XBP 1-Deficiency Abrogates Neointimal Lesion of Injured Vessels Via Cross Talk With the PDGF Signaling

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Objective—Smooth muscle cell (SMC) migration and proliferation play an essential role in neointimal formation after vascular injury. In this study, we intended to investigate whether the X-box–binding protein 1 (XBP1) was involved in these processes.

Approach and Results—In vivo studies on femoral artery injury models revealed that vascular injury triggered an immediate upregulation of *XBP1* expression and splicing in vascular SMCs and that *XBP1* deficiency in SMCs significantly abrogated neointimal formation in the injured vessels. In vitro studies indicated that platelet-derived growth factor-BB triggered *XBP1* splicing in SMCs via the interaction between platelet-derived growth factor receptor β and the inositol-requiring enzyme 1α. The spliced XBP1 (XBP1s) increased SMC migration via PI3K/Akt activation and proliferation via downregulating calponin h1 (CNN1). XBP1s directed the transcription of *mir-1274B* that targeted *CNN1* mRNA degradation. Proteomic analysis of culture media revealed that XBP1s decreased transforming growth factor (TGF)-β family proteins secretion via transcriptional suppression. TGF-β3 but not TGF-β1 or TGF-β2 attenuated XBP1s-induced CNN1 decrease and SMC proliferation.

Conclusions—This study demonstrates for the first time that XBP1 is crucial for SMC proliferation via modulating the platelet-derived growth factor/TGF-β pathways, leading to neointimal formation. **(*Arterioscler Thromb Vasc Biol*. 2015;35:2134-2144. DOI: 10.1161/ATVBAHA.115.305420.)**

Key Words: inositol ■ microRNAs ■ proteomics ■ signal transduction ■ vascular remodeling

Smooth muscle cells (SMCs) are the main cell components in the media of the vessel wall, maintaining the blood vessel tone and pressure. Hyperplasia of SMCs contributes to vascular disease, such as stenosis and atherosclerosis.[1,2] Although the origin of SMCs in the lesion area is controversial,[3–6] SMC migration and proliferation play a central role in disease development. Multiple cytokines or growth factors have been documented to regulate SMC migration and proliferation,[2,7,8] in which platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF)-β play important roles. For stem/progenitor cells differentiation toward SMC lineage, PDGF functions as a differentiation stimulus.[9] In mature SMCs, PDGF functions as a mitogenic factor, triggering SMC dedifferentiation, migration and proliferation.[5,10] Several signaling pathways activated by PDGF in SMC have been reported, including PI3K/Akt, endoplasmic reticulum kinase (ERK), c-Jun, and basic fibroblast growth factor release.[11,12] However, the detailed mechanism on how PDGF receptor activation results in adaptor protein binding is still unclear.

TGF-β is a superfamily of multiple growth factors, including TGF-β1 to TGF-β3, bone morphogenetic proteins, and connective tissue growth factor.[13] TGF-βs can be secreted by almost all cell types, including SMCs, functioning in an autocrine, paracrine, or endocrine manner. Different types of TGF-βs exert different functions in different cell types. On SMCs, TGF-β1 suppresses proliferation while induces differentiation.[14,15] TGF-β2 plays an essential role in the valve formation during embryonic development via endothelial-mesenchymal transition.[16] TGF-β3 negatively regulates TGF-β1–mediated extracellular matrix deposition.[17] During SMC differentiation, the upregulation of calponin h1 (CNN1) expression was observed. Reduced level of CNN1 was observed in human uterine leiomyosarcoma, whereas increasing CNN1 suppressed leiomyosarcoma cell proliferation.[18] Overexpression of CNN1 could suppress SMC proliferation and neointima formation.[19,20]

The X-box–binding protein 1 (XBP1) was originally identified as a stress-inducible transcription factor in both invertebrate and vertebrate cells, and essential for cell survival

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Nonstandard Abbreviations and Acronyms

Ad	adenovirus
CNN1	calponin h1
EC	endothelial cell
ER	endoplasmic reticulum
grp78	glucose response protein 78
IRE1α	inositol-requiring enzyme 1 α
miRNA	microRNA
PDGF	platelet-derived growth factor
PDGFRβ	PDGF receptor β
PKR	protein kinase R
RT-PCR	reverse transcriptase polymerase chain reaction
SMC	smooth muscle cell
TGF-β	transforming growth factor β
UTR	untranslated region
XBP1	X-box–binding protein 1
XBP1s	spliced XBP1
XBP1u	unspliced XBP1

under stress conditions.[21–23] Under ER stress conditions, *XBP1* mRNA undergoes unconventional splicing through an ER-resident kinase that possesses ribonuclease activity, the inositol-requiring enzyme 1 α (IRE1α).[24] Our recent studies and reports from other groups showed that physiological stimuli such as vascular endothelial cell growth factor could trigger *XBP1* splicing in an ER stress response–independent manner.[25–28] In endothelial cells (ECs), *XBP1* splicing plays diverse roles, including cell proliferation,[26] autophagy response,[29] and apoptosis.[30] In SMCs, bone morphogenetic protein-2 was reported to activate *XBP1* splicing,[31] but the exact role of XBP1 in SMCs still remains unclear. In this study, we demonstrated that *XBP1* splicing is crucial in PDGF-BB–induced SMC proliferation and contributes to neointima formation after vascular injury.

Materials and Methods

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

Results

Vascular Injury Increased XBP1 Expression and Splicing

In response to vascular injury, vascular SMCs will be activated undergoing migration, proliferation, and apoptosis.[32] To test whether XBP1 was involved in SMC activation, femoral artery injury model was introduced into the right side of C57Bl/6 mice. The injured vessels were harvested at day 1, and day 3 post surgery, whereas the left uninjured vessels were collected as control. Double immunofluorescence staining with anti–α SMC actin and antispliced XBP1 (XBP1s) or unspliced XBP1 (XBP1u) antibodies were performed on the cryo-sections. As shown in Figure 1A, low levels of XBP1s and XBP1u were detected in the uninjured vessels, but much higher levels of both XBP1s and XBP1u could be detected at the media layer in the injured vessels at days 1 or 3 time points. ECs were observed in the endothelium layer and the adventitia area of the uninjured vessels at days 1 and 3 as revealed by CD31 staining (Figure 1A). There were CD31-positive cells in the adventitia area but not in the endothelium layer of the injured vessels at both days 1 and 3 (Figure 1A).

To further quantitatively compare the expression level of *XBP1s* to that of *XBP1u*, Reverse transcriptase polymerase chain reaction (RT-PCR)/*PstI* digestion assays were performed, using RNA extracted from the vessels. There is a *PstI* restriction enzyme site in the 26 nucleotides intron of the XBP1u mRNA, removal of this intron by unconventional splicing will destroy the *PstI* site. Therefore, the digestion of the RT-PCR product with a primer set flanking the intron can distinguish *XBP1u* (digested) from *XBP1s* (undigested). A schematic illustration of the primer set and the *PstI* site was presented in Figure IA in the online-only Data Supplement. The vascular injury immediately upregulated *XBP1* transcription at day 1, but decreased at day 7 and increased again at later stage ≤28 days (Figure IB in the online-only Data Supplement). The *XBP1* splicing was continuously maintained at higher levels post injury (S/U ratio in Figure IB in the online-only Data Supplement). These results suggest that vascular injury can activate *XBP1* expression and especially splicing in vascular SMCs.

The XBP1 Gene Disruption in SMCs Reduced Neointima Formation

To study the role of XBP1 in injury-induced neointima formation, $XBP1^{loxP/loxP}$ mice were generated by gene targeting[26] and crossbred with *SM22-Cre* transgenic mice to create SMC conditional knockout mice, that is, $SM22\text{-}Cre^{+}/XBP1^{loxP/loxP}$ mice (Figure II in the online-only Data Supplement). Femoral artery injury model was introduced into $XBP1^{loxP/loxP}$ mice (wild-type, $XBP1^{+/+}$) and $SM22\text{-}Cre^{+}/XBP1^{loxP/loxP}$ mice ($XBP1^{-/-}$). As shown in Figure 1B, vascular injury induced neointima formation in $XBP1^{loxP/loxP}$ mice ($XBP1^{+/+}$), which was significantly reduced in $SM22\text{-}Cre^{+}/XBP1^{loxP/loxP}$ mice ($XBP1^{-/-}$). Double immunofluorescence staining displayed XBP1s positive SMCs in the lesion area of the wild-type mice (Figure 1C). Lesion area analysis revealed that in $SM22\text{-}Cre^{+}/XBP1^{loxP/loxP}$ mice ($XBP1^{-/-}$), the lumen area was larger, whereas the intima area and the ratio of intima to media were significantly decreased (Figure 1D). These results suggest that XBP1 expression and splicing in SMCs plays a role in neointimal formation.

PDGF-BB Activated XBP1 Splicing

As indicated above, fewer SMCs were observed in the neointimal lesions in the *XBP1* conditional knockout mice. Vascular injury can trigger platelet aggregation, releasing PDGF in the injured area.[33] PDGF has been demonstrated to be one of the major mitogenic growth factors for adult SMC proliferation. Therefore, we wondered whether the vascular injury–induced *XBP1* splicing was activated by PDGF. To test this, PDGF-BB was added to human aortic SMCs, followed by IRE1α phosphorylation and *XBP1* splicing assessments. As shown in Figure 2A, PDGF-BB treatment transiently increased *XBP1* splicing with a peak at 30 minutes. IRE1α/

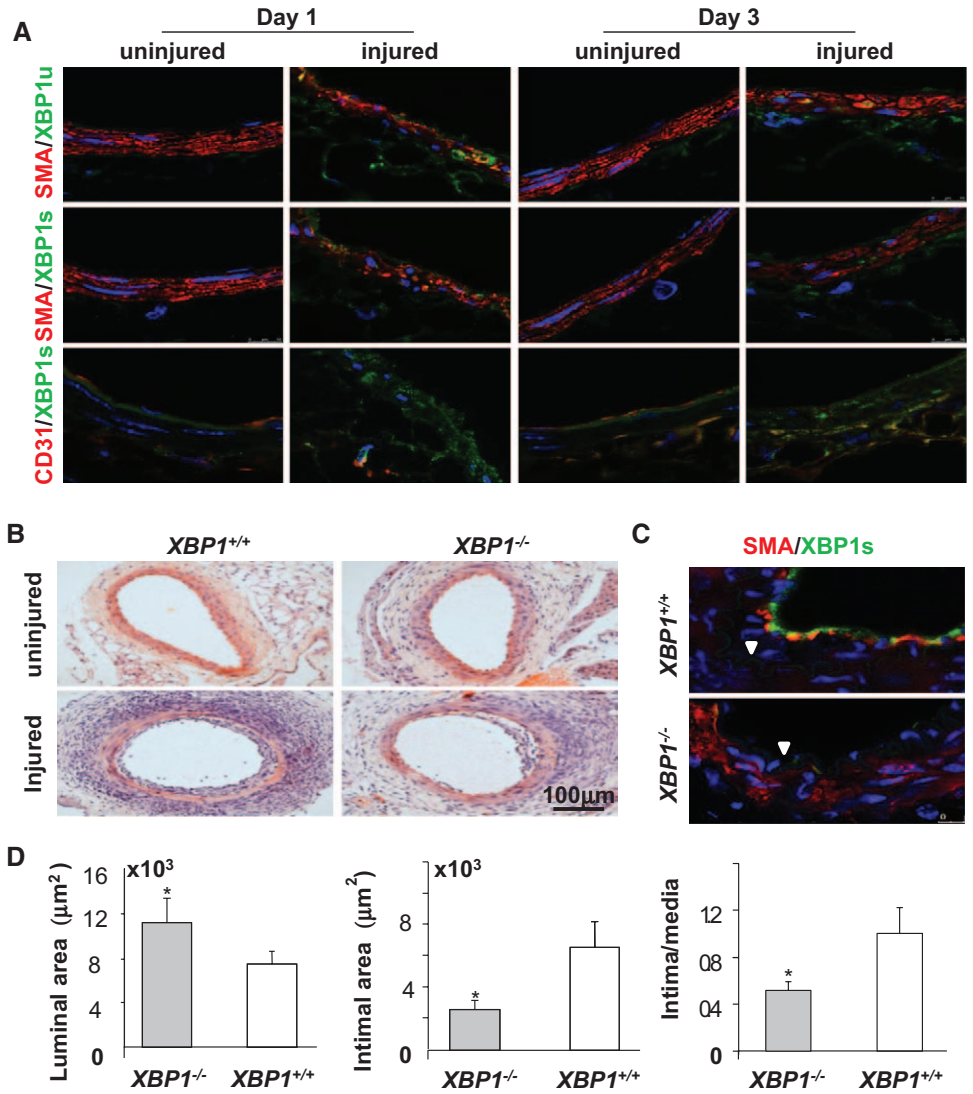

Figure 1. *XBP1* splicing was involved in vascular injury–induced neointima formation. Vascular injury was introduced into the right side of femoral arteries of C57Bl/6 mice (**A**) and the *XBP1*$^{loxP/loxP}$ (*XBP1*$^{+/+}$) and *SM22-Cre*$^{+}$/*XBP1*$^{loxP/loxP}$ (*XBP1*$^{-/-}$) mice (**B** and **C**). The left side uninjured vessels were collected as control. **A**, Vascular injury activated X-box–binding protein 1 (XBP1) expression and splicing. Double immunofluorescence staining were performed on days 1 and 3 vessel cryo-sections with anti–smooth muscle actin (SMA) (red), anti-CD31 (red), anti-XBP1s (green), or anti–unspliced XBP1 (green) antibodies. 4′,6-diamidino-2-phenylindole was used to counterstain the nucleus. **B**–**D**, *XBP1* deficiency in smooth cell muscle decreased the neointima formation. Four weeks post femoral artery injury, the injured vessels and the left side uninjured vessels were harvested and sectioned, followed by hematoxylin and eosin staining (**B**), double immunofluorescence staining with anti-SMA (red) and anti-XBP1s (green) (**C**, arrowheads showed the elastin) and analysis of the average luminal area, intima area, and the ratio of intima/media from 6 mice for each group (**D**). Data presented were representative images or mean of the numbers indicated. *P<0.05.

XBP1 splicing is 1 of the 3 major signaling pathways activated by ER disturbance.[34,35] To test whether PDGF activated *XBP1* splicing through the ER stress response, an ER stress inducer, thapsigargin, was included. As expected, the thapsigargin treatment for 4 hours induced IRE1α phosphorylation, *XBP1* splicing, activating transcription factor 6 cleavage (precursor band decrease) and protein kinase R (PKR)-like ER kinase phosphorylation (Figure 2B), showing a typical ER stress response. However, PDGF treatment only activated IRE1α phosphorylation and *XBP1* splicing without activation of the other 2 signaling pathways (Figure 2B), suggesting that PDGF may activate *XBP1* splicing in an ER stress response–independent manner.

Our previous study and others reported that vascular endothelial cell growth factor activated *XBP1* splicing through vascular endothelial cell growth factor receptor interaction with IRE1α.[26,27] To test whether a similar mechanism was involved, the interaction between PDGF receptor β (PDGFRβ) and IRE1α was first assessed by immunoprecipitation. Indeed, PDGFRβ physically interacted with IRE1α, which was enhanced by the presence of PDGF-BB (Figure 2C). PDGF treatment increased IRE1α phosphorylation at Ser724 site (Figure 2C). Glucose response protein 78 (grp78) is a chaperone molecule, associating with IRE1α under normal conditions. In response to different stimuli, IRE1α phosphorylation can be activated via grp78/IRE1α dissociation dependent or

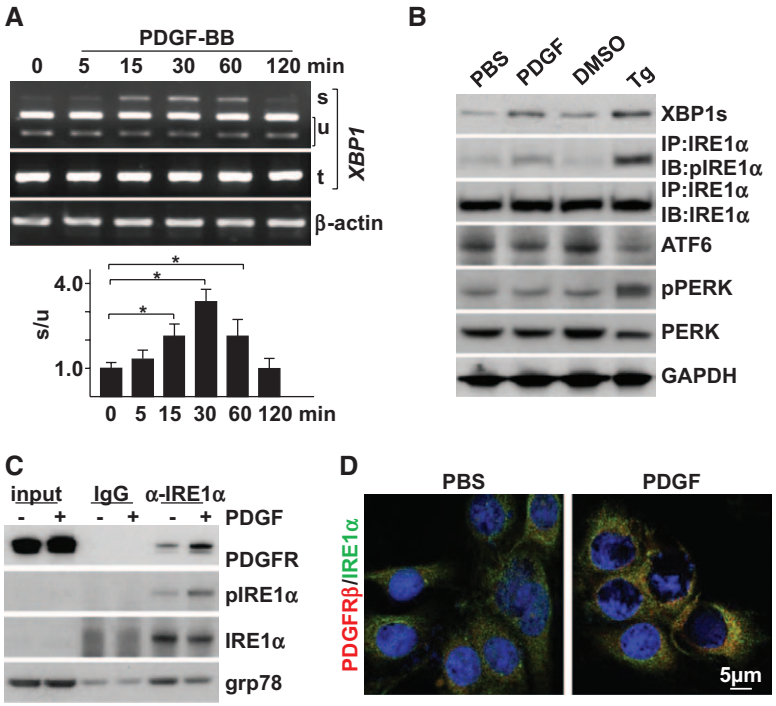

Figure 2. Platelet-derived growth factor (PDGF) activated *XBP1* splicing via interaction between PDGF receptor β (PDGFRβ) and inositol-requiring enzyme (IRE) 1α. **A**, PDGF-BB (2×10^{-5} mg/mL) transiently activated *XBP1* splicing in human aortic smooth muscle cells (HSMCs). **B**, PDGF-induced *XBP1* splicing was independent of endoplasmic reticulum stress. HSMCs were treated with 2×10^{-5} mg/mL PDGF for 30 minutes or 3×10^{-7}mol/L thapsigargin (Tg) for 4 hours, followed by Western blot analysis. Phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO) was included as vehicle control. **C**, PDGF enhanced the interaction between PDGFRβ and IRE1α as revealed by immunoprecipitation. grp78 was included as the positive control. **D**, Confocal images showed the colocalization of PDGFRβ (red) and IRE1α (green) at 30 minutes after PDGF (2×10^{-5} mg/mL) treatment. Data presented were representative images or mean of 3 independent experiments. s indicates spliced *XBP1*; XBP1, X-box–binding protein 1; t, total *XBP1*; and u, unspliced *XBP1*. *$P<0.05$.

independent way.[36,37] To test whether PDGF also induced the dissociation of grp78 and IRE1α, grp78 was detected in the immunoprecipitation samples. Indeed, PDGF increased the dissociation of grp78 and IRE1α (Figure 2C). As grp78 can bind immunoglobulin,[38] a small amount of grp78 could be detected in the IgG control. The interaction of PDGFRβ and IRE1α was further confirmed by immunofluorescence staining (Figure 2D). To define the binding domain in PDGFRβ, the truncated HA-tagged PDGFRβ plasmids were created (Figure IIIA in the online-only Data Supplement) and cotransfected with the truncated FLAG-tagged IRE1α[26] in 293 cells, followed by immunoprecipitation with anti-FLAG antibody and immunoblotting with anti-FLAG and anti-HA antibodies. The kinase domains of both proteins were responsible for their interaction (Figure IIIB in the online-only Data Supplement). These results suggest that PDGF may activate *XBP1* splicing through the interaction between IRE1α and PDGFRβ.

XBP1 Splicing Was Related to SMC Migration and Proliferation

PDGF triggers SMC migration and proliferation.[39] The above data have shown that PDGF can activate *XBP1* splicing. To explore whether XBP1 splicing is involved in PDGF-induced SMC migration and proliferation, cell counting–based proliferation and transwell-based migration assays were performed. Overexpression of XBP1s by adenovirus (Ad)-*XBP1s* gene transfer increased SMC proliferation by 20% when compared with that by Ad-null or Ad-*XBP1u* gene transfer (Figure 3A). However, the knockdown of *XBP1* (*XBP1sh*) or decreased *XBP1* splicing via knockdown of *IRE1α (IRE1αsh*) reduced SMC proliferation (Figure 3B) and abolished PDGF-induced SMC proliferation (Figure 3C). The suppressive effect of the *XBP1* deficiency on SMC proliferation was further confirmed by culturing of SMCs isolated from *XBP1*$^{loxP/loxP}$ (*XBP1*$^{+/+}$) and *SM22-Cre*$^{+}$*/XBP1* $^{loxP/loxP}$ (*XBP1*$^{-/-}$) mice (Figure IVA in the online-only Data Supplement). Ex vivo culture of the arterial rings from *XBP1*$^{loxP/loxP}$ (*XBP1*$^{+/+}$) and *SM22-Cre*$^{+}$*/XBP1* $^{loxP/loxP}$ (*XBP1*$^{-/-}$) mice revealed that the cell outgrowth was significantly retarded in the *XBP1*-deficient SMCs (Figure IVB in the online-only Data Supplement). The cell outgrowth was actually derived from the combined effect of migration and proliferation. The retardation of cell outgrowth in *XBP1*$^{-/-}$ mice not only reflected the suppressive effect on SMC proliferation but also might reflect the decrease of migration. To test this, we first observed the effect of overexpression of XBP1 on SMC migration, using a transwell model. As shown in Figure 3D, overexpression of XBP1s but not XBP1u dramatically increased SMC migration. However, further experiments with SMCs isolated from *XBP1*$^{loxP/loxP}$ (*XBP1*$^{+/+}$) and *SM22-Cre*$^{+}$*/XBP1*$^{loxP/loxP}$ (*XBP1*$^{-/-}$) mice revealed that overexpression of XBP1s had similar effect on the migration of both cell types and that there was no significant difference in the response to PDGF (Figure 3E), suggesting that XBP1 was not necessary for PDGF-induced SMC migration. These results suggest that XBP1 splicing can activate SMC migration and proliferation but is only essential for PDGF-induced proliferation.

XBP1s Increased SMC Migration Via the PI3K/Akt Pathway

It has been reported that PDGF stimulates SMC migration and proliferation through activation of multiple kinases, such as the PI3K/Akt,[40] MEK-ERK,[41] JNK,[42] and p38[43] pathways. The above data indicate that activation of *XBP1* splicing is sufficient to induce SMC migration and proliferation, although it is not necessary for PDGF-induced migration. To test which signaling pathway was involved in XBP1s-induced SMC migration and proliferation, the Akt, ERK, c-jun, and p38 phosphorylation were assessed after the overexpression of XBP1s. As shown in

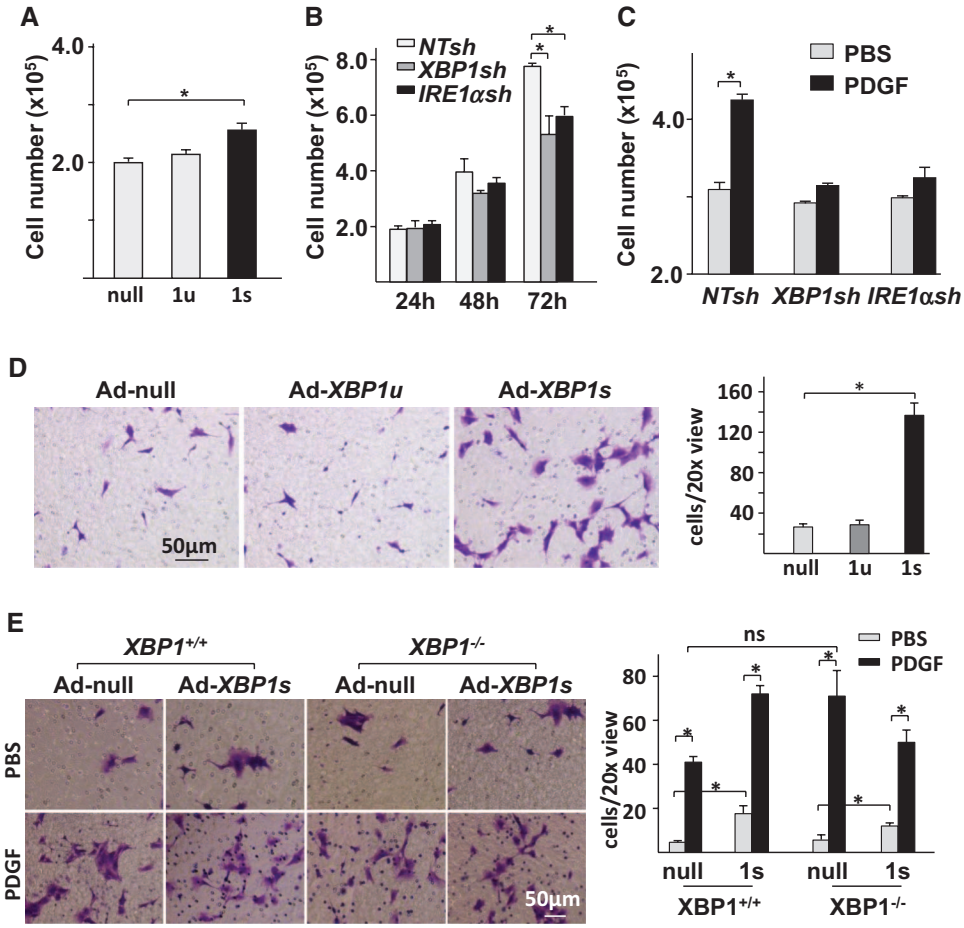

Figure 3. X-box–binding protein 1 (XBP1) splicing triggered smooth muscle cell (SMC) migration and proliferation. **A**, Overexpression of *XBP1s* via adenoviral gene transfer increased SMC proliferation. Cell counting was performed 16 hours post virus infection. **B**, Knockdown of *IRE1*α or *XBP1* by shRNA lentivirus decreased human aortic SMC (HSMC) proliferation. Nontarget shRNA lentivirus (NTsh) was included as control. **C**. Knockdown of *IRE1*α or *XBP1* attenuated platelet-derived growth factor (PDGF)–induced HSMC proliferation. D, Overexpression of XBP1s increased HSMC migration. **E**, XBP1 deficiency in SMC had no effect on PDGF-induced SMC migration. Data presented were representative images or mean of 3 independent experiments. 1u indicates Ad-*XBP1u*; 1s, Ad-*XBP1s*; Ad, adenovirus; and null, Ad-null. **P*<0.05.

Figure VA in the online-only Data Supplement, XBP1s but not XBP1u could activate Akt phosphorylation; ERK phosphorylation was decreased by XBP1s, and there was no change on c-jun and p38 phosphorylation. These results suggest that the PI3K/Akt pathway may be involved. The immunoprecipitation assays revealed that in the presence of XBP1s, more Akt was associated with PI3K (Figure VB in the online-only Data Supplement) and that XBP1s, Akt, and PI3K formed a complex (Figure VC in the online-only Data Supplement). These results suggest that XBP1s can activate the PI3K/Akt signaling pathway. To test whether the PI3K/Akt pathway was involved in XBP1s-induced SMC proliferation and migration, the PI3K/Akt inhibitor LY294002 was included in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based proliferation and the transwell-based migration assays. Interestingly, LY294002 had no effect on XBP1s-induced cell proliferation (Figure VD in the online-only Data Supplement) but significantly suppressed XBP1s-induced SMC migration (Figure VE in the online-only Data Supplement), suggesting that the PI3K/Akt signaling pathway is only involved in SMC migration. As XBP1 deficiency had no effect on PDGF-induced SMC migration, we wondered whether XBP1 was involved in PDGF-induced PI3K/Akt activation. As expected, Western blot analysis revealed that *XBP1* deficiency had no effect on PDGF-induced Akt phosphorylation although the total Akt protein level was decreased in the *XBP1*-deficient cells (Figure VF in the online-only Data Supplement). These results suggest that XBP1s may be sufficient but not necessary to activate the PI3K/Akt signaling pathway that contributes to XBP1s-induced SMC migration and that XBP1s-induced SMC migration, and proliferation are regulated by 2 different pathways.

XBP1s Increased SMC Proliferation Through Downregulation of CNN1

In addition to PI3K/Akt activation, it has been reported that downregulation of CNN1 plays an important role in SMC proliferation.[44] Western blot analysis showed that XBP1s decreased CNN1 and myocardin protein levels but had no effect on α-SMC actin and serum response factor (Figure 4A). Immunofluorescence staining revealed that XBP1s not only decreased CNN1 in the infected cells but also in the adjacent cells (Figure VIA in the online-only Data Supplement),

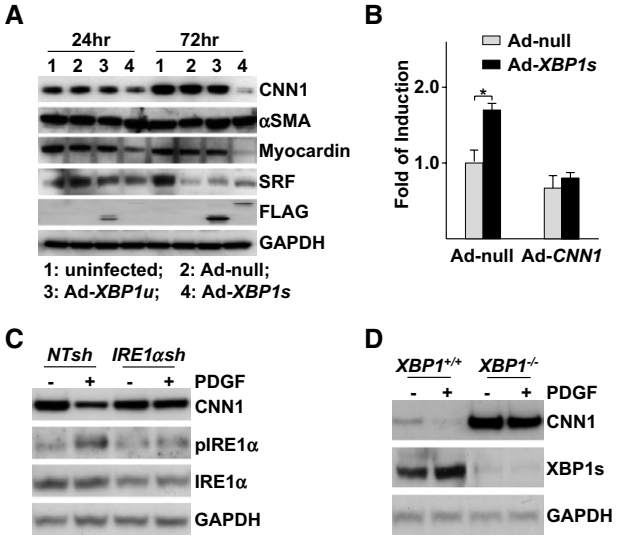

Figure 4. Spliced X-box–binding protein 1 (XBP1s) stimulated smooth muscle cell (SMC) proliferation via downregulating calponin h1 (CNN1). **A**, Overexpression of XBP1s downregulated CNN1 protein level as revealed by Western blot analysis. **B**, Reconstitution of CNN1 via adenovirus (Ad)-*CNN1* gene transfer abolished Ad-*XBP1s*–induced human aortic SMC proliferation as revealed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation assay. **C**, Knockdown of *IRE1*α via shRNA lentivirus ablated platelet-derived growth factor (PDGF)–induced downregulation of CNN1 as revealed by Western blot analysis. Nontarget shRNA lentivirus (NTsh) was included as control. **D**, *XBP1* deficiency abolished PDGF-induced CNN1 decrease in the primary mouse SMCs. SMCs were isolated from *XBP1*$^{loxp/loxp}$ (*XBP1*$^{+/+}$) or *SM22-Cre*$^{+}$/*XBP1*$^{loxp/loxp}$ (*XBP1*$^{-/-}$) mouse arteries and treated with 2×10^{-5} mg/mL PDGF-BB for 24 hours in the absence of serum, followed by Western blot analysis. Note that in *XBP1*$^{-/-}$ cells, there are high levels of CNN1 proteins. Data presented were representative images or mean of 3 independent experiments. **P*<0.05.

suggesting that CNN1 in SMC is regulated by some secreted factors, which are affected by XBP1s. To confirm the involvement of CNN1 in XBP1s-induced SMC proliferation, we created an Ad-*CNN1* viral vector. The coinfection of SMC with Ad-*XBP1s* and Ad-*CNN1* attenuated Ad-*XBP1s*–induced SMC proliferation (Figure 4B). Although LY294002 suppressed XBP1s-induced SMC migration, it had no effect on SMC proliferation. As expected, LY294002 had no effect on XBP1s-induced CNN1 decrease (Figure VIB in the online-only Data Supplement). Further experiments demonstrated that PDGF downregulated CNN1 in human aortic SMCs (Figure 4C) and the cultured mouse arterial SMCs (Figure 4D), which required functional IRE1α and XBP1. SMCs isolated from *XBP1*$^{-/-}$ mice showed retarded proliferation. The CNN1 level in *XBP1*$^{-/-}$ SMCs was dramatically higher than that in wild-type SMCs (Figure 4D; Figure VIC in the online-only Data Supplement). These results suggest that XBP1s may promote SMC proliferation through downregulating CNN1.

XBP1s Downregulated CNN1 Via *mir-1274B*-Mediated mRNA Degradation

The decrease of a protein level can be derived from decreased biosynthesis due to decreased mRNA level, or increased post-translational degradation. To explore how XBP1s downregulated CNN1 protein, we first checked the *CNN1* mRNA level.

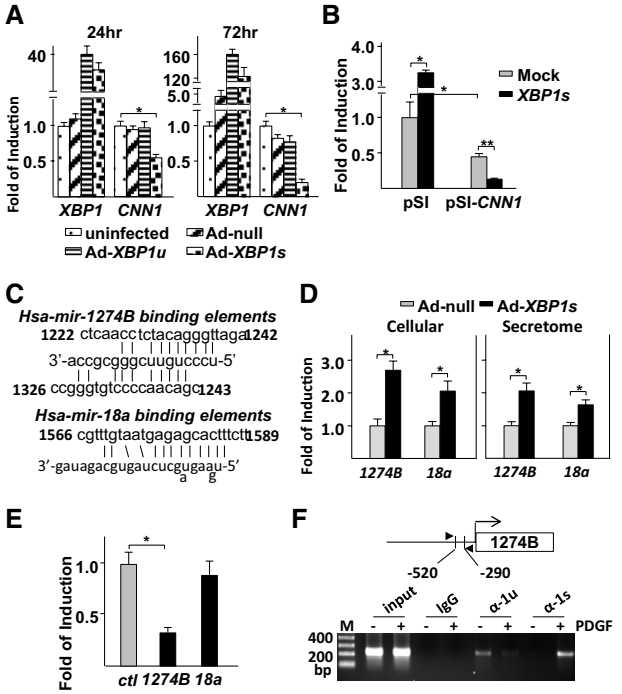

Figure 5. Spliced X-box–binding protein 1 (XBP1s) downregulated calponin h1 (CNN1) via *mir-1274B*–mediated mRNA degradation. **A**, XBP1s not unspliced XBP1 (XBP1u) decreased *CNN1* mRNA level in a time-dependent manner. **B**, Overexpression of XBP1s suppressed pSI-*CNN1* 3′-untranslated region (UTR) reporter gene expression. Mock indicates pShuttle2; pSI, pSi-check2 vector; pSI-*CNN1*, pSi-check2 vector containing *CNN1* 3′-UTR; and *XBP1s*, pShuttle2-FLAG-*XBP1s* plasmid. (**C**, Alignment of *hsa-mir-1274B* and *hsa-mir-18a* to their potential-binding sites in the 3′-UTR of *CNN1* mRNA. Numbers indicate the nucleotide positon (NM_001299). **D**, XBP1s upregulated *mir-1274B* and *mir-18a* expression and secretion. Quantitative polymerase chain reaction was performed to analyze the *mir-1274B* and *mir-18a* in the cellular RNAs or RNAs isolated from conditioned media. **E**, Pre–*mir-1274B* and not pre–*mir-18a* decreased *CNN1* mRNA in transfected smooth cell muscles. Control pre-mir (ctl) was included. **F**, Platelet-derived growth factor (PDGF) increased the binding of XBP1s to the –520 to –290 region in *mir-1274B* promoter as revealed by the anti-XBP1s (α-1s) antibody-mediated chromatin immunoprecipitation assay. Normal IgG and anti-XBP1u (α-1u) were included. Arrow showed the transcription initiation site and direction, arrowheads showed the position of the primer set. Data presented were representative images or mean of 3 independent experiments. Fold of induction was defined as data from the test group was normalized to that of the control group that was set as 1.0. **P*<0.05; ***P*<0.01.

Quantitative RT-PCR analysis showed that overexpression of XBP1s decreased the *CNN1* mRNA level in a time-dependent manner (Figure 5A). Considering that XBP1s is an intact transcription factor, we wondered if XBP1s downregulated *CNN1* transcription. A 1.3-kb *CNN1* gene promoter containing partial exon 1 was amplified by PCR and cloned into pGL3-Luc reporter system. Compared with pGL3-Luc control vector, pGL3-*CNN1*-Luc vector gave rise to high level of luciferase activity in SMCs (30-fold, Figure VIIA in the online-only Data Supplement), indicating the success of the *CNN1* promoter reporter. The overexpression of XBP1s increased pGL3-*CNN1*-Luc reporter expression by a similar effect on the pGL3-Luc basic reporter, 3-fold on both vectors (Figure VIIA in the online-only Data Supplement). In the positive control

grp78-luc reporter system, overexpression of XBP1s increased the reporter expression more than 9-fold as compared to the mock vector. Therefore, we assumed that XBP1s had no effect on pGL3-*CNN1*-Luc reporter expression. These results suggest that XBP1s-induced decrease of *CNN1* may not be through the transcriptional suppression. However, as the cloned reporter is only 1.3-kb, we cannot exclude that XBP1s may suppress *CNN1* transcription through a distal enhancer element.

As XBP1s exerted no inhibitory effect on *CNN1* transcription, the decrease of *CNN1* mRNA might be derived from mRNA degradation. The mRNA stability is mainly regulated by the *cis*-elements in the 3′ terminal untranslated region (UTR).[45] To test this, the 520-bp fragment of the 3′-UTR was amplified and cloned into pSi-check2 vector (Promega), designated as pSI-*CNN1* (Figure VIIB in the online-only Data Supplement). The pSi-check2 (mock) vector or pSI-*CNN1* was cotransfected with pShuttle2-*XBP1s* vector, followed by the reporter analysis. As expected, the insertion of *CNN1* 3′-UTR significantly reduced the ratio of the Renilla/Firefly luciferase activities (Figure 5B). Overexpression of XBP1s significantly increased pSI-check2 vector and decreased pSI-*CNN1* reporter activity (Figure 5B). Further experiments revealed that PDGF treatment also decreased the reporter activity of pSI-*CNN1*, which was blocked by knockdown of *XBP1* or *IRE1*α (Figure VIIIA in the online-only Data Supplement). These results suggest that there may be some XBP1s-regulated *trans*-elements that bind to the *cis*-elements in 3′-UTR, leading to *CNN1* mRNA degradation.

MicroRNAs (miRNAs) play an important role in the mRNA stability.[46] To explore whether the XBP1s-regulated *trans*-elements were miRNAs, miRNA arrays were performed, using Taqman miRNA A/B cards from Life Technologies. A total of 764 miRNAs were analyzed. The XBP1s-regulated miRNAs with 2-fold of changes were listed in Figure VIIIB in the online-only Data Supplement. Through the electronic analysis of the potential miRNA target sequences with RNAwalk software, we found that *mir-18a* (1570–1586, NM_001299) and *mir-1274B* (1226–1239 and 1239–1242, NM_001299) had 1 and 2 potential-binding sites in the 3′-UTR of *CNN1* mRNA, respectively (Figure 5C). Quantitative RT-PCR analysis confirmed that overexpression of *XBP1s* increased both *mir-18a* and *mir-1274B* expression (Figure 5D left). The analysis of miRNAs in cell culture medium revealed that XBP1s also increased the secretion of these 2 miRNAs (Figure 5D right). Transfection of SMCs with miRNA mimics showed that *mir-1274B* but not *mir-18a* decreased the *CNN1* mRNA level (Figure 5E), suggesting that *mir-1274B* is involved in *CNN1* mRNA stability regulation. With the pSI-*CNN1* reporter system, *mir-1274B* exerted a similar effect as *XBP1s* did (Figure IXB and IXC in the online-only Data Supplement). There are 2 potential *mir-1274B*-binding sites in the 3′-UTR of *CNN1* (highlighted sequences in Figure VIIB in the online-only Data Supplement). To confirm the involvement of *mir-1274B* in *CNN1* mRNA regulation, PCR-based mutagenesis was performed to create 2 mutant pSI-*CNN1* plasmids, designated as pSI-*CNN1m1* and pSI-*CNN1m2*, respectively, in which the *mir-1274B*-binding site was mutated by substitution of nucleotides (Figure IXA in the online-only Data Supplement).

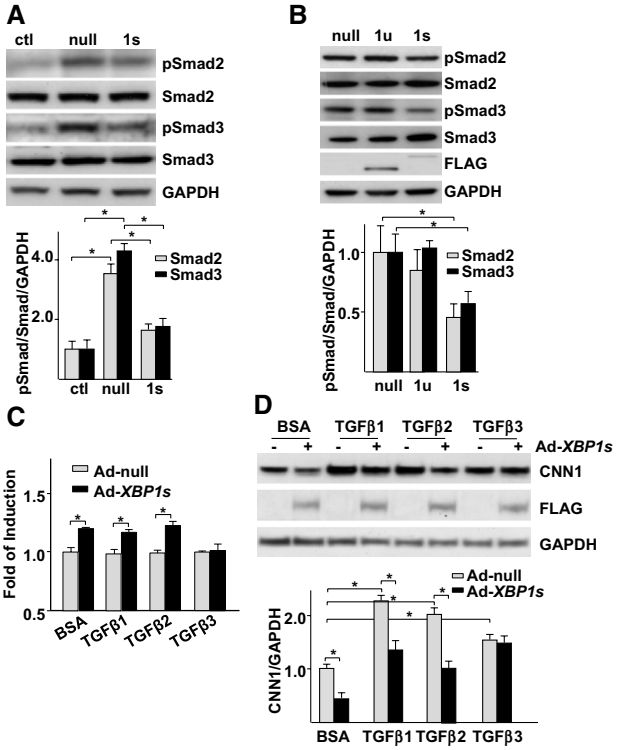

Figure 6. Transforming growth factor (TGF)-β3 ablated X-box–binding protein 1 (XBP1)–induced calponin h1 (CNN1) decrease and smooth muscle cell (SMC) proliferation. **A**, Overexpression of XBP1s decreased TGF-βs secretion. Conditioned media were collected from adenovirus (Ad)-null (null) or Ad-*XBP1s (1s)*–infected SMCs and applied to human umbilical vein endothelial cells, followed by Western blot analysis of the Smad2/3 phosphorylation. Normal culture medium (ctl) was included as control. **Bottom**, The densitometry analysis of the phospho-Smad2/3 against Smad2/3 and GAPDH with that of the control group set as 1.0. **B**, Overexpression of XBP1s decreased the TGF-β signaling in the infected SMCs. Ad-null or Ad-*XBP1u* was included as control. 1u: Ad-*XBP1u*; 1s: Ad-*XBP1s*. **Right**, The densitometry analysis of the phospho-Smad2/3 against Smad2/3 and GAPDH with that of the Ad-null group set as 1.0. **C**, TGF-β3 abolished Ad-*XBP1s*–induced SMC proliferation as revealed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. **D**, TGF-β3 abolished Ad-*XBP1s*–induced decrease of CNN1 protein. **Right**, The densitometry analysis of CNN1 against GAPDH with that of the bovine serum albumin (BSA)/Ad-null group set as 1.0. Data presented were representative images or mean of 3 independent experiments. **P*<0.05.

Compared with pSI-*CNN1*, pSI-*CNN1m1* and pSI-*CNN1m2* had much higher Renilla/Firefly ratio in the mock plasmid (pShuttle2) cotransfected cells (Figure IXB in the online-only Data Supplement), suggesting that *mir-1274B* plays an important role in the degradation of *CNN1* mRNA. Loss of *mir-1274B*-binding sites will stabilize *CNN1* mRNA. As expected, with the pSI-*CNN1m1* and pSI-*CNN1m2* mutant reporters, the overexpression of XBP1s (Figure IXB in the online-only Data Supplement) and *mir-1274B* (Figure IXC in the online-only Data Supplement) had no suppressive effect on the reporter expression any more. In the *mir-1274B* overexpression assay, the negative control pre-mir had some inhibitory effects on the mutant pSI-*CNN1m1* and pSI-*CNN1m2* when compared with that in pShuttle2 experiments (Figure IXC in the online-only Data Supplement). One possibility was that the pre-mir

had an unexpected side effect to induce some factors targeting the mutant-binding sites, resulting in the decreased stability of the mutant reporter mRNA. This caused a significant increase between the pre–*mir-1274B* and pre–*mir-ctl* on the mutant pSI-*CNN1m1* and pSI-*CNN1m2,* showing a positive effect of pre–*mir-1274B* on the mutant reporters (Figure IXC in the online-only Data Supplement). Chromatin immunoprecipitation assays confirmed that XBP1s bound to the –529 to –290 region of the *mir-1274B* promoter in the presence of PDGF (Figure 5F). As expected, XBP1u also bound to this region, which was decreased by PDGF treatment (Figure 5F). These results suggest that XBP1 splicing decreases *CNN1* mRNA stability at least, in part, through the upregulation of *mir-1274B* transcription.

XBP1s Downregulated TGF-β Signaling

We have observed that overexpression of XBP1s not only decreased CNN1 protein level in the infected cells but also in the adjacent cells. This phenomenon suggests that XBP1s may affect the secretion of some factors. To identify the potential factors, proteomics analyses were performed. Conditioned media were collected from Ad-null or Ad-*XBP1u* or Ad-*XBP1s*–infected SMCs, followed by concentration and SDS–PAGE separation. Gel bands were subjected to proteomics analysis.[47] We have detected 64 proteins with difference among the infected cells, categorized into 5 groups (Figure X in the online-only Data Supplement). Interestingly, the TGF-βs family was significantly downregulated by XBP1s but not by XBP1u. To confirm the proteomics data and the involvement of TGF-βs in XBP1s-induced SMC proliferation, we first checked the effect of the conditioned medium from SMCs on the TGF-β signaling in human umbilical vein ECs. It has been well established that TGF-βs exert functions through TGF-β receptors-mediated Smad phosphorylation such as Smad2/Smad3, which in turn, translocate into the nucleus with the assistance of the common Smad mediator, Smad4, and direct target gene transcription.[48] Compared with the normal medium, the conditioned medium from Ad-null–infected SMCs upregulated both Smad2 and Smad3 phosphorylation (Figure 6A). However, the conditioned medium from Ad-XBP1s–infected SMCs significantly reduced both Smad2 and Smad3 phosphorylation when compared with that from Ad-null group (Figure 6A). Further experiments confirmed that overexpression of XBP1s also decreased the TGF-β signaling in SMCs as revealed by the decrease of both Smad 2 and Smad3 phosphorylation (Figure 6B). Quantitative RT-PCR analysis revealed that XBP1s decreased *TGF-β1* to *TGF-β3* mRNA levels, whereas XBP1u decreased *TGF-β1* and *TGF-β2* mRNA levels (Figure XIA in the online-only Data Supplement). Both XBP1u and XBP1s had no effect on the latent TGF-binding protein 1 mRNA level.

TGF-βs are known to suppress SMC proliferation and induce stem/progenitor cells differentiation toward SMC lineage. Thus, we wondered whether the downregulation of TGF-βs contributed to XBP1s-induced SMC proliferation. To test this, the exogenous TGF-βs were included in the proliferation assays. As shown in Figure 6C, TGF-β3 not TGF-β1/TGF-β2 abolished XBP1s-induced SMC proliferation. Western blot analysis revealed that all 3 TGF-βs upregulated CNN1 protein levels but only TGF-β3 ablated XBP1s-induced CNN1 decrease (Figure 6D). Immunofluorescence staining revealed that the TGF-β3 protein level was higher in *XBP1*$^{-/-}$ SMCs when compared with that in *XBP1*$^{+/+}$ SMCs (Figure XIB left in the online-only Data Supplement) and that the exogenous TGF-β3 treatment could restore CNN1 structure in Ad-*XBP1s*–infected SMCs (Figure XIB right in the online-only Data Supplement). These results suggest that the decrease of TGF-β3 may contribute to the decrease of the CNN1 protein level and the increase of SMC proliferation. Quantitative RT-PCR analysis showed that TGF-β3 treatment could not restore *CNN1* mRNA level (Figure XIC in the online-only Data Supplement), suggesting that TGF-β3 may regulate CNN1 protein stabilization through post-translational modification. The inhibitor assays revealed that the proteasome inhibitor ALLN could abolish XBP1s-induced CNN1 decrease (Figure XID in the online-only Data Supplement), suggesting that XBP1s may also activate the proteasome-mediated degradation of CNN1 protein.

Discussion

Here, we observed that XBP1 expression and splicing were significantly upregulated in SMCs in response to vascular injury and that the neointimal lesions in the injured vessels were markedly reduced in the *XBP1*-SMC conditional knockout animals, which was related to the decreased numbers of SMC accumulation. As vascular injury triggers platelet aggregation in the injury area, PDGF is released that could activate SMC proliferation, leading to neointima formation. In this study, we demonstrated for the first time that PDGF activated *XBP1* splicing through the interaction of PDGFRβ and IRE1α, which was sufficient to increase SMC migration and essential for PDGF-induced SMC proliferation. The spliced XBP1 triggered SMC migration via the PI3K/Akt pathway and activated SMC proliferation through downregulating CNN1 protein level via upregulating *mir-1274B*-mediated *CNN1* mRNA degradation or downregulating TGF-β3 expression and secretion. Thus, these findings provide a novel mechanism on SMC proliferation and neointimal formation.

Vascular injury triggers media SMCs activation and inflammatory reactions.[49] The activated SMCs undergo migration, proliferation, and apoptosis.[50] The rapid response of *XBP1* expression and splicing in SMCs to vascular injury suggests that XBP1 may be involved in these processes. Our previous study has demonstrated that *XBP1* splicing can contribute to EC apoptosis and atherosclerosis development.[30] However, in this study, we did not observe SMC apoptosis after XBP1s overexpression. The major role of XBP1 in SMC activation may be migration and proliferation. Considering that in vivo is different from in vitro, we cannot exclude that XBP1 splicing may be involved in SMC apoptosis under vascular injury.

SMC migration from the media to the intima and the following proliferation are key steps in the pathogenesis of vascular diseases, such as atherosclerosis.[51] During these processes, the Akt phosphorylation plays a central role. The Akt phosphorylation is derived from the increased PI3K activation or decreased phosphatase and tensin homolog dephosphorylation.[52,53]

Suppression of PI3K reduces neointima formation,[54] whereas suppression of phosphatase and tensin homolog increases SMC hyperplasia.[55] In this study, we found that XBP1 splicing contributed to neointima formation via regulating SMC migration and proliferation. High levels of *XBP1* expression and splicing could be detected immediately post vascular injury, whereas *XBP1* deficiency in SMC significantly reduced neointima formation. The XBP1s formed a complex with PI3K and Akt, leading to Akt phosphorylation and SMC migration. However, this signaling pathway seemed not involved in XBP1s-induced proliferation. The Akt phosphorylation inhibitor, LY294002, abolished XBP1s-induced SMC migration but exerted no effect on XBP1s-induced proliferation.

PDGF is a well-known mitogenic factor for mature SMC proliferation.[56] Several signaling pathways have been reported to be activated by PDGF/PDGFRβ binding, such as mitogen-activated protein kinases, PI3K/Akt, and basic fibroblast growth factor releasing.[5,11,12] In this study, we have identified a novel signaling pathway after PDGF stimulation, that is, the IRE1α-mediated *XBP1* splicing. Our hypothesis is that on the binding of PDGF, a vesicle containing PDGF/PDGFRβ may be formed via endocytosis with the cytoplasmic domain of PDGFRβ protruding into cytoplasm. The vesicle traffics to ER membrane where the PDGFRβ cytoplasmic domain binds to IRE1α, activating IRE1α dimerization and autophosphorylation, which in turn triggers *XBP1* mRNA splicing. The spliced XBP1 exerts stimulatory effect on SMC proliferation. Knockdown of *IRE1α* or *XBP1* attenuated the basal level and PDGF-induced SMC proliferation, suggesting that IRE1α-mediated *XBP1* splicing plays an important role in SMC proliferation. Indeed, disruption of the *XBP1* gene in SM22 positive cells significantly retarded cell proliferation in vitro and attenuated vascular injury–induced neointima formation. Taken together, vascular injury increases PDGF release that triggers IRE1α phosphorylation and *XBP1* splicing. The spliced XBP1, in turn, activates SMC proliferation, leading to neointima formation.

Different from the vascular endothelial cell growth factor-*XBP1* splicing signaling pathway in ECs,[26] XBP1-induced SMC proliferation is independent of Akt, although overexpression of XBP1s itself could upregulate Akt phosphorylation. Deficiency of XBP1 or knockdown of IRE1α had no effect on PDGF-induced Akt phosphorylation. In SMCs, *XBP1* splicing actually activates proliferation through downregulating CNN1 protein. Reconstitution of CNN1 protein via Ad-*CNN1* gene transfer attenuates XBP1s-induced SMC proliferation, whereas the *XBP1*-deficient SMCs possess high level of CNN1 proteins with reduced proliferation potency. CNN1 is a basic actin-binding protein, featured as a specific marker for SMC differentiation.[57,58] CNN1 may contribute to the maintenance of cytoskeleton in SMC, keeping SMCs at the differentiation and quiescent state. Decrease of CNN1 protein may disrupt this cytoskeleton homeostasis, leading to cell activation. Indeed, reduced level of CNN1 protein was observed in tumor cells and proliferating SMCs, whereas upregulation of CNN1 suppressed SMC proliferation.[18,59,60] Our study also confirmed the negative relationship between CNN1 protein level and SMC proliferation. The slowly proliferating *XBP1*-deficient cells possessed higher level of CNN1, and overexpression of CNN1 via Ad-*CNN1* gene transfer reduced SMC proliferation. The *XBP1* splicing plays an essential negative regulating role in CNN1 expression. Overexpression of XBP1s downregulated CNN1 at mRNA and protein levels, whereas *XBP1* or *IRE1α* deficiency abolished PDGF-induced downregulation of CNN1 protein. Robust level of CNN1 protein was observed in the *XBP1* knockout SMCs. Being a transcription factor, XBP1s seems not involved in *CNN1* transcription. Actually, XBP1s regulates *CNN1* mRNA stability via upregulation of *mir-1274B* and CNN1 protein stability. In the latter process, TGF-β3 may be involved.

miRNAs were identified as negative regulators of mRNA stability or protein translation in the past decade, mainly targeting the 3′-UTR of mRNA molecules.[61] In this study, we found that XBP1s upregulated and downregulated several miRNAs in SMCs, among which *mir-1274B* seemed involved in *CNN1* mRNA stability regulation. Transfection of the *mir-1274B* mimics downregulated *CNN1* mRNA. The *mir-1274B* could also be secreted into culture medium, which might function as a paracrine factor to downregulate *CNN1* mRNA in the adjacent cells. The *mir-1274B* was originally identified from a genome sequencing project, sharing structure with tRNA (lys3).[62] Recent studies showed that *mir-1274B* was upregulated in cutaneous malignant melanoma while downregulated in chemoradiotherapy-treated advanced rectal cancer, implying that *mir-1274B* may be related to tumor growth.[63,64] Report from Shinozuka et al[65] showed that *mir-1274B* was regulated by SnoN/SKIL and negatively related to esophageal cancer cell proliferation. In this study, we demonstrate that *CNN1* is a target of *mir-1274B*, and that XBP1s is involved in the transcriptional regulation of *mir-1274B*. Chromatin immunoprecipitation assay revealed that XBP1s directly bound to the −520 to −290 region in *mir-1274B* promoter.

TGF-β is a superfamily of growth factors, exerting diverse roles in different cell types. It is well established that TGF-βs stimulate stem/progenitor cells differentiation toward SMCs.[66] In this study, we found that XBP1s downregulated the *TGF-β1* to *TGF-β3* mRNA levels, TGF-β signaling and TGF-βs secretion in SMCs. Thus, the XBP1s-induced SMC proliferation may be because of the decrease of TGF-β–mediated differentiation in part. As expected, all the 3 TGF-βs upregulated the CNN1 protein level, suggesting their differentiation roles. However, only TGF-β3 could antagonize XBP1s-induced CNN1 decrease and SMC proliferation. Therefore, TGF-β3 may cross talk with XBP1s to regulate SMC differentiation and proliferation. As proteasome inhibitor ALLN has similar effect as TGF-β3, there may be cross talk between TGF-β3 and proteasome activation. However, the underlying mechanisms in this cross talk need further investigation.

In summary, vascular injury may trigger PDGF release in the injured area. On SMCs, PDGF binds to PDGFRβ, leading to PDGFRβ interaction with IRE1α, which in turn activates IRE1α phosphorylation and *XBP1* mRNA splicing. On one hand, XBP1s activates the PI3K/Akt pathway, increasing SMC migration. On the other hand, XBP1s activates the transcription of *mir-1274B* that targets and increases *CNN1* mRNA degradation, leading to the decrease of CNN1 protein and SMC proliferation. The overall effect contributes to

the neointima formation (Figure XII in the online-only Data Supplement).

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Disclosures

None.

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Significance

This study provides new insights into the mechanisms underlying smooth muscle cell migration and proliferation and neointima formation, in which the X-box–binding protein 1 cross talk with the platelet-derived growth factor signaling pathways plays an essential role. Targeting X-box–binding protein 1 splicing may provide a therapeutic strategy to tackle neointima formation-related vascular diseases.