Cyclooxygenase 2 augments osteoblastic but suppresses chondrocytic differentiation of CD90⁺ skeletal stem cells in fracture sites

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Cyclooxygenase 2 (COX-2) is essential for normal tissue repair. Although COX-2 is known to enhance the differentiation of mesenchymal stem cells (MSCs), how COX-2 regulates MSC differentiation into different tissue-specific progenitors to promote tissue repair remains unknown. Because it has been shown that COX-2 is critical for normal bone repair and local COX-2 overexpression in fracture sites accelerates fracture repair, this study aimed to determine the MSC subsets that are targeted by COX-2. We showed that CD90⁺ mouse skeletal stem cells (mSSCs; i.e., CD45⁻Tie2⁻AlphaV⁻ MSCs) were selectively recruited by macrophage/monocyte chemoattractant protein 1 into fracture sites following local COX-2 overexpression. In addition, local COX-2 overexpression augmented osteoblast differentiation and suppressed chondrocyte differentiation in CD90⁺ mSSCs, which depended on canonical WNT signaling. CD90 depletion data demonstrated that local COX-2 overexpression targeted CD90⁺ mSSCs to accelerate fracture repair. In conclusion, CD90⁺ mSSCs are promising targets for the acceleration of bone repair.

INTRODUCTION
Livelihood is dependent on tissue repair and regeneration after an assault. Recent data suggest that cyclooxygenase 2 (COX-2) deficiency causes delay in the repair and regeneration of many tissues such as bones, muscles, lung, hematopoietic cells, intestines, and liver (1–5). Although COX-2 may affect multiple mechanisms of tissue repair and regeneration, we reason that COX-2–mediated regenerative functions ultimately work directly or indirectly through stem cells that are responsible for tissue repair and regeneration. To support this reasoning, previous studies have well documented the importance of COX-2 for stem cell functions. To provide some examples, early studies have shown that COX-2 is critical for the survival of embryonic stem cells (6). Recent data further demonstrate that COX-2 plays an essential role in the survival of cancer stem cells and hence in the repopulation of cancer cells during chemotherapy and radiotherapy (7, 8). In addition, the role of COX-2 in mesenchymal stem cells (MSCs) has been intensively investigated. It has been reported that COX-2 promotes the differentiation of MSCs into cardiocytes (9) and that COX-2 deficiency impairs bone regeneration mediated by muscle-derived stem cells (1).

With respect to the role of COX-2 in MSC-mediated tissue repair and regeneration, recent data have further demonstrated that MSCs are heterogeneous and contain multiple subsets. However, the mechanisms by which COX-2 regulates the differentiation of MSC subsets to promote the repair and regeneration of different tissues remain unknown. In this regard, one of the tissues that has been intensively studied for the role of COX-2 in the MSC-mediated tissue repair and regeneration is the bone. It has been convincingly demonstrated that COX-2 deficiency causes delay of bone repair (10–12). In addition, data from our laboratory have shown that local COX-2 overexpression in fracture sites accelerates fracture repair (13–15). On the basis of these previous findings, we reason that a detailed understanding of the mechanisms by which local COX-2 overexpression accelerates fracture repair can help the development of novel strategies for the acceleration of bone repair. Moreover, because the bone is one of the few tissues that can repair to their original structures, mechanistic insights on the COX-2 overexpression–mediated acceleration of bone repair can also shed lights on the cause for a lack of complete tissue repair and hence can potentially facilitate the development of novel strategies for improving the repair and regeneration of those tissues that cannot repair to their original architectures.

Accordingly, this study aimed to understand the mechanisms by which local COX-2 overexpression regulates the differentiation of MSC subsets in fracture sites to promote fracture repair. In this regard, our previous findings have demonstrated that local COX-2 overexpression increases the number of osteocalcin (OCN)–expressing osteoblasts, as well as the mRNA expressions of alkaline phosphatase (ALP) and osteopontin in fracture sites (13, 15). In contrast, local COX-2 overexpression reduces callus size, cartilage area, the expression of Sox9 mRNA, and the expression of Col10α1 mRNA in fracture sites (13, 15). These previous findings indicate that local COX-2 overexpression promotes osteoblast differentiation but suppresses chondrocyte differentiation. Accordingly, we hypothesize that local COX-2 overexpression augments the differentiation of MSCs into osteoblast progenitors but suppresses the differentiation of MSCs into chondrocyte progenitors in fracture sites. This study tested this hypothesis.

RESULTS
Identification of the specific mSSC subset that was quantitatively increased in fracture sites following local COX-2 overexpression
Because recent data have demonstrated that skeletal MSC subsets can be distinguished by surface markers in both mice and humans (16–18), as the first step to evaluate our hypothesis, we used the previously reported skeletal MSC surface markers to investigate
MSCs in fracture sites. In this regard, recent data suggest that mouse skeletal stem cells (mSSCs; i.e., CD45−Tie2− AlphaV+ MSCs) can be further divided into two broad categories, i.e., CD90+ and 6C3+ mSSCs (17). Therefore, we proceeded to examine the CD90+ and 6C3+ mSSCs in fracture sites. Accordingly, C57BL/6 (B6) mice were subjected to femur fracture surgery and, on the following day, received in the fracture sites no treatment, a control adeno-associated virus (AAV) vector, or the AAV.COX-2 vector. On days 3, 4, 7, and 10 following the vector treatments, contralateral bones and the bones spanning the fracture sites were harvested and bone marrows were flushed. These bones in which bone marrows have been removed are specifically designated as “intact bones” and “fractured bones,” respectively. Subsequently, we isolated mononuclear cells (MNCs). Hence, these isolated MNCs were not contaminated with bone marrow cells and represented the cells that actively participated in the formation of fracture calluses and in the repair of fractures. We then analyzed the isolated MNCs by fluorescence-activated cell sorting (FACS) (Fig. 1, A and B). Our data showed that, on day 3, the local COX-2 overexpression increased the abundance of mSSCs in the fractured bones (Fig. 1C, top). In addition, the local COX-2 overexpression selectively and significantly increased the abundance of CD90+ but not of 6C3+ mSSCs [Fig. 1, C (middle and bottom) and D]. We also confirmed a previous finding that the expressions of CD90 and 6C3 among mSSCs were mutually exclusive (Fig. 1E) (17). Moreover, our data showed that COX-2 overexpression prolonged the presence of CD90+ mSSCs in the fractured bones (Fig. 1F).

Mechanisms by which CD90+ mSSCs were positioned in fracture sites following local COX-2 overexpression

The foregoing data demonstrate that local COX-2 overexpression not only selectively increases the abundance but also prolongs the presence of CD90+ mSSCs in fracture sites. These findings prompted us to determine the mechanisms by which CD90+ mSSCs were positioned in fracture sites following local COX-2 overexpression. In this regard, monocyte chemoattractant protein 1 (MCP-1) has been previously shown to play a role in the recruitment of MSCs into fracture sites (19). Hence, we first asked whether local COX-2 overexpression increased the expression of MCP-1 in fracture sites. To answer this question, we subjected B6 mice to femur fracture surgery and treated the fractures as described in Fig. 1. Four days after the vector treatments, the fractured bones and contralateral intact bones were harvested and analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) (Fig. 2A). Our data showed that MCP-1 mRNA expression in the fractured bones, when compared...
Study of the MSCs that were targeted by MCP-1

Our observation of the MCP-1–mediated increase in CD90<sup>+</sup> mSSC abundance in fractured bones following local COX-2 overexpression can be due to an increased recruitment of CD90<sup>+</sup> MSCs, followed by a conversion of the CD90<sup>−</sup> MSCs into CD90<sup>+</sup> MSCs or a direct recruitment of CD90<sup>+</sup> MSCs from progenitor niches near fractured bones. To address these two possibilities, in an in vitro migration assay, we determined the ability of CD90<sup>+</sup> and CD90<sup>−</sup> MSCs to migrate toward bone chips that were derived from fractured bones. In this in vitro migration assay system, the bone chips mimicked the in vivo microenvironment of fractured bones. Specifically, we subjected B6 mice to femur fracture surgery and treated the fractures with vectors as described in Fig. 1. Four days after the vector treatments, approximately equal lengths of fractured bones and contralateral intact bones were harvested and cut into small pieces to prepare bone chips. The bone chips were then placed in the lower chambers of a 24-well Transwell culture plate. In addition, an equal number (2.0 × 10<sup>5</sup>) of CD90<sup>+</sup> or CD90<sup>−</sup> bone marrow–derived MSCs (BM-MSCs) was seeded in the upper chambers. We cocultured the MSCs and bone chips for 48 hours, fixed the membranes, and counted the MSCs migrating into the membrane (fig. S1A). Our data showed that, when compared to the membranes from the CD90<sup>−</sup> MSC cultures, those from the CD90<sup>+</sup> MSC cultures showed significantly more migrated MSCs under all conditions (fig. S1, B to D). The data suggest that MCP-1 mainly recruits CD90<sup>+</sup> MSCs from progenitor niches near fractured bones.

Because the receptor for MCP-1 is C-C chemokine receptor type 2 (CCR2), the foregoing in vitro migration data suggest that CD90<sup>+</sup>CCR2<sup>+</sup> MSCs are present in the progenitor niches near fractured bones. To support this notion, previous data have already shown that tissues near the fractured bones, e.g., periostea, endostea, muscles, and bone marrow, all contain CD90<sup>+</sup> MSCs (21–23). To determine whether CD90<sup>+</sup> MSCs in these potential progenitor niches indeed express CCR2 and whether local COX-2 overexpression further increases the abundance of CD90<sup>+</sup>CCR2<sup>+</sup> MSCs, we subjected B6 mice to tibia fracture surgery and treated the fractures with vectors as described...
in Fig. 1. Four days after the vector treatments, the following tissues were collected from the fracture and contralateral sites: bones (fractured bones and intact bones), bone marrows, and muscles. Subsequently, we isolated the MNCs for analysis by FACS (fig. S1E). Our data showed that the MCP-1–responsive CD45−CD90+CCR2+ cells were present in bones, bone marrows, and muscles. In addition, the frequencies of CD45−CD90+CCR2+ cells were significantly increased in the bones and muscles but not in the bone marrows following the local COX-2 overexpression (fig. S1, F and G). Hence, the above data support that MCP-1 targets CD90+MSCs from progenitor niches near fractured bones. In addition, our data suggest that local COX-2 overexpression enhances the recruitment of CD90+MSCs not only by augmenting the expressions of MCP-1 in the fractured bones (Fig. 2) but also by increasing the abundance of MNC–responsive CD45−CD90+CCR2+ cells mainly in periostea, endostea, and muscles but not in the bone marrow.

Mechanisms by which local COX-2 overexpression increased the abundance of CD90+CCR2+MSCs in the progenitor niches near fractured bones

The increasing abundance of CD90+CCR2+MSCs in some progenitor niches such as muscles near the fractured bones following local COX-2 overexpression could be due to an increased proliferation of preexisting CD90+CCR2+MSCs. The other possibility could be an increased proliferation of preexisting CD90−MSCs. To evaluate these possibilities, we labeled CD90− and CD90+BM-MSCs with carboxyfluorescein diacetate succinimidyl ester (CFSE). Subsequently, 1.0 × 10^5 CFSE-labeled CD90−or CD90+MSCs were seeded into the lower chambers of a 24-well Transwell culture plate. In addition, the upper chambers were added with no cells, human embryonic kidney (HEK) 293T cells that were transduced with a control AAV vector, or HEK293T cells that were transduced with the AAV.COX-2 vector. The cells were cultured at 37°C and 5% CO_2. Four days later, we collected and analyzed the CD90− and CD90+MSCs for CFSE dilution by FACS. Our data showed that molecules secreted by COX-2–overexpressed cells promoted the proliferation of CD90+but not of CD90−MSCs (fig. S2A). To support this finding, our data further showed that, at 1, 10, and 100 nM, prostaglandin E2 (PGE2), which is one of the molecules produced by COX-2, significantly increased the proliferation of purified CD90+but not of CD90−MSCs in an in vitro [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) proliferation assay (fig. S2B).

The COX-2–mediated increase in the proliferation of CD90+but not of CD90−MSCs suggests that COX-2 may also promote the conversion of CD90−MSCs into CD90+MSCs. To address this potential, we seeded purified CD90−BM-MSCs in the lower chambers of a 24-well Transwell culture plate. The upper chambers were seeded with 2.0 × 10^5 HEK293T cells that were transduced with either a

![Fig. 3. CD90+ mSSCs isolated from COX-2–overexpressed fractured bones, when compared to controls, displayed significantly augmented and accelerated osteoblast differentiation in vitro. (A) B6 mice received femur fracture surgery and treatments as described in Fig. 1. On day 4 following the treatments, the fractured bones were harvested and the CD90+mSSCs were purified as described in Materials and Methods. The purified CD90+mSSCs were analyzed by RT-qPCR. Data are shown in (B). (B) Data show the mRNA expressions of the following genes: Runx2, OCN, OSX, BMP2, and ALP. (C) The purified CD90+mSSCs in (A) were analyzed by FACS. Data show the frequencies of OSX+ and Runx2+ cells among the CD90+mSSCs (left), as well as the MFIs of the expressions of OSX and Runx2 (right). (D) The purified CD90+mSSCs in (A) were directly cultured in an osteoblast differentiation medium for 21 days. Data are representative images of the cultured cells stained with Alizarin Red. (E) The purified CD90+mSSCs in (A) were first expanded in an MSC medium for 1 week and then cultured in the osteoblast differentiation medium for 21 days. Data are representative images of the cultured cells stained with Alizarin Red. (F) The purified CD90+mSSCs in (A) were cultured in the osteoblast differentiation medium. On weeks 1, 2, and 3, the cultured cells were stained with Alizarin Red. Data show representative images of the cultured cells stained with Alizarin Red. (G) Data show the absorbance at 405 nm of solubilized Alizarin Red–stained cells in (F). Where applicable, data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; n = 3 to 5. Photo credit: Samiksha Wasnik, Loma Linda University.](image-url)
control AAV vector or the AAV.COX-2 vector. The cells were cultured at 37°C and 5% CO₂. On days 1 and 6, we harvested and analyzed the MSCs in the lower chambers for the expression of CD90 by FACS. Our data showed that the cultures of COX-2–overexpressed cells, when compared to those of the control cells, contained significantly increased abundance of CD90+ MSCs on day 6, suggesting an enhanced conversion of CD90− MSCs into CD90+ MSCs (fig. S2, C and D). Collectively, our data suggest that local COX-2 overexpression increases the abundance of CD90+CCR2+ MSCs in the progenitor niches near fractured bones by augmenting the proliferation of CD90+ MSCs, followed by an increased conversion of the CD90−MSCs into CD90+CCR2+MSCs.

The effects of local COX-2 overexpression on the osteoblast differentiation of CD90+MSCs in fractured bones

Thus far, we have shown that local COX-2 overexpression increases the abundance of CD90+ MSCs in fractured bones by MCP-1–mediated cellular recruitment. The next question is whether local COX-2 overexpression has any effects on the osteoblast differentiation of CD90+MSCs in fractured bones. To address this question, we subjected B6 mice to femur fracture surgery and treated the fractures with vectors as described in Fig. 1. Four days after the vector treatments, fractured bones were harvested and CD90+MSCs were purified. We analyzed the purified CD90+MSCs by RT-qPCR (Fig. 3A). Our data showed that CD90+MSCs from COX-2–overexpressed fractured bones, when compared to those from control-treated fractured bones, expressed significantly higher levels of the genes that are known to be essential for osteoblast differentiation, i.e., Runx2, OCN, osterix (OSX), bone morphogenic protein 2 (BMP2), and ALP (Fig. 3B). In another experiment, our FACS data showed that CD90+MSCs from COX-2–overexpressed fractured bones, when compared to those from control-treated fractured bones, contained significantly more Runx2+ and OSX+ cells (Fig. 3C, left). In addition, the local COX-2 overexpression, when compared to the control treatment, also significantly enhanced the mean fluorescence intensities (MFIs) of Runx2 and OSX expressions in CD90+MSCs (Fig. 3C, right).

The foregoing gene expression and FACS data suggest that local COX-2 overexpression augments the osteoblast differentiation of CD90+MSCs in fractured bones. Hence, we proceeded to determine the effects of local COX-2 overexpression on the osteoblast differentiation of CD90+MSCs in fractured bones. Because CD90+MSCs purified from fractured bones were quantitatively low, in most experiments, purified CD90+MSCs were first expanded in cultures for up to 3 weeks. Hence, we first determined the purity and phenotypic stability of purified CD90+MSCs (fig. S3A). Our data showed that purified CD90+MSCs were approximately 96% pure and expressed MSC markers (i.e., CD45+CD34+Sca-1−CD73+CD29+). In addition, the purity and MSC marker expressions were stable for at least 3 weeks (fig. S3, B and C). Using these CD90+MSCs purified from fractured bones, we then asked whether the purified CD90+MSCs could indeed differentiate into osteoblasts in fracture sites. To address this question, we subjected B6.GFP mice to femur fracture surgery. Four days later, fractured bones were harvested for the purification of CD90+MSCs and CD90−mesenchymal cells. Subsequently, the purified CD90+MSCs and CD90−mesenchymal cells were subcutaneously administered into recipient B6 mice near the fracture sites on the second day following the femur fracture surgery. Twenty-one days later, we harvested and analyzed the bones spanning the fracture sites for the expressions of GFP (green fluorescent protein) and OCN (i.e., the marker for osteoblasts) by immunohistochemistry (IHC) (fig. S3D). Our data showed that only the bones administered with the GFP-expressing CD90+MSCs, but not those administered with the GFP-expressing CD90−mesenchymal cells, displayed a clear presence of GFP+ cells (fig. S3E). In addition, an appreciable number of GFP+ cells were contained with OCN, indicating differentiation of the CD90+MSCs into osteoblasts.

Having demonstrated that CD90+MSCs can differentiate into osteoblasts in fracture sites, we used an in vitro nodule assay to determine whether local COX-2 overexpression augmented the osteoblast differentiation of CD90+MSCs in fractured bones. Accordingly, CD90+MSCs purified from fractured bones were cultured in an osteoblast differentiation medium for 21 days. Our data showed that CD90+MSCs purified from COX-2–overexpressed fractured bones, when compared to those from the control-treated fractured bones, displayed enhanced mineralization as determined by Alizarin Red staining (Fig. 3D). In addition, we also cultured the purified CD90+MSCs for 1 week before performing the nodule assay. Our data showed similar results (Fig. 3E). A nodule assay time study showed a significant effect of local COX-2 overexpression at 1 week with continued effect for 3 weeks (Fig. 3, F and G). Because CD90+MSCs purified from fractured bones did not spontaneously differentiate into osteoblasts in the absence of the osteoblast differentiation medium in the nodule assay, we conclude that local COX-2 overexpression significantly augments the osteoblast differentiation potential of CD90+MSCs in fractured bones and that additional signals are required for the ultimate differentiation of CD90+MSCs into osteoblasts.

To further support the role of COX-2 in augmenting the osteoblast differentiation potential of CD90+MSCs, our data showed that soluble factors secreted by COX-2–overexpressed cells augmented the expressions of OCN and OSX in BM-MSCs, which were abolished by the COX-2–specific inhibitor Celecoxib (fig. S4, A and B). Because PGE2 is a major COX-2 enzymatic product, we determined the relative contribution of the four PGE2 receptors (i.e., EP1, EP2, EP3, and EP4) to the COX-2 overexpression–mediated augmentation of osteoblast differentiation in CD90+MSCs in fracture sites. Accordingly, B6 mice were subjected to femur fracture surgery and, beginning on the same day, received a daily subcutaneous dose of an inhibitor for EP1, EP2, EP3, or EP4 near the fracture sites. In addition, on the second day following the fracture surgery, the animals received in the fracture sites a dose of a control AAV vector or the AAV.COX-2 vector. On day 4 following the fracture surgery, we harvested the fractured bones and contralateral intact bones and purified the CD90+MSCs for analysis by RT-qPCR and an in vitro nodule assay for osteoblast differentiation (fig. S4C). The RT-qPCR analysis showed that the expressions of EP1, EP2, EP3, and EP4 in the fractured bones were effectively inhibited by the corresponding inhibitors (fig. S4D). The in vitro nodule assay demonstrated that, although EP2, EP3, and EP4 inhibitors significantly suppressed the mineralization in the CD90+MSCs purified from the control-treated fractured bones, the EP1 inhibitor significantly enhanced the mineralization (fig. S4, E and F). These data are consistent with the previously reported positive roles of EP2, EP3, and EP4 and a negative role of EP1 in wound healing (24–26). Similar to the findings in the control-treated fractured bones, in the COX-2–treated fractured bones, the mineralization of CD90+MSCs was significantly blocked by EP2, EP3, and EP4 inhibitors but not by the EP1 inhibitor (fig. S4, G and H). The foregoing data suggest
that the PGE2 receptors are tightly regulated in CD90⁺ mSSCs during fracture repair.

**The effects of local COX-2 overexpression on the chondrocyte differentiation of CD90⁺ SCs in fractured bones**

In addition to being able to differentiate into osteoblast, CD90⁺ mSSCs from the bone marrow have been shown to differentiate into chondrocytes as well (17). Hence, we sought to determine whether CD90⁺ mSSCs in fractured bones could also differentiate into chondrocytes and, if yes, how local COX-2 overexpression affected their chondrocyte differentiation. To address these questions, we subjected B6 mice to femur fracture surgery and treated the fractures with vectors as described in Fig. 1. Four days after the vector treatments, the fractured bones were harvested and CD90⁺ mSSCs and CD90⁻ mesenchymal cells were purified for analyses by RT-qPCR and an in vitro chondrogenesis assay (Fig. 4A). Our RT-qPCR analyses showed that, in the control-treated fractured bones, the mRNA expressions of Sox9 (P < 0.01), Nkx3.2 (P = 0.07), and Col2a1 (P < 0.01) in the CD90⁺ mSSCs were significantly higher than those in the CD90⁻ mesenchymal cells (Fig. 4B). Because the above three genes are necessary for chondrocyte differentiation, our data suggest that CD90⁺ mSSCs in fractured bones also mediate chondrocyte differentiation during normal fracture repair. In addition, our data showed that the local COX-2 overexpression significantly reduced the mRNA expressions of the above three genes in the CD90⁺ mSSCs (Fig. 4B), which suggests that local COX-2 overexpression suppresses the chondrocyte differentiation of CD90⁺ mSSCs in fractured bones. Further analyses using the in vitro chondrogenesis assay showed that, in the control-treated fractured bones, CD90⁺ mSSCs differentiated into chondrocytes. Consistent with the gene expression data, the chondrocyte differentiation of CD90⁺ mSSCs was suppressed by local COX-2 overexpression (Fig. 4C). Similar to the aforementioned osteoblast differentiation, CD90⁺ mSSCs purified from fractured bones did not spontaneously differentiate into chondrocytes in the absence of the chondrogenic medium. Therefore, our data demonstrate that local COX-2 overexpression suppresses the chondrocyte differentiation potential of CD90⁺ mSSCs in fractured bones.

Our foregoing analyses suggest that CD90⁺ mSSCs in fractured bones contain two subsets among which one subset is osteoblast progenitors and the other is chondrocyte progenitors. In this regard, recent data suggest that CD200 expression may be used for distinguishing CD90⁺ mSSCs into CD200⁻ osteoblast progenitors and CD200⁺ osteoblast progenitors (17). Hence, we analyzed CD200 expression among CD90⁺ mSSCs in fractured bones. Our data showed that local COX-2 overexpression indeed significantly decreased the abundance of CD200⁺ cells among CD90⁺ mSSCs but significantly increased the abundance of CD200⁻ cells among CD90⁺ mSSCs in fractured bones (Fig. 4D).

**Role of WNT signaling in the augmentation of osteoblast differentiation potential of CD90⁺ mSSCs in fractured bones following local COX-2 overexpression**

So far, our data have demonstrated that local COX-2 overexpression augments osteoblast differentiation but suppresses chondrocyte differentiation in fractured bones possibly by promoting the differentiation of CD200⁻CD90⁺ mSSCs (osteoblast progenitors) and by suppressing the differentiation of CD200⁺CD90⁺ mSSCs (chondrocyte progenitors). On the basis of these findings, the next question is the role of WNT signaling in this differentiation process. As shown in Fig. 4E, the mRNA expressions of WNT signaling molecules (i.e., WNT-3a, WNT-7a, β-catenin, T cell factor 1 (TCF-1), and TCF-3) were significantly increased by local COX-2 overexpression (Fig. 4E). The mRNA expressions of WNT signaling molecules were also increased in CD90⁺ mSSCs purified from fractured bones following local COX-2 overexpression (Fig. 5A). Our data showed that local COX-2 overexpression significantly increased the mRNA expressions of WNT signaling molecules (i.e., WNT-3a, WNT-7a, β-catenin, T cell factor 1 (TCF-1), and TCF-3) in CD90⁺ mSSCs purified from fractured bones following local COX-2 overexpression (Fig. 5A). Our data also showed that local COX-2 overexpression significantly reduced the mRNA expressions of WNT signaling negative regulators (i.e., Axin-2 and SOST) in CD90⁺ mSSCs purified from fractured bones following local COX-2 overexpression (Fig. 5B).
significantly increased the mRNA expressions of WNT-1, WNT-3a, WNT-7a, WNT-9, WNT-10, and WNT-16 in the fracture sites (fig. S5).

The above analyses of WNT signaling molecules demonstrated that local COX-2 overexpression enhances WNT signaling in CD90⁺ mSSCs in fractured bones. We hence sought to further determine whether the enhanced WNT signaling was essential for the augmented osteoblast differentiation in CD90⁺ mSSCs following local COX-2 overexpression. Accordingly, we used a β-catenin inhibitor (i.e., ICG001) to block WNT signaling in fracture sites (ICG001 has been previously shown to specifically and effectively block canonical WNT signaling in vivo) (29). Specifically, B6 mice were subjected to femur fracture surgery and, on the same day, were separated into two groups, with one group receiving a daily subcutaneous dose of vehicle and the other group receiving the ICG001 near the fracture sites until bones were harvested. On the following day after the fracture surgery, half of the animals in each group subcutaneously received a control AAV vector and the other half received the AAV.COX-2 vector. Four days after the fracture surgery, we harvested the fractured bones and purified the CD90⁺ mSSCs for analysis by RT-qPCR and an in vitro nodule assay (Fig. 5C). Our data showed that the ICG001 administration reduced the expression of β-catenin mRNA in the CD90⁺ mSSCs by approximately 60% (Fig. 5D). In addition, the ICG001 administration significantly reduced the COX-2 overexpression–mediated up-regulation in the mRNA expressions of Runx2, OSX, and OCN in the CD90⁺ mSSCs in the fractured bones (Fig. 5E). Data from the nodule assay demonstrated that the ICG001 administration completely abolished osteoblast differentiation in the CD90⁺ mSSCs in the fractured bones under both normal and COX-2 overexpression conditions (Fig. 5F). Collectively, we conclude that canonical WNT signaling is required for the preferential osteoblast differentiation of CD90⁺ mSSCs in fractured bones following local COX-2 overexpression.

**Role of CD90⁺ mSSCs in the acceleration of fracture repair following local COX-2 overexpression**

The foregoing analyses indicate that CD90⁺ mSSCs in fractured bones are potentially important for the accelerated fracture repair following local COX-2 overexpression. Hence, we continued to ask what was the role of CD90⁺ mSSCs in the COX-2 overexpression–mediated acceleration of fracture repair. To address this question, we used an anti-COX-2 mAb (clone 30H12) to deplete CD90⁺ cells in fractured bones (30H12 has been shown to specifically and effectively deplete CD90⁺ cells in vivo) (30). First, we decided to confirm whether the clone 30H12 specifically depleted CD90⁺ cells in fractured bones. Accordingly, we subjected B6 mice to tibia fracture surgery. Beginning on the same day of the fracture surgery and on the following two days after the fracture surgery, half of the animals in each group received a control AAV vector and the other half received the AAV.COX-2 vector in the fracture sites. Four days after the fracture surgery, the fractured bones were harvested and the CD90⁺ mSSCs were purified. The purified CD90⁺ mSSCs were analyzed by RT-qPCR and an in vitro nodule assay. Data are shown in (B). (B) mRNA expressions of WNT ligands (WNT3a and WNT7a; top), WNT signaling molecules (β-catenin, TCF-1, and TCF-3; middle), and negative regulators of WNT signaling (Axin-2 and SOST; bottom). (C) B6 mice were subjected to femur fracture surgery and, beginning on the same day, subcutaneously received a daily dose of either vehicle (VC) or ICG001 (an inhibitor for WNT/β-catenin/TCF-mediated transcription) near fracture sites until bones were harvested. On the second day following the fracture surgery, half of the animals in each group received a control AAV vector and the other half received the AAV.COX-2 vector in the fracture sites. Four days after the fracture surgery, the fractured bones were harvested and the CD90⁺ mSSCs were purified. The purified CD90⁺ mSSCs were analyzed by RT-qPCR and an in vitro nodule assay. Data are shown in (D) to (F). (D) Data show the mRNA expression of β-catenin. (E) Data show mRNA expressions of the genes necessary for bone formation, i.e., Runx2, OSX, and OCN. (F) Data show representative images of CD90⁺ mSSCs that were cultured in the osteoblast differentiation medium for 14 and 21 days and stained with Alizarin Red. Where applicable, data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; n = 3 to 5. Photo credit: Samiksha Wasnik, Loma Linda University.

Fig. 5. Blocking of WNT/β-catenin signaling pathway completely abolished COX-2 overexpression–mediated augmentation of CD90⁺ mSSC osteoblast differentiation potential in fractured bones. (A) B6 mice were subjected to femur fracture surgery and, on the second day, received one of the following treatments in the fracture sites: a control AAV vector and the AAV.COX-2 vector. Four days after the fracture surgery, the fractured bones were collected and the CD90⁺ mSSCs were purified as described in Materials and Methods. The purified CD90⁺ mSSCs were analyzed by RT-qPCR. Data are shown in (B). (B) mRNA expressions of WNT ligands (WNT3a and WNT7a; top), WNT signaling molecules (β-catenin, TCF-1, and TCF-3; middle), and negative regulators of WNT signaling (Axin-2 and SOST; bottom). (C) B6 mice were subjected to femur fracture surgery and, beginning on the same day, subcutaneously received a control AAV vector and the other half received the AAV.COX-2 vector. Four days after the fracture surgery, we harvested the fractured bones and purified the CD90⁺ mSSCs for analysis by RT-qPCR and an in vitro nodule assay (Fig. 5C). Our data showed that the ICG001 administration reduced the expression of β-catenin mRNA in the CD90⁺ mSSCs by approximately 60% (Fig. 5D). In addition, the ICG001 administration significantly reduced the COX-2 overexpression–mediated up-regulation in the mRNA expressions of Runx2, OSX, and OCN in the CD90⁺ mSSCs in the fractured bones (Fig. 5E). Data from the nodule assay demonstrated that the ICG001 administration completely abolished osteoblast differentiation in the CD90⁺ mSSCs in the fractured bones under both normal and COX-2 overexpression conditions (Fig. 5F). Collectively, we conclude that canonical WNT signaling is required for the preferential osteoblast differentiation of CD90⁺ mSSCs in fractured bones following local COX-2 overexpression.
consecutively and effectively depletes CD90+ cells in fractured bones. Hence, the fractured bones (Fig. 6B).

The above experiment has confirmed that the clone 30H12 specifically and effectively depletes CD90+ cells in fractured bones. Hence, using the above well-characterized clone 30H12, we then proceeded to determine whether the depletion of CD90+ cells in fractured bones abrogated the COX-2 overexpression-mediated acceleration of fracture repair. Accordingly, we subjected B6 mice to tibia fracture surgery and treated the fractures with either PBS or the clone 30H12, as described in Fig. 6, A and B. In addition, on the second day following the surgery, half of the animals that were administered with PBS or the clone 30H12 received no treatment and the other half received the AAV.COX-2 vector in the fracture sites. Furthermore, a group of animals that received only a control AAV vector in the fracture sites was also included as a control. On days 4, 10, and 21 following the vector treatments, the bones spanning the fracture sites were then examined by x-ray. At the end of the experiment (day 21), the bones spanning the fracture sites were harvested for analyses by micro-computed tomography (µCT) (Fig. 6C). Data from the x-ray analysis on day 21 were consistent with our previous report (13, 15) that local COX-2 overexpression in fracture sites, when compared to the controls, significantly decreased callus sizes (Fig. 6, D and E, and fig. S6). In addition, our data in the current study further showed that the COX-2 overexpression-mediated reduction of callus size was ablated by the clone 30H12 administration. Moreover, µCT analysis demonstrated that, in the fracture sites, the local COX-2 overexpression significantly increased connectivity density, bone volume, bone mineral density, trabecular number, and trabecular thickness but decreased trabecular space, all of which indicated enhanced new bone formation (Fig. 6, F and G). This enhanced new bone formation was completely abolished by the clone 30H12 administration. Hence, we conclude that local COX-2 overexpression targets CD90+ mSSCs to accelerate fracture repair.

**DISCUSSION**

Bone fractures are the most common injuries sustained by humans. In the United States alone, there are approximately 6.3 million fractures annually (31), emphasizing the existence of a large population of patients requiring medical intervention. In contrast to this high demand for medical intervention, there is only one U.S. Food and Drug Administration–approved therapy for delayed healing, i.e., electrical field stimulation (32). Under current therapeutic regimens, approximately 10% of fractures still proceed as delayed healing or nonunion (33, 34), which significantly lowers the quality of life and causes inability for patients to return to their job duties (33). Because delayed fracture repair puts patients under the risk of nonunion, acceleration of normal fracture repair is necessary and is an unmet medical need. In this regard, we have demonstrated that COX-2 gene therapy is one of the most effective treatments for normal rodent bone fractures (13–15). For this reason, the goal of the present study was to determine the MSCs that COX-2 gene therapy acts to accelerate normal fracture repair. This information can be valuable to eventually avoid gene therapy and identify small molecules that can precisely target MSCs for the acceleration of normal fracture repair.

Using tibia and femur fractures, we have now shown that COX-2 gene therapy increases the abundance of CD90+ mSSCs in fractured bones via MCP-1–mediated recruitment (Figs. 1 and 2). In addition, our data have demonstrated that COX-2 gene therapy augments the osteoblast differentiation potential but suppresses the chondrocyte differentiation potential of CD90+ mSSCs in fractured bones (Figs. 3 and 4). Consistent with the above findings, our data have determined that COX-2 gene therapy promotes the differentiation of osteoblast progenitors (i.e., CD90+CD200− mSSCs) but suppresses the differentiation of chondrocyte progenitors (i.e., CD90+CD200+ mSSCs) (Fig. 4). Our data from both tibia and femur fractures were consistent. Moreover, these current findings are in line with our previous observations that COX-2 gene therapy increases the number of OCN-expressing osteoblasts, as well as the mRNA expressions of ALP and osteopontin in fracture sites (13, 15), but reduces the callus size, the cartilage area, the expression of Sox9 mRNA, and the expression of Col11a1 mRNA in fracture sites (13, 15). Collectively, findings from our studies so far support that COX-2 gene therapy augments intramembranous bone formation but suppresses endochondral bone formation in fracture sites.

Although our data have suggested that CD90+ mSSCs are recruited from progenitor niches near fractured bones to selectively augment intramembranous bone formation, we noticed that most CD90+ and CD90− MSCs in muscles and bone marrows did not express AlphaV that is a marker for mSSCs (16). These data indicate that, during the migration from muscles and bone marrows into fractured bones, CD90+ MSCs undergo skeletal lineage commitment into mSSCs. Because most CD90+ MSCs isolated from intact bones also express AlphaV (Fig. 1), our data suggest that signals in bones are sufficient to confer this skeletal lineage commitment of CD90+ MSCs and that this skeletal lineage commitment is independent of local COX-2 overexpression. The signals that confer this skeletal lineage commitment are currently unclear.

On the basis of our findings, we have proposed a model for the COX-2 overexpression–mediated acceleration of fracture repair in long bones (fig. S7). In this model, we propose that, under normal conditions, CD90+CD200+AlphaV+ MSCs undergo proliferation and CD90+ conversion in progenitor niches near fractured bones. Subsequently, the CD90+CD200+AlphaV+ MSCs are recruited into fractured bones by MCP-1. Inside the fractured bones, the CD90+CD200+AlphaV+ MSCs undergo skeletal lineage commitment and become CD90+ mSSCs. Subsequently, the CD90+ mSSCs preferentially differentiate into CD90+CD200+AlphaV+ chondrocyte progenitors with a small fraction of CD90+CD200+AlphaV− osteoblast progenitors. Consequently, the fractured bones are healed predominantly via endochondral bone formation. Under the condition of COX-2 overexpression, local COX-2 overexpression significantly increases the concentrations of COX-2–enzymatic products (i.e., prostanoids) such as PGE2. Consequently, the elevated levels of prostanoids in the progenitor niches near fractured bones significantly increase the proliferation of the CD90+CD200+AlphaV+ MSCs and promote the conversion of the CD90+CD200+AlphaV+ MSCs into CD90+CD200+AlphaV− MSCs. In addition, the elevated levels of prostanoids also significantly increase in the fractured bones the expression of MCP-1, which enhances the migration of CD90+CD200−AlphaV− MSCs into the fractured bones. Inside the fractured bones,
the elevated levels of prostanoids enhance the differentiation of CD90^+ mSSCs into CD90^+CD200^-AlphaV^+ osteoblast progenitors but suppress the differentiation of CD90^+ mSSCs into CD90^+CD200^+AlphaV^+ chondrocyte progenitors by augmenting WNT/\beta\text{-catenin} signaling. As a result, the bone healing is accelerated through enhanced intramembranous bone formation.

Although the current study only focused on the effects of COX-2 gene therapy on mSSCs in fractured bones, COX-2 actions are more pleotropic than those detailed in this study. A previous work (13) indicates that COX-2 also increases bone resorption, which could accelerate fracture healing by increasing the final remodeling of newly deposited bones. In addition, COX-2 also increases angiogenesis, which is critical for the entire repertoire of COX-2–stimulated events on bone formation and resorption (13).

A limitation of this study is that we did not specifically determine whether the COX-2–mediated switch from CD90^+CD200^- mSSCs to CD90^+CD200^+ mSSCs is necessary for the COX-2 overexpression–mediated acceleration of fracture repair. In future studies, transgenic mice in which CD200 expression is overexpressed in CD90^+ mSSCs and/or specifically tagged with fluorescence will be used to address this question.

The importance of the current study is underscored by recent findings that COX-2 is critical for the repair and regeneration of multiple tissues such as the muscles, lung, hematopoietic cells, intestines, and liver (1–5). Accordingly, our findings in this study can potentially help the development of novel strategies for the repair and regeneration of other tissues as well.

MATERIALS AND METHODS

Animals

Twelve-week-old male B6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in Loma Linda University Animal Care Facility. All in vivo animal study protocols were reviewed and approved by both the Institutional Animal Care and Use Committee of Loma Linda University and the Animal Care and Use Review Office of the U.S. Army Medical Research and Materiel Command (USAMRMC) of the U.S. Department of Defense.
Stabilized bone fracture model and virus treatment
Tibial or femur fracture surgery was performed, as described previously (15). Femurs were used for the cell lineage and gene expression analyses, whereas μCT and histological analyses were done in tibial bones. Briefly, to stabilize a bone, a 30-gauge (tibial) or 25-gauge (femur) needle was inserted into the marrow cavity at a position just medial to the patella tendon (fig. S8). Subsequently, fractures were created by a three-point bending technique using an Instron Mechanical tester (Norwood, MA), as described previously (13, 15). After the fracture surgery, bone repair was monitored weekly by x-ray. In addition, x-ray images were analyzed for the measurement of soft callus sizes. Furthermore, μCT analyses were performed at the end of experiments (day 21).

The AAV.COX-2 and AAV control (AAV.LAC-Z or AAV.Empty) viral vectors were constructed, as previously described (15). A total of 5.0 × 10^{11} genomic copies of one of the viruses were injected locally into fracture site (fig. S8).

Isolation and purification of MNCs from fracture sites
Mice were sacrificed at a time indicated in each experiment. Bones spanning fracture sites were collected, muscles were removed, and bone marrows were flushed with PBS. These collected bones spanning fracture sites were designated as fractured bones. In addition, the contralateral bones without muscles and the bone marrows were included as controls and designated as intact bones. Fractured bones and intact bones (about 3 mm) were cut into small pieces and digested with collagenase (2 mg/ml) for 1 hour at 37°C, and MNCs were collected. The MNCs were either further purified for CD90+ mSSCs or directly used for FACS analyses.

To purify CD90+ mSSCs, CD45− cells were first removed using anti-CD45 Microbeads (Miltenyi Biotec). Then, CD90+ cells in the CD45− cells were positively purified using Dynabeads Mouse Pan T (Thy1.2) (Thermo Fisher Scientific), followed by removal of the beads. Our data showed that the bead-free CD45− CD90+ cells in the fractured bones were mostly AlphaV+. Hence, these purified cells were considered to be CD90+ mSSCs, i.e., CD45− Tie2− AlphaV+CD90+ cells.

FACS analysis
Cells were suspended in a FACS buffer [PBS and 0.5% bovine serum albumin (BSA)], incubated with a CD16/CD32 Ab (1 μg/ml; FC block, eBioscience) for 20 min at 4°C, and stained with the fixable viability dye eFluor 780 (eBioscience). The cells were washed twice with the FACS buffer and further stained with desired surface Abs at concentrations per the manufacturers’ instructions at 4°C for 30 min. Surface Abs used in this study included CD45, Tie2, TER119, 6C3, CD90 and CD11b (all from BioLegend); AlphaV (Abcam), CD3 (eBioscience); CD11c (BD Pharmingen); and CCR2 (R&D Systems). Surface stained cells were fixed, permeabilized, and stained for intracellular proteins using a commercial kit (eBioscience). Abs used for the intracellular protein staining include OCN (Santa Cruz Biotechnology) and OSX (Abcam). Concentrations of the Abs were used per the manufacturers’ recommendations. FACS data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

In vitro osteoblast and chondrocyte differentiation
For the in vitro osteoblast differentiation assay, 5.0 × 10^5 CD90+ mSSCs purified from fractured bones were cultured in an MSC differentiation medium (StemPro Osteogenesis Differentiation Kit, Gibco) in a 12-well tissue culture plate. The osteoblast differentiation was normally carried out for 3 weeks. A time course study of in vitro osteoblast differentiation assay was also performed where the cells were examined weekly. At the end of the cell cultures, extracellular calcium deposition was evaluated by Alizarin Red staining. Briefly, the cultured cells were fixed with 4% formaldehyde at room temperature for 20 min. After washing with distilled water for three times, Alizarin Red solution (40 mM; TMS-008-C, Millipore) was added. After 30 min of incubation in Alizarin Red solution, the wells were first rinsed with distilled water for three times and then with PBS for three more times. The wells were examined, and images were taken using either a light microscope (DP72, Olympus) or a digital camera. For quantification of staining (35), the culture wells were incubated in 10% (v/v) acetic acid for 30 min at room temperature, heated at 85°C for 10 min, and followed by acid neutralization with 10% (v/v) ammonium hydroxide. Last, supernatants were read in triplicate at 405 nm in a microplate reader (BioTek Instruments Inc., Winooski, VT).

For in vitro chondrocyte differentiation assay, 5.0 × 10^4 CD90+ mSSCs were seeded as micromass cultures in 50 μl in the center of a 24-well tissue culture plate. After 4 hours of cell adhesion, 500 μl of a chondrogenic medium (StemPro Chondrogenesis Differentiation Kit) was carefully added into each well. The differentiation was carried out for 21 days. On day 21, the micromass cell cultures were fixed in 4% formaldehyde and stained with Alcian blue solution. Images were taken using a light microscope (DP72, Olympus).

Local depletion of CD90+ cells at fracture sites
Male C56BL/6 mice were subjected to tibial fracture surgery. Immediately after the fracture surgery and in the following two consecutive days, the depleting anti-CD90 mAb (clone 30H12, GeneTex), at concentrations of 200, 100, and 100 μg, respectively, was injected subcutaneously near a fracture site.

Radiographic analysis and callus size measurement
Radiographic x-ray images of bones spanning fracture sites were acquired at the time indicated in each experiment in a Packard Faxitron X-Ray Digital Imaging equipment to evaluate the overall healing. X-ray images of day 21 were used for the measurement of callus areas. Soft callus was demarked using the freehand tool in ImageJ software version 1.49v (National Institutes of Health, Bethesda, MD), and area values were obtained and converted to square millimeters via calibration to a scale bar embedded within each image. For each specimen, the soft callus area was measured at three sectional depths that were at least 50 mm apart, and the average value was determined and used for statistical comparisons among groups (36). To validate the callus area measurement, callus index was also determined using μCT three-dimensional images (37).

μCT analysis
Bones spanning fracture sites were harvested at the time indicated in each experiment, fixed in 4% paraformaldehyde, scanned by μCT, and analyzed using a Scanco vivaCT 40 μCT system with a voxel size of 10.5 μm (15, 38). Briefly, each sample was contoured around the callus circumference. The analysis threshold was chosen to distinguish the lower-density woven bones (220 to 570 mg/cm^3) of fracture calluses from the higher-density native cortical bones (570 to 1000 mg/cm^3). The following parameters of new bone formation (lower-density woven bones) were recorded using the analytical software of Scanco (39):
connectivity density, bone volume/volume of interest, bone mineral density, trabecular number, trabecular thickness, and trabecular space.

**Histological analysis**

Bones spanning fracture sites were harvested and fixed in 1% paraformaldehyde containing 0.1% picric acid overnight at 4°C. The samples were embedded in SCEM embedding medium (Section Lab) and surface-covered with the Cryofilm type 2C adhesive film (Section Lab). Frozen sections (thickness, 10 μm) were cut using a Cryostat (CM3050S, Leica).

For IHC staining, the cryosections were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) and blocked with 3% BSA (Sigma-Aldrich) in PBS for 1 hour. The sections were then incubated with an anti-OCN Ab (Abcam). The anti-OCN–stained sections were further stained with an Alexa Fluor 594–conjugated secondary Ab (Abcam). The sections were also stained with an anti-GFP Ab, followed by incubation with Alexa Fluor 488–conjugated secondary Ab (Abcam). Last, the sections were stained with 4’,6-diamidino-2-phenylindole (Abcam). Immunofluorescence images were taken using an Olympus 1X71 inverted fluorescence microscope and were processed using an Olympus cellSens Dimension 1.15 imaging software.

**Quantitative reverse transcription polymerase chain reaction**

Total RNA was extracted using the RNaseasy Mini Kit from Qiagen according to the manufacturer’s protocol. A total of 1 μg of RNA was then used as templates for reverse transcription reaction (High-Capacity cDNA Reverse Transcription Kit, Life Technologies Ltd., Paisley, UK). These generated single-stranded complementary DNAs were then analyzed using the 7900HT Fast Real-Time PCR System (Applied Biosystems) for the desired transcripts using primers specific for the transcripts and a housekeeping gene (i.e., GAPDH). Primers used are shown in table S1.

**Cell proliferation assay**

For CFSE dilution assay, CD90+ and CD90− BM-MSCs were purified and labeled with 5 μM CFSE for 10 min at room temperature. Subsequently, 1.0 × 10^5 CFSE-labeled CD90+ and CD90− MSCs were seeded in the lower chambers of a 24-well Transwell culture plate. In the upper chambers, 2.0 × 10^5 HEK293T cells, which were transduced CD90− to CD90+ in vitro conversion

sorbance in each well (optical density, 570 nm). microplate reader (BioTek Instruments Inc., Winooski, VT) for ab-

was carefully mixed to solubilize crystals and read at 570 μM in a

and 150 hours. At the end of cell culture, the MTT solution was removed

was used as templates for reverse transcription reaction (High-

GAPDH (Applied Biosystems) for the desired transcripts using primers spe-

i = 2.4 nM); item no. 17639, Cayman Chemical\], L-798,106 that is an EP1 antagonist [20 mg/ml (Ki = 2.4 nM); item no. 17639, Cayman Chemical], L-798,106 that is an EP3 antagonist [10 mg/ml (Ki = 0.3 nM); item no. 11129, Cayman Chemical], and ONO-AE3-208 that is an EP4 receptor antagonist [10 mg/ml (Ki = 1.3 nM); item no. 14522, Cayman Chemical]. For in vivo PGE2 receptor inhibition, immediately following fracture surgery, B6 mice subcutaneously received a daily recommended dose of a PGE2 receptor antagonist near fracture sites until bones were harvested. On the next day, mice also received a dose of either a control AAV vector or the AAV.COX-2 vector. Mice were euthanized for analyses 4 days after the fracture surgery.

**In vivo inhibition of WNT/β-catenin signaling**

Immediately after the fracture surgery, mice received a daily subcutaneous injection of vehicle or ICG001 (A8217, APEX BIO, TX) near a fracture site until bones were harvested. On the next day, mice also received a dose of either a control AAV vector or the AAV.COX-2 vector. Mice were euthanized for analyses 4 days after the fracture surgery.

**In vitro migration assay**

A total of 2.0 × 10^5 CD90+ or CD90− BM-MSCs were seeded into the upper chambers, while bone chips were seeded into the lower chambers in a 24-well Transwell culture plate with a membrane with a pore size of 0.8 μm (Transwell Permeable Supports, CORNING). The cells were cultured at 37°C and 5% CO2. Two days later, membranes were fixed in 10% formalin, followed by staining with 0.05% crystal violet solution.

**In vivo MCP-1 blocking**

Male B6 mice were subjected to fracture surgery. Immediately after the fracture surgery and on the following two consecutive days, the neutralizing anti–MCP-1 mAb (clone 2H8; catalog no. BE0185, Bio X Cell, NH), at concentrations of 200, 100, and 100 μg, respectively, was injected subcutaneously near a fracture site.

**Statistical analysis**

CD90+ mSSC abundance data in FACS analyses were analyzed by two-way analysis of variance (ANOVA), followed by post hoc Tukey multiple comparison test. Gene expression data were analyzed by two-tailed t test. μCT data were analyzed using repeated ANOVA, followed by post hoc Dunnett’s multiple comparison test. A P value of <0.05 was considered statistically significant. All analyses were conducted with GraphPad Prism software.

**Supplementary materials**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/7/eaaw2108/DC1

Table S1. Primers used in this study.

Fig. S1. CD90+ MSCs were preferentially recruited into fractured bones from progenitor niches near fracture sites.

Fig. S2. Soluble factors, secreted by COX-2–transduced cells, augmented the proliferation and CD90+ conversion of CD90− MSCs.
expressed common MSC markers, and differentiated into osteoblasts in fracture sites.


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**Acknowledgments:** We thank P. Garcia for assistance with the preparation of this manuscript.

**Funding:** This work was supported by the Telemedicine and Advanced Technology Research Center (TATRC) at the USAMRMC under grant no. W81XWH-12-1-0023 (to D.J.B.). The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

**Author contributions:** S.W., R.L., C.H.R., Y.X., J.Z., and E.E.C. performed the experiments. X.T., D.J.B., X.Q., and K.-H.W.L. interpreted the data. X.T. and D.J.B. conceived, directed, and supervised the study. X.T., D.J.B., and S.W. wrote the manuscript. K.-H.W.L. provided critical reading of the manuscript. All authors reviewed the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 28 November 2018
Accepted 21 June 2019
Published 31 July 2019
10.1126/sciadv.aaw2108