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Discoidin Domain Receptor-1 Regulates Calcific Extracellular Vesicle Release in Vascular Smooth Muscle Cell Fibrocalcific Response via TGF-β Signaling

Jona B. Krohn[1], Joshua D. Hutcheson[2], Eduardo Martínez-Martínez[1], Whitney S. Irvin[1], Carlijn V.C. Bouten[3], Sergio Bertazzo[4], Michelle P. Bendeck[5], Elena Aikawa[1,2]

[1] *Center for Excellence in Vascular Biology and* [2]*Center for Interdisciplinary Cardiovascular Sciences, Harvard Medical School, Boston, MA, USA*
[3] *Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, Netherlands*
[4] *Department of Medical Physics & Biomedical Engineering, University College London, London UK*
[5] *Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada*

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Corresponding author:
Elena Aikawa, MD, PhD
Harvard Medical School, Cardiovascular Medicine
Brigham and Women's Hospital
77 Avenue Louis Pasteur, NRB-741
Boston, MA 02115, USA
Tel. +1-617-730-7755
Fax: +1-617-730-7791
Email: eaikawa@partners.org

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Abstract

Objective: Collagen accumulation and calcification are major determinants of atherosclerotic plaque stability. Extracellular vesicle (EV)-derived microcalcifications in the collagen-poor fibrous cap may promote plaque rupture. In this study, we hypothesize that the collagen receptor discoidin domain receptor-1 (DDR-1) regulates collagen deposition and release of calcifying EVs by vascular smooth muscle cells (vSMCs) through the TGF-β pathway.

Approach and Results: VSMCs from the carotid arteries of DDR-1$^{-/-}$ mice and wild type littermates (n=5-10 per group) were cultured in normal or calcifying media. At days 14 and 21, vSMCs were harvested and EVs isolated for analysis. Compared to wild type, DDR-1$^{-/-}$ vSMCs exhibited a 4-fold increase in EV release ($p<0.001$) with concomitantly elevated alkaline phosphatase (ALP) activity ($p<0.0001$) as a hallmark of EV calcifying potential. The DDR-1$^{-/-}$ phenotype was characterized by increased mineralization (Alizarin Red S and Osteosense, $p<0.001$ and $p=0.002$, respectively) and amorphous collagen deposition ($p<0.001$). We further identified a novel link between DDR-1 and the TGF-β pathway previously implicated in both fibrotic and calcific responses. An increase in TGF-β1 release by DDR-1$^{-/-}$ vSMCs in calcifying media ($p<0.001$) stimulated p38 phosphorylation ($p=0.02$) and suppressed activation of Smad3. Inhibition of either TGF-β receptor-I or phospho-p38 reversed the fibrocalcific DDR-1$^{-/-}$ phenotype, corroborating a causal relationship between DDR-1 and TGF-β in EV-mediated vascular calcification.

Conclusion: DDR-1 interacts with the TGF-β pathway to restrict calcifying EV-mediated mineralization and fibrosis by vSMCs. We therefore establish a novel mechanism of cell-matrix homeostasis in atherosclerotic plaque formation.

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Nonstandard Abbreviations and Acronyms	
DDR-1	discoidin domain receptor-1
WT	wild type
DDR-1$^{-/-}$	discoidin domain receptor-1 knockout
LDL-R$^{-/-}$	low-density lipoprotein receptor knockout
vSMC	vascular smooth muscle cell
EV	extracellular vesicle
NM	normal media
β-GP	β-Glycerophosphate
NPP-1	ectonucleotide pyrophosphatase/phosphodiesterase-1
ECM	extracellular matrix
TGF-β	transforming growth factor β

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Introduction

Vascular calcification is a predictor of cardiovascular events and a major determinant of atherosclerotic plaque stability[1]. While macrocalcifications in the collagen-dense fibrous cap may promote structural stability of the plaque, microcalcifications in collagen-poor areas exert mechanical stress on the surrounding tissue, thus potentiating the risk of rupture and subsequent cardiovascular events[2]. Several determinants that further influence plaque stability have been identified, among which a high number and close spatial proximity of microcalcific deposits as well as a thin collagenous fibrous cap constitute the conditions most favorable for stress-induced rupture[3]. Emerging evidence suggests that vascular wall cells, including smooth muscle cells (vSMCs) and macrophages, release calcifying extracellular vesicles (EV) prone to aggregation in the extracellular matrix (ECM) and formation of calcific foci in the plaque[4,5,6,29]. However, the regulation of collagen production and calcifying EV release by vSMCs is incompletely understood.

Discoidin domain receptors (DDR)-1 and 2 are a family of two receptor tyrosine kinases that exhibit substrate specificity for both fibrillar and non-fibrillar collagens[7]. DDR activity has been implicated in physiological processes such as cell migration[8], differentiation[9] and ECM remodeling[10], whereas dysregulated DDR function has been linked to the progression of fibrosis, arthritis and cancer[11]. DDR-1, composed of five membrane-bound and two secreted isoforms generated by alternative splicing[12], was found to play a complex role in the progression of atherosclerotic plaque formation[13]. By contrast, DDR-2 does not affect vSMC migration, proliferation or ECM remodeling *in vitro*[14]. Ahmad et al. found that DDR-1 influences *in vivo* vascular calcification using DDR-1/LDL-receptor double knockout mice[15]. Recent findings on the role of DDR-1 in fibrocalcific response and the emerging influence of EVs in vascular calcification highlight a possible impact of DDR-1 on the fibrocalcific potential of vSMCs and their release of calcifying EVs. Despite extensive knowledge of the numerous pathways involved in DDR-1 downstream signaling[12], a functional link between DDR-1 signaling and its role in EV-mediated calcification as a predictor of atherosclerotic plaque stability remains to be elucidated.

Transforming Growth Factor-β (TGF-β) and its signaling pathways are strongly associated with vSMC-mediated fibrosis[16] and calcification[17], suggesting a potential regulatory role in vSMC osteogenic differentiation and fibrocalcific response. A canonical pathway featuring the Smad proteins Smad2 and 3 and several non-canonical pathways comprising the MAPkinases Erk1/2, JNK and p38/MAPK are involved in TGF-β receptor activation. Both pathways are known to cross-talk with each other resulting in synergistic or antagonistic effects on potentially desirable biological outcomes[18]. In the context of atherosclerotic plaque progression, TGF-β1 promotes the osteogenic differentiation[19] and calcifying potential[20] of vSMCs *in vitro* and is abundantly expressed in calcified human atheromata[21]. The effect of TGF-β pathways on EV release and the fibro-calcific response in vSMCs, however, remains unclear.

In this study, we hypothesize that DDR-1 signaling regulates EV-mediated vSMC fibrocalcific responses via TGF-β pathways. Wu et al. observed that DDR-1 depletion increases chondrogenic differentiation through enhanced expression of chondrogenic markers like SOX-9 and collagen II in human adipose-derived stem cells[22]. Moreover, the knockdown of DDR-1 in a tumor cell model reportedly stimulated the expression of TGF-β1 on the RNA and protein level[23]. Based on these findings, we demonstrate a functional crosstalk between DDR-1 and the TGF-β pathways involving Smad3 and p38 to regulate the release of

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calcifying EVs by vSMCs. This study introduces a novel mechanism balancing vascular fibrocalcific response in atherosclerotic plaque formation.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

DDR-1 Deficiency Increases the Release of Calcifying EVs in vSMCs

Nanoparticle Tracking Analysis (NTA) of collagenase-digested, purified supernatant from vSMCs after 14 and 21 days of culture showed a uniform size distribution with a mean particle size of 180-200±100nm throughout all samples and time points (Fig.1A). These findings are consistent with previous data on EV size distribution[24]. Compared to wild type littermates, DDR-1$^{-/-}$ vSMCs exhibited a significant increase in the release of EVs by 3.5-fold in normal media ($p=0.0004$) and 4-fold in calcifying media ($p=0.003$) at day 14 and a 2.5-fold increase in both media at day 21 ($p<0.001$; Fig.1B). Transmission electron microscopic (TEM) imaging of vSMCs after 21 days of culture displayed dense clusters of DDR-1$^{-/-}$ vSMCs surrounded by abundant ECM, whereas wild type cells showed sparse cell growth and little ECM production (Fig.1C). TEM images demonstrated an increased number of membrane-bound vesicular bodies in proximity with DDR-1$^{-/-}$ compared to wild type vSMCs. The particles displayed via TEM were found to match the EV size distribution observed using NTA (Supp.Fig.IB), confirming our finding of elevated EV release by DDR-1$^{-/-}$ vSMCs. Further analysis of the calcifying potential of isolated EVs revealed an increase in ALP activity in EVs from DDR-1$^{-/-}$ compared to wild type vSMCs by one order of magnitude in both normal and calcifying media after 14 days ($p<0.0001$) and 21 days ($p<0.01$, Fig.1D). The presence of β-Glycerophosphate in calcifying media, mimicking osteogenic conditions, further induced EV release and ALP activity in EVs from wild type (46.1±23.0 vs. 107.5±7.4ng/well/mg protein, $p<0.05$; Supp.Fig.V) and DDR-1$^{-/-}$ vSMCs (581.3±68.3 vs. 878.2±155.4ng/well/mg protein, $p<0.05$) significantly, thus augmenting their calcifying potential.

DDR-1$^{-/-}$ vSMCs Propagate Calcification Through the Expression of a Pro-Calcific Phenotype

Consistent with elevated EV calcification potential, DDR-1$^{-/-}$ vSMCs exhibited increased ALP activity compared to wild type vSMCs as shown by a colorimetric staining assay after 14 and 21 days of culture (Fig.2A). An ALP activity assay performed on cell lysates concordantly showed a significant increase in the presence of cytoplasmic and cell membrane-bound ALP in DDR-1$^{-/-}$ vSMCs in both normal and calcifying media at both days 14 ($p=0.0003$) and 21 ($p<0.01$, Fig.2B). The addition of β-Glycerophosphate in calcifying media resulted in a linear increase in ALP activity over the 21 days of vSMC culture similar to that observed in EVs, suggesting a direct relationship between ALP expression in vSMCs and EV calcifying potential. Gene expression at 14 days of culture showed a concordant 6- and 9-fold upregulation of ALP mRNA in normal and calcifying media, respectively ($p<0.01$, Fig.2E). Further analysis of osteogenic differentiation in DDR-1$^{-/-}$ vSMCs revealed a 56.1±38.5-fold increase in the synthetic marker osteopontin, along with a 6.6±4.3-fold increase in the

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osteogenic marker Msx2 ($p<0.01$), whereas no significant difference compared to wild type was observed in related markers vimentin and Runx2 (Supp.Fig.VI).
Compared to wild type, DDR-1$^{-/-}$ vSMCs exhibited increased Alizarin Red S-positive mineral deposition. Mineralization of DDR-1$^{-/-}$ cultures in calcifying media visibly progressed from days 14 to 21 with a more than 30-fold increase in Alizarin Red S absorbance compared to wild type at day 21 ($p=0.0005$, Fig.2C, 2D).
Calcific deposition in the ECM is regulated by a balance of inorganic phosphate and pyrophosphate, which is dependent on ALP and its antagonist NPP-1 (Supp.Fig.IA)[25]. ALP produces phosphate for calcium phosphate complexation, whereas NPP-1 generates pyrophosphate, an inhibitor of mineralization. In DDR-1$^{-/-}$ vSMCs, the increase in ALP activity in EVs and ALP expression in vSMCs was accompanied by a concomitant increase in NPP-1 activity in EVs in calcifying media (Supp.Fig.IB) and in cell lysates in both media (Supp.Fig.IC).

DDR-1 Regulates EV-induced Fibrocalcific Response in vSMCs Through TGF-β1 downstream signaling pathways

Members of the TGF-β family modulate vascular fibrosis and calcification[18]. In calcifying media, DDR-1$^{-/-}$ vSMCs release elevated levels of TGF-β1 compared to wild type after 21 days of culture (Fig.3A). TGF-β1 gene expression is doubled relative to wild type in DDR-1$^{-/-}$ vSMCs in both media at day 14 (Fig.3B). Western Blot analysis of TGF-β canonical and non-canonical pathways after 21 days of culture unveiled a near complete suppression of Smad3 phosphorylation ($p<0.0001$, Fig.3C) and a significant decrease in phosphorylated JNK (Supp.Fig.IVB) in DDR-1$^{-/-}$ vSMCs, whereas phosphorylation of p38 was increased significantly ($p=0.02$, Fig.3D). The inverse regulation of canonical and non-canonical TGF-β pathways in DDR-1$^{-/-}$ vSMCs observed *in vitro* was assessed *in vivo* through quantitative immunofluorescence of LDL-R$^{-/-}$ and DDR-1$^{-/-}$ LDL-R$^{-/-}$ aortic sections. The data show a significant increase in phospho-p38 ($p=0.03$) and a trending suppression of phospho-Smad3 in the double knockout ($p=0.06$, Supp.Fig.VIII). No difference between wild type and DDR-1$^{-/-}$ vSMCs was observed in the levels of phospho-Erk1/2 (not shown), Smad2 (Supp.Fig.IVC) and Smad1/5 (Supp.Fig.IVD). The addition of the selective TGF-β receptor-I inhibitor SB431542 abrogated the changes in TGF-β1 release ($p<0.0001$, Supp.Fig.IVA) and TGF-β pathways observed in DDR-1$^{-/-}$ vSMCs, resulting in a complete suppression of Smad3 phosphorylation in wild type and p38 phosphorylation in DDR-1$^{-/-}$ vSMCs ($p<0.0001$ and $p=0.02$, respectively; Fig.3D). These *in vitro* and *in vivo* findings support our hypothesis on a relationship between DDR-1 and TGF-β downstream signaling with manifold implications in vascular calcification.

Inhibition of TGF-β Receptor Type I and p38 Mitigate the Osteogenic Potential of the DDR-1$^{-/-}$ Phenotype

The influence of DDR-1 on p38 and Smad3 phosphorylation as well as on TGF-β1 release by vSMCs raises the question whether these pathways contribute to the osteogenic phenotype exhibited by DDR-1$^{-/-}$ vSMCs and their release of calcifying EVs. After 21 days of culture in calcifying media, the addition of specific TGF-β receptor-I inhibitor SB431542 abrogated the previously observed 30-fold increase in calcific mineral deposition by DDR-1$^{-/-}$ vSMCs ($p<0.0001$, Fig.4A, 4B). Moreover, SB431542 treatment reduced EV release by DDR-1$^{-/-}$ vSMCs ($p<0.0001$, Fig.4C) and mitigated EV calcification potential demonstrated by a decrease in ALP activity ($p<0.01$, Fig.4D). Similarly, SB203580, a selective inhibitor of phospho-p38 MAPkinase downstream signaling, significantly suppressed EV release in DDR-1$^{-/-}$ vSMCs in normal ($p<0.0001$) and calcifying media ($p<0.0001$, Fig.4C) along with a significant decrease in EV ALP activity in normal ($p<0.001$) and calcifying media ($p<0.0001$, Fig. 4D). Thus, inhibition of TGF-β signaling by either SB431542 or SB203580 has an equal impact on major calcification endpoints including EV release and EV calcifying potential

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($p<0.0001$, Fig.4A, 4B). In DDR-1$^{-/-}$ vSMCs, SB431542 and SB203580 consistently attenuated the activity of cytoplasmic and membrane-bound ALP ($p<0.05$) and NPP-1 ($p<0.01$) after 14 days (Supp.Fig. IIIA+B).

DDR-1 Exerts a Negative Feedback on Quantitative Collagen Synthesis in Response to Extracellular Collagen

DDR-1 acts as a sensor for type I collagen in vSMCs, activating a negative feedback loop on collagen synthesis in human vSMCs[10]. In DDR-1$^{-/-}$ vSMCs, lack of collagen feedback from fibrillar collagen in the ECM not only resulted in increased collagen production ($p<0.001$, Fig.5B), but also led to defined changes in the three-dimensional structure of collagen fibers. The fluorescently labeled collagen binding protein CNA35[26] detected a dense network of amorphous collagen fibers devoid of spatial organization in cultures of DDR-1$^{-/-}$ vSMCs after 21 days, whereas collagen produced by wild type vSMCs was found to be less dense ($p<0.001$), more fibrillar and restricted to the immediate vicinity of the vSMCs (Fig.5A). The addition of β-Glycerophosphate in calcifying media increased calcification in DDR$^{-/-}$ vSMCs compared to wild type as detected by a fluorescent calcium tracer[27] ($p=0.002$). Addition of SB431542 reduced collagen synthesis ($p<0.01$) and calcific deposition in calcifying media ($p<0.01$) in DDR-1$^{-/-}$ vSMCs. Thus, TGF-β receptor inhibition re-established wild type conditions in DDR-1$^{-/-}$ vSMCs (Fig.5A, 5B).

DDR-1 Deficiency Triggers TGF-β-mediated Calcific EV Release and Fibrotic Response in vivo

Based on present findings demonstrating a fibrocalcific phenotype of DDR-1$^{-/-}$ vSMCs through an interaction with TGFβ signaling *in vitro*, we investigated the interactions between vSMCs, DDR-1, TGF-β1 and atherosclerotic plaque formation *in vivo*. The aortic arches of LDL-receptor knockout (LDL-R$^{-/-}$) and DDR-1$^{-/-}$ LDL-R$^{-/-}$ mice on a 24-week high fat diet were analyzed for presence and distribution of calcific EVs, collagen and TGF-β1 expression.

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Picrosirius Red (PSR) staining revealed the spatial arrangement and packing density of collagen fibers under polarized light by enhancing natural birefringence. While green colors indicate thin, poorly-packed collagen, increased thickness and packing density causes a shift in birefringence to the reddish-orange spectrum[28]. DDR-1$^{-/-}$ LDL-R$^{-/-}$ aortic arches exhibited a network of thick, densely packed collagen fibers that were nearly undetectable in LDL-R$^{-/-}$ tissue sections (Fig.5C). For quantitative analysis, PSR-stained collagen was categorized into thin and loose (green), medium-sized (yellow) and thick, densely packed fibers (orange-red). Mature, medium-sized fibers were expressed constitutively in the vessel wall of both groups regardless of fibrotic response ($p=0.89$, not shown) and were therefore excluded from analysis. Quantification of thin fibers (green) relative to thick bundles (red-orange) revealed a shift towards thick, densely packed collagen (45.53±19.78% vs. 54.47±19.78% thin fibers, $p=0.01$) in DDR-1$^{-/-}$ LDL-R$^{-/-}$, whereas in LDL-R$^{-/-}$, thin fibers were found to be more abundant (81.90 vs. 18.10±9.60% thick fibers, Fig.5D).

Consistent with *in vitro* findings, immunohistochemistry for TGF-β1 detected positive areas in sections from DDR-1$^{-/-}$ LDL-R$^{-/-}$ mice in proximity to areas positive for thick, densely packed collagen. In LDL-R$^{-/-}$ aortic sections, however, TGF-β1 staining was scarce and rarely found in the vascular interstitium (Fig.5C), supporting our *in vitro* hypothesis on a functional connection between DDR-1 and the TGF-β pathway in the regulation of ECM homeostasis. Similarly, staining for ALP in DDR-1$^{-/-}$ LDL-R$^{-/-}$ sections showed signal in proximity to areas of plaque formation, whereas in the LDL-R$^{-/-}$ control, ALP expression was barely detectable (Supp.Fig.IX).

Analysis of calcific EVs and EV aggregates using density-dependent color scanning electron microscopy (DDC-SEM), a novel method identifying early stages of calcification in the form of calcifying vesicular structures[29], showed a multitude of isolated and aggregating vesicular particles of equal density as adjacent calcific deposits in plaque areas from DDR-1$^{-/-}$ LDL-R$^{-/-}$

mice, bearing strong morphological resemblance to calcifications found in human atheromata (Fig.5E). By contrast, plaques from LDL-R$^{-/-}$ mice presented as mostly fibrotic with only few vesicular structures and calcific deposits of nodular appearance, revealing a pattern of calcific EV release consistent with NTA and TEM analyses.

Discussion

The present findings provide a novel perspective on EV-mediated vascular fibrocalcific response, a crucial determinant of atherosclerotic plaque stability. DDR-1$^{-/-}$ vSMCs exhibited a predominantly osteogenic and synthetic phenotype, concomitant with an increase in all measured endpoints of SMC-mediated fibrocalcific response both *in vitro* and *in vivo*. These included calcifying EV release[4,5,6] and ALP activity[27] as facilitators of mineralization and collagen synthesis as a hallmark of fibrosis, along with enhanced expression of synthetic and osteogenic markers. Microcalcifications in collagen-poor areas of the plaque have proven detrimental to its structural stability, exerting mechanical stress on surrounding tissue and increasing the risk of rupture[2,3]. We recently demonstrated that collagen acts as a scaffold for EV accumulation and formation of macro- or microcalcifications[30], however, the cellular mechanisms regulating the release of calcifying EVs in response to collagen are unknown. The present study introduces DDR-1 as a novel molecular regulator controlling fibrocalcific remodeling of the ECM in atherosclerotic plaques through a previously unknown interaction with TGF-β pathways, thus associating TGF-β1 with EV release in vSMCs. DDR-1 was found to restrict TGF-β1 release and subsequent p38 phosphorylation in vSMCs, thereby limiting EV-mediated calcification and collagen matrix production. Furthermore, this study complements previous work on a DDR-1-activated negative feedback loop on collagen synthesis and remodeling[10,13]. DDR-1 acts as a sensor for ECM collagen and, in response, limits collagen synthesis and calcific EV release by vSMCs, promoting ECM homeostasis. Our findings of enhanced fibro-calcification *in vitro*, increased fibrotic remodeling, ALP expression and an abundance of calcific EVs in the DDR-1$^{-/-}$ model *in vivo* support this conclusion.

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An explanation for the differences between the present study and Ahmad et al.[15] on SMC-mediated calcification is given by the use of different calcifying conditions. While Ahmad et al. induced calcification *in vitro* using a high-phosphate medium prone to spontaneous, ALP-independent mineral formation[31], the present study used media supplemented with β-Glycerophosphate, a specific ALP substrate with low tendency for cell-independent Ca^{2+} complexation[32]. The differences between currently established *in vitro* calcification models may reflect different pathologies[33], the high-phosphate media mimicking the pathogenesis of medial calcification in chronic kidney disease[34], whereas β-Glycerophosphate may promote osteogenic changes by stimulating ALP activity[35], a common mechanism of atherogenic calcification. Indeed, culturing wild type and DDR-1$^{-/-}$ vSMCs in the high-phosphate media used by Ahmad et al. showed a remarkable inversion of the DDR-1$^{-/-}$ phenotype in the characteristic end points of fibrocalcific response including EV release ($p=0.02$, Supp.Fig.VIIA), ALP activity ($p<0.05$, Supp.Fig.VIIB) and TGF-β1 release ($p=0.0002$, Supp.Fig.VIID) but not including TGF-β1 gene expression (Supp.Fig.VIIC). In our *in vivo* model, the abundant presence of accumulating, electron-dense vesicular particles observed using DDC-SEM combined with increased expression of ALP in DDR-1$^{-/-}$ LDL-R$^{-/-}$ vasculature corroborates the role of DDR-1 in early plaque calcification. Future studies are required to elucidate the contribution of stage of plaque maturation as well as varying conditions in the *in vivo* DDR-1$^{-/-}$ model on the differential effects of DDR-1 in fibrocalcific responses observed in our two studies.

The effects of TGF-β1 on atherosclerotic plaque formation are pleiotropic and presumably pathway-dependent[36]. Our study introduces a novel link between DDR-1 and the TGF-β pathway in conjunction with the release of calcifying EVs, establishing a potent regulatory

circuit involving DDR-1 activation, calcified plaque formation and subsequent destabilization. Our findings indicate that DDR-1 crosstalk with TGF-β1 signaling is independent of bone morphogenetic protein (BMP) and osteogenic regulator Runx2[37], while influencing the expression of Msx2, a Runx2-independent promoter of cytokine-mediated osteogenic vascular calcification interacting with the Wnt pathway[38] (Supp.Fig.VI). Under calcifying conditions, DDR-1$^{-/-}$ vSMCs released increased TGF-β1 in the ECM, concomitant with elevated phospho-p38 and suppressed phospho-Smad3 associated with the fibrocalcific DDR-1$^{-/-}$ phenotype *in vitro* and *in vivo*. These findings complement previous work on the positive effect of phospho-p38 on ALP expression and activity[39]. The reversal of enhanced mineral deposition, calcific EV release and ALP activity in DDR-1$^{-/-}$ vSMCs by SB203580 further suggests a causal relationship between p38 phosphorylation and EV-induced fibrocalcific response. We hereby add new evidence to the controversial claims in current literature, either confirming[12,40] or refuting[41] an interaction between DDR-1 and p38 signaling. The near-complete suppression of phospho-Smad3 concomitant with enhanced mineralization observed in DDR-1$^{-/-}$ models both *in vitro* and *in vivo* supports the findings obtained by Shimokado et al. using Smad3$^{-/-}$ vSMCs[42]. TGF-β1 itself is identified as an atherogenic agent, its expression and release related to advanced fibrosis and calcific deposition in human atheromata[43,22]. Moreover, the observed increase in expression and release of TGF-β1 by the DDR-1$^{-/-}$ model *in vitro* and *in vivo* concurs with a previous study on DDR-1 knockdown in a human cancer cell model[23]. Interestingly, the suppression of p38 phosphorylation and TGF-β1 release in DDR-1$^{-/-}$ vSMCs by SB431542 suggests a link between TGF-β receptor activation and the p38 pathway that has been implicated in aortic valve calcification[44]. In our DDR-1$^{-/-}$ LDL-R$^{-/-}$ mouse model, we observed a strong association of TGF-β1-positive areas with PSR-positive fibrotic regions and abundantly present calcific EVs forming compact calcifications similar to those found in human atheromata. This evidence purports a role of TGF-β pathways crucial to the fibrocalcific DDR-1$^{-/-}$ phenotype both *in vitro* and *in vivo*.

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Collectively, we conclude that DDR-1 provides a novel connection between vSMC-induced fibrosis and EV-mediated calcification in early-stage atherosclerotic plaque formation through the adverse regulation of two TGF-β signaling pathways. By restricting TGF-β1 release in vSMCs, DDR-1 suppresses phosphorylation of pro-atherogenic p38 and increases phospho-Smad3, resulting in attenuated fibrosis and calcifying EV release (Fig.6). While further studies are required to elucidate the detailed mechanisms of DDR-1 signaling, the novel role of DDR-1 in maintaining fibrocalcific homeostasis in vSMCs using the TGF-β pathway provides a new perspective on the pathogenesis of plaque formation. DDR-1 hereby exerts a TGF-β1-mediated negative feedback on both fibrotic and calcific responses, preventing early-onset ECM remodeling in the process of plaque formation. The interaction between DDR-1, the TGF-β pathway and the release of calcifying EVs further unveils a range of potential implications in congenital and acquired disorders associated with pathological vascular calcification. Our findings introduce a heretofore-undisclosed mechanism regulating vascular fibrocalcific response and ECM remodeling as major determinants of atherosclerotic plaque stability.

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Disclosures

None.

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Significance

Vascular fibrosis and calcification determine atherosclerotic plaque stability. Microcalcifications formed through the accumulation of calcifying extracellular vesicles (EVs) destabilize the plaque and contribute to the risk of plaque rupture. The interaction of collagen synthesis and calcifying EV release by vascular smooth muscle cells (vSMCs) driving fibrocalcific response in plaque formation is unknown. We identify DDR-1 as a novel molecular switch restricting fibrosis and EV-mediated calcification of the extracellular matrix. By affecting the release of TGF-β1 and several TGF-β signaling pathways in vSMCs, DDR-1 provides negative feedback on collagen synthesis, the release of calcifying EVs and the expression of alkaline phosphatase, diminishing EV calcifying potential. DDR-1 activation thus shuts off excess collagen production and calcific deposition, maintaining homeostasis of the extracellular matrix. Our study introduces the DDR-1-TGF-β axis as the missing link between fibrosis and EV-mediated calcification in atherosclerotic plaque formation.

Figures

Fig.1 Nanoparticle Tracking Analysis (NTA) of EVs released from wild type (WT) and DDR-1$^{-/-}$ vSMCs cultured for 14 and 21 days in normal (NM) and calcifying media supplemented with 10mM β-Glycerophosphate (β-GP). **A**, Size distribution of EVs at days 14 and 21 shows a

characteristic uniform size between 100 and 300nm. **B**, Concentration of EVs released by DDR-1$^{-/-}$ vSMCs is significantly increased compared to WT vSMCs in normal and calcifying media at 14 and 21 days of culture. **C**, TEM imaging of wild type (left) and DDR-1$^{-/-}$ (right) vSMCs after 21 days of culture. EVs in the ECM of DDR-1$^{-/-}$ vSMCs (white arrowheads). Representative images at 11,000x magnification, bar: 500nm. **D**, ALP activity assay in purified EVs reveals increased calcification potential in EVs released from DDR-1$^{-/-}$ vSMCs. Results shown as mean±SD, n=5 for each group, **=p≤0.01, ***=p≤0.001, ****=p≤0.0001.

Fig.2 ALP expression and calcification in wildtype and DDR-1$^{-/-}$ vSMCs. **A**, Representative images of ALP activity staining. **B**, ALP activity assay in cell lysates shows increased activity of cytoplasmic and cell membrane-bound ALP. **C**, Representative images of Alizarin Red S staining in wild type (top) and DDR-1$^{-/-}$ vSMCs (bottom). **D**, Quantification of mineralization measured by Alizarin Red S absorbance at 550nm. Mean±SD, n=5 per group, representative results of 3 independent experiments. **E**, ALP mRNA levels at 14 days of culture relative to wild type normal media (WT NM) and normalized to rplp0, n=10 per group. Statistical differences labeled as follows: *=p≤0.05, **=p≤0.01, ***=p≤0.001, ****=p≤0.0001.

Fig.3 DDR-1 knockout and TGF-β signaling. **A**, concentration of TGF-β1 detected by ELISA in cell media supernatant after 21 days of culture. **B**, TGF-β1 mRNA at 14 days of culture relative to wild type normal media (WT NM) and normalized to rplp0. Representative results of 3 independent experiments in triplicates. **C**, Western Blot analysis for Smad3 (top panel) and p38 (bottom panel) phosphorylation under native conditions (left) and with addition of 2μmol/L SB431542 (right) after 21 days of culture. **D**, quantification of Western Blot band intensities normalized to GAPDH using NIH ImageJ v1.48. Mean±SD, n=10 per group, *=p≤0.05, ***=p≤0.001, ****=p≤0.0001.

Fig.4 SB431542 and SB203580 diminish the osteogenic potential of DDR-1$^{-/-}$ vSMCs. **A**, Alizarin Red S staining after 21 days of culture in native culture media (left), with addition of 2μmol/L SB431542 (middle) or 10μmol/L SB203580 (right). N=5 per group. **B**, Alizarin Red S absorbance of the stained wells of A, normalized to wild type vSMCs cultured in native media. **C**, Relative EV concentration, normalized to wild type. **D**, Relative ALP activity in EVs, normalized to wild type. Mean±SD, n=5 per group, **=p≤0.01, ***=p≤0.001, ****=p≤0.0001.

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Fig.5 DDR-1 acts as a feedback regulator on vascular fibrocalcific response. **A**, Fluorescent labeling of collagen by CNA probe (green) and microcalcific deposits by osteosense (red) produced by wild type (WT) and DDR-1$^{-/-}$ vSMCs in native media (top panel) and with addition of 2μmol/L SB431542 (bottom panel) after 21 days of culture. Bar represents 60μm. Representative images of 4 independent experiments. **B**, % positive area for collagen (green) and microcalcifications (red). Mean±SD, n=5 per group, **=p≤0.01, ***=p≤0.001. **C**, Top panel: Picrosirius Red staining of LDL-R$^{-/-}$ and DDR-1$^{-/-}$ LDL-R$^{-/-}$ aortic arches under polarized microscope at 4x magnification, bar: 200μm. Bottom panel: Immunohistochemical staining for TGF-β1 at 2x magnification. Images correspond to Picrosirius Red stained areas of B. Bar: 100μm. N=6 for each group. For each n, 2 sections were stained for evaluation. **D**, Quantification of relative frequency of thin, loose and thick, densely packed collagen fibers represented by green and orange birefringence using ImageJ v1.48. N=6 per group. **E**, DDC-SEM of plaque areas from LDL-R$^{-/-}$ and DDR-1$^{-/-}$ LDL-R$^{-/-}$ aortic arches, with a human atheroma for comparison. Mineral is presented in orange and ECM is presented in green. Magnification 10,000x, bar: 1μm, n=6 per group.

Fig.6 Ways of DDR-1 interaction with TGF-β signaling through direct suppression of TGF-β1 (1), phosphorylation of p38 (2) or activation of Smad3 (3), leading to downregulation of calcifying EV release and EV-bound ALP by vSMCs. Inverse regulation of Smad3 and p38 activation through possible downstream interaction (4).