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Objective—Decorin and oxidized low-density lipoprotein (Ox-LDL) independently induce osteogenic differentiation of vascular smooth muscle cells (VSMCs). We aimed to determine whether decorin glycosaminoglycan (GAG) chain synthesis contributes to Ox-LDL–induced differentiation and calcification of human VSMCs in vitro.

Methods and Results—Human VSMCs treated with Ox-LDL to induce oxidative stress showed increased alkaline phosphatase (ALP) activity, accelerated mineralization, and a difference in both decorin GAG chain biosynthesis and CS/DS structure compared with untreated controls. Ox-LDL increased mRNA abundance of both xylosyltransferase (XT)-I, the key enzyme responsible for GAG chain biosynthesis and Msx2, a marker of osteogenic differentiation. Furthermore, downregulation of XT-I expression using small interfering RNA blocked Ox-LDL–induced VSMC mineralization. Adenoviral-mediated overexpression of decorin, but not a mutated unglycanated form, accelerated mineralization of VSMCs, suggesting GAG chain addition on decorin is crucial for the process of differentiation. The decorin-induced VSMC osteogenic differentiation involved activation of the transforming growth factor (TGF)-β pathway, because it was attenuated by blocking of TGF-β receptor signaling and because decorin overexpression potentiated phosphorylation of the downstream signaling molecule smad2.

Conclusion—These studies provide direct evidence that oxidative stress–mediated decorin GAG chain synthesis triggers TGF-β signaling and mineralization of VSMCs in vitro. **(*Arterioscler Thromb Vasc Biol*. 2011;31:608-615.)**

Key Words: calcification ■ glycosominoglycan ■ molecular biology ■ oxidized lipids ■ vascular biology

Vascular calcification is a life-threatening complication of cardiovascular disease[1,2] and is an independent risk factor for high morbidity and mortality.[3] Oxidative stress is among the important factors contributing to the progression of vascular calcification[4] and is known to induce cytoskeletal disorganization, as well as downregulate smooth muscle cell gene expression[5] and activate genes that lead to increased deposition of extracellular matrix (ECM) proteins,[6] notably, the profibrotic cytokine transforming growth factor (TGF)-β. However, the molecular mechanism by which oxidative stress accelerates mineralization is still not fully understood.

Many studies have identified a similarity between mineralization of bones and teeth with the pathophysiological ECM calcification that occurs in blood vessels (reviewed by Demer and Tintut[7]). Skeletal ECM mineralizes under physiological conditions and is an active process involving production of specific gene products. Similarly, under pathological conditions, ECM soft tissue mineralization is also thought to be an active process, occurring in response to loss of osteogenic inhibitory molecules, rather than passive mineral precipitation. Knockout mouse studies have shown that many inhibitory factors are secreted ECM proteins, such as osteopontin, matrix Gla protein, and osteoprotegerin. Thus, the ECM can act as a reservoir of bioactive structural proteins that influence cell behavior in response to tissue injury.

One important component of the matrix is the small leucine-rich proteoglycan (SLRP), decorin, consisting of a core protein and single glycosaminoglycan (GAG) chain (chondroitin or dermatan sulfate [CS/DS]). It is expressed in many connective tissues such as developing and adult bone, blood vessels, and skin. Studies have shown that the decorin core protein can bind to TGF-β isoforms, thus regulating TGF-β/ECM interactions.[8] Both TGF-β and decorin have been implicated in promoting vascular calcification,[9,10] although 1 study demonstrated decorin-induced inhibition of matrix mineralization in vitro.[11] Differences in the GAG chain composition of decorin could account for the discrepancy, such as occurs in different tissues,[12] in the development

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of atherosclerosis and calcification progression[13,14] and vascular remodeling,[15] and similar to the pro- and antiatherogenic properties of another proteoglycan perlecan.[16]

Oxidized low-density lipoprotein (Ox-LDL) has been shown to modulate decorin GAG chain synthesis.[17] The GAG chain hinders interaction between decorin core protein and TGF-β,[18] and hence it may be that decorin CS/DS chain synthesis regulates the sequestration/bioavailability of TGF-β in the vessel wall. A key question arising is whether the enzymes xylosyltransferase (XT)-I and XT-II[19] involved in the initial and rate-limiting step in CS/DS chain synthesis, are modulated in oxidative stress–induced vascular calcification. Therefore, we aimed to determine whether GAG chain synthesis on decorin plays an important role in Ox-LDL–induced osteogenic differentiation of VSMCs and to establish the downstream signaling pathway involved.

Methods

An expanded Methods section is available in the online Data Supplement at http://atvb.ahajournals.org.

Cell Culture

Human vascular smooth muscle cells (VSMCs) from tibial arteries from consenting adults were isolated as described in the supplemental Methods section.

LDL Preparation and Oxidation

LDL (density 1.019 to 1.063 g/mL) was isolated from healthy human plasma by sequential ultracentrifugation and oxidized as described (supplemental Methods).

Recombinant Adenoviral Infection of Vascular Smooth Muscle Cells

Cells were seeded in either 25 cm^2 cell culture flasks or 6-well plates at 4×10^4 cells/cm^2 and grown in DMEM supplemented with 10% FBS overnight to reach 80% confluence. Adenovirus encoding decorin (Ad/DCN) and adenovirus encoding GAG-free decorin (Ad/S34A)[20] were used to infect VSMCs at a multiplicity of infection (MOI)=100 as described (supplemental Methods).

Measurement of Calcification

Alizarin red staining and quantification and ALP analysis are available online (supplemental Methods).

Xylosyltransferase Small Interfering RNA Transfection

A total of 1×10^6 cells were transfected with 30 μL of 2.5 μmol/L scrambled negative control, XT-I, XT-II small interfering (si)RNA or 2 μg of pmaxGFP, as described (supplemental Methods) and harvested at the indicated time points.

Quantitative Real-Time PCR

Quantitative PCR was performed using 7900 HT Fast Real-Time PCR system and SYBR green detector (Applied Biosystems) using primers for decorin, XT-I, XT-II, Msx2. Transcripts were normalized to β-actin. Primers used for quantitative RT-PCR were as follows β-actin forward, CCAGCTCACCATGGATGATG; β-actin reverse, GAGCCGTTGTCGACGACG; decorin forward, TCAGCTTTGAGGGCTCCTGT; decorin reverse, ACCAGGGAACCTTTTAATCCG; XT-I forward, CCTGCCTGGAAAGTGATAGCAC; XT-I reverse, GAACACTACCCATCTGCTGGG; XT-II forward, ATCACGAGCAGCACTTCTTTTACAT; XT-II reverse, GGTAGTCGGAACGCTTGTCC; Msx2 forward, TGGATGCAGGAACCCGG; Msx2 reverse, AGGGCTCATATGTCTTGGCG.

Western Blot Analysis

Total protein was extracted from VSMC cultures and quantified using a BCA protein assay kit. Western blot analysis was performed as described in the online supplement. To detect decorin core protein, chondroitinase ABC (0.2 U/mL overnight at 37°C) was used to remove the GAG chain from decorin.

GAG Extraction and Analysis

CS/DS was extracted as described for gags in Pan et al[21] and then chondroitinase ABC–digested, and the resultant disaccharides derivatized with the fluorophore 2-aminoacridone (AMAC, Sigma).[22] Disaccharides were separated by high-performance liquid chromatography on a Kinetex 2.6 μm C18 column (Phenomenex, UK) in a 10% to 30% gradient of methanol in 0.1 mol/L ammonium acetate over 20 minutes at 1 mL/min and quantified according to known standards.

Quantification of Active and Total TGF-β1

TGF-β1 levels in 100 μL of conditioned media were determined using the TGF-β1 EMax Immunoassay kit (Promega), which was performed according to the instructions of the manufacturer. To determine total TGF-β1 levels, the conditioned media were acidified with 1 μL of 1N HCl and neutralized after 15 minutes at 22°C with 1N NaOH.

Results

Ox-LDL Accelerates Calcification of Vascular Smooth Muscle Cells

We investigated Ox-LDL effects on mineralization, and alizarin red staining showed that Ox-LDL accelerated calcification of human VSMCs in a dose-dependant manner, an effect not detected when using native LDL (supplemental Figure I). For all future experiments, 30 μg/mL Ox-LDL was used to treat the cells as our model of induced-oxidative stress. A time-course experiment showed that mineralization was detected as early as day 7 in the presence of Ox-LDL, with increased mineralization at day 14 (Figure 1A). Quantification of alizarin red staining showed a 1.9-fold increase in alizarin red retention in VSMCs after Ox-LDL treatment for 7 days and 3.1-fold increase at day 14 compared with untreated controls (Figure 1B). Culturing VSMCs for 7 and 14 days in the presence of Ox-LDL also increased ALP activity by 18- and 2.3-fold respectively, compared with untreated controls (Figure 1C). In addition, we detected a $\approx$3-fold increase in Msx2 mRNA expression after Ox-LDL treatment at both days 7 and 14 (Figure 1D). Of note, no change in Cbfa1 mRNA abundance was apparent during this time course and under these treatment conditions (data not shown).

GAG Synthesis but Not Decorin Gene Expression in VSMCs Is Altered by Ox-LDL Treatment

Western blot analysis of VSMC protein lysates detected less decorin core protein in Ox-LDL treated cells compared with untreated control cells. However, removing GAG chains with chondroitinase ABC digestion allowed similar levels of core protein in Ox-LDL–treated cells to be detected compared with untreated controls (Figure 2A). Decorin gene expression was also analyzed in lysates from cells grown in the presence and absence of Ox-LDL using quantitative PCR. We detected no change in decorin mRNA expression between the two treatment groups (Figure 2B).

Next, we isolated GAGs from cells grown in the presence and absence of Ox-LDL (supplemental Figure II). Changes in the amount of CS/DS were insignificant, with 22.4 ng/μg

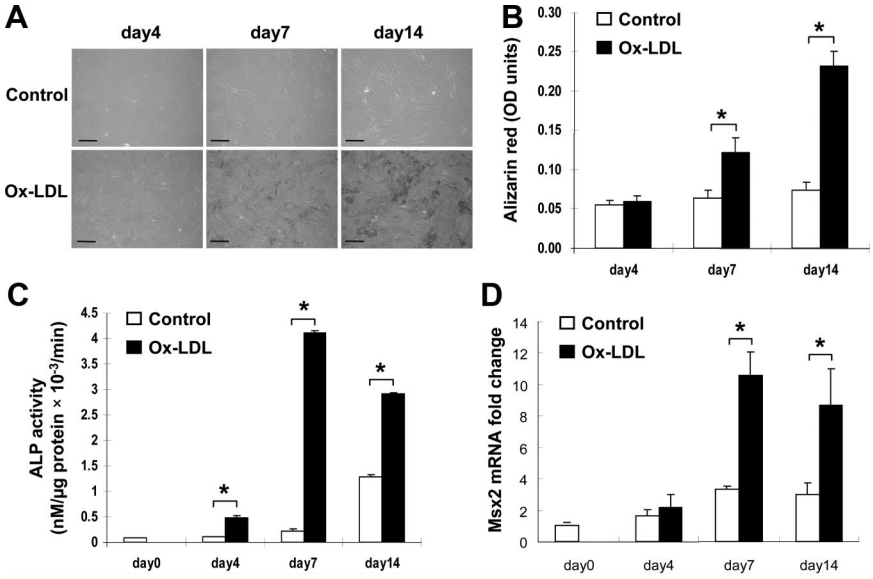

Figure 1. Effect of Ox-LDL on VSMC mineralization. A and B, Alizarin red staining (A) and quantification (B). C, ALP activity, expressed as nmol/mL ρ-nitrophenol converted per microgram of cellular protein per minute. D, Msx2 mRNA expression measured after Ox-LDL treatment. *$P<0.001$. Bar=200 μm.

protein in untreated compared with 19.1 ng/μg protein in Ox-LDL–treated cells. This is consistent with there being no major changes in chain length. However, we detected a significant increase in CS/DS disaccharides containing 4-*O*- and/or 6-*O*-sulfation (Figure 2C).

Ox-LDL Enhances XT-I mRNA Expression in VSMCs

Quantitative PCR analysis showed that osteogenic differentiation of VSMCs was associated with increased XT-I mRNA (Figure 2D) but not XT-II mRNA (Figure 2E) expression and that Ox-LDL further increased XT-I mRNA expression by 2.4-fold at day 4, by 2.7-fold at day 7, and by 3.6-fold at day 14, respectively.

Decorin GAG Chain Is Essential for Ox-LDL–Induced Mineralization of VSMCs

Our data demonstrate that overexpression of wild-type decorin (supplemental Figure III) overtly promotes mineralization in human VSMCs (Figure 3A). However, VSMCs infected with

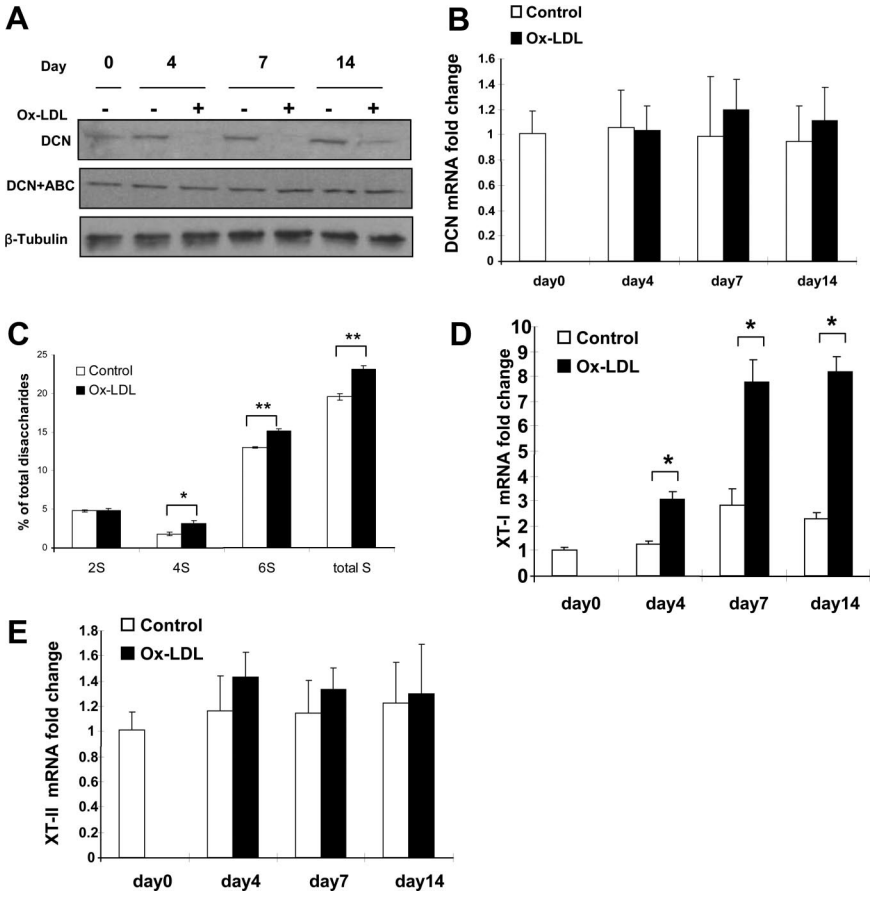

Figure 2. Ox-LDL effects on decorin expression, chondroitin/dermatan sulfate structure, and XT-I mRNA expression in VSMCs. A, Cells were grown with or without 30 μg/mL Ox-LDL over 14 days (n=4). Western blot analysis established decorin core protein expression in VSMCs with or without chondroitinase ABC (ABC) to remove the GAG chain from decorin. β-Tubulin was a loading control. B, Quantitative RT- PCR analysis of decorin mRNA expression in VSMCs expressed as a relative fold change compared with β-actin mRNA. High-performance liquid chromatography showed the disaccharide composition of CS/DS from VSMCs. C, Levels of 2-*O*-sulfated (2S), 4-*O*-sulfated (4S), 6-*O*-sulfated, and total sulfated (total S) disaccharides. *$P<0.05$; **$P<0.005$. D and E, Quantitative PCR was used to analyze XT-I mRNA (D) and XT-II mRNA (E) from VSMCs (n=4) and are presented as relative fold increase over actin mRNA. *$P<0.001$.

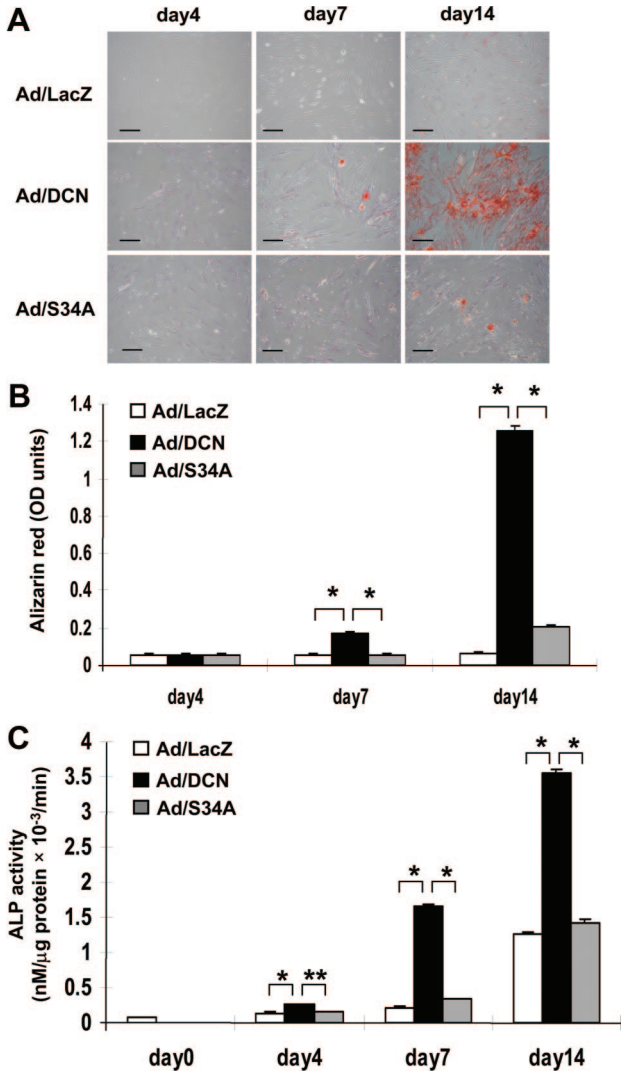

Figure 3. Effect of decorin or mutant decorin overexpression on mineralization of VSMCs. A and B, Alizarin red staining and mineral deposition were quantified in Ad/lacZ-, Ad/DCN-, or Ad/S34A-infected cells grown in osteogenic medium over a 14-day time course (n=3). C, ALP activity was assessed in a parallel experiment and expressed as nmol/mL ρ-nitrophenol converted per microgram of cellular protein per minute. *P<0.001; **P<0.01. Bar=200 μm.

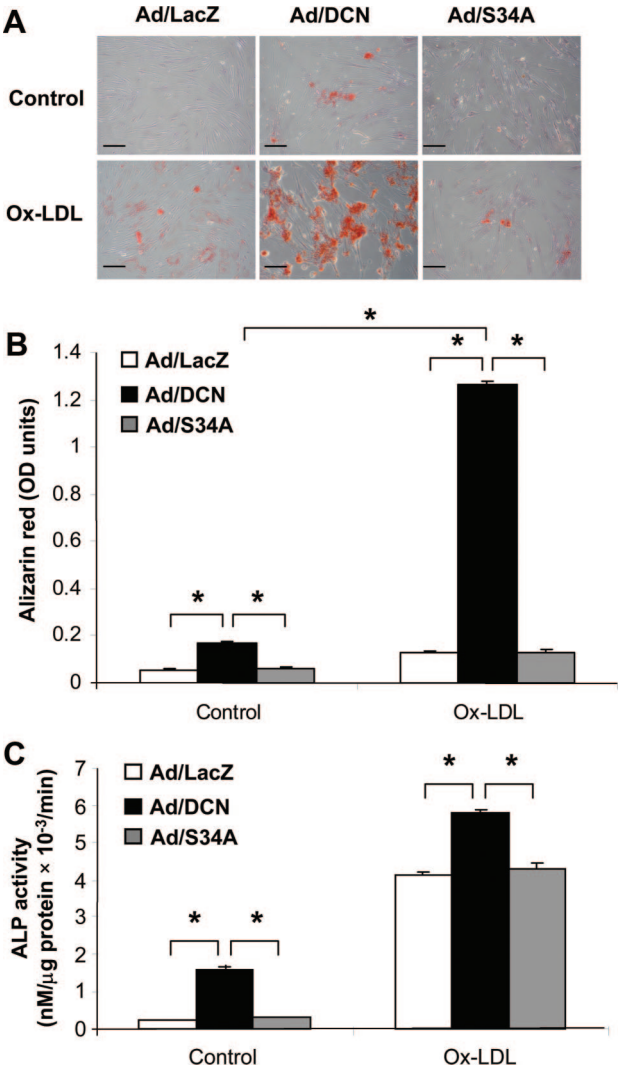

Figure 4. Decorin-induced mineralization of VSMCs is enhanced by Ox-LDL treatment and requires decorin GAG chain. Cells infected with Ad/lacZ, Ad/DCN, or Ad/S34A were grown in osteogenic medium (control) or osteogenic medium containing 30 μg/mL Ox-LDL for 7 days (n=3). A, Cells were stained with alizarin red. Bar=200 μm. B, Mineral deposition was quantified. C, ALP activity was assessed in a parallel experiment. *P<0.001.

Ad/S34A demonstrated 65% and 84% less mineralization compared with Ad/DCN-infected cells at days 7 and 14 respectively (Figure 3B). Similarly, ALP activity was increased by 8.3- and 2.8-fold in decorin-infected cells at day 7 and day 14 respectively, whereas Ad/S34A-infected VSMCs failed to show elevated levels of ALP activity compared with decorin-infected or control Ad/LacZ-infected cells (Figure 3C). Next, to determine combined effects of Ox-LDL and decorin overexpression on ALP activity and subsequent VSMC mineralization, VSMCs were infected with Ad/DCN or Ad/S34A under control (osteogenic) and oxidative stress conditions for 7 days. Cells infected with Ad/DCN together with Ox-LDL treatment, showed a 10-fold increased mineralization compared with control Ad/LacZ-infected cells, under oxidative stress conditions by day 7 in culture, and a 7.5-fold increased mineralization compared with the same cells grown under control osteogenic conditions (Figure 4A and 4B). In contrast, GAG-free decorin was incapable of enhancing mineralization either in the presence or absence of Ox-LDL (Figure 4A and 4B). In cells infected with Ad/LacZ and Ad/S34A, Ox-LDL enhanced ALP activity in line with expectations (Figure 1C).

Of note, ALP activity was significantly increased in cells infected with Ad/DCN under both osteogenic and oxidative stress conditions, compared with Ad/LacZ infected-cells, whereas no similar increase in ALP activity was detected in GAG-free decorin overproducing cells (Figure 4C). These data suggest a key role of the GAG chain of decorin in modulating osteogenic differentiation of VSMCs.

To further verify that GAG chain synthesis plays an important role in Ox-LDL–induced mineralization of VSMCs, XT-I and XT-II siRNAs were then used to transfect VSMCs in the presence of Ox-LDL. Quantitative PCR confirmed significant

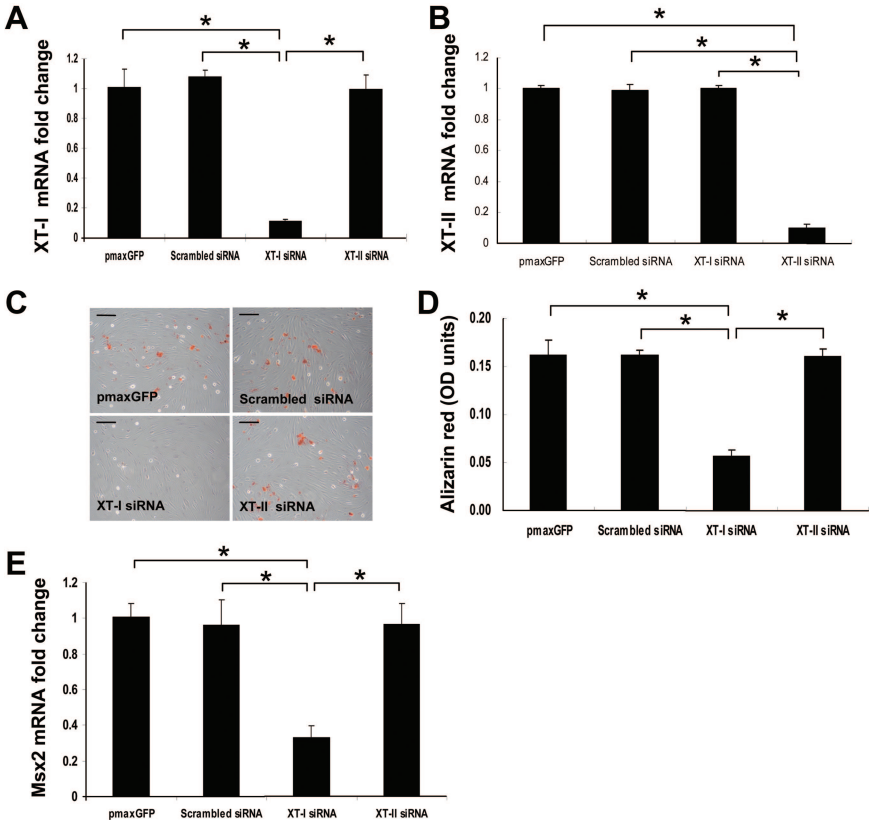

Figure 5. Effect of XT-I and XT-II siRNA on mineralization of VSMCs. Cells were transfected with XT-I or XT-II siRNA at 90% confluence, and 24 hours later, growth medium was replaced by osteogenic medium containing 30 μg/mL Ox-LDL (n=3). A and B, Total RNA was isolated from cells transfected with XT-I or XT-II siRNA at day 2. Quantitative PCR analysis shows successful downregulation of XT-I and XT-II mRNA expression in XT-I or XT-II siRNA-transfected cells. C, Cells were stained with alizarin red. Bar=200 μm. D, Mineral deposition was quantified at day 10. E, Quantitative PCR analysis of Msx2 mRNA expression in cells transfected with XT-I or XT-II siRNA. *$P<0.001$.

downregulation of XT-I and XT-II mRNA expression in respective siRNA-transfected cells compared with other groups (Figure 5A and 5B). Mineralization, detected by alizarin red staining of VSMCs, was reduced by 65% in XT-I siRNA-transfected cells at day 10, as compared with controls but unaffected when XT-II expression was downregulated (Figure 5C and 5D). Furthermore, the osteogenic transcription factor, Msx2 mRNA expression in XT-I, but not XT-II siRNA-transfected cells was downregulated 3-fold compared with control groups (Figure 5E). Taken together, these data suggest that the function of decorin as an inducer of VSMC mineralization is dependent on GAG chain addition by XT-I. In turn, altered posttranslational modification could affect its ability to bind to and sequester TGF-β, although the GAG chain does not seem to be involved directly in TGF-β/decorin interaction.[23]

TGF-β Signaling Is Required for Ox-LDL–Induced Mineralization of VSMCs

We investigated whether TGF-β signaling is required for Ox-LDL–induced mineralization of VSMCs. SB431542 (an inhibitor of TGF-β type I receptor) was used to treat VSMCs in the presence of Ox-LDL. We found that chemical inhibition of TGF-β signaling attenuated the Ox-LDL–induced mineralization of VSMCs (supplemental Figure IVA and IVB). Furthermore, ELISA showed elevated production of active TGF-β1 in Ox-LDL–treated cells versus controls (Figure 6A) with no change in absolute levels of total TGF-β protein after acid activation (Figure 6B). Western blot analysis showed that expression of the downstream target of TGF-β, namely smad2, was unchanged at all time points and in all treatment regimes. However, Ox-LDL treatment of cells stimulated phosphorylation of smad2, an effect which was inhibited by SB431542 (Figure 6C).

Decorin-Induced Mineralization Is Dependent on Activation of TGF-β Signaling

To confirm links among Ox-LDL, decorin, TGF-β, and mineralization, SB431542 was used to treat Ad/DCN-infected VSMCs. Alizarin red staining shows that decorin accelerates mineralization by day 14 compared with control cells, and decorin-induced mineralization of VSMCs is attenuated by treatment with SB431542 (supplemental Figure VA and VB). To establish the essential requirement of GAG chain synthesis on decorin for the activation of the TGF-β pathway, cells were transduced with either Ad/DCN or Ad/S34A. TGF-β1 activation was substantially increased in Ad/DCN-infected cells compared with Ad/LacZ-infected cells (Figure 6D). Active TGF-β1 production was significantly reduced in Ad/S34A-infected compared with Ad/DCN infected cells. However, it was significantly elevated compared with Ad/LacZ controls (Figure 6D). Phosphorylation of smad2 was detected in lysates from cells harvested at each time point after overexpression of decorin (Figure 6E), whereas no phosphorylation of smad2 was detected in the presence of SB431542 (Figure 6E). In line with reduced levels of active TGF-β, less phosphorylated smad2 was detected in Ad/S34A-infected cells compared with Ad/DCN-infected cells (Figure 6F).

Further downstream mechanisms were investigated using quantitative PCR analysis to confirm a link between decorin and osteogenic differentiation. Decorin overexpression was shown to increase Msx2 mRNA expression by ≈4- and 7-fold at days 7 and 14, respectively. However, addition of

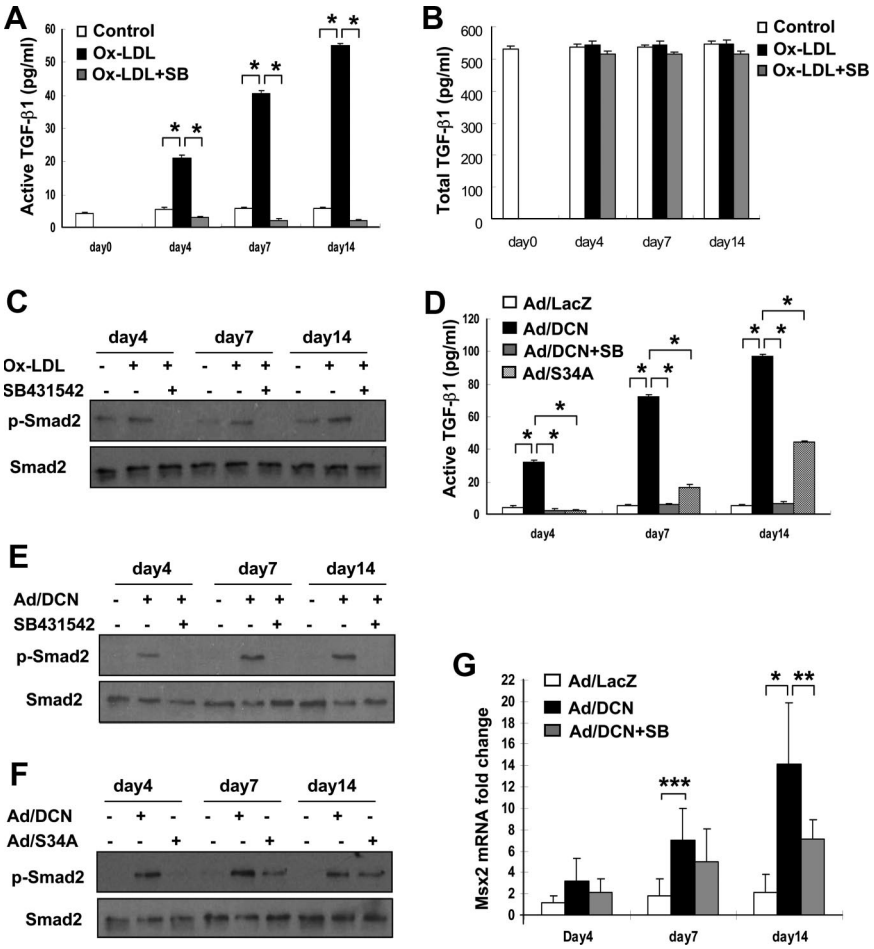

Figure 6. TGF-β signaling is activated during the process of decorin- or Ox-LDL–induced mineralization of VSMCs. A, B, and D, Active (A) and total (B) TGF-β1 levels in conditioned media were measured using EMax immunoassay after treatment of cells with/without 30 μg/mL Ox-LDL and with/without 20 μmol/L TGF-β receptor kinase inhibitor SB431542 (SB) and infected with Ad/lacZ, Ad/DCN, and AdS34A with/without 10μmol/L SB431542 (D). C, E, and F, Western blot analysis of cell lysates showing smad2 and p-smad2 expression in VSMCs under the treatment regimes indicated. G, Quantitative PCR analysis of Msx2 mRNA expression in cells treated with Ad/lacZ, Ad/DCN, and/or SB. $^{*}P<0.001$, $^{**}P<0.01$, $^{***}P<0.05$.

the TGF-β inhibitor SB431542 caused a 50% reduction of Msx2 mRNA expression at day 14 compared with cells overexpressing decorin alone (Figure 6G).

Discussion

In the present study, we demonstrate that decorin GAG chain plays a crucial role as an inducer of VSMC biomineralization and that activation of TGF-β signaling and upregulation of Msx2 underpins Ox-LDL–induced mineralization of VSMCs. We show that Ox-LDL increases ALP activity and accelerates mineralization of VSMCs. These findings are consistent with a previous study in which minimally oxidized-LDL increases ALP activity and mineralization of calcifying vascular cells.[24] Identifying the molecular mechanisms by which vascular calcification occurs has important clinical implications, as therapies can then be tailored to target those patients at most risk.

Decorin consists of a core protein and single GAG chain of CS or DS and both overexpression of decorin,[10] and variable modification of decorin GAG chains in different tissues has been implicated in regulation of mineralization.[14] We now demonstrate that GAG chain synthesis is essential for the procalcification role of decorin under conditions of oxidative stress. GAGs are negatively charged polysaccharides that, when covalently bound to proteins, contribute to the structural integrity and regulatory functions of the ECM. There is a growing recognition of the importance of not only heparin and heparan sulfates but also for the CS/DS chains. We find that less decorin core protein was detected in Ox-LDL–treated cells compared with untreated controls. However, removing GAG chains by ABC chondroitinase digestion yields similar levels of core protein after Ox-LDL–treatment of cells compared with untreated controls. This finding suggests that Ox-LDL has no effect on decorin gene expression per se, but it contributes to the conversion of decorin core protein to its glycanated form by adding GAG chains. XT-I and XT-II are the enzymes responsible for the initiation of GAG chain synthesis, and XT-I levels were shown to be increased during osteogenic differentiation of mesenchymal stem cells.[25] We now show that Ox-LDL increases XT-I but not XT-II mRNA expression, confirming that Ox-LDL alters GAG chain synthesis of vascular proteoglycans, as reported previously.[17] Despite XT-1 upregulation and enhanced GAG attachment to decorin, there was no overall increase in CS/DS levels on all proteoglycans present in VSMCs during oxidative stress.

To determine whether GAG addition specifically on decorin plays a crucial role in bio-mineralization, we compared the effects of Ad/DCN or decorin core protein (Ad/S34A) on mineralization. Ad/DCN induces VSMC mineralization by day 7, with strong mineral staining by day 14, whereas very little, if any, mineralization was detected in cells infected with the unglycanated S34A DCN mutant or in control cells, which take the normal 21 days to mineralize under osteogenic conditions (data not shown). Moreover, Ad/DCN-accelerated VSMC mineralization is further enhanced by concomitant treatment with Ox-LDL, detected as early as day 7 in culture, whereas mutant

decorin, free of GAG chain, is not capable of enhancing VSMC mineralization either in the presence or absence of Ox-LDL. Ad/DCN-induced ALP activity was also further enhanced by Ox-LDL treatment. The finding that XT-I siRNA blocks oxidative stress–induced VSMC mineralization supports our hypothesis that addition of GAG chain on decorin plays a significant role in Ox-LDL–induced mineralization of VSMCs and could affect its ability to bind to and sequester TGF-β. However, these data do not exclude a role of other CS/DS proteoglycans in Ox-LDL–induced SMC mineralization. Consistent with our findings that GAG chain synthesis on decorin is critical for Ox-LDL–induced SMC mineralization, recent studies have shown that oversulfated chondroitin sulfate promotes osteoblast differentiation and mineralization[14] and that increased levels of XT-I have been linked with osteogenic differentiation of mesenchymal stem cells.[25] Our results support studies by Muller et al,[25] who found that XT-I, not XT-II, correlated with osteogenic differentiation of mesenchymal stem cells and that XT-II was not regulated by TGF-β.[25] The increased 4-sulfated CS/DS detected under oxidative stress conditions may also promote mineralization as reported previously.[14] The observed increase in 4-sulfated and 4,6-sulfated CS is also consistent with the reported role of TGF-β in regulating chondroitin 4-*O*-sulfotransferase activity.[26]

It has been shown that decorin regulates TGF-β bioactivity[27] and TGF-β induces VSMC calcification.[9] However, the role of TGF-β in Ox-LDL–induced mineralization and the link between TGF-β and decorin in this pathological process is unclear. To determine whether decorin regulates TGF-β bioactivity, we used Ad/DCN to overexpress decorin in VSMCs and investigated the effect on phosphorylation of smad2, and we assessed the effects of the TGF-β inhibitor SB431542. We show that (1) overexpression of decorin significantly enhances TGF-β activation, without changes in total TGF-β protein levels, and results in subsequent smad2 phosphorylation; (2) decorin-induced TGF-β signaling accelerates osteogenic differentiation of VSMCs; and (3) SB431542 treatment inhibits both smad2 phosphorylation and decorin-induced mineralization of VSMCs.

Binding of decorin to TGF-β has been investigated in some detail but how decorin affects the function of TGF-β in a certain biological setting is less clear. Direct effects on signaling and sequestration of TGF-β have both been proposed.[28,29] Although the finding that intact GAG-carrying decorin promotes TGF-β signaling and osteogenic differentiation could be consistent with the fact that GAG chain addition interferes with TGF-β binding to decorin core protein[18] and hence inhibits sequestration of TGF-β, several of our findings contradict such a model. Firstly, overexpression of decorin core protein (Ad/S34A) should have a dominant-negative effect on Ox-LDL–mediated differentiation/mineralization but has no effect (Figure 4C). Secondly, activation of TGF-β is slightly elevated in Ad/S34A-infected cells compared with the Ad/LacZ controls in Figure 6D, with the smad 2 phosphorylation data shown in Figure 6F being consistent with this finding. These data suggest a partial activation of the pathway with GAG-less decorin at later time points. Both findings would be more consistent with a model whereby intact decorin promotes active TGF-β production, with the GAG chain facilitating an interaction necessary in this process and hence reducing the effective concentration needed in comparison to decorin core protein alone. TGF-β1 and -β2 have been shown to bind to highly sulfated heparan sulfate proteoglycans,[30] and recent research suggests CS/DS can replace heparan sulfate in certain growth factor interactions.[31] Alternatively, it could be that binding of decorin to additional factors, rather than direct binding to TGF-β, activates TGF-β. Furthermore, the fact that inhibition of TGF-β receptor signaling blocks active TGF-β accumulation (Figure 6A and 6D) suggests that TGF-β signaling is needed to induce the observed substantial upregulation of TGF-β bioactivity. This autocrine activation loop is in agreement with Mori et al[32] who also demonstrate TGF-β autoinduction in a human dermal fibroblast transdifferentiation model. The present observation that decorin enhances TGF-β bioactivity in VSMCs without change in total TGF-β protein supports a mechanism of regulation involving posttranslational control of TGF-β activation or active TGF-β availability or enhanced receptor signaling complex formation. One possible mechanism is that the increase in 4-sulfation seen after Ox-LDL treatment is TGF-β–mediated,[8] which in turn could be important for TGF-β activation. If XT-I is regulated by TGF-β and this in turn results in glycanation of decorin and thereby enhanced TGF-β activation, then this could explain the autoactivation loop. In summary, we have shown that Ox-LDL increases XT-I mRNA expression, enabling decorin GAG synthesis and, in turn, elevating TGF-β bioavailability to activate downstream signaling pathways.

Msx2 is a homeodomain transcription factor that has been shown to control osteoblast differentiation and mineralization in the developing skull.[33] Msx2 expression is regulated by TGF-β family members including bone morphogenetic protein (BMP)-2 and TGF-β.[34] Moreover, others have reported that Msx2 promotes vascular calcification.[35] Although some have reported the expression of both Cbfa1 and Msx2 in calcified vessels,[36] others have distinguished endochondral from intramembranous ossification through BMP-2–Cbfa1 and BMP-2–Msx2 signal pathways, respectively.[37] In this study, we show that Ox-LDL activates TGF-β signaling and increases Msx2 mRNA expression but has no effect on Cbfa1 expression. Decorin overexpression also caused an upregulation of Msx2 mRNA expression, which was partially prevented by the TGF-β receptor kinase inhibitor. Furthermore, use of XT-I siRNA blocked Ox-LDL–induced VSMC mineralization, with a concomitant decrease of Msx2 mRNA expression, suggesting that Msx2 is involved in both Ox-LDL– and decorin-induced VSMC calcification. Taken together, we conclude that oxidative stress–mediated mineralization of VSMCs in vitro involves the addition and possible modification of decorin GAG chain, with subsequent activation of TGF-β signaling. Further studies are needed to verify the importance of this pathway in vascular calcification using animal models.

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Disclosures

None.

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Supplemental Material

Decorin Accelerates Vascular Calcification

Methods

Tissue Collection and Human Vascular Smooth Muscle Cell Culture

Patients gave informed consent and approval was received from the Local Research Ethics Committee (07/H1003/196). The study conforms with the Declaration of Helsinki. Vascular smooth muscle cells were isolated using an explant method as described by Endlich *et al.*[1] and used between passage 3-6. Briefly, arteries were placed in saline and kept at 4°C until use (within 1-3 h). The muscle layer was dissected free from the surrounding mucosa, cut into small pieces <3 mm^3, the luminal side of the explants was carefully placed in 6-well plates, in contact with the culture dish (6 to 10 tissue pieces per well) and cells that had migrated from the explants were collected and maintained in regular growth media, Dulbecco's Modification of Eagle's Medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS), at 37°C in a humidified incubator containing 5% CO_2. Explanted tissue pieces were removed 1 week after the first SMCs appeared. To confirm that the cells isolated from human tibial arteries were smooth muscle cells, α-smooth muscle actin (anti-α-SMA, Sigma) positive and von Willebrand factor (anti-vWF, DAKO) negative cells were identified by immunofluorescence as described previously.[2] When the cells reached 80% confluence, growth media was replaced by osteogenic media (DMEM with the addition of 5mmol/L beta-glycerophosphate (Sigma) and 2.6mmol/L calcium). Where indicated, cells were treated with 30µg/ml Ox-LDL.

LDL Preparation and Oxidation

The LDL fraction harvested from human plasma was dialyzed extensively against PBS (pH 7.4) at 4^oC with two buffer changes for 24 hours. Protein concentration of LDL was measured using BCA™ protein assay (Pierce). Oxidation of LDL was performed by incubating LDL with 5 µmol/L $CuSO_4$ at 37°C for 3 hours, terminated by EDTA (24µmol/L). The extent of oxidation was assessed by the

measurement of lipid peroxides using the cholesterol-iodide reagent[3] and the absorbance was measured at 365nm. The oxidised LDL fraction was dialyzed as above and sterilized by passage through a 0.22 µm filter and stored at 4°C for future use. Alizarin red staining showed that Ox-LDL accelerated calcification of human VSMCs in a dose-dependant manner, an effect not detected when using native LDL (Supplementary Figure 1). 30µg/ml ox-LDL was used for all future experiments.

Recombinant Adenoviral Infection of Vascular Smooth Muscle Cells

Cells were seeded in either 25 cm^2 cell culture flasks or 6-well plates at 4 x 10^4 cells/cm^2 and grown in DMEM supplemented with 10% FBS overnight to reach 80% confluence. A dose-response of recombinant adenovirus (RAd) infection was performed using a range of multiplicity of infection (MOI) (0 to 150) to assess the most appropriate multiplicity of infection that should be used to achieve 90% infection without any cell toxicity.[4,5] A multiplicity of infection of 100 was selected for this study.

Induction and Determination of Calcification

Mineralisation of hSMCs was induced using a modification of a method described by Reynolds *et al.*[6], Briefly, hSMCs were cultured in regular growth media for several days until they reached 80% confluence. At that time, media was switched to osteogenic media, *i.e.*, DMEM (high glucose, 4.5 g/L) containing 5% FBS, 10 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 2.6 mmol/L $CaCl_2$, and 5 mmol/L β-glycerophosphate for up to 3 weeks.

i) Alizarin Red Staining

Calcium deposition was assessed in cultures of VSMCs using alizarin red staining. VSMCs were grown in 6-well plates. Cells were washed with PBS three times and fixed in 4% formaldehyde in PBS for 10 minutes at room temperature. The cultures were then washed in PBS and exposed to 2% alizarin red (pH4.2) for 5 minutes. Cells were washed with deionized water three times over a 1 hour

period and phase contrast images were taken immediately. To quantify the extent of mineralization, Alizarin red dye was eluted with 10% formic acid, and the absorbance at 414 nm was determined. Data were expressed as optical density units.

ii) Alkaline Phosphatase Assay

Cells were seeded in 6-well plates at 4 x 10^5 cells/well. Proteins were extracted at the required time-points by freeze-thawing the cells in 0.1% Triton X-100 in PBS. Total cellular proteins were measured using BCA™ protein assay kit (Pierce Biotechnology). Total protein (15 μg) was assayed for alkaline phosphatase (ALP) activity at 405 nm as previously described[7], and calculated as nmol/ml ρ-nitrophenol converted per microgram of protein per minute. The data are from three experiments performed in triplicate and shown as the mean ± S.D.

Western Blot Analysis

Cell lysates (30 ug) were prepared as previously described[2] using lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.1% SDS, 10% glycerol, 1 mM EDTA, 5mM NaF) containing protease inhibitor cocktail and quantified using a BCA™ protein assay kit (Pierce Biotechnology). Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS/PAGE) and proteins were transferred to nitrocellulose membrane (Bio-Rad), and blocked with 5% BSA, 1% non-fat dried milk in Tris-buffered saline with 0.1% Tween 20. Membranes were incubated overnight at 4°C with respective antibodies. After incubation with a secondary antibody conjugated to Horseradish Peroxidase (1:1000, Dako), detection was performed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) and visualised by exposing the membrane to X-ray Hyperfilm (Kodak). Membranes were re-probed following submersion in stripping buffer (100 mmol/L β-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl pH 6.8) for 30 min at 55°C.

Xylosyltransferase siRNA Transfection

The Basic Nucleofector Kit for primary smooth muscle cells (Amaxa, Germany) was used for transfection of human VSMCs. 1×10^6 cells were transfected with 30μl of 2.5μmol/L scrambled

negative control, XT-I, XT-II siRNAs as previously described,[8] or 2μg pmaxGFP according to the manufacturer's instructions. The mixture was transferred into a cuvette for electroporation using the A033 program (Amaxa, Germany). Cells were transferred to a 6-well plate and grown in pre-warmed growth medium for 24 hours, which was then replaced by osteogenic medium containing 30μg/ml Ox-LDL. Efficiency of transfection was assessed by microscopy of GFP fluorescent cells following transfection with pmaxGFP and showed approximately 30% transfection efficiency (data not shown).

Quantitative Real-Time PCR

Total RNA was isolated from the cultured human VSMCs using TRIzol Reagent (Invitrogen) according to manufacturer's instructions and treated with DNase I (Roche). 1 μg RNA was reverse transcribed with oligo$(dT)_{15}$ primer. The relative amounts of mRNA were calculated using the comparative Ct (threshold cycle) method.[9]

Quantification of Active and Total TGF-β1

TGF-β1 levels in conditioned media were determined using the TGF-β1 EMax Immunoassay kit (Promega). 100 μl media were used in the EMax immunoassay, which was performed according to the manufacturer's instructions. The TGF-β1 standard curves generated were linear between 15.6 and 1,000 pg/ml of the TGF-β1 standard supplied by the manufacturer.

Antibodies Used for Western Blot Analysis

Membranes were probed using specific primary antibodies: mouse anti-human β-tubulin (1:500, Santa Cruz), rabbit anti-human decorin (1:500, Santa Cruz), rabbit anti-human p-smad2 (Ser465/467; 1:500, Cell Signalling), and smad2 mouse antibody (L16D3; 1:500, Cell Signalling).

Statistical Analysis

Data are presented as mean ± SD. Statistical analysis was performed by one-way ANOVA and a value of $P<0.05$ was considered significant.

Adenoviral-Mediated Expression of Wild-Type and Mutant Decorin in VSMCs

To further investigate the role of decorin GAG chain in Ox-LDL-induced mineralisation of VSMCs, we used adenoviral-mediated over-expression of a wild-type (Ad/DCN) and a mutant GAG-free decorin (Ad/S34A), generated by replacing Ser34 with Ala.[10] Cells infected with Ad/DCN and Ad/S34A both showed elevated levels of decorin compared to control cells (Supplementary Figure 3).

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Supplementary Figure Legends

Supplementary Figure I. Effect of Ox-LDL on VSMC mineralization. Representative phase-contrast images of VSMCs grown in the presence of different concentrations of native low density lipoprotein (N-LDL) or Ox-LDL (10µg/ml, 20µg/ml, 30µg/ml) for 14 days. Mineralization was assessed by alizarin red staining. Bar=200 µm

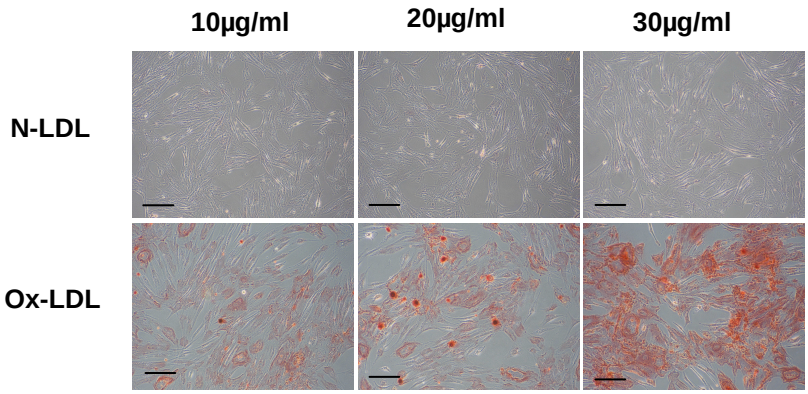
Supplementary Figure II. CD/DS disaccharide chromatogram. HPLC Analysis of CS/DS from VSMCs. (A) Raw data showing a representative chromatogram of CS/DS disaccharides extracted from whole cell lysates of treated and untreated VSMCs. The inset shows expanded view of the peaks from 6 to 16min. (B) 400 pmol CS/DS standards in equal quantities which were then used as reference for quantification. Peak 1, ΔUA(2S)-GalNAc(4S,6S); 2, ΔUA(2S)-GalNAc(4S); 3, ΔUA(2S)-GalNAc(6S); 4, ΔUA-GalNAc(4S,6S); 5, ΔUA(2S)-GalNAc; 6, ΔUA-GalNAc(4S); 7, ΔUA-GalNAc(6S); 8, ΔUA-GalNAc. GalNAc is N-acetylated galactose, which is attached to uronic acid (±2S). (C) CS/DS disaccharide percentage composition. The peak area of each disaccharide was converted to nmol by comparison to CS/DS disaccharide standards of known concentration, then converted to a percentage of total CS. The inset shows an expanded view of disaccharides 1-6 The % of ΔUA-GalNAc(6S) and ΔUA-GalNAc was significantly different in VSMCs treated with ox-LDL (t-test, *$p<0.05$, **$p<0.01$).

Supplementary Figure III. Over-expression of decorin using recombinant adenoviruses. Cells infected with Ad/lacZ, (lane 1) Ad/DCN or Ad/S34A were grown in osteogenic medium for 4 days. VSMC lysates were extracted on day 4. Western blot analysis shows decorin core protein over-expression in both Ad/DCN and Ad/S34A-infected cells. β-Tubulin was used as a protein loading control.

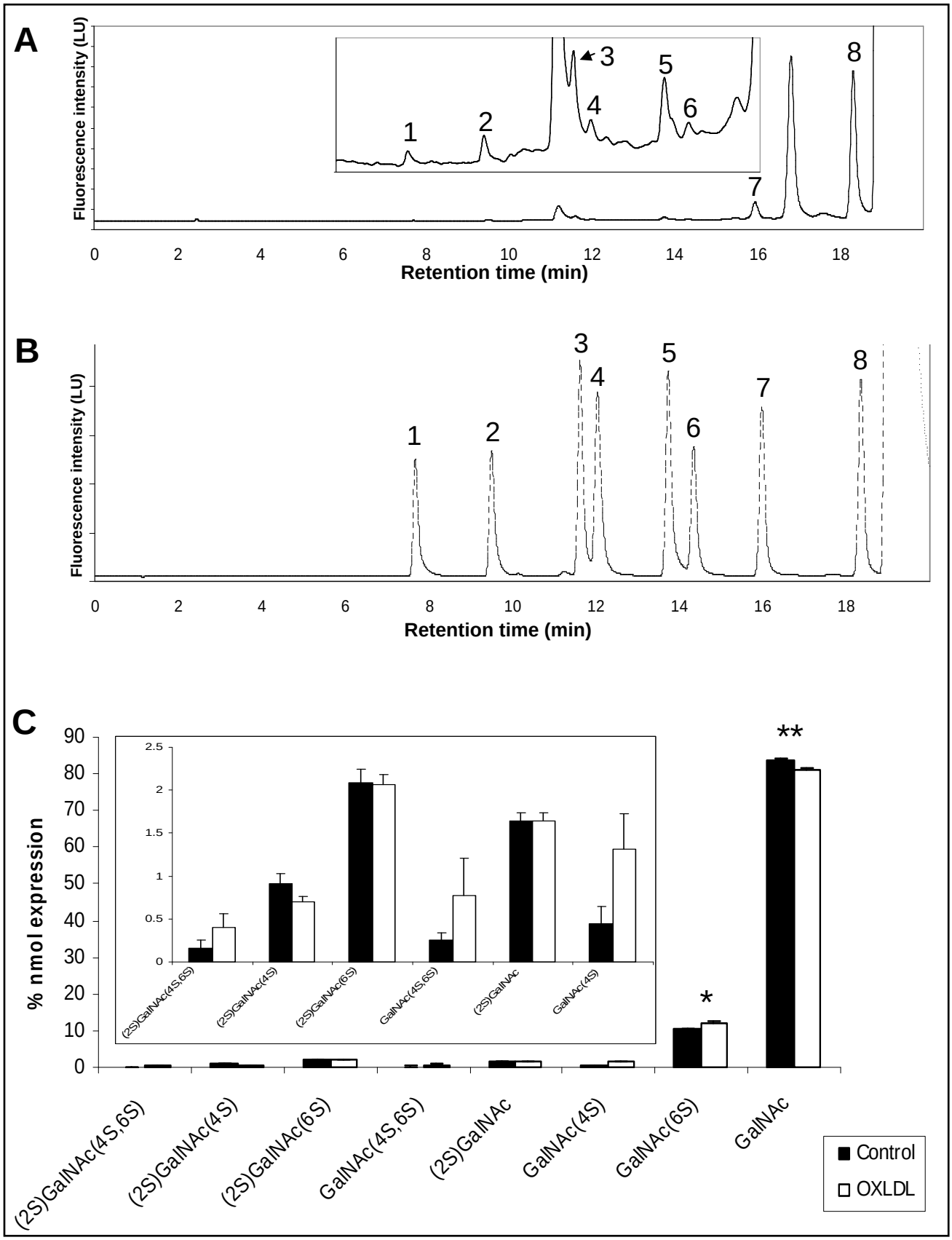
Supplementary Figure IV. Effect of SB431542 on Ox-LDL-induced mineralization of VSMCs. Cells were grown in the presence or absence of 30ug/ml Ox-LDL for 14 days (n=3). 20μmol/L TGF-β receptor kinase inhibitor SB431542 (SB) was used to treat cells in the presence of Ox-LDL. A, Cells were stained with alizarin red. Bar=200 μm B, Mineralization was quantified using absorbance at 414 nm and expressed as optical density units. *P< 0.001.

Supplementary Figure V. Effect of SB431542 on DCN-induced mineralization of VSMCs. Cells infected with Ad/lacZ or Ad/DCN were grown in osteogenic medium over a 14-day time course (n=3). 10μmol/L TGF-β inhibitor SB431542 (SB) was used to treat cells infected with Ad/DCN. A, Cells were stained with alizarin red, Bar=200 μm. B, Mineralization was quantified using absorbance at 414 nm and expressed as optical density units. *P< 0.001.

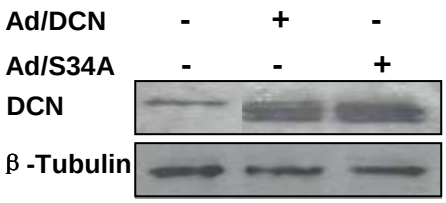
Supplementary Figure I



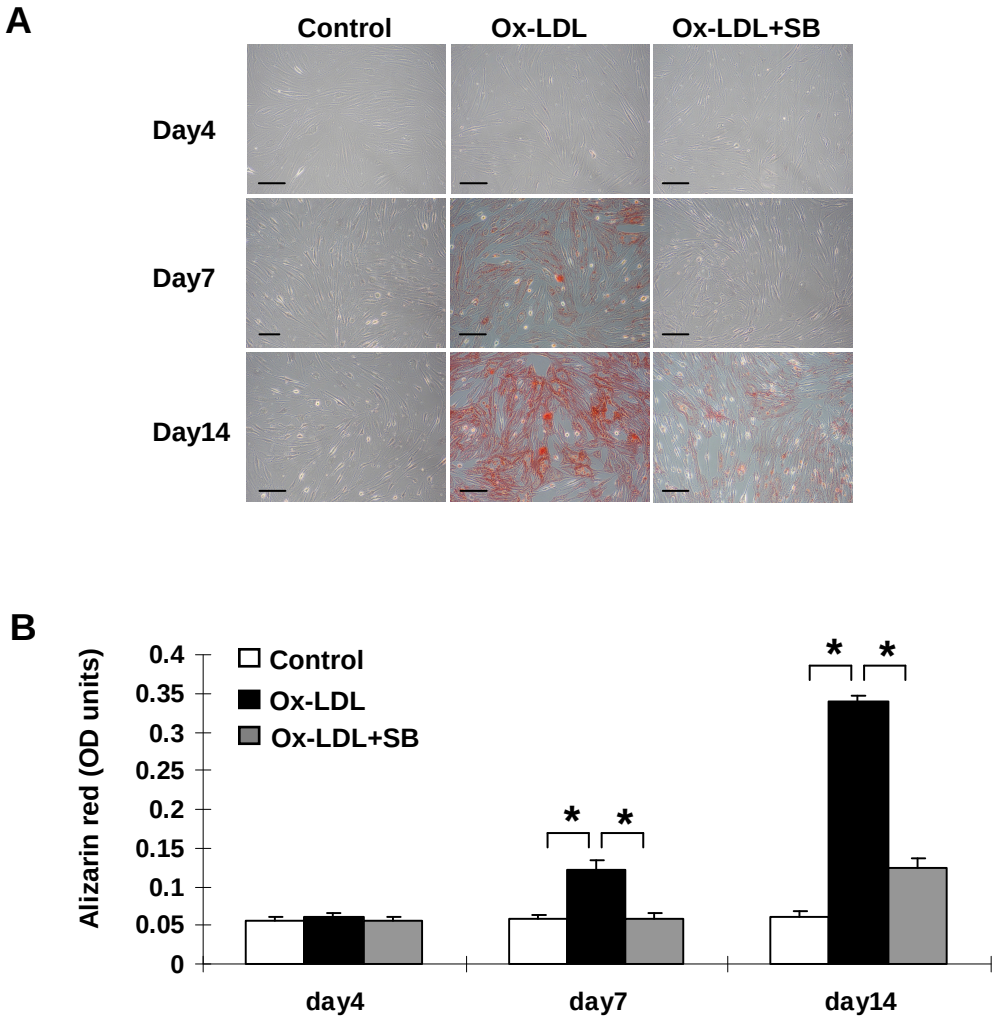
Supplementary Figure II



Supplementary Figure III



Supplementary Figure IV



Supplementary Figure V