MT1-MMP promotes vascular smooth muscle dedifferentiation through LRP1 processing

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Summary

At sites of vessel-wall injury, vascular smooth muscle cells (VSMCs) can dedifferentiate to express an invasive and proliferative phenotype, which contributes to the development of neointimal lesions and vascular disorders. Herein, we demonstrate that the loss of the VSMC differentiated phenotype, as the repression of contractile-protein expression, is correlated with a dramatic upregulation of the membrane-anchored matrix metalloproteinase MT1-MMP (also known as MMP14 and membrane-type 1 matrix metalloproteinase). Matrix metalloproteinase (MMP) inhibitors or MT1-MMP deficiency led to attenuated VSMC dedifferentiation, whereas the phenotypic switch was re-engaged following the restoration of MT1-MMP activity in *MT1-MMP*$^{-/-}$ cells. MT1-MMP-dependent dedifferentiation was mediated by the PDGF-BB–PDGFRβ pathway in parallel with the proteolytic processing of the multifunctional LDL receptor-related protein LRP1 and the dynamic internalization of a PDGFRβ–β3-integrin–MT1-MMP–LRP1 multi-component complex. Importantly, LRP1 silencing allowed the PDGF-BB-induced dedifferentiation program to proceed in the absence of MT1-MMP activity, supporting the role of unprocessed LRP1 as a gatekeeper of VSMC differentiation. Hence, MT1-MMP and LRP1 serve as a new effector–target-molecule axis that controls the PDGF-BB–PDGFRβ-dependent VSMC phenotype and function.

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Key words: Matrix metalloproteinase, Smooth muscle cell, Dedifferentiation, LDL receptor-related protein, PDGFRβ

Introduction

During vascular development, vascular smooth muscle cells (VSMCs) are recruited to newly forming blood vessels, where they contribute to vessel-wall structure and function (von Tell et al., 2006). In a mature arterial wall, a specialized function of differentiated VSMCs is the display of a contractile phenotype, which is dependent on the expression of a range of contractile proteins, including smooth muscle cell (SMC)-specific isoforms of actin (SMA), myosin and calponin (Owens et al., 2004). In contrast to other terminally differentiated cell types, however, mature VSMCs maintain developmental plasticity and are capable of dedifferentiating from a contractile, quiescent state to one characterized by increased migratory and proliferative potential as well as by decreased expression of SMC-specific contractile proteins (Owens et al., 2004). This type of functional plasticity is believed to form the basis for the medial VSMC invasion program, which allows dedifferentiated cells to migrate into the surrounding intima where they can proliferate and synthesize new extracellular matrix (ECM). Through these mechanisms, VSMC dedifferentiation contributes to neointimal lesion formation in cardiovascular disease states (Raines, 2004).

At the sites of vessel-wall injury, endothelial denudation, direct VSMC trauma or inflammation results in the exposure of medial VSMCs to growth factors and inflammatory mediators, which stimulate dedifferentiation (Raines, 2004). Likewise, when VSMCs are exposed to growth-promoting factors after isolation from vessel-wall explants and subsequent culture in vitro, the dedifferentiation program is initiated and the cells progressively suppress contractile-protein expression (Chamley-Campbell et al., 1981; Kawai-Kowase and Owens, 2007; Shanahan et al., 1993). This process, and the associated phenotypic changes, can be further modulated by altered cell-cell contacts and the exposure of VSMCs to various ECM components (Hedin et al., 1988; Koyama et al., 1996), as well as to growth factors belonging to the PDGF, EGF, FGF, TGFβ and IGF families (Hayashi et al., 1999; Kawai-Kowase and Owens, 2007; Owens et al., 2004).

During the process of VSMC dedifferentiation, the activities of a range of ECM-degrading proteinases, including members of the serine proteinase, cysteine proteinase, cathepsin and matrix metalloproteinase (MMP) families are increased (Bendeck et al., 1994; Lijnen et al., 1998; Sukhova et al., 1998; Zempo et al., 1994). Currently, changes in proteinase expression are considered to play a major role in controlling the increased migratory potential and invasiveness of the dedifferentiated VSMCs (Lijnen, 2003; Filippov et al., 2005; Dollery and Libby, 2006). Interestingly, while examining VSMC function in vitro, we unexpectedly observed that the ability of the isolated cells to dedifferentiate was preceded by a dramatic upregulation of the membrane-anchored metalloenzyme membrane-type-1 matrix metalloproteinase (MT1-MMP; also known as MMP14). Importantly, in the absence of MT1-MMP activity, the VSMC dedifferentiation program was largely attenuated and the cells remained locked in a more differentiated state. Further studies demonstrate that MT1-MMP regulates VSMC dedifferentiation by proteolyzing the multifunctional, low-density

lipoprotein (LDL) receptor-related protein LRP1. Following MT1-MMP-dependent LRP1 processing, a PDGF-BB–PDGFRβ pathway is activated, which promotes VSMC dedifferentiation. These findings identify the MT1-MMP–LRP1 axis as a new proteolytic regulatory mechanism of the VSMC differentiation and dedifferentiation programs.

Results

MT1-MMP expression correlates with the spontaneous repression of contractile proteins in cultured VSMCs

The in vitro culture of VSMCs leads to the dedifferentiation of the quiescent, contractile cells and the acquisition of an activated phenotype characterized by the downregulation of contractile proteins, including SMA and calponin (Chamley-Campbell et al., 1981; Kawai-Kowase and Owens, 2007; Shanahan et al., 1993). Interestingly, the levels of MT1-MMP protein were dramatically increased during the isolation and subculture of wild-type VSMCs (18-fold in primary culture and 40-fold in passage #10 cells as compared with VSMCs in medial explants) (Fig. 1A). The major MT1-MMP protein form in VSMCs co-migrated in SDS-PAGE with the active 60-kDa MT1-MMP, which was detectable in HT-1080 cells (Fig. 1A) (Lehti et al., 1998). To define whether this proteinase plays a functional role in the VSMC dedifferentiation program, thoracic aortas were harvested from wild-type and *MT1-MMP*$^{-/-}$ mice (Holmbeck et al., 1999; Zhou et al., 2000), and from SMCs isolated from the respective tissues. The cells were subcultured in vitro under identical conditions, and the levels of contractile proteins assessed. Whereas comparable levels of SMA were detected in the *MT1-MMP*$^{+/+}$ and *MT1-MMP*$^{-/-}$ aortas and the corresponding first-passage VSMCs, only the SMA content of wild-type VSMCs declined as a function of passage number (Fig. 1B). By contrast, *MT1-MMP*$^{-/-}$ VSMCs retained contractile-protein expression during subculture, as indicated by the intense SMA staining observed in passage #10 *MT1-MMP*$^{-/-}$ cells (Fig. 1B). Relative to *MT1-MMP*$^{+/+}$ VSMCs, the mRNA levels for the smooth-muscle contractile proteins SMA, SM22α and calponin were elevated 2.4±0.4-, 4.1±0.4- and 3.8±0.2-fold, respectively, in *MT1-MMP*$^{-/-}$ cells, as assessed by quantitative real-time PCR (n=3; mean ±1 s.d.). These results reveal an unexpected relationship between MT1-MMP expression and the loss of the VSMC contractile phenotype.

MT1-MMP promotes PDGF-BB–PDGFRβ-dependent downregulation of contractile-protein expression

Considering the reported roles of growth factors such as EGF and PDGF-BB in inducing the dedifferentiation of cultured VSMCs (Kawai-Kowase and Owens, 2007), we next analyzed the effects of synthetic inhibitors directed against EGFR and PDGFRβ tyrosine kinases, as well as against MMPs, on the regulation of contractile-protein expression. Importantly, when wild-type VSMCs were continuously cultured in the presence of either the PDGFRβ kinase inhibitor AG1296 or the wide-spectrum MMP inhibitor BB-94, the expression of SMA and calponin was maintained at 1.8 to 3.2 times higher levels than in cells cultured without any inhibitors (n=3; P<0.001) (Fig. 2A). By contrast, the EGFR tyrosine-kinase inhibitor AG1478 had a negligible effect on the dedifferentiation-associated suppression of the genes encoding these proteins (Fig. 2A). Interestingly, none of these inhibitors altered significantly the relatively high expression of SMA or calponin in passaged *MT1-MMP*$^{-/-}$ VSMCs (Fig. 2A). As such, these results are consistent with dual contributions of both PDGFRβ and MT1-MMP in the suppression of VSMC contractile proteins during dedifferentiation.

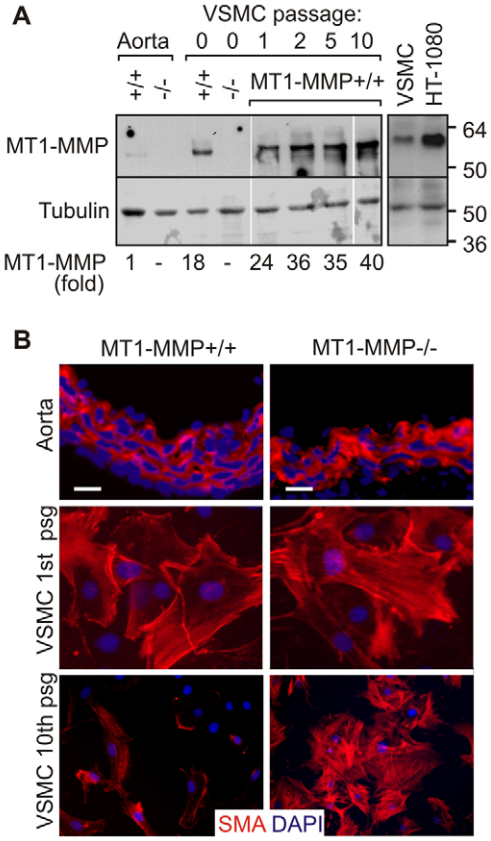

Fig. 1. Correlation between MT1-MMP expression and VSMC contractile-protein expression. (A) MT1-MMP levels were assessed by immunoblotting protein extracts from the aortas of 4-day-old wild-type (+/+) and *MT1-MMP*$^{-/-}$ (–/–) mice, and from the corresponding isolated VSMCs at the indicated passages using antibodies against the catalytic domain of MT1-MMP. Extracts from human HT-1080 fibrosarcoma cells that expressed constitutively activated MT1-MMP were analyzed as controls side-by-side with the tenth-passage VSMCs. β-tubulin served as a loading control. Mean values of relative MT1-MMP levels are expressed below each lane (n=2). Relative mobilities of the molecular-mass markers are indicated in kDa. (B) Aortic frozen sections of 4-day-old wild-type (*MT1-MMP*$^{+/+}$) and *MT1-MMP*$^{-/-}$ mice, and the corresponding isolated passage-1 and -10 VSMCs from the aortas of wild-type and *MT1-MMP*$^{-/-}$ mice, were analyzed for SMA by immunofluorescence. Nuclei were visualized by DAPI. Note the high SMA expression in the tenth-passage *MT1-MMP*$^{-/-}$ VSMCs. Scale bars: 20 μm.

To assess directly the role of MT1-MMP in the regulation of PDGF-BB-induced VSMC dedifferentiation, early-passage wild-type and *MT1-MMP*$^{-/-}$ VSMCs were exposed to PDGF-BB. After incubation for 48 hours, the levels of SMA and calponin, and of the dedifferentiation-associated intermediate-filament protein vimentin, were assessed by immunoblotting. The addition of PDGF-BB decreased the relative protein levels of both SMA and calponin, and slightly increased the levels of vimentin, in wild-type VSMCs (Fig. 2B). By contrast, the expression of these downstream protein targets were unaffected by PDGF-BB treatment of *MT1-MMP*$^{-/-}$ VSMCs (Fig. 2B). Likewise, the treatment of wild-type cells with recombinant tissue inhibitor of metalloproteinases 2 (TIMP2), an efficient endogenous inhibitor of membrane type (MT)-MMPs, resulted in impaired PDGF-BB-mediated regulation of SMA and calponin (Fig. 2B). These results were further supported by the loss of calponin in the actin filaments of PDGF-BB-treated wild-type VSMCs, but not in the corresponding *MT1-MMP*$^{-/-}$ VSMCs

(Fig. 2C). As expected, the restored expression of wild-type MT1-MMP, but not a catalytically inactive mutant (E240A), in *MT1-MMP*$^{-/-}$ VSMCs allowed for a similar downregulation of calponin expression (Fig. 2D). Taken together, these results indicate that MT1-MMP activity plays a regulatory role in the PDGF-PDGFRβ-mediated suppression of VSMC contractile proteins during dedifferentiation.

MT1-MMP induces LRP1 processing and multimolecular-complex formation

Previous studies have demonstrated that LRP1 acts as a negative regulator of the PDGF-PDGFRβ pathway in VSMCs (Boucher et al., 2003). Given the potential susceptibility of LRP1 to MT1-MMP-dependent processing (Rozanov et al., 2004), LRP1 protein levels were assessed in wild-type and *MT1-MMP*$^{-/-}$ VSMCs. Importantly, the relative levels of the 515-kDa α-chain of LRP1 were markedly decreased following the 48-hour PDGF-BB stimulation in wild-type VSMCs in an MMP-dependent manner (Fig. 3A). Using maximal detection sensitivity, several minor processing products of LRP1 α-chain were detectable at the same time as the decreased levels of 515-kDa α-chain (supplementary material Fig. S1). By contrast, the levels of the LRP1 α-chain were not altered in *MT1-MMP*$^{-/-}$ cells cultured under identical conditions (Fig. 3A), consistent with MT1-MMP-dependent LRP1 processing in VSMCs. Coinciding with the reduced cellular LRP1-α-chain levels in PDGF-BB-stimulated wild-type cells, the levels of a soluble α-chain fragment were increased in the corresponding conditioned medium as assessed by immunoprecipitation (Fig. 3B). The levels of the 85-kDa β-chain of LRP1 in wild-type or *MT1-MMP*$^{-/-}$ cells were not significantly affected by PDGF-BB or GM6001 (Fig. 3A).

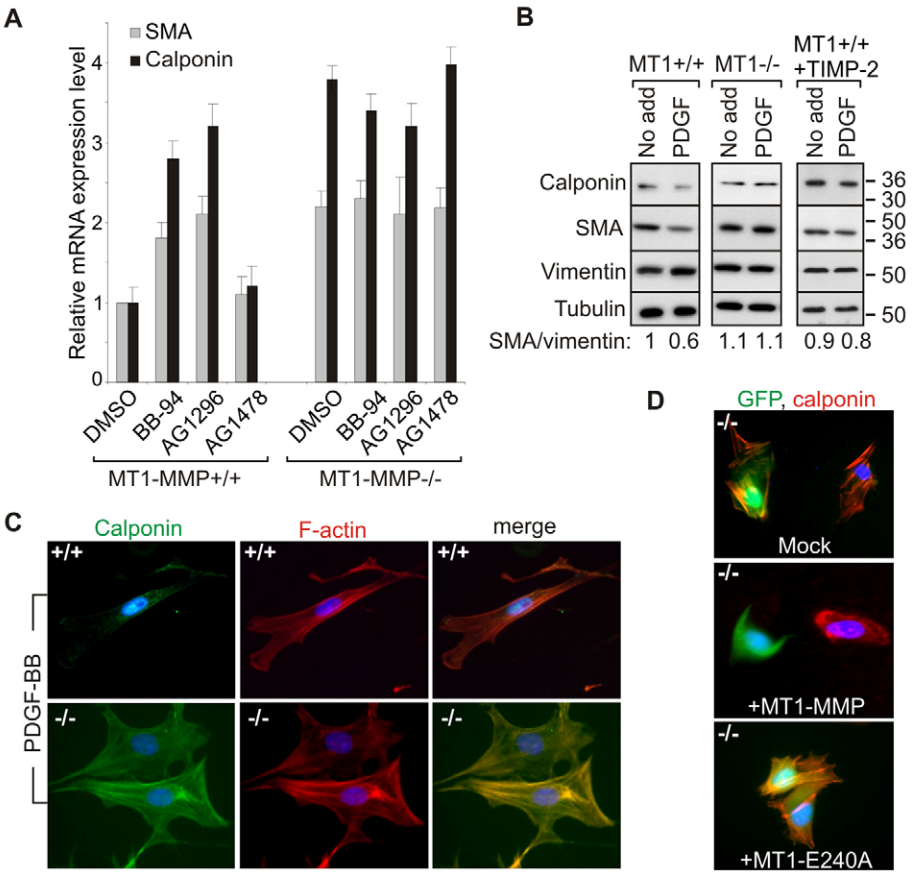

Fig. 2. MT1-MMP promotes PDGFRβ- and PDGF-BB-dependent suppression of VSMC contractile proteins. (A) Quantitative assessment of SMA and calponin mRNA expression by real-time PCR. mRNA was isolated from subconfluent tenth-passage VSMCs cultured with the wide-spectrum MMP inhibitor BB-94 (10 μM), PDGFRβ kinase inhibitor AG1296 (10 μM) and EGFR tyrosine-kinase inhibitor AG1478 (10 μM) as indicated. The expression data are normalized against mouse TBP and presented relative to the mRNA expression in mock (0.1% DMSO)-treated wild-type cells (mean ±1 s.d., *n*=3). (B) Early-passage wild-type (*MT1*$^{+/+}$) and *MT1-MMP*$^{-/-}$ VSMCs were cultured on polymerized collagen I in the presence of TIMP2 (4 μg/ml) for 48 hours as indicated, and exposed to PDGF-BB (25 ng/ml) under serum-free conditions. After 48 hours, the cells were lysed and the lysates analyzed by immunoblotting for the relative levels of calponin, SMA and vimentin. β-tubulin served as a loading control. Mean values of the ratios between normalized SMA and vimentin protein levels are presented below each lane (*n*=3). Relative mobilities of the molecular-mass markers are indicated in kDa. (C) Calponin expression was assessed by immunofluorescence staining of cells exposed to PDGF-BB as above. Filamentous actin was visualized with phalloidin (F-actin) and nuclei with DAPI. (D) *MT1-MMP*$^{-/-}$ VSMCs were transfected with EGFP expression-vector alone (Mock), or with either expression construct for wild-type MT1-MMP (MT1-MMP) or the catalytically inactive mutant (MT1-E240A) as indicated. Calponin expression (red) was assessed by immunofluorescence after 48 hours of treatment with PDGF-BB.

To assess the impact of MT1-MMP on PDGF-BB-induced interactions of LRP1, VSMCs were treated with PDGF-BB for 15 minutes followed by immunoprecipitation with anti-LRP1 antibodies. PDGF-BB treatment resulted in the specific co-precipitation of a phosphorylated ~180-kDa protein, which most probably corresponds to the activated PDGFRβ, with LRP1 in wild-type cells, as detected by immunoblotting for phosphotyrosine residues (Fig. 4A). This interaction was dependent on MT1-MMP activity as it was not detected in either MMP-inhibitor-treated *MT1-MMP*$^{+/+}$ VSMCs or *MT1-MMP*$^{-/-}$ cells (Fig. 4A). A faint protein band of ~85 kDa, which might correspond to phosphorylated LRP1 (Boucher et al., 2002; Loukinova et al., 2002), was barely detectable, and no clear correlation between the levels of this band and MT1-MMP activity could be established (Fig. 4A). A lower-molecular-weight fragment of LRP1 β-chain was observed in wild-type cells, but not in *MT1-MMP*$^{-/-}$ cells, following a 3-hour PDGF-BB stimulation (Fig. 4B), consistent with the induction of further proteolytic processing of LRP1 (May et al., 2002).

The PDGFRβ pathway can also be regulated by interacting cell-surface integrins (Schneller et al., 1997). Furthermore, both functional and physical interactions of MT1-MMP with β3- and β1-integrins have been reported (Deryugina et al., 2000; Galvez et al., 2002). Therefore, we determined the levels of these integrins and their interactions with PDGFRβ, LRP1 and MT1-MMP in wild-type and *MT1-MMP*$^{-/-}$ VSMCs. The total level of these integrins in cell extracts (Fig. 4C), and the weak association between PDGFRβ and β1 integrin (Fig. 4D), were not affected by MT1-

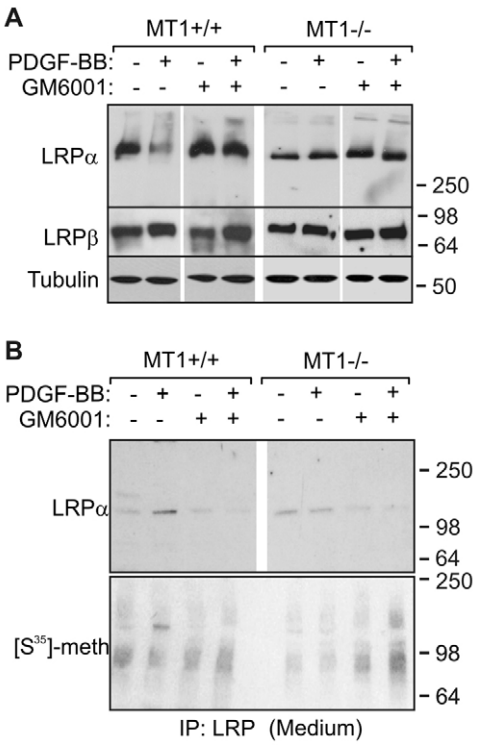

Fig. 3. MT1-MMP induces LRP1 processing. (A) Wild-type (*MT1*$^{+/+}$) and *MT1-MMP*$^{-/-}$ (*MT1*$^{-/-}$) VSMCs were cultured on polymerized collagen I for 48 hours in the presence of MMP inhibitor (GM6001, 10 μM) as indicated and treated with PDGF-BB (25 ng/ml) under serum-free conditions. Total cell lysates were analyzed by immunoblotting for both the α- and β-chains of LRP1. (B) The cells were treated with PDGF-BB for 48 hours. For the last 24 hours of PDGF-BB treatment, the cells were labeled with [^{35}S]-methionine (50 μCi/ml) as indicated. Soluble LRP1 fragments were detected from the conditioned medium by immunoprecipitation with polyclonal rabbit anti-LRPα antibodies followed by immunoblotting with mouse monoclonal anti-LRPα antibodies (LRPα) or by autoradiography ([^{35}S]-meth). Relative mobilities of the molecular-mass markers are indicated in kDa.

MMP, as assessed by immunoblotting and immunoprecipitation assays. Stable β3-integrin–PDGFRβ interactions were detected in wild-type VSMCs in parallel with the co-precipitation of MT1-MMP and β3 integrin in a PDGF-BB-independent manner (Fig. 4D). By contrast, the levels of PDGFRβ were reduced in the corresponding β3-integrin complexes isolated from *MT1-MMP*$^{-/-}$ VSMCs (Fig. 4D). Interestingly, PDGF-BB stimulation induced a strong interaction between LRP1 and β3 integrin in wild-type cells, but not in *MT1-MMP*$^{-/-}$ VSMCs (Fig. 4D).

MT1-MMP enhances PDGFRβ internalization in caveolae

Given the observed MT1-MMP-dependent PDGFRβ-membrane interactions, potential changes in the subcellular distribution of PDGFRβ were next characterized by immunofluorescence analysis. Consistent with the reported caveolae localization of PDGFRβ in VSMCs (Liu et al., 1996; Peterson et al., 2003), PDGFRβ largely colocalized with caveolin 1 in both wild-type and *MT1-MMP*$^{-/-}$ VSMCs (Fig. 5A). However, the patched intracellular localization of PDGFRβ that was detected in wild-type cells was decreased in *MT1-MMP*$^{-/-}$ cells (Fig. 5A), suggesting that PDGFRβ internalization might have been impaired. To directly determine the efficiency of PDGFRβ internalization, serum-starved wild-type and *MT1-MMP*$^{-/-}$ VSMCs were incubated with anti-PDGFRβ antibodies on ice and transferred to 37°C for 30 minutes in the presence of PDGF-BB to allow for ligand-induced internalization of PDGFRβ and the bound antibodies. After blocking the remaining anti-PDGFRβ antibodies on the cell surface, the internalized PDGFRβ was detected. In contrast to the efficient internalization of PDGFRβ in wild-type cells, the rapid internalization of PDGFRβ was markedly impaired in *MT1-MMP*$^{-/-}$ VSMCs (Fig. 5B).

The coincidental induction of PDGFRβ internalization (Fig. 5B) and PDGFRβ-LRP1 interactions (Fig. 4A,D) by PDGF-BB treatment suggested that these receptors would colocalize in wild-type cells at the sites of internalization. Immunofluorescence analysis revealed specific colocalization of endocytosed PDGFRβ with LRP1 β-chain in the intracellular vesicular compartments of PDGF-BB-treated wild-type cells (Fig. 6A). By contrast, this PDGF-BB-induced intracellular colocalization was barely detectable in *MT1-MMP*$^{-/-}$ VSMCs (Fig. 6A). Given the reported mechanism by which PDGFRβ is stabilized via the LRP1-dependent inhibition of PDGFRβ ubiquitylation and degradation after PDGF-BB-induced endocytosis (Takayama et al., 2005), the levels of total, internalized and ubiquitylated PDGFRβ were assessed. Immunoprecipitation assays coupled with cell-surface biotinylation and PDGF-BB-induced internalization confirmed a markedly impaired PDGFRβ endocytosis in PDGF-BB-stimulated *MT1-MMP*$^{-/-}$ VSMCs (Fig. 6B). Interestingly, the relative levels of ubiquitylated PDGFRβ were not altered in *MT1-MMP*$^{-/-}$ cells (Fig. 6B) despite the fact that the PDGF-BB-induced endocytosis of PDGFRβ is frequently associated with the receptor downregulation through polyubiquitin-directed degradation (Takayama et al., 2005). The total levels of both internalized and non-internalized PDGFRβ in wild-type and *MT1-MMP*$^{-/-}$ cells, respectively, also decreased only slightly at comparable rates in the course of a 2-hour incubation with PDGF-BB (Fig. 6C). The proteolytic activity of MT1-MMP thus promotes, co-incidentally with LRP1 processing, dynamic PDGF-BB-induced and caveolae-mediated internalization of PDGFRβ multi-receptor complexes.

LRP1 knockdown rescues the suppression of contractile-protein expression by PDGF-BB–PDGFRβ-activated *MT1-MMP*$^{-/-}$ VSMCs

The phenotype of mice harboring a smooth-muscle-specific *LRP1* gene inactivation has revealed the role of this receptor as a negative regulator of PDGFRβ signaling (Boucher et al., 2003). Therefore, we considered the possibility that MT1-MMP-dependent proteolysis of LRP1 would be a prerequisite for efficient PDGFRβ function. To test this hypothesis and the potential positive contribution of MT1-MMP-dependent LRP1 and β3 integrin interactions for modulating PDGFRβ function, the expression of either LRP1 or β3 integrin was downregulated by specific siRNAs. The knockdown of LRP1 or β3 integrin with >80% efficiency did not notably affect PDGFRβ levels in either wild-type or *MT1-MMP*$^{-/-}$ VSMCs (Fig. 7A). Furthermore, β3-integrin silencing in neither wild-type cells nor *MT1-MMP*$^{-/-}$ VSMCs affected the suppression of contractile proteins in response to PDGF-BB treatment (Fig. 7B). By contrast, in a fashion consistent with the predicted negative regulatory effect of LRP1 on PDGFRβ function in MT1-MMP-deficient cells, LRP1 knockdown rescued the ability of *MT1-MMP*$^{-/-}$ VSMCs to downregulate calponin and SMA (Fig. 7B,C). *MT1-MMP*$^{+/+}$ VSMCs underwent a normal dedifferentiation program in the presence or absence of LRP1 expression (Fig. 7B,C). Taken together, these results demonstrate that MT1-MMP controls VSMC phenotype by relieving a LRP1-dependent block in the PDGF-BB–PDGFRβ

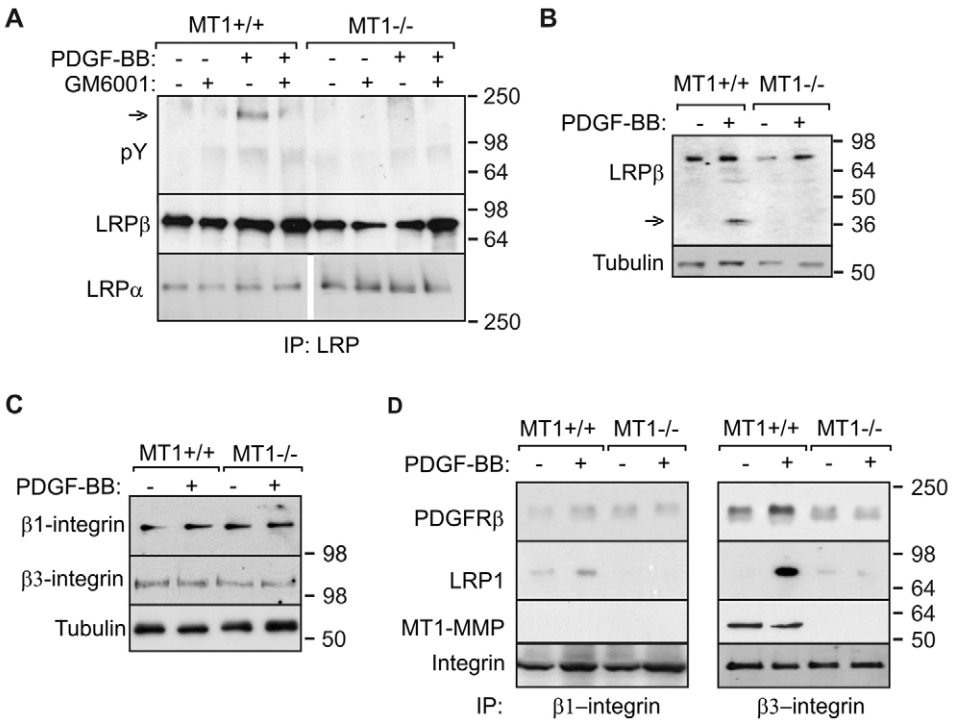

Fig. 4. MT1-MMP promotes interactions between PDGFRβ, β3 integrin and LRP1. (A) VSMCs were treated with PDGF-BB for 30 minutes and lysed. The lysates were subjected to immunoprecipitation with polyclonal rabbit anti-LRP1 antibodies. Tyrosine-phosphorylated proteins in the immunoprecipitates were detected by anti-phosphotyrosine antibodies (pY) and LRP1 by mouse monoclonal anti-LRPβ and anti-LRPα antibodies. Arrow indicates the phosphorylated protein that co-precipitates with LRP1 and most probably represents PDGFRβ. (B) LRPβ protein levels, as detected by immunoblotting, in the lysates of VSMCs that were treated with PDGF-BB for 3 hours. β-tubulin served as a loading control. Arrow indicates the cleaved fragment of LRP1 β-chain. (C) Wild-type (*MT1*$^{+/+}$) and MT1-MMP-null (*MT1*$^{-/-}$) VSMCs were cultured on polymerized collagen I and were serum starved for 24 hours. The cells were then treated with PDGF-BB (10 ng/ml) for 30 minutes and lysed. The levels of β1- and β3-integrins in the cell lysates were detected by immunoblotting. β-tubulin served as a loading control. (D) The cell lysates were prepared as above and subjected to immunoprecipitation with polyclonal rabbit anti-β1- and β3-integrin antibodies. PDGFRβ, LRPβ, MT1-MMP, and β1- and β3-integrin in the immunoprecipitates were detected by immunoblotting.

signaling cascade that controls the cellular dedifferentiation program.

Discussion

ECM-degrading MMPs contribute to the invasion of dedifferentiated VSMCs into the intimal space and thus to the formation of neointimal lesions (Dollery and Libby, 2006; Lijnen, 2003). The specific function of MT1-MMP in these processes has been demonstrated by decreased ligation-induced neointimal hyperplasia in *MT1-MMP*$^{+/-}$ heterozygote mice (Filippov et al., 2005). Although previous studies have established the essential role of MT1-MMP in the degradation of interstitial collagen and VSMC invasion in the arterial wall (Filippov et al., 2005), we were intrigued by the unexpectedly close relationship between MT1-MMP upregulation and the spontaneous suppression of contractile proteins in cultured VSMCs. This connection raised the possibility of a yet unrecognized role for MT1-MMP in the regulation of the phenotypic dedifferentiation program in addition to its direct pro-invasive ECM-degrading activities. Consistent with this new function for MT1-MMP, we found that the repression of contractile genes in isolated VSMCs was inhibited by both synthetic MMP inhibitors and MT1-MMP deficiency. Accordingly, the sustained expression of differentiation-associated contractile genes was not affected by MMP inhibitors in *MT1-MMP*$^{-/-}$ cells. Furthermore, the phenotypic dedifferentiation was rescued by the restored expression of active MT1-MMP in null cells, indicating that the proteolytic MT1-MMP activity promotes VSMC dedifferentiation.

Growth factors such as PDGF-BB and EGF are considered as potent inducers of VSMC dedifferentiation both in vivo and in vitro (Kawai-Kowase and Owens, 2007; Raines, 2004), but the contributions of specific serum factors for the spontaneous dedifferentiation of isolated VSMCs are not clear. We found that, under current culture conditions, the phenotypic switch had a dual dependence on PDGFRβ and MT1-MMP activities. This suggests that MT1-MMP enhances the dedifferentiation program through proteolytic augmentation of the PDGF-BB–PDGFRβ pathway. The activity of this pathway and the phenotypic outcome can be modulated by different ECM molecules, soluble factors and membrane co-receptors (Boucher et al., 2003; Hedin et al., 1988; Hobson et al., 2001; Koyama et al., 1996; Schneller et al., 1997). Because MT1-MMP is an efficient pericellular ECM-degrading proteinase that can also cleave various cell-surface proteins and receptors (Itoh and Seiki, 2006), it could potentially affect the PDGF-BB–PDGFRβ pathway through numerous mechanisms. Among the known MT1-MMP substrates are basement-membrane components (Hotary et al., 2006) and interstitial collagens (Filippov et al., 2005; Holmbeck et al., 1999; Holmbeck et al., 2004; Schneider et al., 2008), which are both associated with the phenotypic regulation and PDGF-BB responsiveness of VSMCs (Koyama et al., 1996; Thyberg and Hultgardh-Nilsson, 1994). Furthermore, MT1-MMP might release active soluble factors from the ECM or cells (Page-McCaw et al., 2007). However, wild-type conditioned media or ECM had negligible effects on the PDGF-BB responsiveness of *MT1-MMP*$^{-/-}$ VSMCs (K.L., S.J.W. and

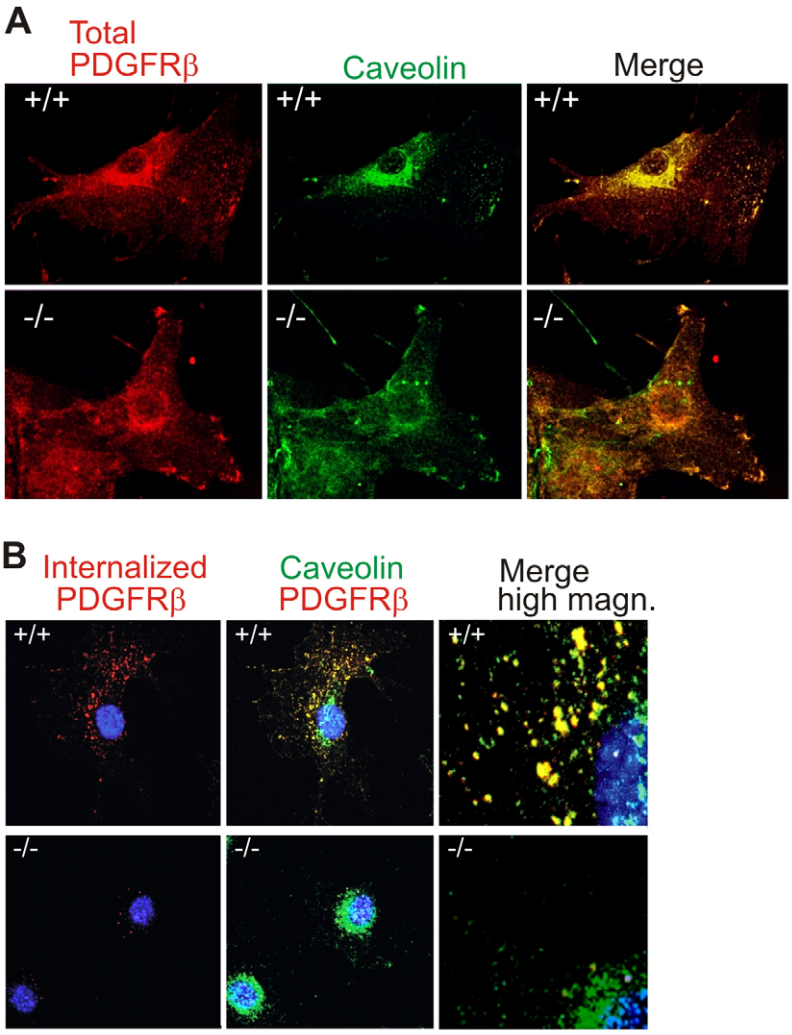

Fig. 5. MT1-MMP is required for efficient ligand-induced internalization of PDGFRβ in VSMCs. (A) VSMCs on polymerized collagen I were fixed and permeabilized for immunofluorescence staining using antibodies against PDGFRβ and caveolin 1. PDGFRβ colocalized with caveolin 1 (caveolae) in both wild-type (+/+) and MT1-MMP-null (–/–) cells, but specific intracellular caveolin-1 structures did not contain PDGFRβ in *MT1-MMP*$^{-/-}$ VSMCs. (B) To detect internalization, quiescent VSMCs were labeled with anti-PDGFRβ antibodies for 1 hour at 4°C. Unbound antibodies were removed and the cells shifted to 37°C for 30 minutes in the presence of PDGF-BB (10 ng/ml). After blocking the anti-PDGFRβ antibodies that remained on the cell surface with unlabelled secondary antibodies, the cells were fixed, permeabilized and stained with anti-caveolin-1 antibodies followed by secondary Alexa-Fluor-488- and Alexa-Fluor-594-conjugated antibodies. The merged high-magnification image depicts colocalization between internalized PDGFRβ and caveolin 1 in yellow. Note the efficient PDGFRβ internalization in caveolin-positive structures of wild type (+/+) VSMCs in contrast to minor PDGFRβ internalization in MT1-MMP-null (–/–) cells.

J.K.-O., unpublished observation). Stable modifications of soluble or ECM proteins are therefore not likely to underlie the MT1-MMP-dependent phenotypic regulation. Considering the regulation of the PDGF-BB–PDGFRβ pathway by a variety of membrane co-receptors (Boucher et al., 2003; Hedin et al., 1988; Hobson et al., 2001; Koyama et al., 1996; Schneller et al., 1997), these observations are consistent with either the production of positive PDGFRβ-membrane co-effectors or the loss of negative co-effectors as a consequence of MT1-MMP proteolysis.

We have previously found that MT1-MMP interacts with PDGFRβ in VSMC membrane complexes and enhances mitogenic and chemotactic PDGF-BB signaling through a yet undefined proteolytic mechanism (Lehti et al., 2005). In VSMC membranes, PDGFRβ also interacts with LRP1, which has been characterized as an MT1-MMP substrate in vitro (Rozanov et al., 2004) and a negative regulator of PDGFRβ in VSMCs in vivo (Boucher et al., 2003). Using isolated *MT1-MMP*$^{-/-}$ VSMCs, we now provide evidence for the release of VSMCs from LRP-mediated negative regulation by MT1-MMP-dependent LRP1 cleavage. In support of this conclusion, we found that: (1) endogenous MT1-MMP activity enhanced LRP1 processing in dedifferentiating VSMCs, and (2) siRNA-mediated LRP1 knockdown rescued the PDGF-BB-stimulated contractile-protein repression in *MT1-MMP*$^{-/-}$ cells. These results thus identify MT1-MMP and LRP1 as a new effector-target pair that controls the function and PDGF-BB responsiveness of VSMCs.

Strong PDGFRβ–LRP1-β-chain interactions were, however, detected only in the cells that expressed active MT1-MMP and responded efficiently to PDGF-BB in conjunction with the proteolytic processing of LRP1. This suggests that mechanisms other than stable LRP1-PDGFRβ interactions might contribute to the defective dedifferentiation of *MT1-MMP*$^{-/-}$ VSMCs. Indeed, LRP1 is a large, multifunctional receptor that also serves as an endocytic receptor that regulates the levels of various extracellular ligands through delivery to lysosomal degradation (Lillis et al., 2005). LRP1 can also alter the trafficking and compartmentalization of various membrane receptors and growth factors, as well as directly mediate intracellular signaling (Gonias et al., 2004; Lillis et al., 2005). Accordingly, the negative regulation of PDGFRβ has been reported to depend on ligand binding (e.g. ApoE) to LRP1 (Boucher and Gotthardt, 2004; Zhu and Hui, 2003). This is consistent with the current observation of efficient PDGFRβ function in complexes in which MT1-MMP-dependent LRP1 ectodomain shedding would inhibit ligand binding.

LRP1 reduces the ubiquitin-mediated degradation of endocytosed PDGFRβ in fibroblasts by sequestering Cbl, a ubiquitin E3 ligase (Takayama et al., 2005). We found that, in spite of enhanced endocytosis, the degradation of PDGFRβ in the multi-molecule complexes was not faster in MT1-MMP-expressing VSMCs relative to the null cells in which stable LRP1-PDGFRβ interactions were not detected. Therefore, after MT1-MMP induction and LRP1 processing in wild-type VSMCs, the LRP1 β-chain might function as a positive co-receptor that diminishes PDGFRβ degradation. Current results also indicate that MT1-MMP augments interactions of LRP1 and PDGFRβ with β3 integrin. Although the β3-integrin–PDGFRβ interaction have been shown to enhance PDGF-BB signaling (Schneller et al., 1997), β3-integrin knockdown did not notably affect the suppression of VSMC contractile proteins. Thus, the integrin did not play a crucial role in the VSMC dedifferentiation under current experimental conditions. However, considering the abundance of potential MT1-MMP substrates in the VSMCs, cleavage of MT1-MMP substrates other than LRP1 might contribute to VSMC phenotype and function in the presence of a complex mixture of serum and ECM factors.

Collectively, our results support a new mechanism for VSMC-phenotype regulation in which MT1-MMP-dependent LRP1 processing and association of PDGFRβ, β3-integrin and LRP1 complexes leads to efficient signal transduction through PDGFRβ and its downstream pathways (Fig. 8). This promotes the suppression of contractile genes, and the induction of pro-migratory and pro-mitogenic genes (Kawai-Kowase and Owens, 2007). In a mature arterial wall, the differentiated VSMCs display stable adhesive interactions with the surrounding basement membrane. In conjunction with their gene expression profile, this renders them relatively refractive to PDGF-BB and related stimuli (Kawai-Kowase and Owens, 2007). Cooperative signals from a wide range of growth-promoting factors, such as inflammatory cytokines (Chen et al., 2006) or the VSMC contact with type I collagen (Owens et al., 2004), enhances PDGF-BB responsiveness of VSMCs as well as the induction of VSMC dedifferentiation at sites of injury (Kawai-Kowase and Owens, 2007). Importantly, MT1-MMP is upregulated by similar vascular-growth stimuli, including IL1 and TNFα, oxidized LDLs, type I collagen, increased blood flow and vessel-wall stretch (Haas et al., 1999; Rajavashisth et al., 1999). Considering also the high levels of LRP1 expression at the sites of vascular injury (Lillis et al., 2005), we propose that MT1-MMP-dependent LRP1 processing is likely to contribute to VSMC dedifferentiation and function at sites of vascular injury. With the similar time window of strong MT1-MMP expression (Apte et al., 1997) and PDGF-BB–PDGFRβ dependence in developing mouse vasculature, as well as the defective recruitment of brain and retinal mural cells in MT1-MMP-null mice (Lehti et al., 2005), these mechanisms might also help to understand the function of VSMCs in neovessel stabilization.

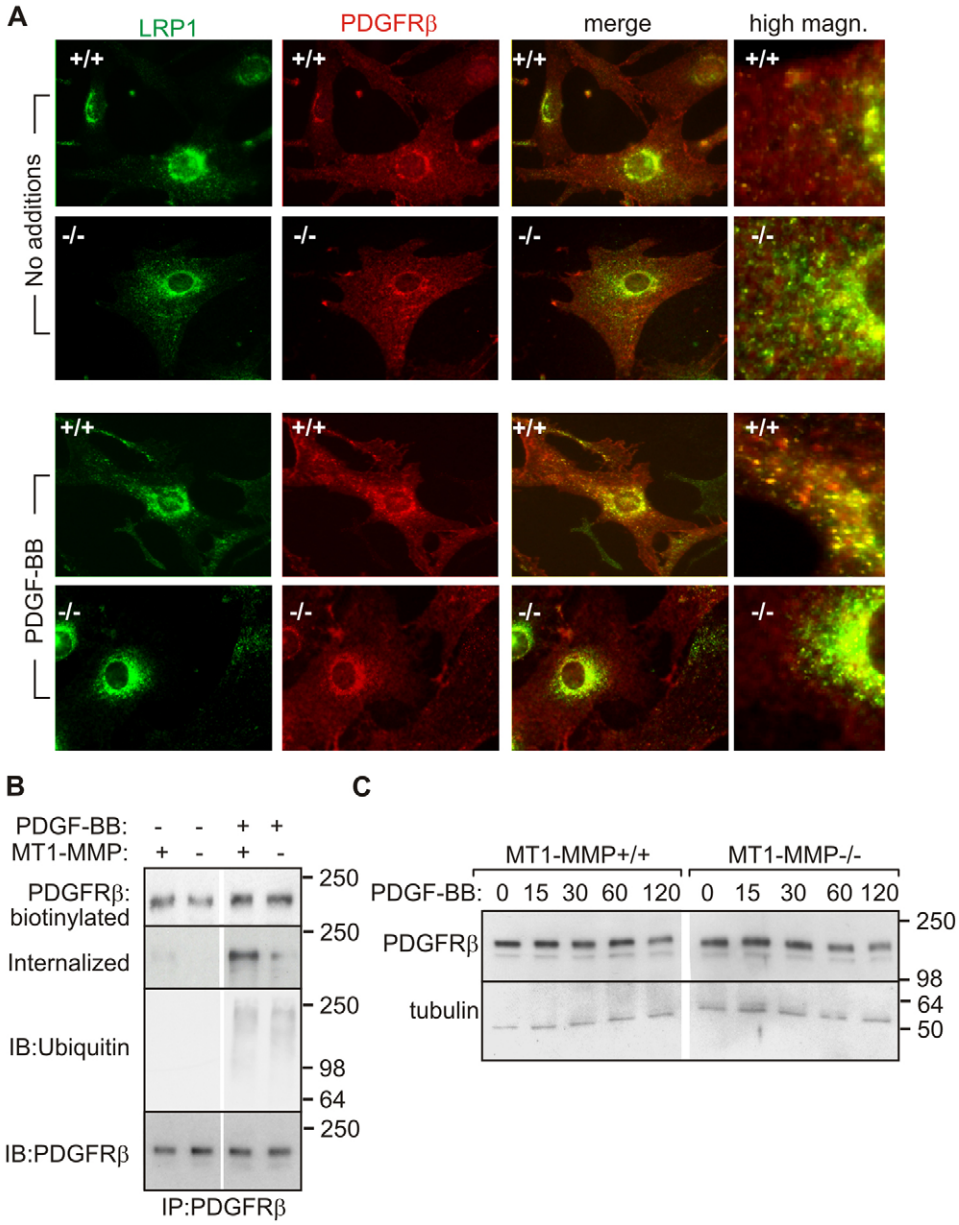

Fig. 6. PDGFRβ remains stable in PDGF-BB-induced and -internalized LRP1 complexes in MT1-MMP-expressing cells. (A) Control (+/+) and MT1-MMP-null (–/–) VSMCs on collagen were treated with PDGF-BB for 30 minutes and the subcellular localization of LRP1 and PDGFRβ analyzed by immunofluorescence. Note the intracellular colocalization of these receptors in PDGF-BB-treated wild-type VSMCs. (B) VSMCs were surface biotinylated and shifted to 37°C for 30 minutes in the presence of PDGF-BB to allow PDGFRβ internalization. The biotinylated PDGFRβ remaining on the cell surface was left intact (biotinylated) or removed by reduction (internalized). Total biotinylated and internalized receptors were then detected in PDGFRβ immunoprecipitates by streptavidin conjugate. Total and ubiquitylated PDGFRβ in precipitates was detected by immunoblotting. (C) VSMCs were stimulated with PDGF-BB for the indicated periods of time (minutes) and lysed for analysis. Total PDGFRβ protein levels were assessed by immunoblotting. Relative mobilities of the molecular-mass markers are indicated in kDa.

Materials and Methods

Cell culture

Aortic VSMCs were isolated from mice with targeted deletion in the *MT1-MMP* gene (Holmbeck et al., 1999; Zhou et al., 2000) and from wild-type littermates by a combination of partial proteinase digestion and cell outgrowth from medial explants as described (Lehti et al., 2005). Briefly, the thoracic aortae were dissected into fragments, endothelial cells removed by gentle scraping and the media explants stripped of adventitia. Tissues were partially digested with collagenase type 2 (1.5 mg/ml; Worthington) and plated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, and 2 mM L-glutamine to allow the migration of VSMCs from the partially dissociated explants. Isolated VSMCs were maintained in complete DMEM and used for experiments between passages 1-6 unless otherwise indicated.

Real-time PCR

Total RNA from VSMCs was extracted with the RNeasy Mini Kit (Qiagen). Reverse transcription of RNA (500 ng) was performed with the RT Kit (Invitrogen). SMA, SM22α and calponin expression was quantified on GeneAmp 7500 Sequence Detector thermal cycler (Applied Biosystems) using TaqMan Universal PCR Master Mix and

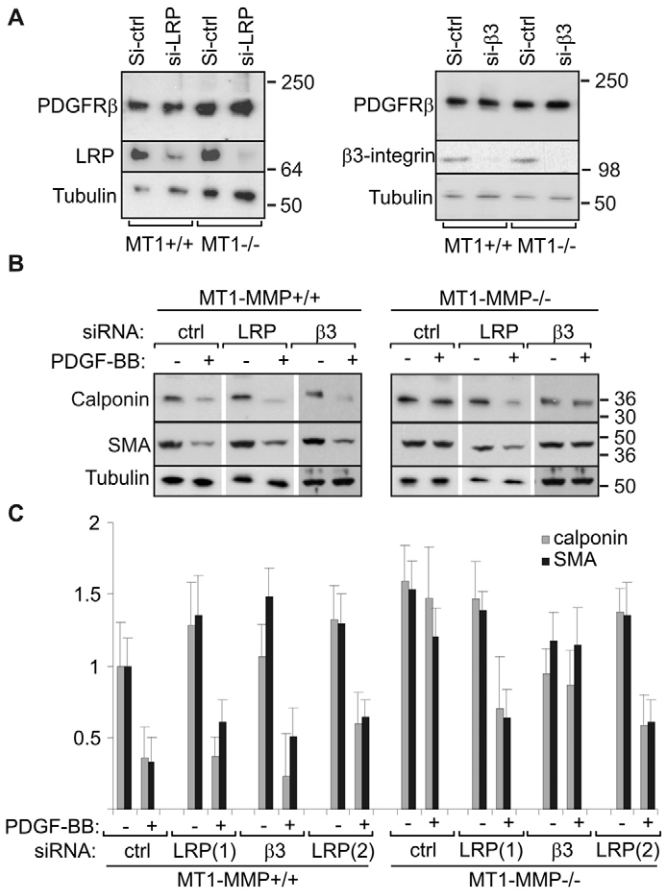

Fig. 7. LRP1 knockdown by siRNA rescues PDGF-BB-mediated dedifferentiation of *MT1-MMP*$^{-/-}$ VSMCs. (A) Wild-type (*MT1*$^{+/+}$) and *MT1-MMP*$^{-/-}$ (*MT1*$^{-/-}$) VSMCs were plated on type I collagen and transfected with control, β3-integrin (right panels) or LRP1 (left panels) siRNAs as indicated. The cells were then exposed to PDGF-BB (25 ng/ml) under serum-free conditions for 48 hours and lysed. The relative levels of LRP1, β3 integrin and PDGFRβ in the lysates were assessed by immunoblotting. β-tubulin served as a loading control. (B) The relative levels of calponin and SMA were assessed by immunoblotting. Similar results were obtained with two different siRNA oligomers targeting LRP1 [LRP(1) and LRP(2)] and β3 integrin. (C) Quantitative assessment of SMA and calponin protein levels normalized against β-tubulin. The data are expressed as relative values (mean ±1 s.d., *n*=3).

validated primers (Mn00725412.S1, Mn00787032.m1; Applied Biosystems). Gene expression was normalized to TATA-binding protein (TBP) and *GAPDH* mRNA expression.

cDNA constructs, siRNAs and transfections

Expression vectors encoding full-length human *MT1-MMP* cDNA and cDNA encoding the MT1-MMP mutant MT1-E240A (MT1-MMP with inactivating E240 to A substitution in the active site) have been described (Lehti et al., 2000). Subconfluent VSMCs were transiently transfected with the vectors using FuGENE 6 (Roche). The transfection efficiency was monitored and transfected cells identified by EGFP expression. FITC-conjugated or unconjugated siRNA oligonucleotides targeted against 21-nucleotide sequences of MT1-MMP, LRP1 and β3 integrin (HP GenomeWide siRNAs; S100177807, S100176743, S100176722, SI01078490 and SI01078497; Qiagen), and non-silencing control siRNA (Qiagen) were transfected using Lipofectamine 2000 (Invitrogen). A transfection efficiency of >90% of FITC-conjugated siRNAs was confirmed 24 hours after transfection. Knockdown efficiency was monitored by immunoblotting and real-time PCR after 48 hours.

Cell treatments, immunoblotting and immunoprecipitation

VSMCs were plated atop type I collagen gels (acid extracted from rat tail; Sigma) in DMEM with 10% FBS for 48 hours. VSMCs were then serum-starved in DMEM with 0.1% bovine serum albumin (BSA) for 48 hours followed by incubation with human recombinant PDGF-BB (25 ng/ml), IGF1 (5 ng/ml) and IL1β (10 ng/ml; all from R&D Systems), or vehicle for 0-48 hours. The cell lysates were prepared as described (Lehti et al., 2005). In selected experiments, VSMCs were cultured or pre-treated and stimulated in the presence of human recombinant TIMP2 (4-5 μg/ml, R&D Systems), the synthetic MMP inhibitors BB-94 or GM6001 (5-10 μM final concentration in 0.1% DMSO), PDGFRβ kinase inhibitor (AG1296, 10 μm) and EGFR tyrosine-kinase inhibitor (AG1478, 10 μm; all from Calbiochem except BB-94 kindly provided by British Biotech). Protein concentrations of the clarified cell and tissue extracts were determined by BCA-protein assay kit (Pierce), and equal amounts of protein were subjected to SDS-PAGE, or immunoprecipitation with rabbit polyclonal antibodies to PDGFRβ (Santa-Cruz), β1 integrin (Chemicon), β3 integrin (Chemicon) and LRP1 (Dudley K. Strickland, University of Maryland School of Medicine, Rockville, MD) as described (Lehti et al., 1998). Immunoblotting was carried out with primary antibodies against β-tubulin (Cell Signaling Technology), phosphotyrosine (Upstate-Millipore), PDGFRβ (R&D Systems), β1 integrin (Chemicon), β3 integrin (Chemicon), the catalytic domain of MT1-MMP (Ab-3) (Lehti et al., 2000), LRP1 (rabbit polyclonal 2629 against α-chain, mouse monoclonal 8G1 against α-chain, and 5A6 against β-chain; Dudley K. Strickland and Abcam), SMA (Sigma) and calponin (Sigma). Soluble LRP1 fragments were also detected by autoradiography after metabolic labeling of the cells for 24 hours with 50 μCi/ml [^{35}S]-methionine (<1000 Ci/mmol) in methionine-free MEM. Scanned images were quantified using Scion Image software.

Immunofluorescence and immunohistochemistry

Thoracic aortas were dissected from 4-day-old *MT1-MMP*$^{-/-}$ and littermate control mice, and fixed with 4% paraformaldehyde. Aortic frozen sections (10 μm) were prepared and post-fixed with acetone:methanol (1:1) followed by immunostaining with Cy3-conjugated mouse monoclonal anti-αSMA antibodies (Sigma) and visualization of nuclei with DAPI. Isolated VSMCs were fixed, permeabilized and stained with primary antibodies against αSMA and calponin as described (Lehti et al., 2005). Texas-red-conjugated phalloidin (Molecular Probes) was used to visualize polymerized actin. Following mounting in Vectashield with DAPI for nuclear staining (Vector Laboratories), samples were examined with a Zeiss Axioplan 2 microscope and Zeiss LSM 510 Meta laser scanning confocal microscope. Confocal

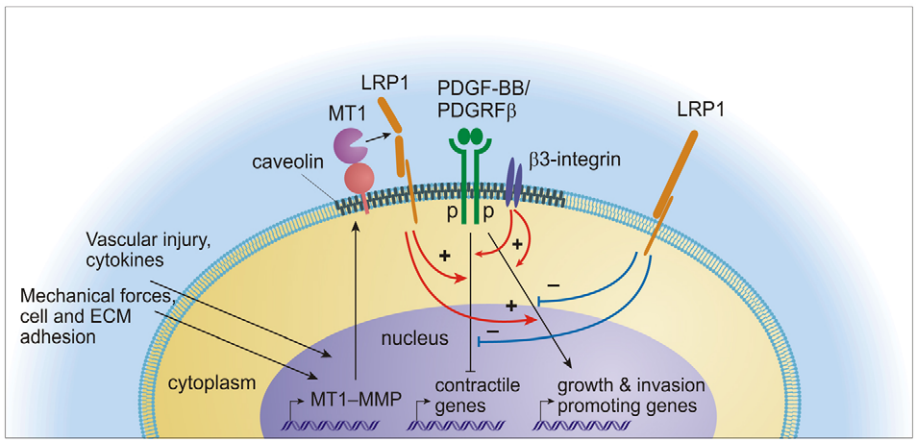

Fig. 8. Schematic representation of the MT1-MMP-dependent regulation of PDGFRβ-membrane interactions and VSMC gene expression. Our results support the mechanism of VSMC-phenotype regulation in which MT1-MMP induction after vascular injury or exposure to growth stimuli results in LRP processing and the dynamic association of complexes of PDGFRβ, β3 integrin and LRP1. These complexes can be actively mobilized through endocytosis coincidently with efficient signal transduction through downstream pathways to the nuclei (Tallquist and Kazlauskas, 2004). There, the altered interactions of transcription factors, including serum response factor and myocardin, lead to the suppression of contractile genes, and to the induction of pro-migratory and pro-mitogenic genes (Kawai-Kowase and Owens, 2007).

images were stored as digital files with Zeiss LSM software and viewed with Corel Photo Paint.

Internalization assays

Cell-surface proteins were biotinylated with 0.5 mg/ml Sulfo-NHS-SS-biotin (Pierce) as described (Lehti et al., 1998) and shifted to 37°C for 30 minutes. After removal of the remaining biotin from the cell surface with oxidized glutathione, the cells were lysed and lysates subjected to immunoprecipitation with anti-PDGFRβ antibodies followed by SDS-PAGE and detection of biotinylated receptor in the precipitates by horseradish-peroxidase-conjugated streptavidin. For antibody-internalization assay, the living cells on glass coverslips were washed with cold phosphate buffered saline (PBS) and incubated with anti-PDGFRβ antibodies (R&D Systems) on ice for 30 minutes. Antibodies were subsequently removed and cells washed prior to shift to 37°C. Anti-PDGFRβ antibodies that remained on the cell surface were coupled with unlabelled secondary antibodies. Cells were then fixed using 3% paraformaldehyde (PFA). Non-specific binding sites were saturated with BSA (5% solution in PBS). The cells were then permeabilized with 0.1% Triton X-100 in PBS for the detection of internalized anti-PDGFRβ antibodies and endocytosis markers with primary antibodies against EEA1, clathrin and caveolin (Transduction Laboratories) and Alexa-Fluor-594- and -488-conjugated secondary antibodies (Molecular Probes), and mounted using Vectashield (Vector Laboratories) containing DAPI to stain the nuclei. Images were obtained as described for immunofluorescence.

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