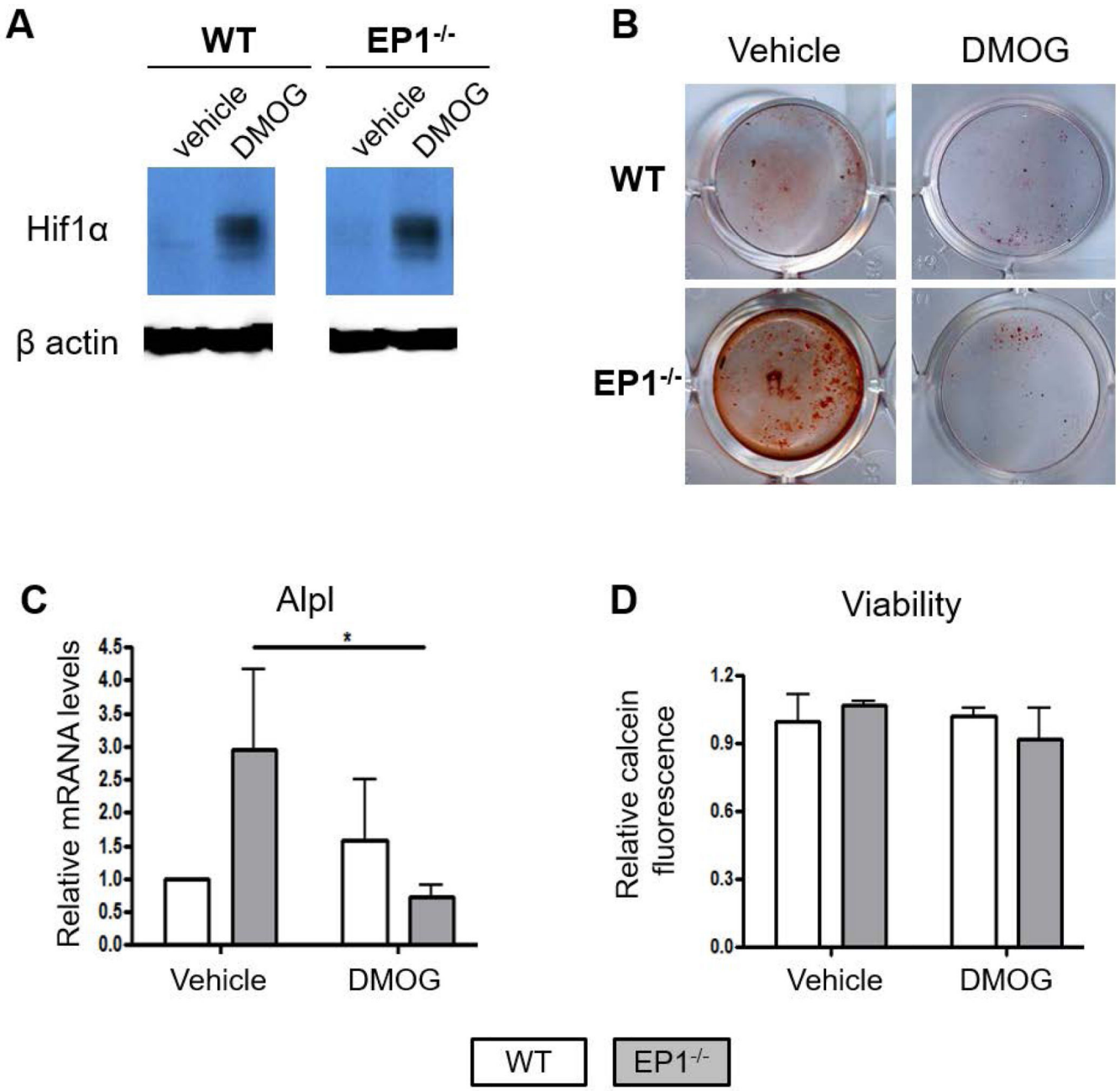

Figure 6. EP1$^{-/-}$ BMSCs enhanced differentiation is HIF1α dependent

A) Western blot images of BMSCs from WT mice treated with either vehicle or DMOG. (B) Representative Alizarin Red (AR) staining of WT or EP1$^{-/-}$ BMSCs cultured in osteogenic medium supplemented with either vehicle or DMOG. (C) RT-qPCR of *Alpl* gene expression in presence of DMOG (N=6). (D) Viability of cells after treatment with DMOG as measured using calcein fluorescence (N=3). Two-way ANOVA with multiple comparison analysis performed for statistical analysis D. (*) = $p<0.05$.

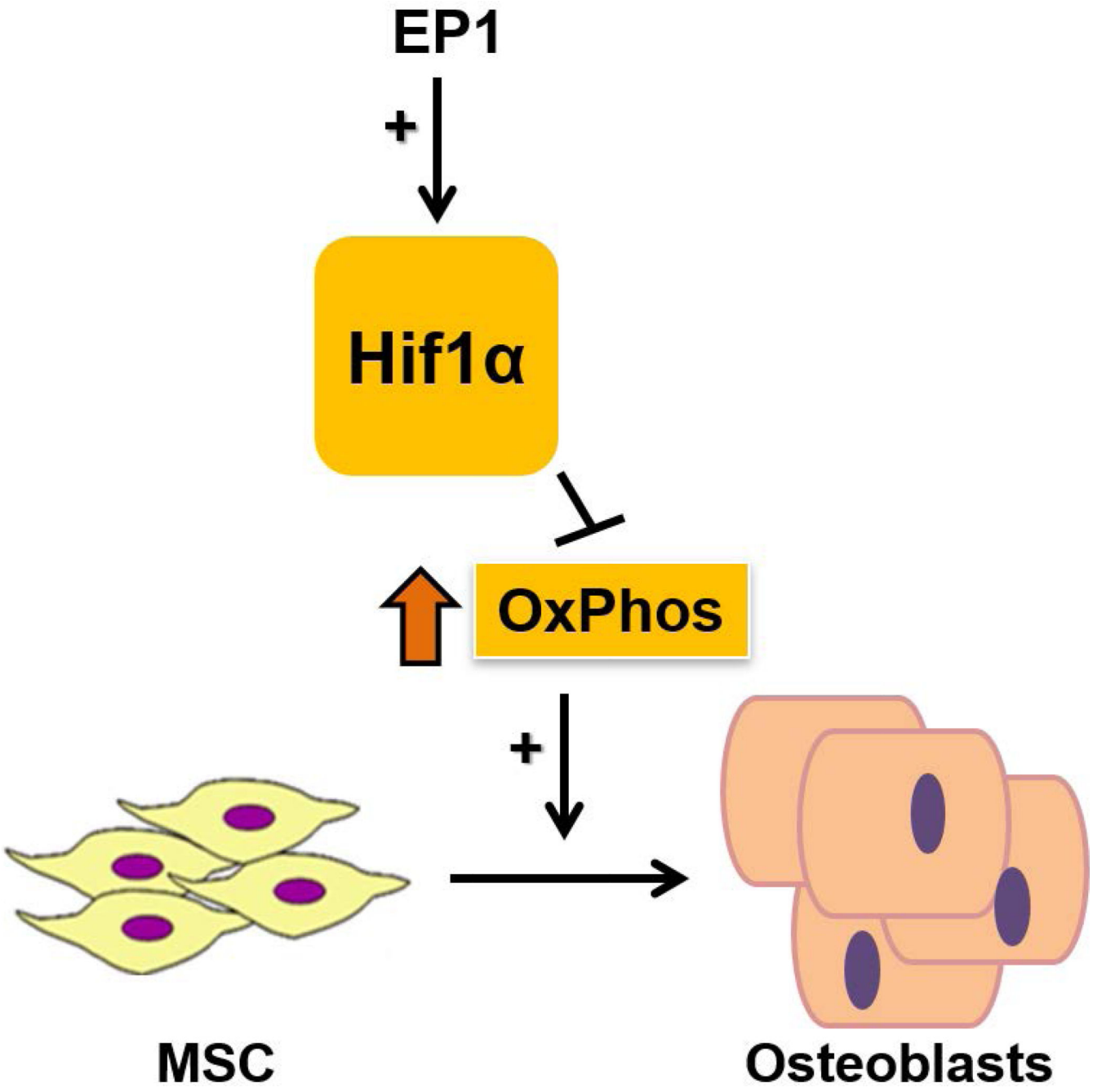

Figure 7. Proposed model

We propose that EP1 regulates Hif1α activity which in turns maintains a lower OxPhos rate in BMSCs. However, by deletion of EP1, lower Hif1α activity leads to higher OxPhos which allows higher osteogenic differentiation rate.