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Msx2 Promotes Osteogenesis and Suppresses Adipogenic Differentiation of Multipotent Mesenchymal Progenitors*

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Abstract

In the aorta, diabetes activates an osteogenic program that includes expression of bone morphogenetic protein-2 (BMP2) and the osteoblast homeoprotein Msx2. To evaluate BMP2-Msx2 signaling in vascular calcification, we studied primary aortic myofibroblasts. These cells express vascular smooth muscle cell (VSMC) markers, respond to BMP2 by upregulating Msx2, and undergo osteogenic differentiation with BMP2 treatment or transduction with a virus encoding Msx2. The osteoblast factor osterix (Osx) is upregulated 10-fold by Msx2 but Runx2 mRNA is unchanged; the early osteoblast marker alkaline phosphatase increases 50-fold with mineralized nodule formation enhanced 30-fold. Adipocyte markers are concomitantly suppressed. To better understand Msx2 actions on osteogenesis vs. adipogenesis, mechanistic studies were extended to C3H10T1/2 mesenchymal cells. Msx2 enhances osteogenic differentiation in synergy with BMP2. Osteogenic actions depend upon intrinsic Msx2 DNA binding; the gain-of-function variant Msx2(P148H) directs enhanced mineralization, while the binding-deficient variant Msx2(T147A) is inactive. Adipogenesis (lipid accumulation, PPARγ expression) is inhibited by Msx2. By contrast, suppression of adipogenesis does not require Msx2 DNA binding; inhibition occurs in part via protein-protein interactions with C/EBPα that control PPARγ transcription. Thus, Msx2 regulates osteogenic vs. adipogenic differentiation of aortic myofibroblasts. Myofibroblasts capable of both fates can be diverted to the osteogenic lineage by BMP2-Msx2 signaling, and contribute to vascular calcification.
Introduction

Mineral deposition in the skeleton is regulated by morphogenetic, metabolic, mechanical, inflammatory, and endocrine factors. With aging, abnormalities in orthotopic (e.g., bone formation) and heterotopic arterial vascular calcification are observed with very high prevalence (1) -- the latter enhanced by hyperglycemia, hyperlipidemia, and chronic renal insufficiency (1),(2). At least three variants of vascular calcification have been described: (a) Calcification of necrotic, intimal atherosclerotic plaques; (b) medial artery calcification; and (c) calcific sclerosis of the aortic valve. Vascular calcification is a highly significant complication of diabetes, and has emerged as a powerful predictor of cardiovascular morbidity and mortality (2). The molecular mechanisms that perturb normal vascular calcium metabolism are only beginning to be understood (1,3,4). Demer was the first to show that vascular calcification may progress via molecular processes similar to osteogenesis (5). This group showed that the powerful bone morphogen, bone morphogenetic protein 2 (BMP2) is expressed in calcified atherosclerotic plaques of humans (5). Bostrom et al further demonstrated that aortic calcification in response to matrix Gla protein (MGP) deficiency was most likely via BMP2 signaling; MGP can abrogate alkaline phosphatase (ALP) induction by inhibiting BMP2 association with the BMP receptor (6). Thus, these studies point to a role for BMP2 in vascular calcification.

Recently, we reported the use of the LDLR -/- mouse as a model of diet–induced vascular calcification in response to diabetes and dyslipidemia; mineral deposition is most notable early on in valve leaflets (7). RT-PCR analyses demonstrated diet–induced upregulation of aortic Msx2, a homeodomain transcription factor that controls osteoblast
differentiation and mineralization in the developing skull (8). In situ hybridization showed that Msx2 is expressed in a subset of aortic adventitial myofibroblasts and valve fibrosal cells(7). Since the Msx2 gene is a direct gene target of BMP2(9), we have studied the function of BMP2-Msx2 signaling in aortic osteogenesis. We show that aortic myofibroblasts are multipotent, capable of expressing VSMC, osteoblast, and adipocyte genes. Msx2 drives osteogenic differentiation of myofibroblasts without hindering VSMC phenotype, but suppresses adipogenesis. Unlike osteogenic activation, suppression of adipogenesis is independent of intrinsic Msx2 DNA binding, occur in part via antagonistic protein-protein interactions with C/EBPα that precludes transactivation of key target genes such as PPARγ. Thus, BMP2-Msx2 signaling promotes osteogenic differentiation of aortic myofibroblasts. Msx2 can regulate the lineage fate of multipotent myofibroblasts via distinct cell-autonomous mechanisms that are either dependent (osteogenesis) or independent (adipogenesis) of intrinsic Msx2 DNA binding activity.

**Experimental Procedures**

*Animals, cell culture, and retroviruses* ---LDLR -/- mice and C57BL/6 mice were maintained as detailed (10), following procedures approved by the Washington University Animal Studies Committee. Aortic RNA was isolated from 10 week old LDLR -/- mice (4 per group) maintained on mouse chow, a diabetogenic high fat diet with cholesterol (Fat + Chol), or a high fat diabetogenic diet without cholesterol (Fat) for 5 weeks as previously detailed (7). Primary mouse aortic myofibroblasts (adventitial cells) were isolated from 6 week old male C57BL/6 mice as previously described (10).
C3H10T1/2 cells (11,12) and CV1 cells were obtained from American Type Cell Culture and cultured in Basal Eagle’s medium containing 10% serum. Our N-terminal Met-FLAG tagged wild-type Msx2, Msx2(P148H), or Msx2(T147A) cDNAs (13) were subcloned into the NeoI and BamHI sites of SFG retroviral vector (14). As a negative control, SFG-LacZ encoding β-galactosidase was generated in the same fashion (15,16). The SFG retrovirus has been shown to have no effect on osteoblast differentiation (15,16). Pseudotyped retroviral particles were prepared as previously detailed (14,15). First passage transduced cells were used for all the assays.

Osteogenic gene expression and mineralization assays -- RNA extraction and RT-PCR was performed as detailed (7,17). The primers for Msx2, OPN, and GAPD were reported previously (7,10). For real-time fluorescence RT-PCR, relative mRNA levels were expressed as percent of 18S ribosomal RNA levels. Amplimers used and designed with Primer Express Software V1.0 (ABI PE Biosystems, Palo Alto, CA) were: Msx2, 5’-ACC ACG TCC CAG CTT CTA GC-3’ and 5’-GCT CTG CGA TGG AGA GGT ACT G –3’; BMP2, 5’-CAC CGT GCT CAG CTT CCA-3’ and 5’-TCG GGA AGT TTT CCC ACT CA-3’; Osx, 5’-CCC TTC TCA AGC ACC AAT GG-3’ and 5’-AAG GGT GGG TAG TCA TTT GCA TA-3’; ALP, 5’-ACA CCA ATG TAG CCA AGA ATG TCA-3’ and 5’-GAT TCG GGC AGC GGT TAC T-3’; Runx2 5’-CCG TGG CCT TCA AGG TTG T-3’ and 5’-TTC ATA ACA GCG GAG GCA TTT-3’; PPARγ2, 5’-ACC ACT CGC ATT CCT TTG AC-3’ and 5’- TGG GTC AGC TCT TGT GAA TG-3’; adipsin, 5’-TGC ATC AAC TCA GAG GTG TCA ATC A-3’ and 5’-TGC GCA GAT TGC AGG TTG T-3’; VSMC α-Actin, 5’-CGG GAG AAA ATG ACC CAG ATT AT-3’ and
5'-GGA CAG CAC AGC CTG AAT AGC-3'; SM22α, 5'- GAG GGA TCG AAG CCA GTG AA-3' and 5'-TGA GCC ACC TGT TCC ATC TG-3'; and 18S, 5'-CGG CTA CCA CAT CCA AGG AA-3' and 5'-GCT GGA ATT ACC GCG GCT-3'. Western blot analyses, ALP activity assays, and Alizarin red staining of calcium deposition were performed as described previously(18). The PPARγ antibody H-100 was purchased from Santa Cruz Biotechnology (Cat # 7196; Santa Cruz, CA). The tubulin anti-body TU-01 was purchased from Zymed (South San Francisco, CA). For mineralized nodule assays, myofibroblasts were maintained in growth medium supplemented with 50 µg/ml ascorbic acid, and 10 mM β-glycerol phosphate for 25 days. At the end of the culture period, cultures were stained for mineral deposition using the von Kossa method as previously described (7). Digital photomicrographs were captured with a Spot Enhanced camera mounted on a Zeiss Axiovert S100 microscope, and von Kossa stained osteogenic colonies quantified using Kodak 1D automated image analysis software version 3.5.3.

Eukaryotic Expression Constructs, PPARγLUC Promoter- Reporter Constructs, Transient Transfection Assays, and GST pull-down assays – Expression constructs for Msx2, Msx2(T147A), Msx2(P148H), and Msx2(Δ132-148) have been previously described and characterized (13, 17). Mouse C/EBPα cDNA was obtained by RT-PCR amplification (that also introduced convenient 5'- and 3'- linkers) of the mRNA obtained from adipocytes generated from C3H10T1/2 cells (vide infra) and subcloned into the KpnI – BamHI sites of pcDNA3 (Invitrogen, Carlsbad, CA) using techniques previously detailed (13). Amplimers used were 5'-GAT GGT ACC ATG GAG TCG GCC GAC TTC TAC-3' and 5'-GAC GGA TCC CTA GTC CTG GCT GCT GCG-3'. The LIP and
LAP forms of C/EBP-β (19) were the kind gift of Dr. Linda Sandell (Washington University, St. Louis). The synthesis of 700 PPARγ_LUC (luciferase reporter in KpnI – MluI sites of pGL2 Basic (Promega, Madison, WI; mouse 0.7 kb PPARγ promoter fragment –615 to +66; (20)) was obtained by PCR using mouse genomic DNA as a template, applying techniques previously described (10). Amplimers used were 5’-GAC GGT ACC TTT ATA GAA TTT GGA TAG CAG-3’ and 5’-GAT CAC GCG TAA CAG CAT AAA ACA GAG ATT TG-3’. All constructs were sequenced to verify fidelity (ABI Prism Dye Terminator Kit, Foster City, CA). For transfection, either C3H10T1/2 mural pleuripotent mesenchymal cells (11,12,20) or CV1 fibroblastic cells were transfected using Lipofectamine (Invitrogen) as outlined in Figure legends. A CMV β-galactosidase plasmid was included as an internal control for transfection efficiency. The ratio of C/EBP to Msx2 expression construct was systematically varied as outlined in the figure legends. Empty pcDNA3 expression vector was used to maintain constant DNA concentrations in all transient transfections. For experiments designed to demonstrate specificity for particular C/EBP family members, the C/EBP to Msx2 expression plasmid ratio was 10:1. One day following transfection, cultures were re-fed with growth medium and cellular luciferase and β-galactosidase activities were measured 24 h – 48 h later as previously described (13,21). The purification of GST fusion proteins expressed in E. coli and coupled in vitro transcription / translation (Promega, Madison, WI) of [35S] Met radiolabeled transcription factors has been previously detailed (13,21). Pull-down assays, SDS-PAGE, and autoradiography were performed as recently detailed (17).
Adipogenesis assays – Confluent cultures of transduced C3H10T1/2 cells were subjected to adipogenic medium containing 0.1 µM dexamethasone, 50 µM indomethacin, and 5 µg/ml insulin (DII medium) for 14 days. The progression of adipogenesis was monitored under light microscope. At the end of culture period, cells were stained for lipid droplets using Oil Red-O stain as described (12).

Statistics -- Statistical analyses were performed using Student’s unpaired t-test or one-way ANOVA as previously detailed (22). Each experiment was performed at least twice and the representative data were presented as mean ± SEM of independent replicates (n ≥ 3).
Results

**BMP2-Msx2 signaling promotes osteogenic differentiation of vascular myofibroblasts.** -- We previously demonstrated that high fat diabetogenic diets induce calcific vasculopathy and aortic Msx2 and osteopontin expression in LDLR-/- mice (7). To understand the mechanisms leading to aortic calcification, we analyzed effects of high fat diets on the expression of BMP2, a potent osteogenic agent (23) that activates Msx2 expression (9) and has been identified in human atherosclerotic plaques (5). As compared to mouse chow, high fat diabetogenic diets -- either with (Fat + Chol) or without (Fat) cholesterol supplementation – concomitantly upregulated aortic BMP2 and Msx2 mRNA accumulation (Fig. 1A; also ref. (7)). Msx2 expression in aorta is primarily localized to a sub-population of adventitial and valvular fibrosal cells that also expressed VSMC α-actin (7). To confirm that BMP2-Msx2 signaling can facilitate aortic calcification, we studied effects of BMP2 and Msx2 on the commitment of aortic myofibroblasts to osteogenic differentiation. Primary aortic myofibroblasts (VSMC α-actin + ) were prepared as previously detailed (10). As shown in Figure 1B, BMP2 treatment of myofibroblasts for 8 days upregulated ALP activity, an early mineralization marker indicating commitment to osteogenic differentiation (16,18). Concomitantly, BMP2 dose-dependently upregulated Msx2 mRNA in myofibroblasts, quantified by real-time fluorescence RT-PCR (Fig. 1C). To evaluate the role of Msx2 in myofibroblast function, we used the pantropic VSV-G protein pseudotyped retrovirus (14-16) to transduce myofibroblasts with either the LacZ control virus or virus expressing FLAG tagged Msx2. Staining for β-galactosidase activity (Fig. 2A), analysis of Msx2 mRNA accumulation (Fig. 2B) and Western blot for FLAG-Msx2 (Fig. 2C) confirmed the
efficient transduction of >90% of cells and robust expression of Msx2. Like BMP2 treatment, Msx2 transduction upregulated mRNA accumulation (Fig. 2D; 50–fold) and ALP activity (Fig. 2E; 10-fold). Thus, components of the BMP2-Msx2 signaling cascade necessary for the initiation of osteoblast lineage differentiation are present in vascular myofibroblasts.

**Msx2 expression stimulates osteogenesis of myofibroblasts** — Osx is a BMP2-induced transcription factor that directs osteoblast-specific differentiation, upregulates ALP expression, and is necessary for mineralization (24). To show that Msx2 enhances osteogenesis in myofibroblasts, we evaluated effects of Msx2 on Osx expression and mineralized nodule formation. As shown in Figure 3A, Msx2 upregulated Osx 10-fold; by contrast, expression of Runx2 — a transcription factor that demarcates the bipotential osteoblast and chondrocyte progenitor (24) — was unchanged (Fig. 3B). Mineralized nodule formation was also increased 30-fold by Msx2 (Fig. 3C and 3D). VSMC phenotypic markers SM22α and VSMC α-actin were not suppressed (in fact increased ca. 3– to 4-fold; Fig. 3E). By contrast, the adipogenic marker, PPARγ was suppressed >90% by Msx2 (Fig. 3F). Thus, Msx2 enhances osteogenesis of aortic myofibroblasts as evidenced by Osx expression, ALP activation, and enhanced mineralized nodule formation. Adipogenic markers such as PPARγ are concomitantly suppressed (vide infra).

**Msx2 enhances osteogenic differentiation of C3H10T1/2 cells via mechanisms dependent upon intrinsic Msx2 DNA binding activity** — C3H10T1/2 is a well-characterized multipotential cell line for studying mesenchymal cell differentiation (12,20). To better
understand the molecular mechanisms whereby Msx2 regulates osteogenic vs. adipogenic differentiation, we therefore evaluated the effects of Msx2 on C3H10T1/2 lineage potential. C3H10T1/2 cells were transduced with either SFG-LacZ (control) or SFG-Msx2, and osteogenic differentiation stimulated with BMP2 (100 ng/ml) as detailed in methods. Consistent with our results in transduced primary aortic myofibroblasts, Msx2 markedly upregulated ALP activity (Fig. 4A), and mRNA accumulation (Fig. 4B), and increased culture mineralization (Figure 4C; Alizarin Red stain for calcium) in C3H10T1/2 cells.

Previously, we generated and biochemically characterized two variants of Msx2 -- Msx2(P148H) and Msx2(T147A) – that exhibit enhanced DNA binding (3-fold improvement in $K_a$) and no DNA binding, respectively (13). Of note, Msx2(P148H) corresponds to a gain-of-function variant that causes precocious calvarial mineralization in humans. In order to evaluate the contributions of DNA binding to the pro-osteogenic actions of Msx2, we compared the activity of SFG-Msx2(P148H) and SFG-Msx2(T147A) on osteogenic differentiation in the C3H10T1/2 cell system, assessing mineralization with alizarin red and differentiation with alkaline phosphatase staining. A time course with and without BMP2 (100 ng/ml supplementation) was undertaken to accentuate any functional differences. As shown in Figure 5, both Msx2 and Msx2(P148H) enhanced matrix mineralization after 19 days in culture; mineralization was synergistically enhanced by the concomitant treatment with BMP2, with accelerated kinetics in cells expressing Msx2(P148H), noticed by day 13 in culture. By contrast, Msx2(T147A) – a Msx2 variant that completely lacks intrinsic DNA binding activity (13) – was not capable of enhancing osteogenic differentiation in this assay either in the
presence or absence of BMP2 treatment (even though it was capable of suppressing adipogenesis; vide infra); staining with Alizarin red was equivalent to that of SFG-LacZ control cultures. Analysis of ALP mRNA accumulation (Fig. 6A), enzyme activity (Fig. 6B), and histochemical staining (data not shown) confirmed the important role for DNA binding in Msx2 pro-osteogenic actions in this assay. Thus, Msx2 augments osteogenic differentiation of multipotent mesenchymal cells via a mechanism dependent upon and proportional to the intrinsic Msx2 DNA binding activity.

Msx2 suppresses adipogenic differentiation of C3H10T1/2 cells via mechanisms independent of intrinsic Msx2 DNA binding activity -- Mesenchymal cells such as bone marrow stromal cells can give rise to both osteogenic and adipogenic lineages (25,26). To better understand the molecular mechanisms whereby Msx2 suppresses adipogenic differentiation, we therefore evaluated the effects of Msx2 on C3H10T1/2 lineage potential. C3H10T1/2 cells were transduced with either SFG-LacZ (control) or SFG-Msx2, and adipogenic differentiation stimulated with dexamethasone, insulin, and indomethacin (DII) as detailed in “Experimental Procedures.” Consistent with our results in transduced primary aortic myofibroblasts, Msx2 markedly suppressed cellular lipid vesicle accumulation under adipogenic conditions (Fig. 7A), which was confirmed by Oil Red O staining (Fig. 7B). The adipocyte differentiation markers PPARγ (Figure 7C) and adipsin (Figure 7E) were down-regulated by Msx2 under basal condition and after stimulation with DII. Inhibition of PPARγ by Msx2 was further confirmed by Western blot analysis (Fig. 7D); PPARγ protein accumulation was suppressed, while tubulin levels were not regulated by Msx2. Similar results were obtained in pro-adipocytic 3T3L1 cells
(data not shown). In order to evaluate the contributions of DNA binding to the ant adipogenic actions of Msx2, we compared the activity of SFG-Msx2(P148H) and SFG-Msx2(T147A) on adipogenic differentiation in the C3H10T1/2 cell system, assessing by oil red O staining cellular lipid accumulation. As shown in Figure 8, all three variants – wild type Msx2, Msx2(P148H) (increased DNA binding), and Msx2(T147A) (no DNA binding) (13) -- were capable of suppressing adipogenic differentiation. Moreover, the activity of the PPARγ promoter (LUC reporter) – entrained to adipogenic differentiation via protein-DNA interactions at well-defined tandem C/EBP binding cognates (20) – was markedly suppressed in Msx2, Msx2(P148H), and Msx2(T147A) co-transfected cells by 90% (Figure 8B), consistent with the reduction in adipogenic differentiation. Thus, the actions of Msx2 in pleuripotential C3H10T1/2 cells confirm that Msx2 suppresses adipogenesis. However, unlike the augmentation of osteogenic differentiation (Fig. 5 and Fig. 6), suppression of adipogenesis occurs via a molecular mechanism that is independent of intrinsic Msx2 DNA binding activity (Figure 8).

Msx2 participate in protein–protein interactions with C/EBPα that inhibit transcriptional activation of the PPARγ promoter -- Adipogenesis is dependent upon transcriptional programs elicited by members of the C/EBP family and PPARγ, a fatty acid activated nuclear receptor that promotes adipogenic differentiation (19,27). The mouse PPARγ promoter is a target of C/EBP activity entrained to adipogenic differentiation via series of C/EBP cognates located ca. 0.3 kb upstream of the transcription initiation site (20). Since Msx2 suppresses PPARγ expression (Figure 5), we evaluated potential function and physical interactions between Msx2 and C/EBPα. As
shown in Figure 9A, protein – protein interactions between Msx2 and C/EBPα were readily identified in GST pulldown assays; radiolabeled C/EBPα binds to GST-Msx2 (lanes 4 and 5) but not to GST resin (lanes 2 and 3). The interaction is specific, since C/EBPα does not directly bind to GST-Runx2 (lanes 6 and 7). The functional consequence of the interaction was next addressed. As shown in Figure 9B, expression of C/EBPα activates PPARγ promoter (luciferase reporter) activity in transient co-transfection assays. Msx2 suppresses C/EBPα activation of the PPARγ promoter in a concentration-dependent fashion (Fig. 9B). Consistent with our results using transduced C3H10T1/2 cells (Fig. 8), all three variants – wild type Msx2, Msx2(P148H) (increased DNA binding), and Msx2(T147A) (no DNA binding) (13) -- inhibit C/EBPα activation of the PPARγ promoter (Figure 9C). By contrast, Msx2(Δ132-148), an inactive Msx2 variant (13, 17), does not suppress C/EBPα activation of PPARγ(Fig. 9C). We wished to assess the specificity of this functionally antagonistic interaction between Msx2 and other C/EBP family members. Therefore, a series of C/EBP expression plasmids were tested for the capacity to reverse Msx2-dependent suppression of the PPARγ promoter. The pcDNA3-C/EBP plasmids were used at 10-fold higher concentrations than the pcDNA-Msx2 expression plasmid to accentuate functional differences between the C/EBP family members in these experiments. As shown in Figure 9D, Msx2 co-expression suppresses the activity of the PPARγ promoter, which is overcome by C/EBPα, and to a lesser extent, by C/EBPβ / LAP (Fig. 9D). This effect is specific, since other C/EBP members such as LIP and C/EBPδ could not overcome Msx2-dependent suppression (Fig. 9D). Thus, Msx2 inhibits expression of the adipogenic differentiation program in part via inhibitory protein – protein interactions with C/EBPα.
Discussion

Vascular calcification has emerged as an important harbinger of cardiovascular morbidity and mortality, particularly so in the setting of diabetes (2,28) – an increasingly prevalent disease achieving epidemic proportions (29). Decreased vascular compliance and increased arterial matrix remodeling in response to calcium deposition may contribute to disease pathophysiology and amputation risk (2,30). As compared to our understanding of orthotopic mineral metabolism, regulation of heterotopic vascular calcification is poorly understood (3). The hierarchy of pathophysiologically relevant signals that control vascular inflammation, smooth muscle proliferation, matrix remodeling, osteogenic differentiation, and mineral deposition remains to be determined via detailed study of appropriate in vivo and ex vivo model systems.

We previously reported that high fat diabetogenic diets promote hypercholesterolemia and vascular calcification in LDLR -/- mice, with concomitant upregulation of aortic Msx2 gene expression (7). By in situ hybridization, Msx2 is localized to adventitial cells and aortic valve fibrosal cells in a pattern overlapping that of VSMC α-actin (7). We speculated that this cell population might be a microvascular smooth muscle cell called a pericyte (31). Of note, Tyson recently demonstrated the expression of Msx2 (by in situ hybridization) in the microvasculature associated with human calcified arteries -- co-expressed with VSMC α-actin (32). Pericytes, like bone marrow stromal cells, express VSMC α-actin and Stro-1 (31), a characteristic marker of pleuripotential myofibroblasts (33); indeed, the multipotent bone marrow stromal cell may be considered a tissue-specific pericytic VSMC, supported by recent histoanatomic
studies (34). Thus, vascular calcification proceeds in part via an active ossification program that recruits vascular progenitors to the osteogenic lineage.

In this work, we extend our previous observations by providing direct evidence that Msx2 is a transcription factor that can program the osteogenic cell fate of vascular myofibroblasts. The primary aortic myofibroblasts we study are capable of expressing VSMC, osteoblast, and adipocyte genes. Msx2 promotes the osteogenic gene program in myofibroblasts, as evidenced by upregulation of Osx, the early osteoblast marker ALP, and mineralized nodule formation. Osx was first identified as a BMP2–induced transcript, and is an osteoblast-specific transcription factor necessary for ALP-dependent bone formation (24). The upregulation of Osx by Msx2 is intriguing, particularly since Msx2 did not alter expression of Runx2—a key regulator of the bipotential osteoblast and chondrocyte progenitor. Moreover, we have identified that certain targets of Runx2—dependent transactivation—such as the very late osteoblast differentiation marker osteocalcin—are down-regulated by Msx2 actions via inhibitory protein-protein interactions ((17), and unpublished observations). This suggests that the molecular mechanisms of Msx2-dependent vascular ossification may occur via a pathway overlapping yet distinct from those regulated by Runx2. Indeed, Chan, Karsenty, and colleagues were the first to suggest the existence of an ossification program that proceeds independent of Runx2/Cbfa1 activity (35); they recently identified a Wnt signaling system that (a) proceeds via the LRP5 receptor (36), (b) controls ossification and skeletal mass, and (c) is independent of Runx2 expression levels (35). Since Msx2 is one downstream target of Wnt signaling in embryonic cells (37), it is tempting to speculate that the pathway we are studying in myofibroblasts may significantly interdigitate with the
Runx2/ Cbfa1-independent osteogenic system activated by LRP5. Studies are underway to directly test this notion.

We observe marked synergy between Msx2 and BMP2 signaling; synergy with BMP2 was time-dependent, elaborated only after several weeks of treatment with BMP2 in culture. Unlike transcriptional suppression, this time-dependent activation of osteogenesis by Msx2 requires and is proportional to the intrinsic DNA binding functions of Msx2. Given this result, it is tempting to speculate that BMP2 upregulates an Msx2 co-activator. Potential candidates include members of the LEF1/TCF, Dlx or Smad transcription factor family. Future studies will detail the novel mechanisms whereby Msx2 activates expression of gene targets such as Osx during osteogenic differentiation.

In these analyses, we identify a novel biological activity of Msx2 – suppression of adipogenic differentiation. This occurs in part via the suppression of PPARγ expression, an important ligand-regulated adipogenic differentiation factor (19,20). Unlike the pro-osteogenic actions, suppression of adipogenesis is independent of intrinsic Msx2 DNA binding activity. Consistent with our studies of transcriptional suppression of the osteocalcin gene (late osteoblast phenotypic marker that inhibits mineralization; Refs. (21,38)), the suppression of PPARγ gene expression occurs via inhibitory protein-protein interactions. Recent studies of the enamel-specific amelogenin gene have also implicated a role for antagonistic protein-protein interactions between Msx2 and C/EBP family members (39). Of note, the weaker transactivator C/EBPβ appears to play a more important role early on during adipogenic commitment(19). Given the stage-specific roles for C/EBP family members during adipogenesis(19) and the differing capacity to reverse Msx2 inhibition of PPARγ transcription, regulatory influences of Msx2
expression may vary during adipocyte maturation. Very recent observations suggest that Msx2-transduced cells secrete an adipostatic activity (our unpublished data). Future studies will examine the relative contributions of cell-autonomous and paracrine signals to Msx2 control of mesenchymal cell fate.

During development, the Msx family members Msx1 and Msx2 play important, partially redundant roles in craniofacial morphogenesis and mineralization(8). However, Maas and colleagues have identified that Msx2 exerts a functionally non-redundant role in the global control of skeletal osteogenesis(8); Msx2-/- mice exhibit a generalized skeletal osteoblast deficiency and low-turnover osteoporosis syndrome – consistent with our observations that Msx2 can promote osteogenic differentiation of mesenchymal progenitors. The regulation of mesenchymal cell lineage allocation by Msx gene family members has been previously suggested by the work of Keating (40). Inducible expression of Msx1 in C2C12 muscle cells results in the de-differentiation of committed myoblasts; when Msx1 is subsequently down-regulated, these cells can then re-differentiate along osteogenic, chondrogenic, adipogenic, and myogenic lineages. In both primary aortic myofibroblasts and C3H10T1/2 cells, Msx2 alters cellular fate, augmenting osteogenesis while suppressing adipogenesis. Abate-Shen has demonstrated that, unlike Msx2, Msx1 suppresses ALP expression in C3H10T1/2 cells (41). Aortic expression of both Msx1 and Msx2 is up-regulated in response to high fat diets in the LDLR-/- mouse (7); given the above, it will be important to identify if these Msx family members exhibit distinct functions with respect to vascular mesenchymal cell plasticity and osteogenic lineage allocation.
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Abbreviations used are: ALP, alkaline phosphatase; BMP2, bone morphogenetic proteins 2; C/EBP, CCAAT / enhancer binding protein; Chol, cholesterol supplementation of a high fat diet; CMV, cytomegalovirus immediate early promoter; DII, dexamethasone /insulin /indomethacin; FCS, fetal calf serum; Fat, high fat diabetogenic diet; GAPD, glyceraldehyde phosphate dehydrogenase; Gla, γ-carboxylated glutamic acid; GST, glutathione – S – transferase; LacZ, SFG retrovirus expressing β-galactosidase; LEF1/TCF; lymphoid enhancer factor 1 / T- cell factor; LDLR, low density lipoprotein receptor; LAP, liver activated protein; LIP; liver inhibitory protein; LRP, LDLR related protein; LUC, luciferase gene; MGP, matrix Gla protein; OPN, osteopontin; Osx, osterix; PPAR, peroxisome proliferator activated receptor; RT-PCR, reverse transcription polymerase chain reaction; VSMC, vascular smooth muscle cells; VSV, vesicular stomatitis virus.
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Figure Legends

Figure 1. Aortic BMP2 and Msx2 expression promote osteogenic commitment of myofibroblasts. (A), High fat diabetogenic diets containing cholesterol (lane 2; Fat + Chol) or lacking cholesterol (lane 3; Fat) upregulate expression of BMP2, Msx2, and OPN mRNA in aortas as compared to the low-fat mouse chow diet (lane 1). Msx2 expression in aorta is primarily localized to a sub-population of adventitial and valvular fibrosal cells that also expressed VSMC α-actin (Ref. (7)). Panels (B) and (C), Primary aortic myofibroblasts were prepared and characterized as recently detailed (10). Incubation of primary aortic myofibroblasts with BMP2 (100 ng/ml) for 8 days concomitantly increases ALP activity (B) and Msx2 mRNA accumulation (C). *p<0.001 vs. Vehicle Control. See text for details.

Figure 2. Transduction of Msx2 into primary aortic myofibroblasts upregulates expression and activity of alkaline phosphatase, an early osteoblast phenotypic marker. (A), Aortic myofibroblasts are efficiently transduced by pseudotyped SFG retrovirus as shown by >90% cells exhibiting β-galactosidase (LacZ) activity by histochemical stain. Msx2 mRNA (B) and Msx2 protein (C) is expressed by myofibroblasts transduced with SFG-Msx2 virus as demonstrated by Western blot for FLAG-tagged Msx2 (C). (D) SFG-Msx2 transduced cells express higher levels of ALP mRNA accumulation (D) and ALP activity (E). *p<0.001 vs. LacZ control cells. See text for details.
Figure 3. Msx2 promotes osteogenesis differentiation of aortic myofibroblasts while suppressing the adipogenic marker, PPARγ. Msx2 stimulates the expression of Osx (A) without affecting Runx2 mRNA levels (B). Msx2 enhances osteogenic mineralized colony formation in aortic myofibroblast cultures as shown by von Kossa staining (C), and as quantified by image analysis (D). Msx2 stimulates the accumulation of SM22α and VSMC α-actin mRNA (E) – smooth muscle cell markers -- but suppresses PPAR-γ mRNA accumulation (F). *p<0.001 vs. LacZ cells. See text for details.

Figure 4. Msx2 stimulates osteogenesis in C3H10T1/2 cells. SFGMsx2- transduced C3H10T1/2 cells exhibit higher ALP activity (A) and ALP mRNA accumulation (B). After culturing in medium containing ascorbic acid, β-glycerolphosphate, and BMP2 (100 ng/ml) for 18 days, Msx2 transduced cells show enhanced matrix calcification as visualized by Alizarin red staining (C). *p<0.001 vs. LacZ cells.

Figure 5. Msx2 induced osteogenic differentiation of C3H10T1/2 cells is enhanced by BMP2 treatment and requires intrinsic Msx2 DNA binding activity. C3H10T1/2 cells were transduced with SFG retroviruses encoding wild type Msx2, the gain-of-function variant Msx2(P148H), or the bind deficient variant Msx2(T147A) as indicated. Osteogenic differentiation of pools of clones was assessed either in the presence or absence of 100 ng/ml BMP2 as indicated. Calcification was visualized by Alizarin red staining of cultures, and comparison
was made with cells transduced with the control virus expressing LacZ. Note that both Msx2 and Msx2(P148H) transduced cells exhibit greater Alizarin red staining -- markedly enhanced by BMP2 treatment. Mineralization was robustly accelerated in BMP2-treated cultures expressing the DNA binding gain-of-function variant Msx2(P148H), observed after 13 days in culture. Further note that transduction with the Msx2(T147A) variant cannot enhance mineralization either in the presence or absence of BMP2. See text for details.

Figure 6. Msx2 induced ALP mRNA accumulation and enzyme activity requires intrinsic Msx2 DNA binding activity. C3H10T1/2 cells were transduced with SFG retroviruses encoding wild type Msx2, the gain-of-function variant Msx2(P148H), or the bind deficient variant Msx2(T147A) as indicated. Osteogenic differentiation of pools of clones was assessed either in the presence or absence of 100 ng/ml BMP2 as indicated. Alkaline phosphatase gene expression (panel A) and enzyme activity (panel B) was measured as described in “Experimental Procedures,” and comparison was made with cells transduced with the control virus expressing LacZ. Note that both Msx2 and Msx2(P148H) transduced cells exhibit greater alkaline phosphatase mRNA accumulation and enzyme activity as compared to control LacZ cultures. Further note that transduction with the Msx2(T147A) variant cannot enhance ALP expression.

*a*p<0.01 vs. corresponding LacZ level.  
bp<0.001 vs. cells transduced with Msx2.
Figure 7. **Msx2 inhibits adipogenesis of C3H10T1/2 cells.** (A) Msx2 prevents cytosolic lipid accumulation after incubation in the adipogenic medium (DII) for 4 and 9 days. Note the marked reduction in refractile cellular lipid droplets in cells transduced with SFG-Msx2 vs. the SFG-LacZ control. (B) Oil Red O-staining of the cultures confirms the accumulation of lipid in the LacZ cells but not in cells expressing Msx2. (C) Expression of Msx2 inhibits PPARγ mRNA accumulation in both basal and DII-stimulated conditions. (D) Inhibition of PPARγ expression by Msx2 is further demonstrated at the protein level by Western blot. Tubulin levels are unchanged. (E) Msx2 also inhibits the expression of adipin in basal and DII-stimulated conditions. p<0.001 vs. corresponding LacZ level. p<0.001 vs. the vehicle treated LacZ cell.

Figure 8. **Msx2 suppression of adipogenic differentiation and PPARγ transcription is independent of intrinsic Msx2 DNA binding activity.** C3H10T1/2 cells were transduced with SFG retroviruses encoding wild type Msx2, the gain-of-function variant Msx2(P148H), the bind deficient variant Msx2(T147A), or the control virus SFG-LacZ as indicated. Panel A, Adipogenic differentiation of pools of clones was assessed by oil Red O-staining of lipid accumulation as described in the text. Note that lipid accumulation is suppressed in cells transduced with Msx2, Msx2(P148H), or Msx2(T147A). Panel B, Activity of the PPARγ promoter in MC3T3E1 cells co-transfected with the indicated pcDNA-Msx2 variant expression plasmids. Note that the 0.7 kb PPARγ promoter activity (-615 to +66) is markedly reduced in cells expressing Msx2, Msx2(P148H), or
Msx2(T147A). Similar results were obtained using C3H10T1/2 cells transduced with viruses encoding these same Msx2 proteins (data not shown). *p<0.001 vs. pcDNA3 transfected control cells. See text for details.

Figure 9. Msx2 inhibition of PPARγ is mediated via physical and functional interactions with C/EBPα. (A), GST pulldown assays were used to identify potential protein-protein interactions between [35S]C/EBPα and GST-Msx2. Lane 1, 10% of input. Note that [35S]C/EBPα robustly interacts with GST-Msx2 resin (lanes 4 and 5), but not GST (lanes 2 and 3), or GST-Runx2 (lanes 6 and 7). (B), PPARγ promoter activation by CEBPα is inhibited by Msx2. CV1 fibroblasts were transfected and regulation of transcription by Msx2 assayed as previously detailed (see ref. 17) with the indicated input of expression plasmid DNA in each culture well. Empty pcDNA expression plasmid was used to maintain constant DNA concentration, and CMV - β-galactosidase was used to control for transfection efficiency in all experiments shown in panels B, C, and D. in all transfections. Note that CEBPα (125 ng input) activates basal PPARγLUC activity. Further note that Msx2 dose-dependently suppresses C/EBPα – dependent activation (C/EBPα input maintained at 125 ng throughout). a*p<0.01 from basal control level; b*p<0.01 from C/EBPα – stimulated PPARγLUC activity observed in the absence of Msx2. (C), Note that Msx2, Msx2(T147A), and Msx2(P148H) are all capable of suppressing C/EBPα activation of the PPARγ promoter. An inactive Msx2 variant, Msx2(Δ132-148), cannot suppress PPARγLUC activity. In these experiments, 125 ng of C/EBPα expression plasmid

29
and 300 ng of Msx2 expression plasmid were used per well (ratio of 1 : 2.4).  

\(^a\) \(p \leq 0.01\) from basal control level;  \(^b\) \(p \leq 0.01\) from C/EBP\(\alpha\) – stimulated \(PPAR\gamma\)\(LUC\) activity observed in the absence of Msx2 variants.  (D) Suppression of the \(PPAR\gamma\) promoter by Msx2 is reversed by over-expression of specific C/EBP family members. To accentuate reversibility and specificity, the pcDNA-C/EBP expression plasmids were used at 10-fold higher concentrations than the pcDNA-Msx2 expression plasmid in these experiments (2000 ng of C/EBP expression construct, 200 ng of Msx2 expression construct). Msx2 co-expression in transient transfection assays suppresses basal \(PPAR\gamma\)\(LUC\) activity. Further note that the over-expression of either C/EBP\(\alpha\) or the activating form of C/EBP\(\beta\), a.k.a. LAP, reverses Msx2-dependent transcriptional suppression. Finally, note that LIP and C/EBP\(\delta\) have no effect on Msx2-dependent suppression of \(PPAR\gamma\) transcription.

\(^a\) \(p < 0.001\) vs. corresponding pcDNA vector control (CON).  \(^b\) \(p < 0.001\) vs. cells transfected with the pcDNA3-Msx2 expressing construct. See text for details.

**Figure 10. Msx2 Regulation of Aortic Myofibroblast Differentiation: A working model.** As in bone marrow stromal cells, the vascular myofibroblast has the capacity to adopt several cells fates. Msx2 acts as a molecular gatekeeper, promoting osteogenic differentiation while inhibiting adipogenic differentiation via protein-protein interaction with C/EBP\(\alpha\). Osteogenic differentiation requires and is proportional to intrinsic Msx2 DNA binding activity. The mechanism whereby Msx2 activates \(Osx\) transcription in synergy with BMP2-elaborated signals is under investigation. Effects of Msx2 on Runx2- dependent
chondrogenic mineralization are predicted to be inhibitory, but have yet to be directly tested in vascular progenitors.
Figure 1
Figure 2
Figure 3

A

B

C

D

Figure 3
Figure 3 (cont)
Figure 4

A

Alkaline Phosphatase Activity (nmol/min/mg protein)

LacZ | Msx2

Cell Lines

B

Relative Alkaline Phosphatase mRNA Levels (% of 18S)

LZ | Msx2

Cell Lines

C

Alizarin Red Stain

LacZ

Msx2
- BMP2

| Days | 13 | 19 |
|------|----|----|
| LacZ |    |    |
| Msx2 |    |    |
| P148H|    |    |
| T147A|    |    |

+ BMP2

| 13 | 19 |
|----|----|
| LacZ|    |
| Msx2|    |
| P148H|   |
| T147A|   |

Alizarin Red Stain

Figure 5
**Figure 6**

**A**

![Graph A: Relative Alkaline Phosphatase mRNA Levels](image)

**B**

![Graph B: Alkaline Phosphatase Activity](image)

Legend:
- a, b

Cell Lines: LacZ, Msx2, P148H, T147A
Figure 7
Figure 8

A

LacZ
Msx2
P148H
T147A

Oil Red O Stain

B

780bp PPARgammaLuc Activity

Plasmids

pcDNA3  Mxs2  P148H  T147A

* * *
Figure 9A

| Input   | GST   | GST-Msx2 | GST-Runx2 |
|---------|-------|----------|-----------|
| C/EBP-α |       |          |           |

1 2 3 4 5 6 7
Figure 9B
Figure 9C

pcDNA3-C/EBP : pcDNA3-Msx2 = 1 : 2.4
Figure 9D

pcDNA3-C/EBP: pcDNA3-Msx2 = 10 : 1
Msx2 Regulation of Aortic Myofibroblast Differentiation: Working Model

Vascular Myofibroblast

? Runx2/Cbfa1

?? Msx2

?? BMP2 signal

C/EBP-α/β

PPAR-γ

Adipocyte

Osx

Osteoblast

Osteogenic Mineralization

Chondrocyte

Chondrogenic Mineralization

Figure 10
Msx2 promotes osteogenesis and suppresses adipogenic differentiation of multipotent mesenchymal progenitors
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