NFI-C Regulates Osteoblast Differentiation via Control of Osterix Expression

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ABSTRACT

In bone marrow, bone marrow stromal cells (BMSCs) have the capacity to differentiate into osteoblasts and adipocytes. Age-related osteoporosis is associated with a reciprocal decrease of osteogenesis and an increase of adipogenesis in bone marrow. In this study, we demonstrate that disruption of nuclear factor I-C (NFI-C) impairs osteoblast differentiation and bone formation, and increases bone marrow adipocytes. Interestingly, NFI-C controls postnatal bone formation but does not influence prenatal bone development. We also found decreased NFI-C expression in osteogenic cells from human osteoporotic patients. Notably, transplantation of *Nfic*-overexpressing BMSCs stimulates osteoblast differentiation and new bone formation, but inhibits adipocyte differentiation by suppressing PPARγ expression in *Nfic*$^{-/-}$ mice showing an age-related osteoporosis-like phenotype. Finally, NFI-C directly regulates Osterix expression but acts downstream of the BMP-2-Runx2 pathway. These results suggest that NFI-C acts as a transcriptional switch in cell fate determination between osteoblast and adipocyte differentiation in BMSCs. Therefore, regulation of NFI-C expression in BMSCs could be a novel therapeutic approach for treating age-related osteoporosis.

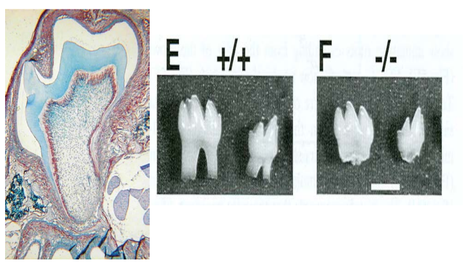
DISCLOSURE OF POTENTIAL CONFLICTS OF INTERESTS
The authors declare no conflicts of interest.

INTRODUCTION

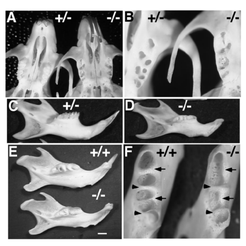
Bone marrow stromal cells (BMSCs) have the capacity to differentiate into osteoblasts and adipocytes. Osteogenesis is regulated by several growth and transcription factors, such as transforming growth factor β (TGF- β), bone morphogenetic proteins (BMPs), Wnt, Hedgehog, Runx2, Osterix (Osx), and β-catenin, whereas adipogenesis is controlled by peroxisome proliferator-activated receptor gamma (PPARγ). With aging, BMSCs become inclined to undergo differentiation into adipocytes, resulting in an increased number of adipocytes and a decreased number of osteoblasts in bone marrow. However, the mechanism underlying this differentiation switch remains unknown.

The nuclear factor I (NFI) family members of transcription factors are expressed from four highly conserved genes in mammals (named *Nfia*, *Nfib*, *Nfic*, and *Nfix*). All four NFI genes are expressed in human osteoblasts and osteoblast-like cell lines. In particular, *Nfic* mRNA is highly expressed in normal osteoblasts compared with other NFI family members. In addition, *Nfic*$^{-/-}$ mice showed defects in alveolar bone formation in molar tooth sockets. However, the exact role of NFI-C in osteoblast differentiation and bone formation remains unclear. In the present study, we investigated the role of NFI-C in osteoblast differentiation and bone formation during osteogenesis.

Nfic mRNA expression in normal tooth



Abnormal alveolar bone formation in *Nfic*-deficient mice.



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METHODS

- **Animals**
  - *Nfic*$^{-/-}$ mice were kindly provided by Dr. Richard M. Gronostajski.
- **Micro-CT and Histomorphometric Analyses**
  - Analyzed by micro-CT with a SkyScan scanner and the associated software. Histomorphometric analyses - using the OsteoMeasure histomorphometry system.
- **Histology Analyse**
  - H&E, von Kossa, TRAP, and IHC staining.
- **Cell Culture**
  - BMSCs were isolated in tibia and femur of 6-week-old WT and *Nfic*$^{-/-}$ mice.
    Osteogenic differentiation - α-MEM + 5% FBS, ascorbic acid (50 μg/ml), and β-glycerophosphate (10 mM).
    Adipogenic differentiation - DMEM + 10% FBS, insulin (10 μg/ml), dexamethasone (1 μM), and 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM).
- **Bone Marrow Cavity Transplantation of BMSCs**
  - *Nfic*$^{-/-}$ BMSCs were labeled with GFP using a retrovirus, and then cultured for 24 hr with *Nfic* or *Osx* retrovirus.
  - The cells were injected *Nfic*- or *Osx*-overexpressing BMSCs, or corresponding mock-infected (GFP-labeled) BMSCs (1×10^6 cells/femur in 10 μl of α-MEM) into the bone marrow cavity of the left femur**.**
  - Analyzed using micro-CT.
- **RT-PCR and Real-time PCR Analyses**
- **Western Blot Analyses**
- **ChIP Assays**
- **Gene-Expression Profiling**
  - Publicly available gene expression datasets were downloaded from GEO (accession number GSE35959). NFI-C mRNA expression (Probeset ID 213298_at) was analyzed between hBMSCs of osteoporotic patients (hBMSCs-OP; 79–94 years old) and hBMSCs of the age-matched control group. (hBMSCs-Old donors; 79–89 years old, n = 4).
- **Statistical Analyses**
  - All quantitative data are presented as the mean ± S.D.
  - Statistical differences were analyzed by using Student's t tests (*, P < .05).

RESULTS

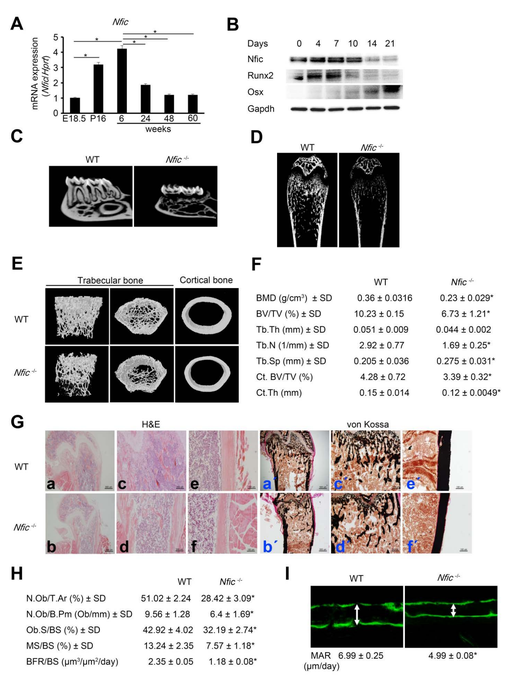

Figure 1. *Nfic* disruption impairs bone formation during postnatal osteogenesis. (A): Nfic expression was evaluated using real-time PCR analyses in BMSCs derived from aged mice. **(B)**: Western blot analyses. **(C)**: Representative micro-CT image of the mandible and **(D)**: the distal femur. **(E)**: 3D micro-CT images. **(F)**: Micro-CT quantification. **(G)**: H&E staining (a-f) and von Kossa staining (a´-f´). **(H)**: Histomorphometric analyses. **(I)**: Mineral apposition rates (MAR).

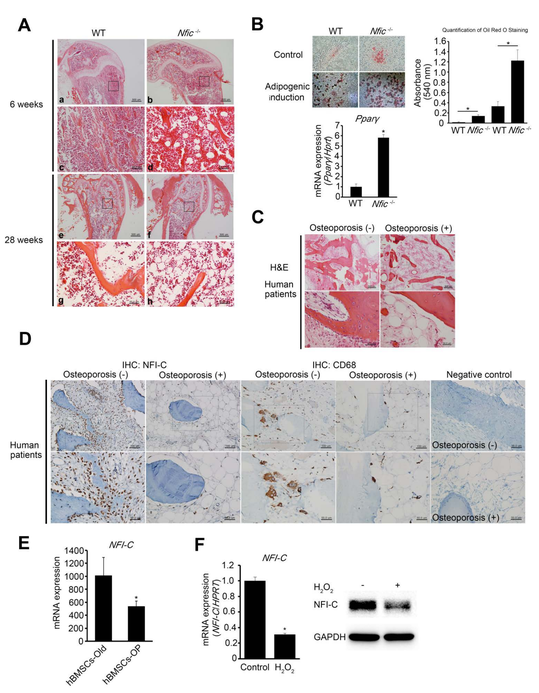

Figure 2. *Nfic*-deficiency increases bone marrow fat as seen in osteoporotic patients. (A): H&E staining. **(B)**: Representative Oil Red O staining images (upper left panel) and quantification of oil red O staining (upper right panel). **(C)**: H&E and **(D)**: IHC staining from bone specimens of an osteoporotic patient. **(E)**: Expression of *NFI-C* mRNA was analyzed from gene expression dataset GSE35959 deposited in GEO. **(F)**: Effect of H_2O_2 on NFI-C expression in hBMSCs.

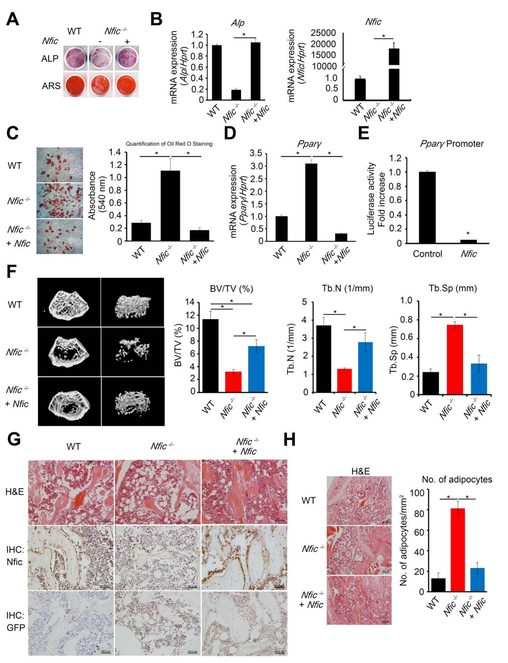

Figure 3. *Nfic* accelerates osteoblast differentiation and suppresses adipocyte differentiation. (A): ALP staining and Alizarin red S staining. **(B)**: *Alp* (left panel) and *Nfic* (right panel) expression. **(C)**: Representative Oil Red O staining images (left panel) and quantification of oil red O staining (right panel). **(D)**: *Pparγ* expression. **(E)**: *Pparγ* promoter activity. **(F)**: Representative micro-CT images and micro-CT quantification of the distal femurs in WT and *Nfic*$^{-/-}$ mice transplanted with *Nfic*-overexpressing BMSCs or mock-infected BMSCs at 10 weeks of age. **(G)**: Histological analyses. **(H)**: H&E staining (left panel) and number of adipocytes (right panel).

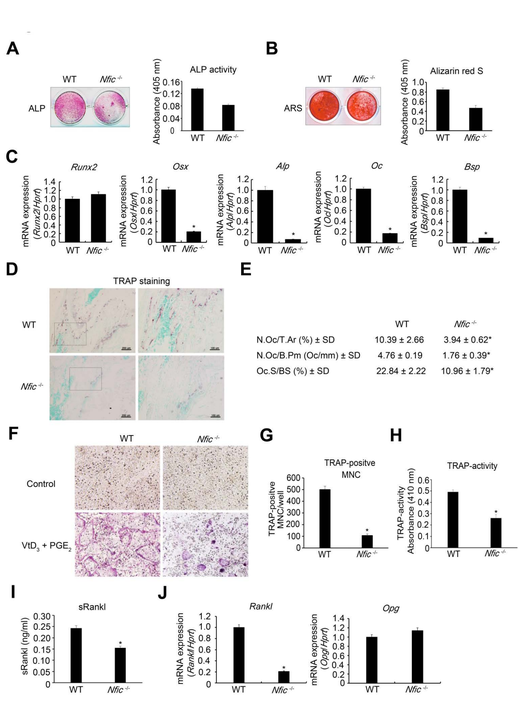

Figure 4. *Nfic* disruption impairs osteoblast differentiation and reduces osteoclast activity. (A): ALP staining and activity. **(B)**: Alizarin red S staining (ARS). **(C)**: *Runx2*, *Osx*, *Alp*, *Oc*, and *Bsp* expression. **(D)**: TRAP staining of femurs from WT and *Nfic*$^{-/-}$ mice aged 6 weeks. **(E)**: Histomorphometric analyses. **(F)**: WT BMMs were co-cultured with WT and *Nfic*$^{-/-}$ primary osteoblasts for 6 days in the absence or presence of $VitD_3$ and PGE_2, fixed, and stained for TRAP. **(G)**: TRAP-positive multinucleated cells (MNCs) were counted in D. **(H)**: TRAP activity was quantified in cell lysates. **(I)**: sRankl levels were measured in cell culture media using ELISA kits. **(J)**: *Rankl* and *Opg* expression.

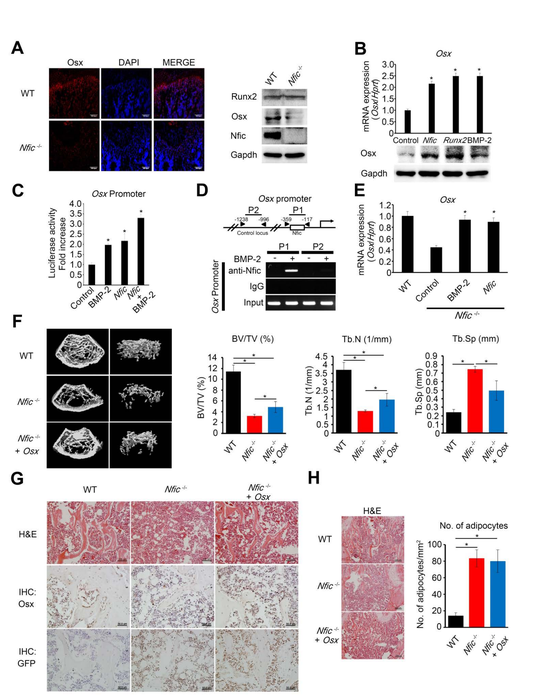

Figure 5. *Nfic* mediates BMP2-*Runx2*-induced *Osx* expression. (A): Immunofluorescence staining of Osx (red). Western blot analyses. **(B)**: Real-time PCR and western blot analyses. **(C)**: *Osx* promoter activity. **(D)**: ChIP analyses. P1 primers: putative Nfic-binding motif. P2 primers: negative control locus. **(E)**: *Nfic*$^{-/-}$ osteoblasts were treated with BMP-2 (300 ng/ml) and/or transfected with the *Nfic* expression vector. *Osx* expression was analyzed using real-time PCR. $n = 3$, *, $P < .05$. **(F)**: Representative micro-CT images and micro-CT quantification of the distal femurs in WT and *Nfic*$^{-/-}$ mice transplanted with *Osx*-overexpressing BMSCs or mock-infected BMSCs at 10 weeks of age. **(G)**: Histological analyses. **(H)**: H&E staining (left panel) and number of adipocytes.

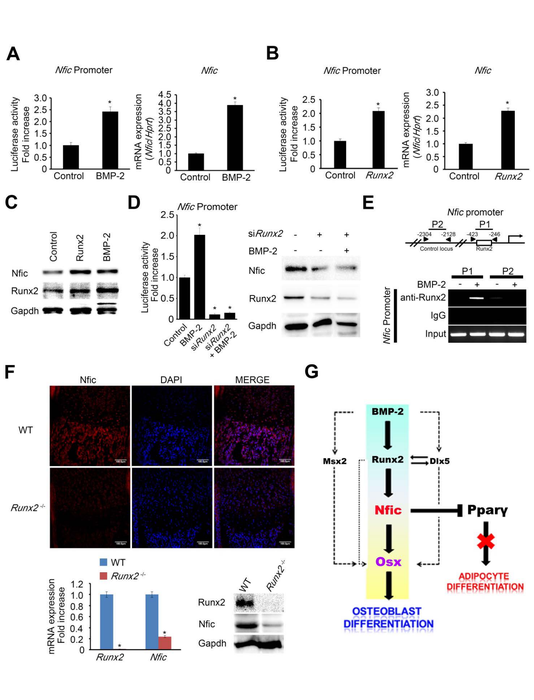

Figure 6. *Runx2* mediates BMP-2-induced *Nfic* expression. (A and **B)**: *Nfic* promoter activity and mRNA expression. **(C)**: Western blot analyses. **(D)**: *Nfic* promoter activity. **(E)**: ChIP analyses. P1 primers: putative Runx2-binding motif. P2 primers: negative control locus. **(F)**: Immunofluorescence staining of Nfic (Red) in femurs from E18.5 WT and *Runx2*$^{-/-}$ mice. Total RNA and protein isolated in calvarial bone from WT and *Runx2*$^{-/-}$ mice. *Runx2* and *Nfic* expression was assessed using real-time PCR and western blot analyses. **(G)**: A model of role of Nfic during osteoblast and adipocyte differentiation.

SUMMARY & CONCLUSION

1. **We found an age-related decrease in *Nfic* expression in BMSCs.**
2. ***Nfic*$^{-/-}$ mice show an age-related osteoporosis-like phenotype with decreased osteoblast differentiation and increased adipocyte differentiation.**
3. ***Nfic* overexpression reduced adipocyte differentiation through suppression of PPARγ, but increased osteoblast differentiation in *Nfic*$^{-/-}$ BMSCs.**
4. **Transplantation of *Nfic*-overexpressing BMSCs rescued an osteoporosis-like phenotype in *Nfic*$^{-/-}$ mice.**
5. **Nfic directly regulates Osx expression through the BMP-2 signaling pathway during osteoblast differentiation.**
6. **Runx2 acts upstream of Nfic and regulates Nfic expression through the BMP-2 signaling pathway.**

These findings suggest that NFI-C is an important factor regulating the balance between osteoblast and adipocyte differentiation in BMSCs.

Taken together, our data suggest that NFI-C is a new candidate gene that causes osteoporosis. Therefore, regulation of NFI-C expression in BMSCs could be a novel therapeutic approach for treating osteoporosis.