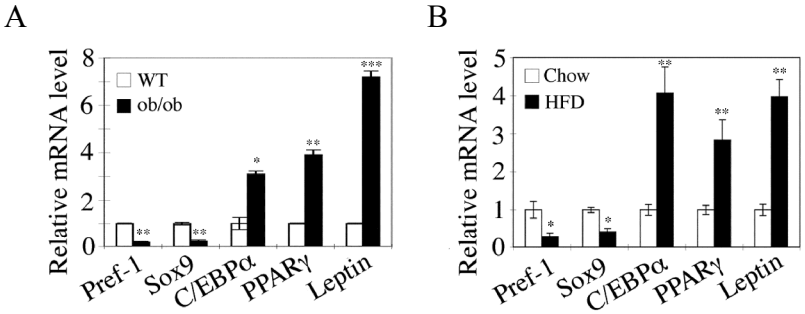
## Supplemental Material

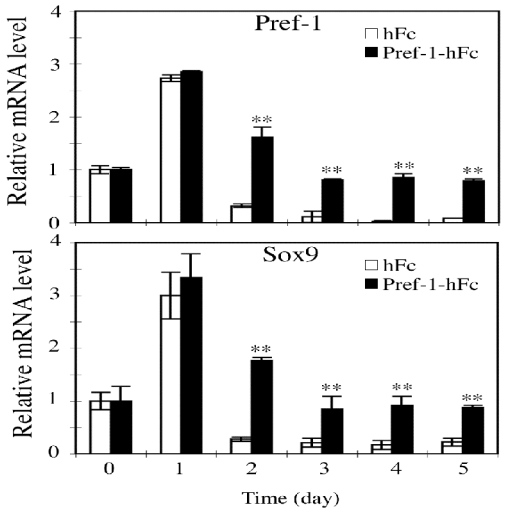
**Suppl. Figures.**

**Suppl. Figure 1**. Primer sets used for the cDNA probes were Col II: 5'-cacactggtaagtggggcaagac-3' and 5'-ggattgtgttgtttcaggattcggg-3'; Col X: 5'-cctgggttagatggaaacag-3' and 5'-aatctcatcaaatgggatggg-3'; Runx2: 5'-gcatcctatcagttcccaatgg-3' and 5'-caatatggccgcgaaacaga-3'; ALPase: 5'-tggagatggacaaattcccc-3' and 5'-tctcgttatccgagtaccag-3'; osteocalcin: 5'-acctagcagacaccatgagg-3' and 5'-ggcagcacaggtcctaaatag-3'.

**Suppl. Figure 2**. Expression of Sox9 in adipose tissue. (A) Gonadal fat pads were isolated from 4 month-old age matched C57BL/6 and *ob/ob* mice (n = 4). Total RNA were extracted and used for RT-qPCR. (B) Total RNA from gonadal fat pads of 12 wk-chow and high fat diet fed C57BL/6 mice were used for RT-qPCR (n = 4).

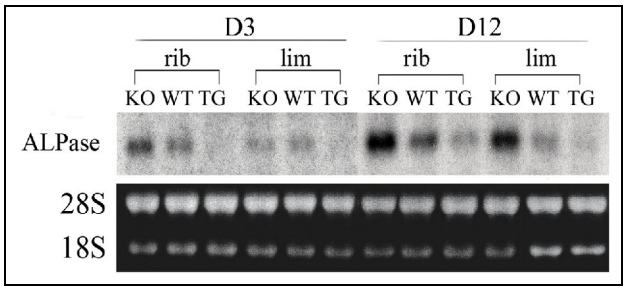



**Suppl. Figure 3**. Expression pattern of Pref-1 and Sox9 during MEF differentiation into adipocytes in the presence of Pref-1-hFc or control hFc.

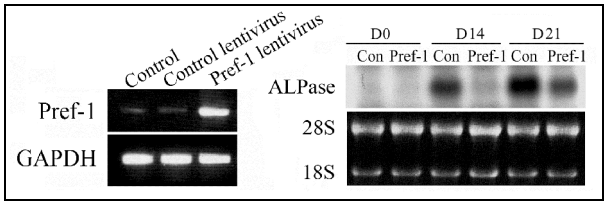



**Suppl. Figure 4**. Primary cells isolated from ribs and limbs were cultured in the presence of osteogenic agents. RNA were prepared at Day 3 or Day 12 for Northern blot analysis

for ALPase. ALPase expression was inversely correlated with Pref-1 expression in these cells undergoing osteogenesis.




**Suppl. Figure 5**. Wild-type calvarial mesenchymal cells were infected with Pref-1 lentivirus or control empty lentivirus. After 48-hr infection, Pref-1 expression was examined by RT-PCR. Infected cells were then subjected to osteogenesis assay. Northern blot analysis shows that ALPase expression after osteogenic treatment was markedly lower in cells infected with Pref-1 lentivirus, compared to the cells infected with control lentivirus at Day 14 and Day 21. These results demonstrate the inhibitory effect of Pref-1 on osteoblast differentiation in vitro.




## Suppl. Materials and Methods

### Cell culture

For micromass chondrogenesis, cells in 1:1 DMEM/Ham F-12 media were cultured in induction media containing 50 μg/ml ascorbic acid, $10^{-8}$ M DEX and 50 ng/ml BMP2 for 2 wks. For osteogenesis, cells were cultured in α-MEM media with 10% FBS to confluence, and subjected to osteogenesis by adding β-glycerophosphate (10 mM) and ascorbic acid (50 μg/ml) with 50 ng/ml BMP2 for 14-21 days. For adipocyte differentiation, 2 day post-confluent cells were treated with differentiation inducing media containing 1 mM DEX, 0.5 mM MIX and 1.67 mM insulin in DMEM with 10% FBS and maintained for 8 days. At 80% confluence, cells were infected with lentivirus for Pref-1 or Sox9 expression for 18 hrs. Media were then replaced by DMEM with 10% FBS for further experiments.

### Histological staining

Tissues were fixed in 4% PFA in PBS, decalcified in 0.1M EDTA for 2 wks at room temperature before embedding in paraffin and sectioning at 5 μm. Hematoxylin/eosin (HE), alcian blue, and von Kossa/nuclear fast red staining were

performed. For von Kossa staining, cells were first fixed in 4% PFA in PBS, washed, and stained with 5% silver nitrate. Cells in culture were washed first in water, then in 5% sodium thiosulfate, and rinsed in water. For alcian blue staining, cells in culture were washed with PBS, stained in 1% alcian blue and 3% acetic acid for 2 hrs before washing. Oil red O staining was performed as described previously (Kim et al., 2007).

**Northern blot analysis, RT-PCR and RT-qPCR**

Total RNA prepared with Trizol (Invitrogen) was subjected to agarose-formaldehyde gel electrophoresis and blotted onto Hybond N membranes (Amersham). The membranes were hybridized with $^{32}$P-labeled cDNAs for mouse Col II, Col X, Runx2, Ihh, ALPase, and osteocalcin. Primer sets used for RT-PCR to produce the cDNA probes were described in supplemental data section. Hybridization was performed as described previously (Wang and Sul, 2006). For RT-qPCR, 1 μg of total RNA was reverse transcribed using SuperScript II (Invitrogen). The cDNAs were mixed with TaqMan Universal PCR Master Mix (Applied Biosystems), specific primers and TaqMan MGB probes for Sox9, Sox5, Sox6, Runx2, Ihh, Col II, C/EBPα and PPARγ and analyzed by using ABI7900. The obtained CT values for these genes were normalized to those of rodent GAPDH expression by the ΔΔCT method. The mean CT was converted to relative expression value by the equation, $2^{-\Delta\Delta Ct}$, and the range was calculated by the equation, $2^{-(\Delta\Delta CT + stdev\Delta\Delta CT)}$.

**Western blot analysis**

Pref-1 null MEFs were pretreated with 30 mM PD98059, 10 mM U0126 or control 0.1% DMSO for 30 min, and cultured in the presence of Pref-1-hFc and inhibitors for 3 hrs for the measurement of Sox9 expression or for 30 min for ERK phosphorylation (Figure 1Bb). Or, Pref-1 null MEF were transfected with ERK1/2 siRNA or control siRNA. After 72 hrs of transfection, cells were treated with Pref-1-hFc or control hFc for 30 min for ERK phosphorylation or for 3 hrs for Sox9 expression (Figure 1Bc). Protein samples were subjected to SDS-10% polyacrylamide gel electrophoresis and transferred to Protran membranes (Schleicher & Schuell). After blocking with 4% nonfat dry milk in Tris-buffered saline-Tween buffer, the membranes were incubated with rabbit anti-Sox9, anti-GAPDH (Santa Cruz), anti-ERK1/2 polyclonal antibodies and anti-phosphorylated-ERK1/2 monoclonal antibody (Cell Signaling), followed by the appropriate HRP-conjugated secondary antibodies (Bio-Rad). Signals were detected by chemiluminescence (Perkin Elmer).

**Measurement of alkaline phosphatase (ALPase) activity**

Cells were washed with 10 mM Tris-HCl, pH 7.2, and scraped into extraction buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Triton X-100 and protease inhibitor cocktail). Cells were then sonicated for 15 sec on ice. The ALPase activity of the supernatants was determined by measuring the $OD_{405}$ after incubation with 1 M diethanolamine pH 9.8, 0.5 mM $MgCl_2$, 10 mM L-homoarginine, 0.5 mg/ml BSA and 12 mM p-nitrophenyl phosphate as the substrate. Protein concentration was determined by the Bradford method. The ALPase activity was expressed as nanomoles of p-nitrophenyl produced per min per milligram of protein (nmol/mg protein/min).

**Electrophoretic mobility shift assay (EMSA)**

Sox9 protein were in vitro translated using Promega TNT® Translation Systems and used in EMSA. Thirty bp sense and antisense oligonucleotides containing potential Sox9 core binding sequence CAAT were labeled by $\gamma$ $^{32}$P-dCTP. Mutant competitors were the oligonucleotides mutated CAAT to CCAT. Samples were separated by 5% native polyacrylamide gel electrophoresis before exposure to film. The sequence of sense and antisense oligonucleotides for C/EBPβ promoter (Bp) and C/EBPδ promoter (Dp) were as follow:

Bp-1005/-975: 5'-GGGTCAATGGGTCGGGGGTCAGCCCCTGACA
and 5'-TGTCAGGGGCTGACCCCCGACCCATTGACCC;
Bp-770/-740: 5'-CCACCGCAATCACCTGAGAAGCCTCTGGGCA
and 5'-TGCCCAGAGGCTTCTCAGGTGATTGCGGTGG;
BP-675/-645: 5'-CTACTAACAATACCAACACTGTCCACGGAGG
and 5'-CCTCCGTGGACAGTGTTGGTATTGTTAGTAG;
BP -65/-35: 5'-GCCGGGCAATGACGCGCACCGACCGGGTGGC
and GCCACCCGGTCGGTGCGCGTCATTGCCCGGC;
Dp-1950/-1920: 5'-GGAACCTCCAATATGTTCCATATGTGACATT
and 5'-AATGTCACATATGGAACATATTGGAGGTTCC;
Dp-1770/-1740: 5'-AGATTAACTCAATTTATAGTTTAGGAAATTA
and 5'-TAATTTCCTAAACTATAAATTGAGTTAATCT
Dp1430/-1400: 5'-GGTGCGGCCCAATAGTGGCCACGGTAGCCTG
and 5'-CAGGCTACCGTGGCCACTATTGGGCCGCACC
Dp-1160/-1130 : 5'-AACCCCAGAGGCAATTAGTTACAATAGCAAC
and 5'-GTTGCTATTGTAACTAATTGCCTCTGGGGTT
Mutant BP-675/-645: 5'-CTACTAACCATACCAACACTGTCCACGGAGG
and 5'-CCTCCGTGGACAGTGTTGGTATGGTTAGTAG;
Mutant Dp-1770/-1740: 5'-AGATTAACTCCATTTATAGTTTAGGAAATTA
and 5'-TAATTTCCTAAACTATAAATTGAGTTAATCT