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Autocrine Regulation of Interferon γ in Mesenchymal Stem Cells plays a Role in Early Osteoblastogenesis

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ABSTRACT

Interferon gamma (IFNγ) is a strong inhibitor of osteoclast differentiation and activity. However, its role in osteoblastogenesis has not been carefully examined. Using microarray expression analysis, we found that several IFNγ inducible genes were upregulated during early phases of osteoblast differentiation of human mesenchymal stem cells (hMSC). We therefore hypothesized that IFNγ may play a role in this process. We first observed a strong and transient increase in IFNγ production following hMSC induction to differentiate into osteoblasts. We next blocked this endogenous production using a knockdown approach with siRNA and observed a strong inhibition of hMSC differentiation into osteoblasts with a concomitant decrease in Runx2, a factor indispensable for osteoblast development. Additionally, exogenous addition of IFNγ accelerated hMSC differentiation into osteoblasts in a dose dependent manner and induced higher levels of Runx2 expression during the early phase of differentiation. We next examined IFNγ signaling *in vivo* in IFNγ receptor-1 knock-out (IFNγR1$^{-/-}$) mice. Compared to their wild-type littermates, IFNγR1$^{-/-}$ mice exhibited a reduction in bone mineral density. As in the *in vitro* experiments, MSC obtained from IFNγR1$^{-/-}$ mice showed a lower capacity to differentiate into osteoblasts. In summary, we demonstrate that the presence of IFNγ plays an important role during the commitment of MSC into the osteoblast lineage both *in vitro* and *in vivo*, and that this process can be accelerated by exogenous addition of IFNγ. These data therefore support a new role for IFNγ as an autocrine regulator of hMSC differentiation and as a potential new target of bone forming cells *in vivo*.

INTRODUCTION

Maintenance of skeletal integrity is a complex phenomenon characterized by timely regulation between bone formation and bone resorption [1]. Osteoblast formation occurs within the bone marrow by differentiation of pluripotent mesenchymal stem cells (MSC) [2-4]. Regulation of osteoblastogenesis is critical in the acquisition and maintenance of bone mass throughout life [1,2,4]. MSC obtained from the bone marrow and exposed to ascorbic acid *in vitro* produce a collagenous extracellular matrix

and express specific genes associated with the osteoblastic phenotypes such as alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OCN) [3]. The transcription factors involved in the process of differentiation of MSC into osteoblasts are a subject of intense research since their identification could provide us with potential targets for bone formation. Microarray studies of osteoblast differentiation in mouse and human models *in vitro* have identified novel transcription factors that may be important in the establishment and maintenance of differentiation [5]. Most models are based on temporal changes post-induction of MSC that included a proliferative phase (wk 1), a collagen matrix deposition phase (wk 2), and a mineralization phase (wk 3) [6].

In this study, we initially used human MSC (hMSC) that display a stable phenotype, remain monolayer *in vitro,* and can be induced to differentiate into adipocytes, chondrocytes, or osteoblasts [3]. We performed a functional analysis of gene expression changes when hMSC are induced to differentiate into osteoblasts. Our data indicated that several IFNγ inducible genes were expressed transiently during the proliferative phase by hMSC undergoing osteoblastic differentiation *in vitro*. Considering that there is a known interplay between interferon and bone cells, mostly with the osteoclast lineage [1,7], and that the role of IFNγ in osteoblast differentiation and function remains unknown, we assessed the role of IFNγ in osteoblastogenesis both *in vitro* and *in vivo*.

In this study we looked at IFNγ production when hMSC were induced to differentiate into osteoblasts and at the consequences of disrupting IFNγ production by siRNA in hMSC prior to their commitment to osteoblastogenesis. We also examined the effect of exogenous addition of IFNγ to cultured cells as well as the expression of transcription factors required in osteoblast differentiation [8,9]. Finally, we tested whether, as in our *in vitro* experiments, absence of IFNγ signaling affects osteoblastogenesis *in vivo*. Our data indicate that IFNγ plays an important role in

hMSC commitment to osteoblastogenesis both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Osteogenic Differentiation of hMSC in vitro
The induction of osteogenic differentiation of hMSC was described previously [3]. Briefly, hMSC (BioWhittaker, Walkersville, MD, USA) were plated at a density of 5×10^5 cells per well in 150 cm^2 dishes containing mesenchymal stem cell growth medium (GM) (BioWhittaker, Walkersville, MD, USA) with 10% fetal calf serum (FCS) and incubated at 37ºC. After the cells reached 60% confluence, media was replaced with either GM or osteoblastogenesis induction medium (OM) (prepared with MSCGM, 10% FCS, 0.2mM dexamethasone, 10 mmol/L β glycerol phosphate and 50μg/mL ascorbic acid) for 21 days. Media was changed every three days. Media obtained at the beginning of the 1st, 2nd and 3rd wk of differentiation as well as conditioned media were collected for measurement of IFNγ. Concentrations of IFNγ in the conditioned media were measured from both GM and OM treated cells using Human NT-4 DuoSet ELISA Development Kit (R&D Systems, Minneapolis, MI, USA).

cDNA Microarray Analysis of hMSC
Total RNA was extracted from hMSC treated with either growth or osteogenic medium after the 1st and 3rd wk of differentiation using an Easy-Kit mini prep (Qiagen, Valencia, CA, USA). Generation of cDNA, fluorescent labeling, hybridization to the gene chip and data analysis were performed by the Genomics Laboratory at McGill University as previously described [10]. We examined 12,000 human genes and expressed sequences tags (ESTs) on the array Human Genome U95A (Affymetrix, Inc. Santa Clara, CA, USA) and analyzed the results using the MicroDB™ Software (Affymetrix, Inc. Santa Clara, CA, USA). Expression values of the differentiated and non-differentiated hMSC were compared using Student's t test. Genes with significant changes

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were then grouped depending on their known function. The biological function of each gene product was obtained from literature searches in medical databases. This experiment was repeated twice and significant changes in gene expression determined by the method of biological duplicates as previously described [11,12].

Treatment of hMSC with IFNγ under conditions of osteoblastic differentiation

hMSC were plated in 4 cm^2 dishes containing growth medium at a density of 4×10^4 cells per dish. After 48 h, media was replaced with growth or osteogenic medium containing either IFNγ (Sigma-Aldrich, St. Louis, MO, USA) at different concentrations (1, 10 and 100 ng/ml), or vehicle. Media was changed every 3 days. After 1 wk of treatment, media was aspirated and cells were stained for both ALP by using TT-blue+ (Sigma-Aldrich, St. Louis, MO, USA) and mineralization by using Alizarin red (Sigma-Aldrich, St. Louis, MO, USA). The number of ALP positive cells per field was quantified in 10 different fields per well. Matrix mineralization was quantified by extracting the Alizarin red staining with 100mM cetypyridinium chloride (Sigma, St. Louis, MO, USA) at room temperature for 3 h. The absorbance of the extracted Alizarin red S staining was measured at 570 nm. Data represented are expressed as units of Alizarin red per mg of protein in each culture normalized to the number of cells per well.

IFNγ Knockdown by siRNA

Knockdown of the IFNγ was obtained by gene siRNA in differentiating hMSC as previously described [13]. Briefly, hMSC were grown in MSCGM containing 10% FCS until 60% confluence in 6 well plates. Medium was then removed and replaced with serum free MSCGM and transfected with 300 nmol of siRNA using the siRNA oligo transfection kit (sc-29528, Santa Cruz, CA) according to the manufacturer's directions. We used a double stranded siRNA oligonucleotide against human IFNγ (sc-39606) and a negative control siRNA (sc-37007) from Santa Cruz Biotechnology Inc. (Santa Cruz,

CA). Cells were incubated for 5-8 hrs in serum free MSCGM and the medium then replaced with osteogenic medium. siRNA transfection was repeated every 3 days and cells treated for up to 14 days. Specific siRNAs directed against human IFNγ was a pool of the following 3 separate strands: Sense strand (a):5'-CGAAGAGCAUCCCAGUAAUtt-3' (position 616-634); sense strand (b):5'CUGUGACUGUCUCACUUAAtt-3' (position 807-826); and sense strand (c):5'-GCAAGGCUAUGUGAUUACAtt-3' (position 849-867) (Figure 2B). The positions of the siRNAs strands were obtained through GenBank mRNA accession number: NM-000619.2. Their specificity was verified in the non-redundant human DNA database using a BLAST algorithm (accessed through the National Center for Biotechnology Information). Control siRNA did not lead to any specific degradation of known cellular mRNA and was selected because it exhibited no cellular toxicity.

Semi-quantitative Real Time-Polymerase Chain Reaction (RT-PCR)

At the time points indicated cells were collected and washed twice in phosphate-buffered saline (PBS). Poly(A)$^+$ mRNA was isolated with the QuickPrep Micro mRNA purification kit according to the manufacturer's specifications (GE Healthcare Bio-Sciences Inc., Baie d'Urfe, AC, Canada), dissolved in DEPC treated water and subjected to DNAse I treatment. 2 μg of poly(A)$^+$ mRNA was reverse transcribed using Qiagen One Step RT-PCR Enzyme Mix (Qiagen, Valencia, CA, USA). The resulting cDNA was amplified by 35 polymerase chain reaction cycles with an annealing temperature of 58°C. Oligonucleotide primers for IFNγ amplification were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The predicted size of the IFNγ product was 545 bp (Figure 2B). Genes were randomly selected from the list of genes with significant change after the 1st and 3rd wk of differentiation (see Additional Information). Primers were synthesized by Alpha DNA Inc. (Montreal, Quebec). Selected primers for the 1st wk included IFN-inducible protein 35 (IFI

35)(5′–CTCTGCTCTGATCACCTTTGATCAC–3′upstream and 5′–GCTTCTGGAAGTGGATCTCCAGGA–3′downstream); Interleukin 10 receptor (IL10r)(5′–GAACCTGACTTTCACAGCTCAGTAC–3′upstream and 5′–TCAGGTGCTGTGGAAGAGAATTC–3′downstream);Growth related oncogene 1 (GRO1)(5′–GAACATCCAAAGTGTGAACGTGAAG–3′upstream and 5′–ATTTGCTTGGATCCGCCAGCCTCTA–3′downstream); and importin (5′–GAGAATTGCAGAATTGGCCTGACCT–3′upstream and 5′–CTATGTCTGAGTACTTCATGCCA–3′downstream). For the third wk, selected primers included: Transforming growth factor β receptor 2 (TGFBR2) (5′–TACATCGAAGGAGAGCCATTCGC–3′upstream and 5′–TGCAGCACACTCGATATGGACCAG–3′downstream); LDL receptor related protein 5 (LRP5)(5′–GTCGTAGTCGATGGCAATGGCGT–3′upstream and 5′–ACGGACTCAGAGACCAACCGCATC–3′downstream); osteopontin (OPN) (5′–ACTCTGGTCATCCAGCTGACTCGT–3′upstream and 5′–CTCCTAGGCATCACCTGTGCCATA–3′downstream); osteocalcin (OCN) (5′–TGGCCGCACTTTGCATCGCTGG–3′upstream and 5′–CGATAGGCCTCCTGAAAGCCGATG–3′downstream); and runt-related transcription factor 2 (Runx2) (5′–TGGCCGCACTTTGCATCGCTGG–3′upstream and 5′–CGATAGGCCTCCTGAAAGCCGATG–3′downstream).

Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin was used as controls. The expected sizes of the amplification products were between 400 and 800 base pair.

Amplified products were analyzed by either 1.5% or 2% agarose gel electrophoresis. The signals were quantified by densitometry (Bio-Rad laboratories, Hercules, CA, USA) and normalized according to GAPDH or β-actin density.

Western Blot Analysis
Cells were collected at the times indicated, lysed, separated by gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking, the membrane was incubated overnight at 4°C using an antibody directed against Runx2, IFNγ, OCN, IFNγ receptor 1 (IFNγR1) (Santa Cruz Antibodies, Santa Cruz, CA, USA), or tubulin (Sigma, St. Louis, MO, USA), and the bound antibodies were detected with the corresponding secondary antibodies conjugated with horseradish peroxidase. Blots were developed by enhanced chemiluminescence using Lumi-GLO reagents (Kirkegaard & Perry, Gaithersburg, MD, USA). The signals were quantified by densitometry and expression ratios were normalized according to tubulin density.

Cell Viability and Proliferation Assays
To assess whether treatment of differentiating hMSC with either IFNγ or IFNγ knockdown had an effect on their proliferation and survival, cells were plated onto 24-well culture and treated with either with IFNγ or IFNγ siRNA as previously described. After treatment cells were released by 0.25% v/v trypsin-ethylenediaminotetraacetic acid treatment, and resuspended in 10% FCS-containing media. After centrifugation (1,000 x g for 5 min.) cells were resuspended in 10% v/v FCS-containing media mixed with 0.4% w/v trypan blue in 1:1 ratio and a viable cell count performed using a hemacytometer (nonviable cells were stained with trypan blue).

For proliferation analysis, MSC cells were seeded at a density of 4×10^2 cells/well in 96-well cluster plates (Falcon, Becton-Dickinson, NJ, USA). Cells were induced to differentiate and treated with either IFNγ or IFNγ siRNA as previously described. Cell proliferation was quantified by MTS-formazan analysis (CellTiter

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96® AQueous Cell Proliferation Assay, Promega, Madison, WI, USA). Briefly, a stock solution of MTS was dissolved in PBS at a concentration of 5mg/ml and was added in a 1:10 ratio (MTS/DMEM) to each well, incubated at $37^{o}C$ for 2h, and the optical density determined at a wavelength of 490 nm on a microplate reader model 3550 (Biorad, Hercules, CA, USA). In preliminary experiments the absorbance was found to be directly proportional to the number of cells over a wide range (2×10^2 to 5×10^4 cells/well). The percent proliferation was defined as [(experimental absorbance - blank absorbance)/control absorbance - blank absorbance)] x 100, where the control absorbance is the optical density obtained for 1×10^4 cells/well (number of cells plated at the start of the experiment), and blank absorbance is the optical density determined in wells containing medium and MTS alone.

Animals
We purchased IFNγR1$^{-/-}$ mice (strain name 129-Ifngr^{tm1} on a C57BL/6 background) from Jackson Laboratory (Bar Harbor, ME, USA). As a control strain we purchased the strain name 129S1/SvImJ on a C57BL/6 background from the same source. Mice were housed in cages in a limited access room. Animal husbandry adhered to Canadian Council on Animal Care Standards, and all protocols were approved by the McGill University Health Center Animal Care Utilization Committee.

Bone Mass Measurements by Dual Energy X-ray Absortiometry
Hip and spine bone mineral density (BMD) were measured at 4, 8, and 12 wks using a PIXIMUS bone densitometer (PIXIMUS ^{TM}, GE medical systems, Schenectady, NY, USA). A quality control phantom was used to calibrate the densitometer prior to each experiment.

Ex-vivo Cultures of Bone Marrow Cells
Bone marrow cells were prepared and induced to differentiate into osteoblast as previously described [14]. Briefly, one side tibiae from 4-and 8-wk-old IFNGR$^{-/-}$ and IFNGR$^{+/+}$ mice ($n=$ 12 per group per time point) were flushed using a 21-gauge needle attached to a 10 ml syringe filled with Dulbecco's modified Eagle's medium (DME) (GIBCO BRL, Gaithersburg, MD, USA). The bone marrow cells were filtered through a cell strainer with a 70-micron nylon mesh (BD Bioscience, Bedford, MA, USA) and plated in 10 cm^2 tissue culture dishes. The cells were incubated in growth medium at 37°C with 5% humidified CO$_2$ and isolated by their adherence to tissue culture plastic. Medium was aspirated and replaced with fresh medium every 2 to 3 days to remove non-adherent cells. The adherent MSC were grown to confluency for about 7 days and defined as MSC at passage 0, harvested with 0.25% trypsin and 1 mM EDTA for 5 min at 37ºC, diluted 1:3 in growth medium, plated and grown to confluency for further expansion. After 2nd and 3rd passages, MSC were used for subsequent experiments.

To induce differentiation, a total of 10^4 cells were diluted in osteogenic medium and plated in 24 dishes per group, each 4 cm^2. Media was aspirated and replaced with fresh osteogenic medium every 3 days. At 21 days, medium was removed and cultures were fixed in 10% v/v formol/saline solution for 5 min. Colony forming units-osteoblasts (CFU-OB) were detected by Alizarin Red (pH 7.4) staining. The total number of CFU-OB per dish was counted macroscopically with a flat bed scanner fitted with a transparency adapter.

Statistical Methods
Statistical analysis – All data are expressed as mean ± SD of three replicate determinations. Unless otherwise stated, all experiments were repeated three times. Statistical analysis was performed by one-way ANOVA or Student's t-test. A probability value of $p < 0.05$ was considered statistically significant.

RESULTS

IFNγ Inducible Genes are upregulated during hMSC differentiation into osteoblasts in vitro – We exposed hMSC to either growth or osteogenic medium for a total of 3 wks. Subsequently, we examined 12,000 human genes in the array Human Genome U95A (Affymetrix, Inc. Santa Clara, CA, USA) to assess gene expression during the proliferative (wk 1) and mineralization (wk 3) phases of osteoblastic differentiation. The scatter plots of gene expression levels were determined for each time point at wk 1 and wk 3 by averaging mRNA expression levels from growth and osteogenic media exposed cells plotted in a double-logarithmic scale (see Supplemental Data). Genes identified as differentially expressed between growth and osteogenic medium treated cells had a minimum of 2.5-fold increase or decrease in gene expression. At week one and three, a subset of markers of early osteoblast differentiation [5, 15-20] as well as markers of mature osteoblasts [20-21] were differentially expressed in response to osteogenic medium (Figure 1). In a set of 103 genes during the proliferative phase (wk 1), a predominant expression of markers of early osteoblast differentiation was found (Figure 1A). Furthermore, at wk 3 of differentiation, in a set of 104 genes, a strong expression of the markers of mature osteoblasts [20-21], including ALP, osteopontin, OCN and bone sialoprotein2 (BSP-2), was found (Figure 1B). In addition, a set of genes known as IFNγ inducible genes [22-26] displayed striking and transient changes in gene expression at wk 1 (Figure 1A), and returned to normal at wk 3 (Figure 1B). Finally, to estimate the reliability of our microarray results, we compared our results with those obtained by RT-PCR normalized for GAPDH on randomly selected genes at each time point (Figure 1, C and D).

IFNγ is produced following induction of hMSC to the osteoblastic lineage and regulates their differentiation in an autocrine manner – After identification that IFNγ inducible genes were upregulated during early osteoblastogenesis, we then assessed the capacity of hMSC to produce IFNγ prior to, and following, exposure to osteogenic medium. Concentrations of IFNγ were almost undetectable in the conditioned medium of uncommitted hMSC at wks 1 and 2 (Figure 2A), but increased significantly in the 3rd wk, confirming previous reports of IFNγ production by confluent MSC [27,28]. In contrast, conditioned hMSC exposed to osteogenic medium showed a dramatic increase of IFNγ production at wk 1, and returned to baseline at wk 2 (Figure 2 A) ($p< 0.01$).

We then assessed the effect of IFNγ blockade on the ability of hMSC to differentiate into osteoblasts. IFNγ blockade did not affect cell survival of proliferation (data no shown). We compared hMSC differentiation into osteoblasts in cells treated with control siRNA and IFNγ siRNA. IFNγ mRNA levels remained stable throughout the experiment when treated with control siRNA (Figure 2C, left panel). In contrast addition of IFNγ siRNA induced a rapid and sustained inhibition of IFNγ mRNA for the duration of the experiment (Figure 2C, right panel). This sustained inhibition was also seen with IFNγ protein levels (Figure 2D). IFNγ blockade in the presence of osteogenic medium resulted in a strong inhibition of differentiation as shown prior to (Figure 2E) and after staining (Figure 2F) with Alizarin red.

IFNγ induces osteoblastogenesis in a dose dependent manner – The expression of IFNγR1 was significantly increased by exposure of hMSC to OM and by treatment with IFNγ as compared to GM-treated hMSC ($p<0.01$) (Figure 3A and B). This increase occurred early (at week 1) and was sustained for the duration of the experiment (Figure 3B). In addition, when hMSC were induced to differentiate with OM no significant difference in cell proliferation between IFNγ- and vehicle-treated cells was found (Figure 3C). We then examined the capacity of IFNγ to alter the differentiation of hMSC into osteoblasts. IFNγ was added simultaneously with OM. At wk 1 post addition

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of IFNγ to hMSC, a significant dose-dependent and early increase of ALP expressing cells was observed as compared with untreated differentiating cells (76% ± 4 vs. 8% ± 2, $p<0.01$), (Figure 3, D and E). Higher doses of IFNγ did not cause further increase in the percentage of ALP expressing cells (data no shown). Similar results were obtained using Alizarin red staining, a measure of the ability of osteoblasts to mineralize (Figure 3, E and G). Quantification of alizarin red staining showed that hMSC treated with IFNγ mineralized earlier in a dose dependent manner ($p<0.01$) (Figure 3G). Additionally, when hMSC were treated with increasing concentrations of IFNγ in growth medium alone, no effect on either ALP expression or mineralization was observed (Figure 3D and F).

Expression of osteogenic transcription factors following IFNγ knockdown – We first assessed whether the high levels of early osteoblast differentiation and mineralization induced by IFNγ in OM-treated hMSC were concomitant with changes in the expression of Runx2. hMSC treated with GM either alone or with IFNγ (100ng/ml) expressed low levels of Runx2 at wk1,2 and 3 (Figure 4 A and B). As expected [8], levels of Runx2 expression progressively increased after exposure of hMSC to OM (Figure 4 A and B). In contrast, addition of IFNγ to the OM induced a significant increase in expression of Runx2 at wk 1 compared to growth medium alone-treated cells. IFNγ-treated cells continued to display a strong induction of Runx2 at wk 2 as compared to OM alone and decreased at wk3 to the same levels observed with OM (Figure 4A and B).

We next assessed the effect of IFNγ blockade on the expression of Runx2 and OCN. Concomitant with lower levels of osteoblast differentiation and mineralization, a dramatic decrease in the expression of Runx2 and OCN was observed in cells treated with IFNγ siRNA as compared to control siRNA (Figure 4C and D).

IFNγ Signaling Regulates Osteoblast Differentiation in vivo

We then determined the effect of IFNγ signaling on osteoblast differentiation *in vivo* in a mouse model in which the IFNγ receptor had been disrupted by homologous recombination, the IFNγR1$^{-/-}$ model. These animals are normal at birth, are fertile, have a normal growth rate and body weight, and cannot be differentiated from the control IFNγR1$^{+/+}$ mice, except for subtle changes in the immune system [29,30]. Initially, we examined BMD changes in IFNγR1$^{-/-}$ mice and in the control IFNγR1$^{+/+}$ mice. BMD measured at the spine and femora indicated that IFNγR1$^{-/-}$ mice had a significantly lower bone mass in the spine (41% reduction) compared to IFNγR1$^{+/+}$ mice ($0.038g/cm^2 ± 0.004$ vs. 0.064 g/cm^2 ± 0.005, $p<0.01$) and in the femur (31% reduction) ($0.032g/cm^2 ± 0.005$ vs. 0.046 g/cm^2 ± 0.002 vs. $p<0.01$) (Figure 5A). Subsequently, to assess whether, as in our *in vitro* experiments, MSC lose their capacity to differentiate into osteoblasts in absence of IFNγ signaling *in vivo*, we cultured bone marrow cells derived from 4- and 8-wk-old IFNγR1$^{-/-}$ and IFNγR1$^{+/+}$ mice in osteogenic medium. After 3 wks of treatment, the number of CFU-OB derived from IFNγR1$^{-/-}$ mice was significantly lower than the number derived from 4-wk-old IFNγR1$^{+/+}$ mice (6 ± 2 vs. 21 ± 5, $p<0.01$) and 8-wk-old IFNγR1$^{+/+}$ mice (12 ± 5 vs. 46 ± 2, $p<0.01$) (Figure 5, B and C). Similarly, bone marrow cells derived from 4- and 8- week old IFNγR1$^{+/+}$ mice and treated for 14 days with IFNγ siRNA had a significant reduction in the number of CFU-OB as compared to bone marrow cells treated with control siRNA (data not shown).

DISCUSSION

In this study, we identified a new role for IFNγ in MSC committed to differentiate into the osteoblastic lineage both *in vitro* and *in vivo*. We first determined the changes in gene expression that take place during the differentiation of hMSC into osteoblasts. We demonstrated that in addition to the expression of previously

described signaling pathways of osteoblast differentiation [5], a strong and transient induction of a new signaling pathway, IFNγ inducible genes, occurred during the proliferative phase of hMSC differentiation.

Among these IFNγ inducible genes, IFI-16 and absent in melanoma 2 belong to the IFNγ-induced nuclear protein family NIH-200, which is known to play an important role in cell differentiation and embryonic development [22,23]. Earlier studies had shown that after exposure to IFNγ, IFI-16 and absent in melanoma 2 interact with Stat heterodimer and are translocated to the nucleus by karyopherin where they associate with casein kinase 2 [23,26], a protein involved in osteogenic differentiation of hMSC [21, 26]. Our study also demonstrates that karyopherin, also known as importin beta 2, is upregulated at the end of the proliferative phase.

The pattern of gene expression observed therefore supports the coordinated and early activation of these IFNγ inducible genes during the commitment of hMSC to the osteoblastic lineage. The transient nature of IFNγ inducible-genes expression was striking during the proliferative phase (wk 1) and returned to baseline during the mineralization phase. These changes were suggestive of a potential role of IFNγ signaling at the early stage of the differentiation process of hMSC into osteoblasts and triggered us to investigate the regulation of IFNγ during this process. In fact, IFNγ production by proliferating and osteoblastic stromal cells has been reported earlier [27,28]. However, the production of IFNγ by MSC undergoing osteogenic differentiation was considered as a response to the presence of factors released by immune cells into the media [27].

An important finding from our study was the novel observation of a sharp and transient increase in IFNγ production by hMSC induced to differentiate into osteoblasts in the absence of either immune cells or their released factors.

This was concomitant with the transient expression of IFN-inducible genes, which showed a significantly higher expression in hMSC committed to differentiate into osteoblasts as compared to non-committed hMSC at week 1. It is important to note that this difference in gene expression does not imply that IFNγ-responsive genes have not been activated in undifferentiated (non-committed) hMSC at week 3, but rather indicates no significant difference in IFNγ-responsive genes relative expression between OM and GM-treated cells at week 3. Consistent with previous reports [27,28], we found that hMSC express receptors for IFNγ (IFNγR1) and that induction of osteogenic differentiation is accompanied by a rapid and sustained increase in their expression suggesting a synergistic interaction between IFNγR1 expression and the autocrine production of its ligand. Addition of IFNγ further enhanced this rapid and sustained increase of IFNγR1 expression only in the presence of osteogenic medium further supporting this synergistic interaction. Interestingly, IFNγR1 expression was initially low in non-osteogenic conditions (GM medium) and increased rapidly reaching a maximum at week 2 suggesting that the late (delayed) autocrine production of IFNγ by hMSC may be responsible for this delayed increase in IFNγR1 expression. Taken together, these findings suggest that modulation of IFNγR1 plays an important role for hMSC differentiation and acts synergistically with IFNγ in an IFNγ autocrine loop to modulate osteoblast differentiation. The critical role of this autocrine loop was then demonstrated by blocking endogenous IFNγ production by siRNA in differentiating hMSC and also by inducing the differentiation of MSC, obtained from the osteopenic IFNγR1$^{-/-}$ mice, into the osteoblastic lineage. We expected that both approaches used here would block endogenous IFNγ production prior to the induction with the osteogenic medium. We found that absence of IFNγ not only inhibited the early peak of IFNγ production by hMSC observed at wk 1 *in vitro*, but also significantly inhibited MSC differentiation into osteoblasts at wk3, both *in vitro* and *in vivo*. This evidence

indicates that expression of IFNγ by MSC is an important early step during their commitment to the osteoblast lineage.

Furthermore, to assess the potential effect of exogenous treatment with IFNγ on osteoblastogenesis, we next added IFNγ to osteogenic medium-treated hMSC committed to differentiate into osteoblasts. At week 1, IFNγ accelerated hMSC differentiation into osteoblasts in a dose-dependent manner only in the presence of osteogenic medium, indicating that and optimal osteogenic milieu is necessary for IFNγ effect to occur. This effect was not dependent on cell proliferation, indicating that IFNγ stimulates differentiation without affecting proliferation in the presence of osteogenic medium.

Another important finding in this study was the observed changes on Runx2 expression in early osteoblastogenesis prior to and following IFNγ knockdown. Previous studies have indirectly linked IFNγ and Runx2 through the crosstalk between Runx2 and Stat1, a transcription factor regulated by IFNγ [5, 31-33]. Unphosphorylated Stat1 physically interacts with Runx2 to inhibit its function [5, 32]. However, a direct link between IFNγ and Runx2 in the absence of immune cells or immune cells-released factors has not been reported. In our study, Runx2 expression, was strongly inhibited following knockdown of endogenous IFNγ production supporting a mechanistic link between IFNγ signaling and Runx2. Additionally OCN, an osteogenic protein downstream of the Runx2-activated pathway [18], was also inhibited after IFNγ knockdown.

Addition of IFNγ to MSC growth medium did not show an effect on Runx2 expression over the levels seen with growth medium alone. In contrast, the strongest induction of Runx2 occurred at wk1 following addition of IFNγ to osteogenic media. This strong induction was sustained at wk2 but decreased abruptly at wk3. The strong initial increase is consistent with Runx2-mediated osteoblastogenesis with IFNγ.

The mechanism underlying the progressive decrease in Runx2 expression at weeks 2 and 3 is unclear but could be related to down-regulation following completion of the differentiation process. Taken together, these results strongly suggest that IFNγ signaling is a key regulator of hMSC differentiation into osteoblast acting at least in part through the Runx2-related pathway. Further studies looking at the link between Runx2 and IFNγ signaling are required.

The significance of our findings relay in the importance that osteimmunology has acquired in recent years. Although IFNγ is considered to play an important role in bone turnover, it has been proposed that its role is predominantly in the regulation osteoclastic activity [34]. IFNγ is a strong inhibitor of osteoclastogenesis *in vitro* [7, 34] but stimulates osteoclastogenesis *in vivo* [35]. A significant lack of knowledge exists regarding the role of IFNγ on osteoblastogenesis and bone formation both *in vitro* and *in vivo*.

Overall, our results suggest that, in addition to its reported role on osteoclastogenesis and bone resorption [31,34-36], IFNγ may also play an important role in osteoblastogenesis and bone formation both *in vitro* and *in vivo*. In addition, our finding that hMSC secrete IFNγ in the absence of immune cells and that its inhibition by siRNA blocks hMSC differentiation into osteoblasts suggest that the autocrine secretion of IFNγ by hMSC is an essential step in the early commitment of hMSC towards osteoblastogenesis *in vitro*. Considering the importance of the cross talk between the immune system and skeletal homeostasis [1,7] our data showing an effect of IFNγ on hMSC commitment to the osteoblast lineage add yet another level to the complexity of IFNγ role in bone biology.

In conclusion, our data support the concept of an IFNγ autocrine loop as an important early step for hMSC commitment to the osteoblast lineage. Furthermore, the observation that activation of IFNγ signaling further enhances hMSC differentiation into osteoblasts indicates that

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additional studies are necessary to determine its overall contribution to skeletal homeostasis.

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Figure 1. Differentially expressed genes between hMSC exposed to either growth medium or osteogenic medium after the 1st and 3rd week of differentiation. (A and B) Bars represent the fold change of transcript levels of a particular gene between osteogenic medium (OM) and growth medium (GM) treated cells (mean of 2 experiments using biological duplication) (*p*< 0.01). Abbreviations for gene names: IFI-16, interferon-inducible protein; PTHR1, parathyroid hormone receptor 1; human PDGFR, human platelet-derived growth factor alpha-receptor; TGFBRII, transforming growth factor, beta receptor 11; ALP, alkaline phosphatase; OCN, osteocalcin; OPN, osteopontin; LRP-5, LDL receptor related protein 5. (C and D) Randomly selected genes, which showed a significant change in the microarray analysis (see supplemental data), were tested by semi-quantitative RT-PCR and their level of expression was quantified by densitometry and normalized according to GAPDH density. The numbers indicate the fold inductions of gene expression by microarray analysis (Affy) as compared to semi-quantitative RT-PCR.
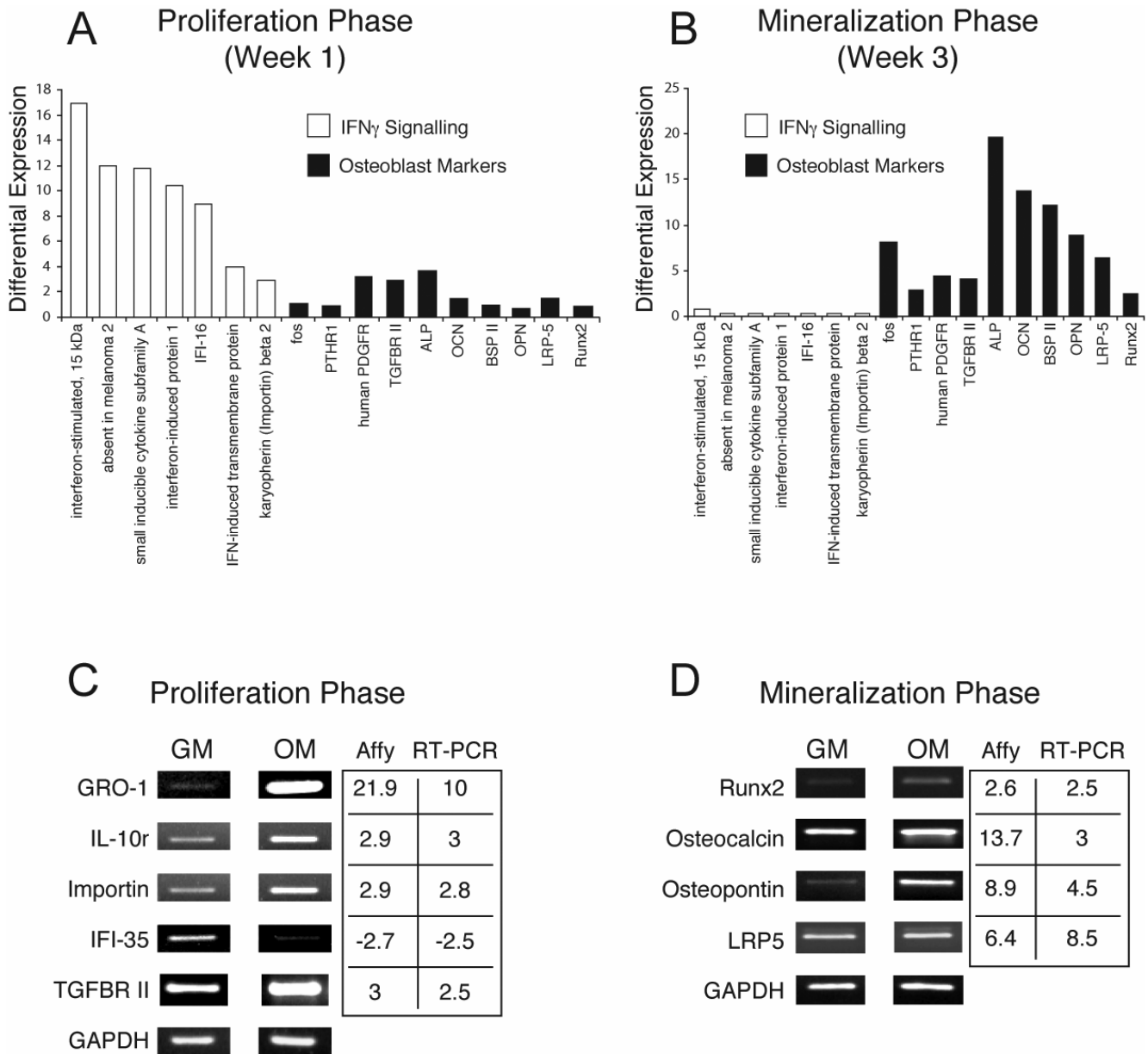
Figure 2. Autocrine production of IFNγ and its role on osteogenic differentiation of hMSC

(A) hMSC treated with osteogenic medium (OM) produce high levels of IFNγ during the 1st wk of differentiation followed by a drop in production during the 2nd and 3rd wks. In contrast, hMSC treated with growth medium (GM) had an increase of IFNγ secretion into the media after the 3rd wk in culture. (B, C) mRNA expression of IFNγ following treatment of MSC with IFNγ siRNA or control siRNA. (B) Partial IFNγ mRNA structure and schematic representation of the three IFNγ siRNA target sequences: a, b, and c. Arrows indicate the direction of PCR. Semi-quantitative IFNγ mRNA expression (C) was measured by RT-PCR and β-actin served as an internal control. hMSC transfected with control siRNA (C, left panel) or IFNγ siRNA (C, right panel) at day 3 (lane 2), day 6 (lane 3), day 9 (lane 4) and day 14 (lane 5). Line 1 represents untransfected control. Note the strong and sustained inhibition of IFNγ mRNA.

(D) Protein expression of IFNγ after either IFNγ siRNA or control siRNA treatment. MSC were treated for 14 days in the presence of osteogenic medium (OM). Treatment with IFNγ siRNA inhibited IFNγ levels by about 80% as shown in panel D. β-tubulin expression is shown in the lower panel. The relative intensity is presented in the bar graph as a ratio of β-tubulin expression. Results are representative of three separate experiments. * represents a significant difference from control siRNA, $p<0.01$.

(E) Phase contrast microscopy of MSC treated with osteogenic medium and IFNγ siRNA (or control siRNA) for 14 days, (F) MSC were stained with alizarin red and observed under a light microscope. There is a marked reduction of mineralization after IFNγ siRNA treatment of osteogenic differentiating hMSC (F).
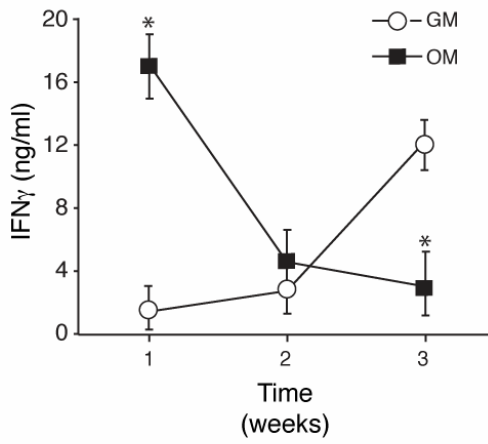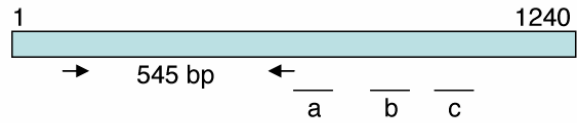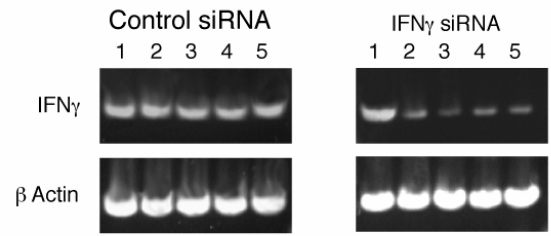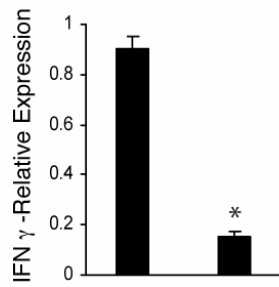
A

IFNγ (ng/ml)

GM
OM

*

*

Time
(weeks)

B

1   1240

545 bp

a   b   c

C

Control siRNA   IFNγ siRNA

1 2 3 4 5   1 2 3 4 5

IFNγ

β Actin

D

Control
siRNA

IFNγ
siRNA

IFNγ

β-Tubulin
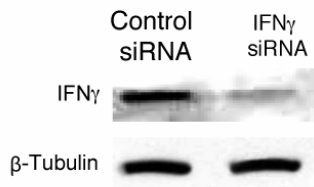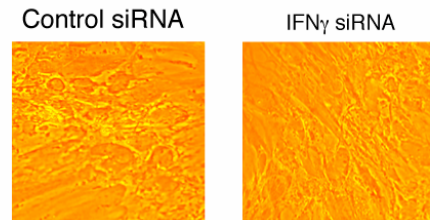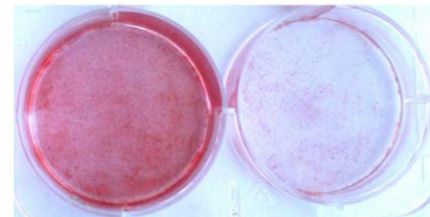
IFN γ - Relative Expression

*

E

Control siRNA   IFNγ siRNA

F

Figure 3. Effect of exogenous addition of IFNγ on osteogenic differentiation of hMSC.

(A,B) Treatment of hMSC with IFNγ both in growth (GM) and osteogenic media (OM) induced significantly higher levels of IFNγR1 as compared to vehicle treated hMSC (GM). The levels of protein expression were significantly higher in GM-treated hMSC at week 3 as compared with week 1 closely correlating with the presence of IFNγ in the media (Figure 2A). The levels of expression of IFNγR1 were significantly increased in hMSC treated with either OM alone or in the presence of IFNγ. * represents a significant difference between week 1 and week 3, $p<0.01$; ** represents a significant difference between week 1 and week 3 in hMSC treated with GM $p<0.001$.

(C) No significant differences in cell proliferation following treatment with increasing doses of IFNγ were noted.

(D-G) At week 1, addition of IFNγ in osteogenic medium-treated hMSC accelerates their osteogenic differentiation in a dose dependent manner as shown by alkaline phosphatase staining (D and F) and the capacity of the differentiated osteoblasts to mineralize after the first week of treatment with IFNγ (E and G). The absorbance of the extracted Alizarin red S staining was measured at 570 nm and adjusted to the number of cells (G). At week 1, alizarin red staining quantification (G) shows a significantly higher mineralization in the OM+IFNγ-treated as compared with GM+IFNγ-treated cells. Six wells were analyzed per experimental condition. Results are representative of three separate experiments. * represents a significant difference from GM, $p<0.01$.
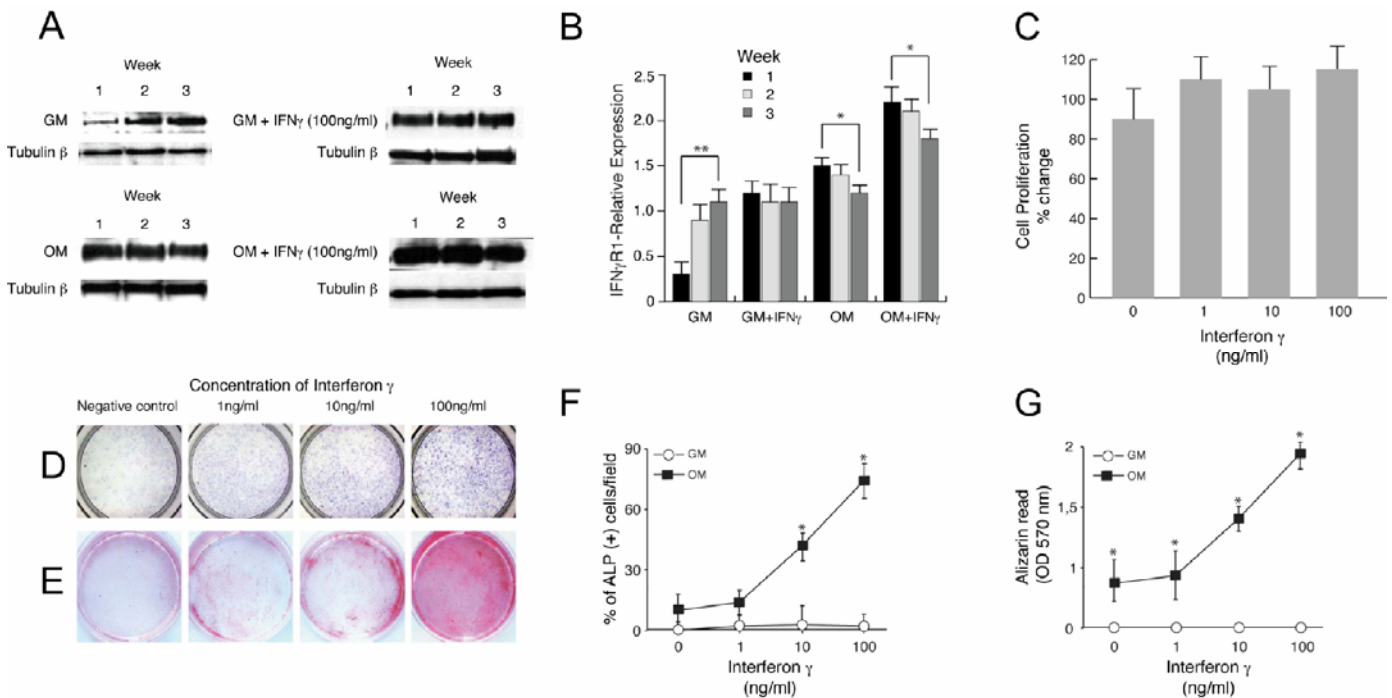
Figure 4. Effect of IFNγ knockdown on expression of osteogenic transcription factors

(A) Osteogenic medium (OM) alone induces progressive expression of Runx2 from wk 1 to wk whereas no change overtime was observed with growth medium (GM) containing either IFNγ or vehicle alone. Addition of IFNγ to the OM induced a significant increase in expression of Runx2 at wk 1 compared to both OM- and GM-treated cells, which decreased significantly at wk2 and wk3. The blots are representative of at least three separate experiments. (B) Histogram of relative Runx2 expression from 3 different experiments from the proliferation phase (week 1) to the mineralization phase (week 3). * represents a significant difference from GM either with or without treatment with IFNγ vs. OM containing either IFNγ or vehicle, $p<0.01$; σ represents a significant difference between OM+ IFNγ and OM-treated cells, $p<0.01$; ϕ represents a significant difference between week 1 and week 3 in OM+ IFNγ-treated cells, $p<0.01$; ψ represents a significant difference between week 2 and week 3 in OM-treated cells.

(C) Protein expression of Runx2 and osteocalcin (OCN) after IFNγ siRNA, or control siRNA treatment. MSC were treated for 14 days in the presence of osteogenic medium (OM). Treatment with IFNγ siRNA inhibited Runx2 and osteocalcin levels by about 80% as shown in panel D. β-tubulin expression is shown in the lower panel. (D) The relative intensity to these proteins is presented in the bar graph as a ratio of β-tubulin expression. Results are representative of three separate experiments. * represents a significant difference from control siRNA, $p<0.01$.
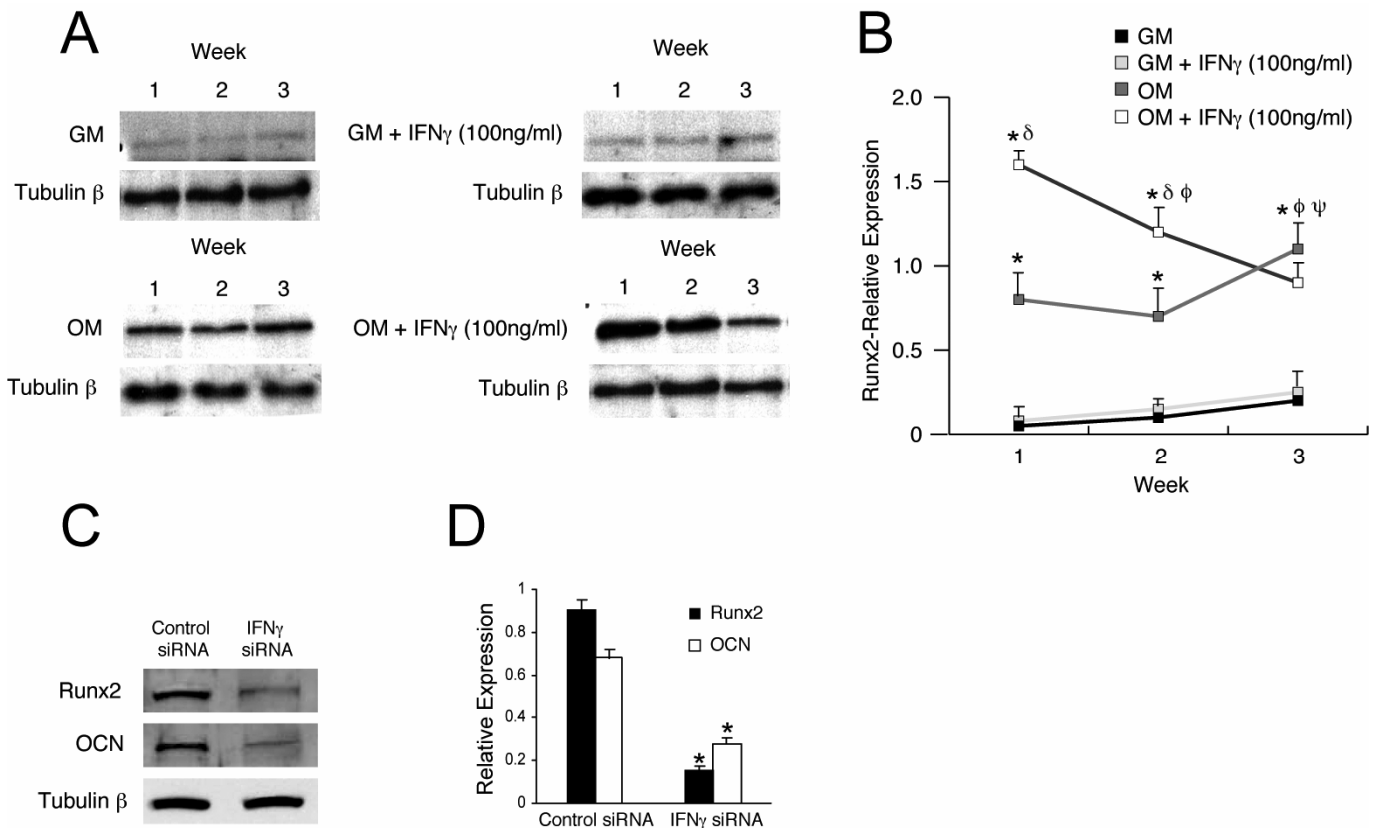
**Figure 5. Effect of IFNγ signaling disruption on bone mineral density and MSC differentiation into osteoblasts.**

(A) Bone mineral density of IFNγR1$^{-/-}$ and IFNγR1$^{+/+}$ mice over time. IFNγR1$^{-/-}$ mice have a lower bone mass as compared to IFNγR1$^{+/+}$ mice in both spine and femur at 4, 8 and 12 wks (*p<0.01). (B) Formation of CFU-OB in ex-vivo cultures of bone marrow cells from 4- and 8-wk-old IFNγR1$^{+/+}$ (left panels) and IFNγR1$^{-/-}$ (right panels). Bone forming nodules (CFU-OB) were much more abundant after 3 wks of induction of differentiation of bone marrow cells treated with osteogenic medium and derived from IFNγR1$^{+/+}$ mice compared to IFNγR1$^{-/-}$ mice. (C) Quantification of CFU-OB. IFNγR1$^{-/-}$ mice (■) show a decreased number of CFU-OB per plate as compared to their IFNγR1$^{+/+}$ control mice (□) (* p< 0.01).
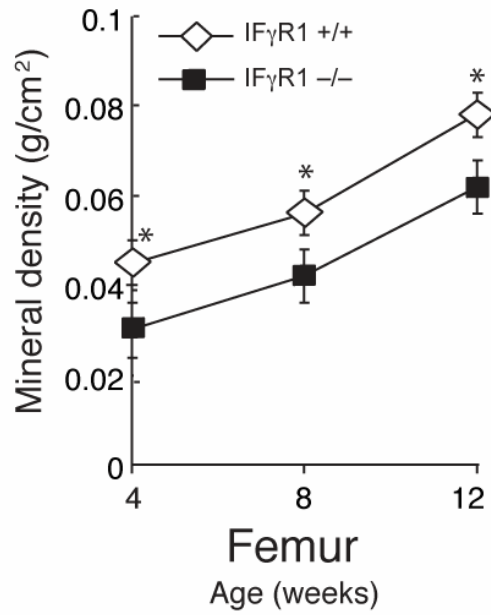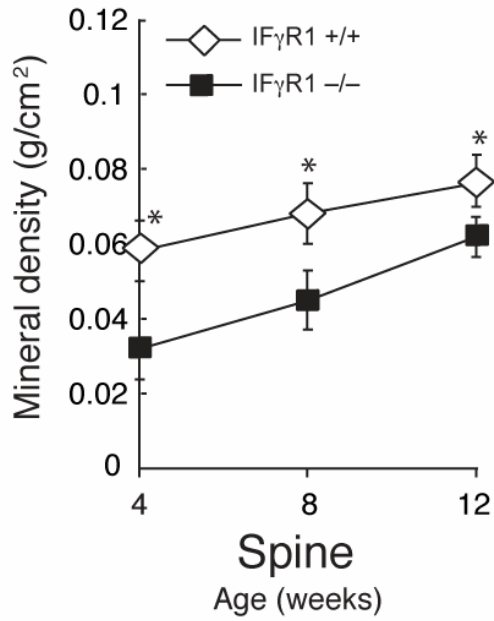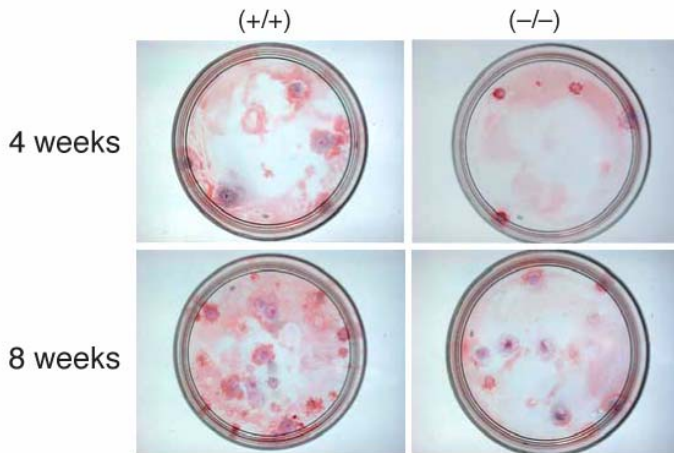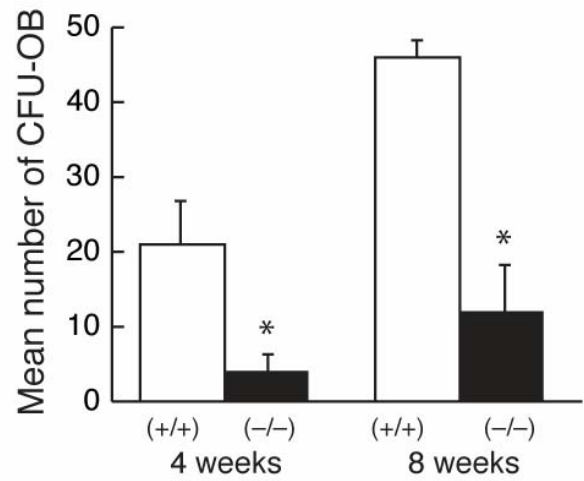
**Autocrine Regulation of Interferon {gamma} in Mesenchymal Stem Cells plays a Role
in Early Osteoblastogenesis**

Gustavo Duque, Dao Chao Huang, Michael Macoritto, Daniel Rivas, Xian Fang Yang,
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