Targeted Reduction of Vascular Msx1 And Msx2 Mitigates Arteriosclerotic Calcification And Aortic Stiffness In LDLR-Deficient Mice Fed Diabetogenic Diets

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ABSTRACT

When fed high fat diets, male LDLR-/- mice develop obesity, hyperlipidemia, hyperglycemia, and arteriosclerotic calcification. An osteogenic Msx-Wnt regulatory program is concomitantly upregulated in the vasculature. To better understand the mechanisms of diabetic arteriosclerosis, we generated SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice, assessing the impact of Msx1+Msx2 gene deletion in vascular myofibroblast and smooth muscle cells. Aortic Msx2 and Msx1 were decreased by 95% and 34% in SM22Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- animals, respectively, vs. Msx1(fl/fl);Msx2(fl/fl);LDLR-/- controls. Aortic calcium was reduced by 31% and pulse wave velocity – an index of stiffness - was decreased in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice vs. controls. Fasting blood glucose and lipids did not differ, yet SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- siblings became more obese. Aortic adventitial myofibroblasts from SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice exhibited reduced osteogenic gene expression and mineralizing potential with concomitant reduction in multiple Wnt genes. Sonic hedgehog (Shh) and Sca1 – markers of aortic osteogenic progenitors – were also reduced, paralleling a 78% reduction in alkaline phosphatase (TNAP)-positive adventitial myofibroblasts. RNAi interference revealed that while Msx1+Msx2 support TNAP and Wnt7b expression, Msx1 selectively maintains Shh and Msx2 sustains Wnt2, Wnt5a, and Sca1 expression in aortic adventitial myofibroblast cultures. Thus, Msx1 and Msx2 support vascular mineralization by directing the osteogenic programming of aortic progenitors in diabetic arteriosclerosis.
INTRODUCTION

Vascular calcification increasingly afflicts our aging population, driven by the dysmetabolic milieu of diabetes, dyslipidemia and uremia (1-4). In type 2 diabetes, arterial vascular calcification primarily occurs within the tunica media with contributions from atherosclerotic plaques as they accrue. Both medial and atherosclerotic calcification increase vascular stiffness thus impairing Windkessel physiology, the elasticity of conduit vessels that enables smooth distal tissue perfusion throughout the cardiac cycle(3; 5). During systole, compliant conduit vessels capture kinetic energy as potential energy, then release this energy during diastole as a mechanism that sustains perfusion pressure with cardiac relaxation(5). When conduit vessels lose elasticity, cardiac afterload and myocardial oxygen consumption are increased, tissue perfusion becomes increasingly pulsatile in distal vascular beds --- and risks for end-organ barotrauma and ischemia are increased during systole and diastole, respectively(5). The net consequence is increased cardiovascular morbidity and mortality via stroke, MI, congestive heart failure, and lower extremity amputation(1; 2).

When fed high fat diets, male LDLR-/- mice develop obesity, hyperlipidemia, insulin-resistant diabetes, and arterial calcification(6-9). These responses phenocopy the arteriosclerotic pathobiology observed in patients with diabetes(1). Msx1 and Msx2 are homeodomain transcription factors indispensable for craniofacial bone formation (10) and cardiac valve morphogenesis(11; 12), and the osteogenic Msx gene regulatory program is concomitantly upregulated in calcifying arteries of diabetic mice and humans with diabetes, dyslipidemia, and/or uremia-induced vascular disease(1; 2; 13). Thus,
mineralization programs regulated by Msx genes are activated during vascular calcium accrual in the setting of type 2 diabetes.

In previous studies, we demonstrated that augmenting aortic Msx2 gene expression – either via TNF-dependent pro-inflammatory signals or via direct Msx2 transgenic strategies – worsens arterial calcification(8; 9; 14). We wished to address whether reducing aortic Msx gene expression mitigated arteriosclerotic calcification as additional proof for the role of this gene regulatory pathway in arterial disease biology. Implementing Cre-lox technology and the SM22-Cre transgenic mouse(15), we show that targeted reductions in vascular smooth muscle Msx2 and Msx1(10; 16; 17) reduce arterial calcification and improve arterial compliance in diabetic LDLR-/- mice – with concomitant reductions in the mineralizing potential of vascular osteoprogenitors.
MATERIALS AND METHODS

Cell culture reagents, biochemicals, antibodies, and immunohistochemistry

Molecular, biochemical, genotyping, and histological methods have been previously detailed(7-9; 18). The indicated, inventoried Taqman Gene Expression assays were purchased from Life Technologies for quantifying mRNA accumulation by real-time fluorescence RT-qPCR. Genotyping primers were ordered from Life Technologies. Amplimer pairs are as follows.  SM22-Cre:  5’-CAG ACA CCG AAG CTA CTC TCC TTC C-3’ and 5’-CGC ATA ACC AGT GAA ACA GCA TTG C-3’ (500 bp). Msx1: 5’-ACA CTA TGC TTG ATG TGG TCC CAG GGC-3’ and 5’-GGG CTC GGC CAA TCA AAT TAG AGA G-3’ (WT = 165 bp; flox = 233 bp); Msx2: 5’-GTT TCA TGA CCT CAT TAC TCA CGC TG-3’ and 5’-GGT ACC TTT GTC AAA TCT GTG AG-3’(WT = 158 bp, flox = 226); LDLR-/-:  5’-ACC CCA AGA CGT GCT CCC AGG ATG-3’ and 5’-CGC AGT GCT CCT CAT CTG ACT TGT C-3’ for the genomic site of insertion (intact WT = 383 bp) and 5’-AGG ATC TCG TCG TGA CCC ATG GCG A -3’ and 5’-GAG CGG CGA TAC CGT AAA GCA CGA GG-3’ for neomycin (200 bp). ELISA kits quantifying matrix metabolism markers desmosine (American Research Products, CSB-E14196M) and type I collagen propeptide P1NP (IDS Inc., AC-33F1) were purchased from commercial sources.  Lipofectamine RNAiMax, ON-TARGETplus control and SMARTpool siRNAs targeting Msx1 and Msx2 were purchased from Life Technologies. GSK3β /phospho-GSK3β antibodies were from Cell Signaling Technologies(8; 9; 14).
Generation and evaluation of SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice

Procedures for handling mice were approved by Washington University and Sanford-Burnham Institutional Animal Care and Use Committees. LDLR-/-B6.129S7-Ldlrtm1Her/J (19) and SM22-CreTg(Tagln-cre)1Her/J (15) mice were obtained from Jackson Laboratory. Msx1(fl/fl);Msx2(fl/fl) mice and have been described (20), and were bred onto the LDLR-/- background. Experimental SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/-, SM22-Cre;Msx2(fl/fl);LDLR-/-, and Msx1(fl/fl);Msx2(fl/fl);LDLR-/- control animals, were obtained via the breeding scheme outlined in Figure 1. At 5 to 10 weeks of age, animals were weighed. Male sibling cohorts (N = 4 to 13 / genotype as indicated) of Msx1(fl/fl);Msx2(fl/fl);LDLR-/- and SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice with equivalent starting weights were challenged with high fat western diet (HFD; Harland Teklad TD88137) for 2 months. At the end of dietary challenge, thoracic aortas were harvested, weighed, and extracted for calcium, collagen, or total RNA, implementing methods previously detailed(9; 21).

Assessment of aortic stiffness by aortic pulse wave velocity (PWV)

Cohorts of male Msx1(fl/fl);Msx2(fl/fl);LDLR-/- and SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice were challenged with HFD for 3 months. Aortic arch PWV was determined as described(21) using a modification of the transit time method(22), implementing a Vevo770 Ultrasound System with a 30 MHz transducer (VisualSonics Inc, Toronto, Canada). Under isofluorane anesthesia, the ascending aorta, aortic arch and proximal portion of the descending aorta were imaged in one 2-D imaging plane from the right superior parasternal view. The pulse wave Doppler sample volume
was placed first near the aortic valve to record blood flow velocity in the proximal aorta, then promptly moved to the visualized portion of the descending aorta without changing the imaging plane in order to record blood flow velocity in the descending aorta. The curvilinear distance between the proximal and distal points of the aortic velocity interrogation (D2-D1; in mm) was measured using the exact coordinates of the Doppler sample volumes. The time delay between the onset of flow velocity in the distal and proximal portions of the aorta (T2-T1; in msec) was measured relative to the simultaneously recorded ECG signal. PWV was calculated as the ratio of D2-D1 to T2-T1, expressed as m/s.

**Preparation of primary aortic adventitial mesenchymal cell cultures**

Aortic adventitial myofibroblasts were prepared using a modification of published methods(14; 23). Aortas were isolated from Msx1(fl/fl);Msx2(fl/fl);LDLR-/- and SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice by dissection from diaphragm to aortic outflow, processed, and cell isolates pooled (8-10 male mice per genotype). After rinsing twice in PBS supplemented with penicillin-streptomycin-fungizone (PSF; 200 U/ml -200 ug/ml - 2.5 ug/ml) with intervening blotting on Kimwipes to remove blood, each aorta was rinsed 3 times in fresh DMEM supplemented with PSF. Individual aortas were then minced into 4 fragments. Two aortas per genotype were placed in a 50 mL conical screw cap tube, then digested at 37 °C / 5% CO2 incubator in 4 ml of type I collagenase (Worthington Biochemical Corporation, cat. # LS004149, 1 mg/ml), DNase I (Sigma-Aldrich, D5025, 60 U/ml), and hyaluronidase (Sigma-Aldrich, H3506, 0.5 mg/ml) in DMEM with PSF using a sterile flea stir bar to provide gentle agitation. Two sequential
1-hour digestions were performed and combined, and cells pelleted at 1500 rpm for 5 minutes. The cell pellet was re-suspended in DMEM with 10% FBS supplemented with PSF, and then plated onto 10-cm tissue culture plates coated with rat tail type I collagen (6 ug/cm2). After 3 days, half of the medium was removed from each culture and replaced with fresh growth medium. Two days later, cells were rinsed once with growth medium lacking PSF, then maintained in growth media with penicillin-streptomycin (100 IU/ml-100 ug/ml) and fed every 3 days until confluence. Only passage 1 and passage 2 cultures were used for experimentation. Our methods for preparation of SFG-LacZ and SFG-Wnt7b retroviruses and myofibroblast transduction have been described(18). RNA interference (RNAi) in primary adventitial cultures was performed in 12-well cluster dishes (100,000 cells / well) in triplicate with 100 nM non-targeting control siRNA (NTC), 50 nM Msx1 siRNA + 50 nM NTC, 50 nM Msx2 siRNA + 50 nM NTC, or 50 nM Msx1 + 50 nM Msx2 siRNA using RNAiMAX from Life Technologies, following the manufacturer’s protocol. Two days later RNA was isolated for RT-qPCR using methods detailed(18).

**Alkaline phosphatase staining of aortic primary cell cultures**

Alkaline phosphatase-positive cells in aortic adventitial cell cultures were visualized as previously described, but using the Vector Red fluorescent substrate(14; 18). Briefly, aortic mesenchymal cells were cultured on a type I collagen pre-coated- 4-chamber slide (60,000 cells/chamber) for a total of 8 days. During the final 6 days, cells were treated with β-glycerophosphate (5 mM) and ascorbic acid (50 µg/ml). Cells were then washed three times with TBS (20 mM Tris HCl, 1.5 M NaCl, pH7.5), fixed with 4%
paraformaldehyde in TBS for 4 min, then rinsed 3 times with TBS. Staining with Vector Red reagent (Vector Red Alkaline Phosphatase Substrate Kit I, Vector Laboratories, Cat. # SK-5100) was performed for 1 hour in the dark per the manufacturer’s protocol. After washing 3 times with TBS and twice with distilled water, nuclei were stained with DAPI (Prolong Gold Antifade Reagent, Molecular Probes, Cat. # P36931), and slides coverslipped. Photomicrographs of alkaline phosphatase-positive fluorescent cells (TX2 filter cube), nuclei (DAPI filter cube), and phase contrast images were taken using a Leica DM 4000B fluorescence microscope at 200X magnification.

**Calcium staining of aortic primary cell cultures**

Aortic adventitial cells were seeded in type I collagen-coated 24-well cluster plates (15,000 cells/well, 3 wells per genotype). The next day and every other day thereafter, cells were fed with DMEM medium containing 10% FBS, ascorbic acid (50 μg/ml) and β-glycerophosphate (5 mM) for a total of 12 days. Mineralized matrix was detected by Alizarin Red S staining as described (14; 18) and images (12-16 images/well) were captured by Nikon Eclipse Ti microscope at 100X magnification. Alizarin red-stained areas were quantified using NIH Image J analysis as previously detailed(14).

**Statistics**

All experiments have been performed with n = 3 to 12 independent replicates per group. Statistical analyses were performed using GraphPad Instat Software (version 3.06), implementing standard parametric or non-parametric methods (2 tailed testing) when indicated. Graphic data are presented as the mean ± SEM.
RESULTS

Reductions in aortic Msx1 and Msx2 mitigate arteriosclerotic calcification responses in LDLR-deficient mice fed high fat diabetogenic diets

Msx1 and Msx2 are highly related members of the NK-like homeodomain transcription factor family(11; 12). Msx1 and Msx2 play quantitatively distinct but functionally redundant roles in neural crest biology, craniofacial and heart valve morphogenesis, and skeletal and ectodermal organ growth at sites where dynamic epithelial-mesenchymal interactions occur(11; 12). Our previous studies demonstrated that a HFD upregulates Msx1 and Msx2 in the aortic myofibroblasts and smooth muscle cells in male LDLR-/- mice(6; 23), presaging subsequent cardiovascular calcification and the expression of several osteogenic Wnt ligands including Wnt3, Wnt5, and Wnt7 family members(7). Moreover, transgenic augmentation of aortic Msx2 expression promoted calcification in the tunica media in mice fed HFD, with mineralization proceeding via activation of an osteogenic Wnt cascade(7). To better understand the role of endogenous Msx1 and Msx2 in vascular disease processes including arteriosclerotic calcification, we implemented Cre-Lox technology to reduce aortic Msx1 and Msx2 tone in genetically engineered mice possessing floxed (fl) Msx alleles(20). The SM22-Cre transgene was utilized to deliver Cre recombinase to VSMCs and aortic myofibroblasts(15). Utilizing the breeding scheme outlined in Figure 1, SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- and Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice were generated, and male sibling cohorts studied. Msx1 and Msx2 mRNAs were significantly reduced in total aortic RNA from SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice vs. Msx1(fl/fl);Msx2(fl/fl);LDLR-/- controls, with concomitant reductions in osterix (Osx) – a zinc finger transcription factor
upregulated by Msx2(23; 24) and necessary for osteogenic mineralization(25) (Figure 2A). While the abundance of Runx1, Runx2, and Sox9 messages were unaltered in total aortic RNA, expression of Shh – a marker of the vascular multipotent mesenchymal progenitor(26) – was concomitantly reduced with vascular Msx gene deficiency. We next challenged sibling male cohorts with the diabetogenic HFD for 2 months, and assessed the impact upon aortic calcium accumulation. As compared to Msx1(fl/fl);Msx2(fl/fl);LDLR-/ controls, SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/ mice exhibited a significant 31% reduction in total aortic calcium accrual (Figure 2B). Alizarin red staining for aortic calcium confirmed that changes in mineral deposition largely localized within the tunica media (Figure 2C-2D). By contrast, total aortic collagen accumulation (Figure 2E) and ascending aorta wall thickness (Figure 2F) were unaltered. SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/ mice were significantly more obese than Msx1(fl/fl);Msx2(fl/fl);LDLR-/ controls (Figure 3A), but bone mineral density and bone mineral content by DXA were also unaltered (Figure 3B). No significant differences were observed with respect to diet-induced changes in fasting plasma glucose, triglycerides, or cholesterol (Figure 3C). Furthermore, plasma haptoglobin and 8-F-isoprostanes -- markers of systemic inflammation and oxidative stress upregulated by diabetogenic HFD(8) -- did not differ with vascular Msx gene deletion (Figure 3D), indicating genetic manipulation of vascular Msx genes had not mitigated the pro-inflammatory metabolic milieu. Thus, aortic Msx1 and Msx2 expression in aortic VSMCs and myofibroblasts support arteriosclerotic calcification in LDLR-/ mice fed diabetogenic HFD.
**Reductions in aortic Msx1 and Msx2 reduce arteriosclerotic vascular stiffening**

Arterial calcification is a harbinger of vascular stiffness, a composite of material properties and geometric properties that alter the distensibility of conduit vessels during the cardiac cycle (3). From the Moens-Korteweg relationship(27), the product of the elastic modulus and the arterial wall thickness to lumen diameter ratio is proportional to the square of the conduit vessel PWV; thus PWV is an index of vessel stiffness arising from contributions of vessel material properties (e.g., calcification, fibrosis) and geometric properties (wall thickness, diameter)(3). We therefore assessed aortic arch PWV in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/ mice and Msx1(fl/fl);Msx2(fl/fl);LDLR-/ controls following 3 months of HFD, implementing Doppler echocardiography as non-invasive and clinically useful method to assess aortic stiffness(22). As shown in Figure 4A, aortic arch PWV was significantly reduced by 30% in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/ mice (p < 0.05 vs. controls) indicating reduced vascular stiffness. Importantly, aortic diameters did not differ between Cre-minus and Cre-plus cohorts at the ascending aorta (1.3 +/- 0.05 mm vs. 1.4 +/- 0.05 mm), aortic arch (1.1 +/- 0.05 vs. 1.2 +/- 0.03 mm), or descending aorta (0.9 +/- 0.02 vs. 1.0 mm +/- 0.04 mm; all p = NS; Figure 4B). Plasma markers of matrix remodeling and synthesis -- viz., the elastin degradation product desmosine (28) and type I procollagen N-telopeptide (9) -- did not significantly differ between Msx1(fl/fl);Msx2(fl/fl);LDLR-/ and SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/ animals (Supplement Figure S1). Thus, reducing arterial Msx1 and Msx2 expression mitigates vascular stiffness in a murine model of diet-induced diabetic arteriosclerosis. Reductions in vascular calcification -- but
not changes in vascular diameter, wall thickness, or fibrosis – accompany reductions in vascular stiffness.

**Osteogenic gene regulatory programs and Wnt gene expression are reduced in aortic myofibroblasts isolated from SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR/− mice**

Reductions in arteriosclerotic calcification and vascular stiffness with reduced Shh expression suggested that key components of vascular mesenchymal progenitor differentiation were perturbed in aortic myofibroblasts from SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR/− mice. Therefore, we studied expression of transcriptional markers of early osteogenic, adipogenic, chondrogenic, and myogenic differentiation in aortic adventitial myofibroblasts stimulated with a dexamethasone cocktail used to enhance mesenchymal cell differentiation from progenitors(29). As shown in Figure 5A, primary aortic myofibroblasts generated from male SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR/− mice exhibit deficiencies in Dlx5, PPARG, Sox9, and myocardin (Myocd) gene expression with concomitant Cre-dependent reductions in Msx1 and Msx2. An impact at an early stage of osteogenic differentiation is likely, since levels of BSP (bone sialoprotein) and OCN (osteocalcin) – the latter a gene of mature osteoblasts directly inhibited by Msx2(30) -- were both reduced in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR/− cultures (Figure 5B). Consistent with this notion, Shh and Sca1, markers of adventitial osteoprogenitors(26), were concomitantly diminished with combined Msx1+Msx2 deficiency (Figure 5B). Expression of leptin and OPN (osteopontin) were unaffected (Figure 5B).
Increasing Msx2 tone in adventitial myofibroblasts by either viral transduction or transgene expression upregulates a cohort of genes encoding Wnt ligands. Therefore, we examined the impact of Msx1 + Msx2 deficiency on the expression of all 19 Wnt ligands. As shown in Figures 5C and 5D, the relative expression of Wnt1, Wnt2, Wnt2b, Wnt3, Wnt4, Wnt5a, Wnt5b, Wnt7a, Wnt7b, Wnt8b, Wnt9a, and Wnt11 were all reduced by ≥ 50% in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- cultures. Wnt10a and Wnt10b were unaffected. Axin2, a genomic target entrained to canonical Wnt/β-catenin signaling in multiple cell types (31), was also downregulated (Figure 5D). GSK3β phosphorylation – another index of activated Wnt signaling (32), is concomitantly reduced in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- cultures as well (Figure 5E and 5F). Thus, combined Msx1 and Msx2 deficiency in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- aortic myofibroblasts reduces elaboration of differentiated mesenchymal cell transcripts, including the expression of osteogenic and Wnt gene regulatory programs.

Alkaline phosphatase-positive and Alizarin red-positive osteogenic nodules are reduced in aortic myofibroblasts isolated from SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice

We and others previously demonstrated that aortic adventitial myofibroblasts and calcifying vascular cells residing within the tunica media exhibit osteogenic, myogenic, and limited adipogenic potential when cultured (23; 26; 33). Moreover, transduction with a retrovirus driving expression of Msx2 promotes osteogenesis in aortic myofibroblasts (23). However, the consequences of depleting endogenous Msx signaling upon vascular mesenchymal osteogenic differentiation are unknown. Therefore, we
prepared primary aortic myofibroblasts from male SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/− and Msx1(fl/fl);Msx2(fl/fl);LDLR-/− sibling controls, cultured them under mineralizing conditions, and quantified alkaline phosphatase and mineralizing Alizarin red osteogenic colonies as previously described (14; 23). As shown in Figures 6A and 6B, the numbers of alkaline phosphatase – positive adventitial myofibroblasts was decreased by 78% in aortas of Msx-deficient mice as compared to controls. Moreover, implementing Alizarin red staining to quantify calcified colonies, mineralization was shown to be concomitantly decreased by 43% (Figures 6C and 6D). Reduced calcification was due in part to diminished Wnt gene bioactivity, since transduction of SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/− cells with a retrovirus driving Wnt7b expression restored Alizarin red staining (Supplement Figure S2). Thus, the combined Msx1 and Msx2 deficiency in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/− aortas reduces the osteogenic mineralization potential of vascular myofibroblasts.

**Msx1 and Msx2 play overlapping yet distinct roles in support of osteogenic programs elaborated by aortic adventitial myofibroblasts.**

In a separate experimental cohort, conditional deletion of the Msx2 gene alone was insufficient to reduce aortic calcium accumulation in response to HFD challenge (1.2 +/- 0.2 vs. 1.1 +/- 0.2 ug calcium/ gm aorta in Msx2(fl/fl);LDLR-/− vs. SM22-Cre;Msx2(fl/fl);LDLR-/− mice; n = 9-11/genotype; p=NS), indicating some functional redundancy. To better understand the individual contributions of Msx1 and Msx2 to adventitial myofibroblast biology, we implemented RNAi to reduce Msx1, Msx2, or Msx1+Msx2 expression in primary cell cultures, quantifying the impact upon osteogenic
lineage programming, *TNAP*(34) and cardiogenic (32)*Wnt* (*Wnt2, Wnt5a, Wnt7b, Wnt11*) gene expression. As shown in Figure 7A, RNAi selectively and specifically reduced *Msx* gene expression in transfected aortic adventitial myofibroblast cultures. *TNAP* and *Wnt7b* messages were down-regulated by siRNA targeting either *Msx1* or *Msx2*. By contrast, *Wnt5a* and *Wnt2* expression were down-regulated primarily in response to *Msx2* depletion. Unlike cultures from *SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/-* mice, *Wnt11* expression was not acutely altered by RNAi targeting *Msx1+Msx2*. Majesky previously identified a *Shh-*, *Sca1-*, and *Msx1*-expressing multipotent adventitial cell population capable of osteogenic differentiatiation(26). We therefore assessed the impact of *Msx1* and *Msx2* RNAi on expression of these specific adventitial progenitor markers, further encompassing *CD90+CD105* as general markers of mesenchymal stem cell populations (26), and *Sox9* as a marker of the osteochondroprogenitor(35). As shown in Figure 7B, siRNA targeting *Msx1* selectively reduced *Shh* expression. By contrast, siRNA targeting *Msx2* preferentially down-regulated *Sca1* and *Sox9*, while *CD90* and *CD105* were unaffected. Thus, *Msx1* and *Msx2* program aortic adventitial osteoprogenitors via overlapping yet distinct mechanisms that control *Wnt* and *Shh* gene expression.
DISCUSSION

*Msx1* and *Msx2* play fundamental roles in signaling cascades controlling craniofacial morphogenesis and epithelial-mesenchymal interactions driving odontogenesis, cardiac cushion formation, and breast development(10-12). *Msx2* is also upregulated in aortic valve and vascular myofibroblasts during the progression of diet-induced diabetes in preclinical models of vascular calcification(6; 7). Atherogenic stimuli such as TNF, IL1β, and lipid products derived from oxidized LDL all upregulate *Msx2* in vascular mesenchymal cells(8; 14; 36). Furthermore, transgenic over-expression of *Msx2* in the vessel wall promotes vascular calcification and activation of Wnt-dependent osteogenic gene regulatory programs(7). Importantly, *Msx2* is elaborated in sclerotic vessel(1; 2) and valve(13) segments in humans afflicted with arteriosclerosis. However, the contributions of endogenous *Msx* genes to cardiovascular disease during post-natal life, including vascular calcification, have not been previously examined. Due to the functionally redundant roles of *Msx1* and *Msx2* in vital developmental processes(10-12; 17), we addressed the impact of combined *Msx1* and *Msx2* vascular deficiency on arteriosclerotic disease in *LDLR/-* mice.

Consistent with our results demonstrating the activation of aortic osteogenic mineralization in transgenic mice over-expressing *Msx2*(7), reductions in vascular *Msx* tone elicit reciprocal changes; aortic calcium accrual is reduced, alongside reductions in vascular stiffness, in *SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR/-* mice vs. *Msx1(fl/fl);Msx2(fl/fl);LDLR/-* controls. Reductions in aortic calcification and stiffness occurred in the absence of significant improvements in metabolic status. Aortic collagen
accumulation and vascular wall thickness – other contributors to vascular stiffness (3) – were unaltered. This suggests a fundamental contribution of endogenous Msx gene tone to vascular calcium and osteoprogenitor homeostasis. Consistent with this notion, ex vivo analysis of vascular adventitial myofibroblasts from SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR−/− revealed reductions in Alizarin red calcium staining and expression of bone-specific markers. Because Msx1 and Msx2 are cell-autonomous inhibitors of OCN gene transcription (6; 30)– but OCN mRNA levels were reduced with Msx deficiency -- we considered the possibility that reductions in Msx tone might reduce osteoprogenitor numbers. This was confirmed by enumerating alkaline phosphatase-positive cells arising in the adventitial myofibroblast population. Majesky first identified that Shh demarcates the renewable mesenchymal progenitors with osteogenic potential within the aorta (26). We therefore examined the impact of Msx1+Msx2 deficiency on Shh expression, and identified significant reductions in Shh in aortic myofibroblast cultures. Concomitant reductions in multiple Wnts not only confirm our previous studies indicating that Wnt genes are entrained to Msx tone, but also suggest that the Wnt ligand milieu may be critical to development and maintenance of osteoprogenitors. Canonical Wnt signals can also upregulate both Msx1 and Msx2 gene expression (37), introducing the notion of feed-forward regulation. Thus, endogenous Msx signaling contributes to vascular calcification, directing pro-sclerotic gene regulatory programs that drive osteoprogenitor development and differentiation (7; 23; 24).

RNAi confirmed the role of Msx genes in TNAP and Wnt expression. Paracrine Wnt signals are important for the elaboration of osteogenic TNAP expression downstream of BMP2 and Shh (38). Surprisingly, while commonalities exist in Msx1 and Msx2
regulation of TNAP and Wnt7b, Msx1 appears to play a uniquely important role in support of Shh expression. By contrast, Msx2 is more important in the elaboration of Wnt5a and Wnt2. Taken together, these data suggest that Msx1 and Msx2 differentially support Shh and Wnt signaling pathways to promote the osteogenic programming of adventitial progenitors. Future studies will identify genomic complexes targeted by Msx1 vs. Msx2.

Of note, global Msx1+Msx2 deficiency results in embryonic lethality and abnormal cardiac morphogenesis due to cardiac neural crest hyperproliferation(12). Additionally, hypoplastic myocardial atrioventricular (AV) cushions in Msx1-/--;Msx2-/- embryos arise from impaired endothelial-mesenchymal transitioning (EnMT) necessary for AV valve formation(11). Thus, the cardiac defects of Msx1-/--;Msx2-/- embryos relate to aberrant tissue remodeling and hemodynamics arising from perturbed EnMT and cell type - specific proliferative control(11; 12). We speculate that the cardiovascular developmental consequences of Msx deficiency may be more significant in the endothelial lineage than in the VSMC lineage.

There are limitations to our studies. Our data newly establish the role for endogenous vascular Msx genes in diabetic arteriosclerosis, vessel stiffness, and the osteogenic potential of vascular myofibroblasts – and confirm the impact of Msx1+Msx2 on Wnt gene expression. However, the specific Wnt ligands programmed by Msx1+Msx2 as necessary for osteoprogenitor development and differentiation have yet to be characterized. Multiple non-canonical as well as canonical Wnt ligands are regulated. Wnt11, Wnt5a, and Wnt7b are capable of provoking both non-canonical and canonical signals dependent upon context(39), and these ligands participate in both osteogenesis
and fibrogenesis (9; 40; 41). Other important Wnts are also expressed by aortic myofibroblasts (42; 43), and the increased body fat observed in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice following HFD challenge likely arises in part due to reduced Wnt tone (44). Finally, Msx1+Msx2 deficiency did not completely eliminate vascular calcium accrual. As Giachelli, Chen, and colleagues showed, a Runx2-positive cell population is responsible for approximately 80% of the vascular osteogenic cells within the vessel wall (45), driving osteogenic mineralization in response to oxidative stress (46). Msx1+Msx2 deficiency did not abrogate vascular Runx2 expression, and we speculate that Runx2 osteogenic programming contributes to residual disease. Nevertheless, our data demonstrate that significant reductions in vascular calcification -- with concomitant reductions in arterial stiffness -- can be achieved via reductions in arterial Msx signaling. Future studies will identify the specific Wnt ligands required for programming osteogenic differentiation and vascular calcification in conjunction with Msx1+Msx2. Targeted inhibition of these ligands and their cognate receptors may phenocopy, in part, the beneficial actions of myofibroblast Msx-deficiency in arteriosclerotic disease and thus offer a therapeutic strategy.

**AUTHOR CONTRIBUTIONS**

S.L.C. researched data and reviewed/edited the manuscript. A.B. researched data and reviewed/edited the manuscript. J.S.S. researched data and reviewed/edited the manuscript. B.R. researched data. K.K. researched data. Y.B.A. researched data. A.K. researched data and reviewed/edited the manuscript. M.M researched data. R.M. reviewed/edited the manuscript. D.A.T. researched data and wrote the manuscript.
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FIGURE LEGENDS

Figure 1: Flow diagram of breeding strategy implemented to generate experimental
SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice and Msx1(fl/fl);Msx2(fl/fl);LDLR-/- controls. Msx1(fl/fl);Msx2(fl/fl)(20), LDLR-/- (19), and SM22-Cre(15) transgenic mice on the C57BL/6 background were generated as described, then bred as indicated to generate male sibling cohorts for subsequent dietary challenge and characterization. See Materials and Methods for the specific PCR amplimers used for genotyping.

Figure 2: Conditional deletion of Msx1 and Msx2 in the VSMC lineage reduces arteriosclerotic calcification in LDLR-/- mice fed diabetogenic high fat diets. Panel A, RT-qPCR of aortic RNA verified Cre-dependent reductions in aortic Msx1 and Msx2 gene expression in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice. While Runx genes were not affected, Shh – a marker of the adventitial osteoporogenitor – and the osteogenic zinc finger transcription factor Osx were significantly reduced (n = 4-5 per genotype). Panel B, biochemical measurement of aortic calcium content following acidic extraction revealed significantly reduced aortic calcification in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice (n = 9-13 per genotype). Panel C, Alizarin red S staining revealed calcification within the tunica media. Panel D, higher power magnification of aortic wall in panel C. Scale bars = 100 microns. Panel E, no differences were noted in aortic collagen content (n = 7-8 per genotype). Panel F, histomorphometric wall thickness was also not affected by vascular Msx deficiency (n=3 per genotype). *, p < 0.05. ***, p < 0.001.
Figure 3: Conditional deletion of Msx1 and Msx2 in the VSMC lineage augments fat mass -- but does not alter bone mass or diet-induced changes in fasting glucose or lipid profiles -- in LDLR-/- mice. Panel A, body weight and composition assessed by dual electron X-ray absorptiometry revealed increased body fat in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice vs. Msx1(fl/fl);Msx2(fl/fl);LDLR-/- controls when fed HFD for 2 months. Panel B, no difference in bone mineral content or areal density were observed. Panel C, fasting glucose, triglycerides, and cholesterol were unaltered. Panel D, fasting plasma 8-isoprostane and haptoglobin levels were also unaltered (n=9-13/genotype). **, p < 0.01.

Figure 4: Aortic pulse wave velocity, a measure of arterial stiffness, is reduced in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice. Male Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice and SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- male sibling cohorts were challenged with HFD for 3 months, then evaluated by Doppler echocardiography to quantify aortic stiffness as previously described using pulse wave velocity (PWV). Panel A, as compared to Msx1(fl/fl);Msx2(fl/fl);LDLR-/- controls, SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice exhibited significantly reduced aortic arch PWV (n = 4 per genotype). Panel B, aortic diameters did not differ between genotypes as assessed by echo. Recall that while calcification was decreased, no change in aortic wall thickness or collagen content was observed (Figure 2). See text for discussion.

Figure 5: Primary aortic myofibroblasts from SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice exhibit reductions in differentiation-
induced expression of early osteochondrogenic, adipogenic, and myogenic transcriptional regulators with concomitant deficiency in multiple Wnt gene programs. Primary aortic adventitial myofibroblasts were isolated then induced to undergo differentiation implementing a modification of a dexamethasone-based protocol used to drive mesenchymal differentiation(29). RNA was extracted for quantitative RT-qPCR for the gene indicated (18S normalized). Panel A, note that increases in early osteogenic (Msx1, Msx2, Dlx5)(47; 48), osteochondrogenic (Sox9)(35), adipogenic (PPARG) (49), and smooth muscle myogenic(Myocd) transcriptional regulators were reduced in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/ myofibroblasts vs. Msx1(fl/fl);Msx2(fl/fl);LDLR-/- controls. Panel B, Shh and Sca1 -- markers of the early multipotent adventitial progenitor(26) – were also reduced in Mxs-deficient myofibroblasts. Concomitant reductions in osteoblast-specific differentiation markers (BSP, OCN) were noted, while OPN and leptin were unaffected. Panels C and D, the expression of multiple Wnt ligands, including those previously demonstrated to be upregulated by Msx2 over-expression(7), was reduced in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/ myofibroblasts. The expression levels of Wnt5a and Wnt11, the two most abundant transcripts, have been scaled by a factor of 1/3rd as indicated to facilitate presentation of all transcripts. The Wnt/β-catenin target gene Axin2 was simultaneously down-regulated, while Wnt10a and Wnt10b were unaffected by Mxs deficiency. Panels E and F, Ser-9 phospho-GSK3β, an independent index of activated Wnt signaling, was also significantly reduced in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/ myofibroblasts as revealed by Western blot. *,§,#: p < 0.05, < 0.01, and < 0.001 respectively vs. differentiated Cre-negative control.
Figure 6: Primary aortic myofibroblasts from SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice exhibit reductions in alkaline phosphatase-positive osteoprogenitors and Alizarin red S calcium staining. Primary aortic adventitial myofibroblasts were isolated from male SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice and male Msx1(fl/fl);Msx2(fl/fl);LDLR-/- controls, cultured under osteogenic conditions, then analyzed for alkaline phosphatase (Alk Phosphatase) cell numbers and calcium deposition by Alizarin red S staining as previously described(14). Alkaline phosphatase-positive cell numbers were reduced in SM22-Cre;Msx1(fl/fl);Msx2(fl/fl);LDLR-/- mice (panels A and B; n = 31-33 fields per genotype in two independent cultures), as was Alizarin red staining (panels C and D). Scale bar = 25 microns.

Figure 7: Msx1 and Msx2 program aortic adventitial osteoprogenitors via overlapping yet distinct mechanisms that control Wnt and Shh gene expression. Primary aortic adventitial cells were transfected with either a control non-targeting siRNA, or siRNA targeting Msx1 or Msx2 as indicated. RNA was extracted 2 days later, and gene expression analyzed by RT-qPCR. Panel A, note that while Msx1 and Msx2 both support TNAP and Wnt7b expression, Wnt2 and Wnt5a were dependent upon Msx2. Panel B, the aortic adventitial progenitor markers Shh and Sca1 differentially responded to Msx RNAi, with Msx1 supporting Shh expression. Sca1 and Sox9 mRNAs were selectively downregulated by RNAi targeting Msx2. CD90 and CD105 were unaffected.
See text for details. *, §, #: p < 0.05, < 0.01, and < 0.001 respectively vs. non-targeting siRNA control.