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X-box binding protein 1–mediated COL4A1s secretion regulates communication between vascular smooth muscle and stem/progenitor cells

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Vascular smooth muscle cells (VSMCs) contribute to the deposition of extracellular matrix proteins (ECMs), including Type IV collagen, in the vessel wall. ECMs coordinate communication among different cell types, but mechanisms underlying this communication remain unclear. Our previous studies have demonstrated that X-box binding protein 1 (XBP1) is activated and contributes to VSMC phenotypic transition in response to vascular injury. In this study, we investigated the participation of XBP1 in the communication between VSMCs and vascular progenitor cells (VPCs). Immunofluorescence and immunohistology staining revealed that *Xbp1* gene was essential for type IV collagen alpha 1 (COL4A1) expression during mouse embryonic development and vessel wall ECM deposition and stem cell antigen 1-positive (Sca1$^+$)-VPC recruitment in response to vascular injury. The Western blot analysis elucidated an *Xbp1* gene dose-dependent effect on COL4A1 expression and that the spliced XBP1 protein (XBP1s) increased protease-mediated COL4A1 degradation as revealed by Zymography. RT-PCR analysis revealed that XBP1s in VSMCs not only upregulated *COL4A1/2* transcription but also induced the occurrence of a novel transcript variant, soluble type IV collagen alpha 1 (*COL4A1s*), in which the front part of exon 4 is joined with the rear part of exon 42. Chromatin-immunoprecipitation, DNA/protein pulldown and *in vitro* transcription demonstrated that XBP1s binds to exon 4 and exon 42, directing the transcription from exon 4 to exon 42. This leads to transcription complex bypassing the internal sequences, producing a shortened COL4A1s protein that increased Sca1$^+$-VPC migration. Taken together, these results suggest that activated VSMCs may recruit Sca1$^+$-VPCs *via* XBP1s-mediated COL4A1s secretion, leading to vascular injury repair or neointima formation.

Vascular smooth muscle cells (VSMCs), the predominant cells in tunica media of artery, retain remarkable plasticity in the postnatal period and can switch between differentiated and dedifferentiated phenotypes in response to physiological and pathological cues such as vascular injury, hypertension, and atherosclerosis (1–3). Recent studies from various groups have demonstrated that the local resident vascular progenitor cells (VPCs) from tunica adventitia can differentiate into VSMCs, contributing to neointima formation (4, 5). However, the interaction between the existing activated VSMCs and VPCs recruitment remains unclear.

The type IV collagen is one of the major components of the basement membrane, where it provides functions such as membrane stability and physical barrier between endothelium and VSMCs (6). Besides the basement membrane, type IV collagen also exists in the extracellular matrix of different tissues. It is ubiquitously expressed and secreted by many cell types including VSMCs. A noncollagenous (NC) domain exists in the C-terminal of type IV collagen alpha chains including COL4A1, which can be released *via* matrix metalloproteinase (MMP)-mediated degradation and facilitate signal transduction *via* integrins (7, 8). The type IV collagen is also widely used as flask-coating reagents for stem/progenitor cell differentiation toward vascular cell lineages (9, 10). Thus, type IV collagen is involved in VSMC and VPC intercellular communication.

The X-box binding protein 1 (XBP1) is a transcription factor that is upregulated under stress conditions to promote cell survival (11). Under these conditions, the unspliced *XBP1* mRNA undergoes unconventional splicing in the cytosol (12, 13). The inositol-requiring enzyme 1 alpha (*IRE1α*)

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‡ These authors contributed equally to this study and should be considered joint first author.

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recognizes two stem-loop structures on *XBP1* mRNA and cleaves using its ribonuclease activity (14, 15) giving rise to active spliced *XBP1* (*XBP1s*) mRNA. Upon translation and subsequent translocation of the XBP1s protein into the nucleus, it activates the transcription of genes such as endoplasmic reticulum chaperones to promote cell survival (16). Our previous studies have demonstrated that XBP1s plays an important role during neointima formation leading to atherosclerosis development by acting on vascular endothelial and smooth muscle cells (17, 18).

In this study, we report that the activation of XBP1 splicing in VSMCs trigger the production of a soluble type IV collagen alpha 1 (COL4A1s), which may contribute to VPC recruitment to the injury site.

Results

XBP1 is essential for COL4A1 expression during embryonic development and vascular remodeling

It is well known that VSMCs undergo contractile to synthetic phenotype transition following vascular injury, contributing to extracellular matrix deposition in the vessel wall. Our previous study has shown that *XBP1* splicing is involved in vascular injury–mediated VSMCs activation (18). To examine whether there exists a relationship between XBP1, COL4A1, and VPC migration, we detected COL4A1 expression and VPC migration in XBP1-deficient mouse models. As shown in Figure 1*A* (upper and middle panel), the platinum wire scratching-mediated endothelium denudation induced neointima formation, during which alpha smooth muscle actin and stem cell antigen 1-positive cells were recruited to the neointima area. *XBP1* deficiency in VSMCs decreased those positive cells recruitment and neointima formation. Importantly, *XBP1* deficiency in VSMCs significantly reduced COL4A1 expression in the vessel wall (Fig. 1*A*, lower panel). Further experiments with E10.5 embryos from $XBP1^{+/-}$ X $XBP1^{+/-}$ crossbreeding, respectively, revealed that *XBP1* deficiency decreased COL4A1 expression in a gene dose-dependent manner, as demonstrated by immunohistological staining (Fig. 1*B*), quantitative RT-PCR (Fig. 1*C*), and Western blot analysis (Fig. 1*D*). Taken together, these results suggest that XBP1 plays an important role in the regulation of COL4A1 mRNA and protein expressions.

Overexpression of the spliced XBP1 increased the occurrence of short COL4A1 protein

Our results above show that the XBP1 gene is essential for COL4A1 expression during embryonic development and in response to vascular injury. The two genes encoding *COL4A1* and *COL4A2* are located head-to-head in chromosome 13, sharing the 127 bp promoter (19, 20). The quantitative RT-PCR analysis revealed that overexpression of the XBP1s increased both *COL4A1* and *Col4A2* mRNA levels (Fig. 2*A*). Western blot with anti-COL4A1 antibody detected some smaller bands in both conditioned medium (i, ii, iii, and iv) and cell lysates (i*, ii*, iii*, and iv*) (Fig. 2*B*). The 100 kDa COL4A1 band is much less in cell lysate as compared with that in conditioned medium (Fig. 2*B*), which may be due to secretion. Strikingly, the 100 kDa band in XBP1s group existed in very

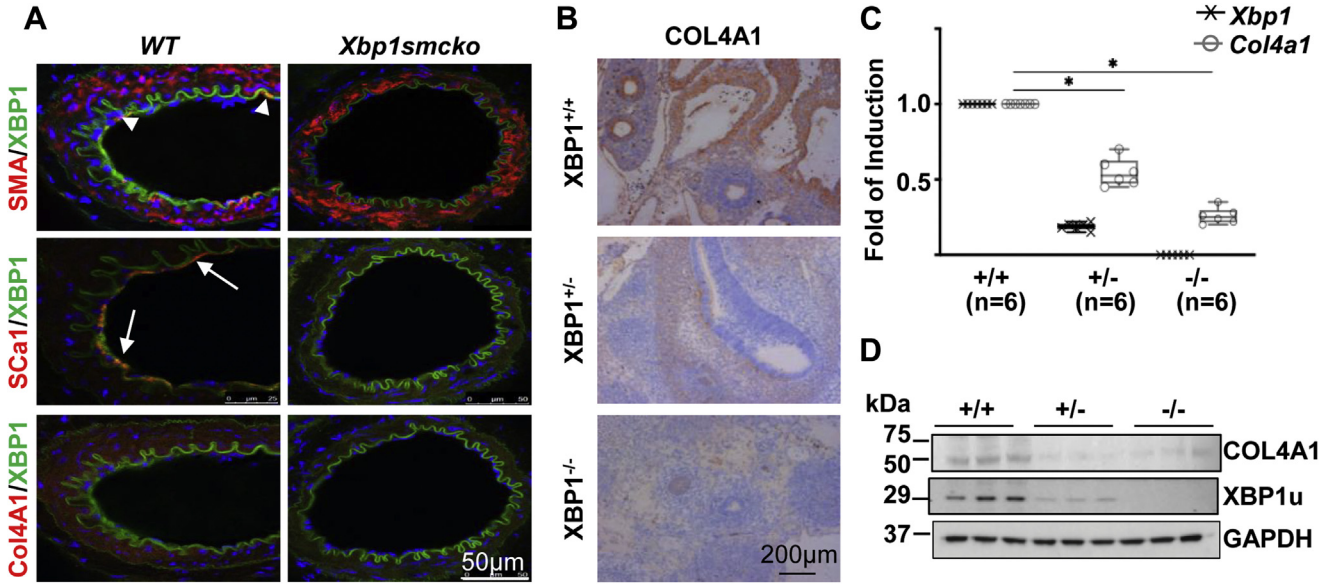

Figure 1. XBP1s was essential for COL4A1 expression during embryonic development and vascular remodeling. *A*, *Xbp1* deficiency in VSMCs decreased COL4A1 expression in the vessel wall and SMC/VPC recruitment in the lesion area. Femoral artery injury model was introduced into wild type (WT, $Xbp1^{loxP/loxP}$) and smooth muscle cell–specific knockout (*Xbp1smcko, SM22-Cre/*$XBP1^{loxP/loxP}$) mice. Double immunofluorescence staining with antibodies against XBP1 (*green*) and COL4A1 (*red*) or Sca1 (*red*) or SMA (*red*) were performed on cryo-sections isolated 2 weeks postsurgery. *Arrow* and *Arrowhead* indicate migrated VPCs and SMCs, respectively. *B–D*, *Xbp1* deficiency decreased COL4A1 expression in a gene dose dependent manner. Embryos were harvested from the crossbreeding between global knockout heterozygous $Xbp1^{+/-}$ at E10.5 stages, followed by immunostaining with anti-COL4A1 antibody on cryosections (*B*), quantitative RT-PCR (*C*, fold of induction was defined as the ratio of the gene mRNA level of the transgenic mice to that of WT mice set at 1.0), and Western blot analysis (*D*). Data presented as box and whisker plots or representative images shown from six embryos or mice per group. *: $p <$ 0.05. COLA1, type IV collagen alpha 1; $Sca1^{+}$, stem cell antigen 1-positive; VPC, vascular progenitor cell;VSMC, vascular smooth muscle cell; XBP1, X-box binding protein 1; XBP1s, spliced XBP1 protein.

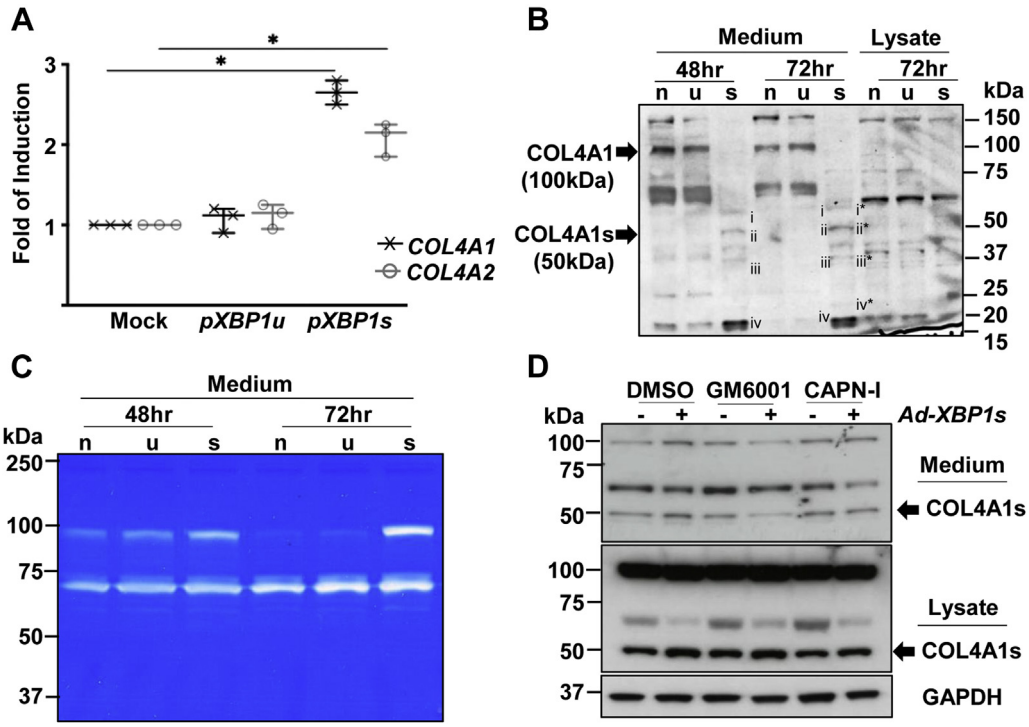

Figure 2. Spliced XBP1 induced a novel COL4A1s isoform. *A*, overexpression of XBP1s increased both *COL4A1* and *Col4A2* mRNA levels. HVSMCs were transfected with *pShuttle2* (Mock), *pShuttle2-XBP1u* (*pXBP1u*) and *pShuttle2-XBP1s* (*pXBP1s*) for 36 h, followed by quantitative RT-PCR. Fold of induction was defined as the ratio of COL4A1 or Col4A2 to GAPDH (internal control) to that of mock group set at 1.0. *B*, overexpression of XBP1s induced multiple smaller bands of COL4A1 in the conditioned medium (i, ii, iii, and iv). Similar size bands were also observed in cell lysate (i*, ii*, iii*, and iv*). n: *Ad-null*, u: *Ad-XBP1u*, s: *Ad-XBP1s*. The 100 kDa COL4A1 and 50 kDa COL4A1s were indicated. *C*, overexpression of XBP1s activated proteases in conditioned medium as revealed by zymography. n: *Ad-null*, u: *Ad-XBP1u*, s: *Ad-XBP1s*. *D*, protease inhibitors did not inhibit the occurrence of short 50 kDa COL4A1 protein. HVSMCs were infected with *Ad-XBP1s* at 10 multiplicity of infection in the presence of matrix metalloproteinase inhibitor (GM6001, 10 μmol/L) or calpain inhibitor (CAPN-I, 10 μmol/L) for 48 h, followed by Western blot analysis (conditioned medium or cell lysate). Data presented as box and whisker plots or representative images shown from three independent experiments. *: $p < 0.05$. COLA1s, soluble type IV collagen alpha 1; HVSMC, human smooth muscle cell line; XBP1s, spliced XBP1 protein; XBP1, X-box binding protein 1.

little amount with increased smaller bands as compared with other two groups, indicating that XBP1s may increase the degradation of COL4A1 noncollagenous (NC) domain in medium (21, 22). However, a 50 kDa COL4A1s protein band (ii) was upregulated in XBP1s overexpressed conditioned medium (Fig. 2*B*), which is different in size from the bands derived from the degradation of the NC domain of COL4A1. Further Zymography assays revealed that overexpression of XBP1s increased proteases secretion (Fig. 2*C*). MMP and Calpain (CAPN) are proteases with molecular weight around 100 kDa which can degrade type IV collagen (21, 22). Thus, we examined whether MMP inhibitor GM6001 or CAPN inhibitor CAPN-I could abolish this XBP1s-induced short 50 kDa COL4A1s protein expression. The Western blot analysis of both conditioned medium and cell lysate showed that the induction of this short COL4A1 protein was not inhibited by these protease inhibitors (Fig. 2*D*). There is difference in COL4A1 bands in conditioned medium and cell lysates between Figure 2, *B* and *D*, which may be derived from the effect of dimethyl sulfoxide (0.1%v/v) that needs further investigation. However, the upregulation of the 50 kDa band by XBP1s was consistent in both conditions. Taken together, these results suggest that the 50 kDa COL4A1s protein may be derived from mechanisms other than proteases-mediated degradation.

Overexpression of XBP1s induced the transcription of a soluble isoform

Several proteases have shown to degrade COL4A1 NC domain which has a molecular weight <40 kDa (8, 21). Thus, we wondered whether the XBP1s-induced 50 kDa protein band was a novel COL4A1 isoform. To investigate, we designed a primer set targeting the first exon (exon 1) and the last exon (exon 52) of *COL4A1,* respectively, to perform a routine RT-PCR. A 1.6 kb band, which was increased by XBP1s overexpression, was amplified instead of the expected 5kb band of *COL4A1* (Fig. 3*A*). The subsequent DNA sequencing demonstrated that this PCR product was derived from a novel transcript variant, in which the front part of exon 4 and the rear part of exon 42 was joined together with the open reading frame unchanged (Fig. 3*B*). This novel transcript variant can also undergo translation to produce a 553 amino acid protein, designated as soluble COL4A1s where the signal peptide, 7S domain, and NC domain remain unchanged while the internal helical domain is shortened (Fig. 3*B*). Following 293 cells transfection with *pS2-COL4A1s* vector containing COL4A1s coding sequence and FLAG-tag downstream of 7S domain (Fig. 3*C*, upper), the conditioned medium was collected for incubation with anti-FLAG agarose beads (10 μl and 50 μl) (Fig. 3*C*, lower). The Western blot analysis using

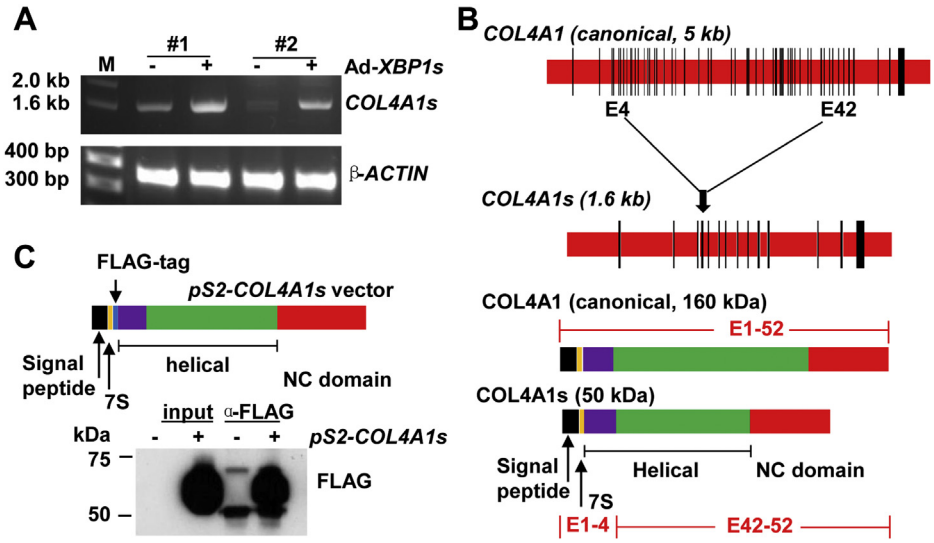

Figure 3. XBP1s induced a soluble COL4A1s isoform. *A*, overexpression of XBP1s induced a short *COL4A1* transcript variant as revealed by RT-PCR with a primer set located in exon 1 and exon 52 (#1 and #2, two independent experiments). *B*, a schematic illustration of COL4A1 and COL4A1s mRNA and protein structures. COL4A1s is derived from the joining of the front part of exon 4 and the rear part of exon 42 with internal parts removed. COL4A1s protein retains the signal peptide, 7S domain and NC domain with internal helix domain shortened. *C*, the engineered COL4A1s isoform could be expressed and purified from 293 cells. The *upper panel* show schematic illustration of the COL4A1s vector *pS2-COL4A1s* containing a FLAG-tag downstream of the 7S domain. The *lower panel* show that COL4A1s with FLAG-tag could be secreted in the conditioned medium by *pS2-COL4A1s* transfected 293 cells and concentrated by anti-FLAG antibody pulldown with pS2 vector as control. One milliliter conditioned medium was concentrated with Ultracel-10k (input) and anti-FLAG-magnetic beads (α-FLAG), respectively, followed by Western blot analysis with anti-FLAG antibody. Representative images are from three independent experiments. COLA1s, soluble type IV collagen alpha 1; NC, noncollagenous; XBP1s, spliced XBP1 protein; XBP1, X-box binding protein 1.

anti-FLAG antibody detected 50 kDa COL4A1s protein from these anti-FLAG agarose beads. The full-length mRNA sequence of this novel transcript variant (start and stop codons highlighted in red color) and its amino acid sequence are provided in Figure 4, *A* and *B*, respectively. Additionally, these sequences have also been submitted to GenBank database (Accession: MN310603 and QIP67963 for mRNA and protein sequences, respectively)

The novel transcript variant COL4A1s might be derived through an intron-bypass mechanism

To understand the mechanisms underlying the occurrence of *COL4A1s* transcript variant, we analyzed the DNA sequence within exon 4 and 42. We found that there are nearly identical sequences existing in exon 4 and 42, designated as loxP-like sequences lplE4 and lplE42, respectively (Fig. 5*A*). These loxP-like sequences can form stem-loop structure (Fig. 5*B*), like that by XBP1 mRNA (11). Thus, we hypothesized that *COL4A1s* occurred either through a) IRE1α-mediated unconventional splicing as observed in XBP1 mRNA splicing (11) or b) through an intron-bypass mechanism similar as *Cre-loxP* recombination. To investigate, we first performed a modified chromatin-immunoprecipitation assay, in which IRE1α and XBP1 bound RNA and DNA were isolated using anti-IRE1α and anti-XBP1 antibodies. This was followed by reverse transcription and routine PCR analysis with primer sets flanking exon 4 and exon 42, respectively. As shown in Figure 5*C*, both IRE1α and XBP1s proteins bound to exon 4 but not exon 42 region of COL4A1 mRNA. As expected, XBP1s but not IRE1α bound to exon 4 and exon 42 regions in the chromatin (Fig. 5*D*). Moreover, the binding capacity of IRE1α and XBP1s to mRNA and DNA sequence of exon 4 was enhanced in the presence of platelet growth factor (PDGF) stimulation. The DNA binding was verified a DNA-protein pulldown assay. The biotin-labeled lplE4 (btn-E4) and lplE42 (btn-E42) double stranded DNA probes were incubated with *Ad-XBP1s*-infected human smooth muscle cell line (HVSMC) cell lysate and pulled down by streptavidin-magnetic beads, followed by Western blot with anti-FLAG (to detect XBP1s) and anti-IRE1α antibodies. As shown in Figure 5*E*, both lplE4 and lplE42 elements bound to XBP1s but not to IRE1α as expected. Afterward, we created a reporter vector, pSI-lplE4-Ren-lplE42 (Fig. 5*F*, upper), where the lplE4 and lplE42 elements were inserted upstream and downstream respectively of Renilla luciferase gene (1.0kb) in pSI-check2 vector. The unconventional splicing or recombination between the lplE4 and lplE42 elements will excise Renilla, leading to a decrease in its luciferase activity. Indeed, overexpression of XBP1s decreased Renilla luciferase activity that could not be rescued by the addition of IRE1α inhibitor, 4μ8c (Fig. 5*F*, lower). This suggests that the IRE1α-mediated unconventional splicing mechanism may be not involved. To examine the intron-bypass mechanism hypothesis, we created another vector, pSI-lplE4-LacZ-lplE42, where the Renilla luciferase gene of pSI-lplE4-Ren-lplE42 was replaced by the LacZ coding sequence (3.5 kb), increasing the sequence length between the two elements (Fig. 5*G*, upper). Using these plasmids, we performed an *in vitro* transcription assay with nuclear extracts from *Ad-XBP1s*-infected HVSMCs, followed by RNA extraction and RT-PCR with a primer set flanking the two elements (p1 and p2 in Fig. 5*G*, upper). If a recombination occurs between these two elements, the primer set will amplify an 80 bp

A

***COL4A1s* mRNA sequence**

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   1 gcttggagcc gccgcacccg ggacggtgcg tagcgctgga agtccggcct tccgagagct agctgtccgc cgcggccccc
  81 gcacgccggg cagccgtccc tcgccgcctc gggcgcgcca ccatgggccc ccggctcagc gtctggctgc tgctgctgcc
 161 cgccgccctt ctgctccacg aggagcacag ccgggccgct gcgaagggtg gctgtgctgg ctctggctgt ggcaaatgtg
 241 actgccatgg agtgaaggga caaaagggtg aaagaggcct cccggggtta caaggtgtca ttgggtttcc tggaatgcaa
 321 ggacctgagg ggccacaggg accaccagga caaaagggtg atactggaga accaggaatt cctggatcca aaggagagca
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1681 tagagaggag cgagatgttc aagaagccta cgccgtccac cttgaaggca ggggagctgc gcacgcacgt cagccgctgc
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3201 a
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B

COL4A1s amino acid sequence

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PGIPGSKGEQGFMGPPGPQGQPGLPGSPGHATEGPKGDRGPQGQP
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CPSGWSSLWIGYSFVMHTSAGAEGSGQALASPGSCLEEFRSAPFIEC
HGRGTCNYYANAYSFWLATIERSEMFKKPTPSTLKAGELRTHVSRCQ
VCMRRT
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Figure 4. The full-length mRNA (*A*) and amino acid (*B*) sequence of the COL4A1s isoform. The start and stop codons were highlighted in red color. These sequences have been release by GenBank database (Accession: MN310603 and QIP67963 for mRNA and Protein sequences, respectively).

PCR product. Indeed, nuclear extracts from *Ad-XBP1s*-infected HVSMC produced an 80 bp PCR product from pSI-lplE4-LacZ-lplE42 (Fig. 5*G*, lower) but not from pSI-lplE4-RenlplE42 (data not shown). These results suggest that the occurrence of recombination between the lplE4 and lplE42 elements may be dependent on size of the internal sequence.

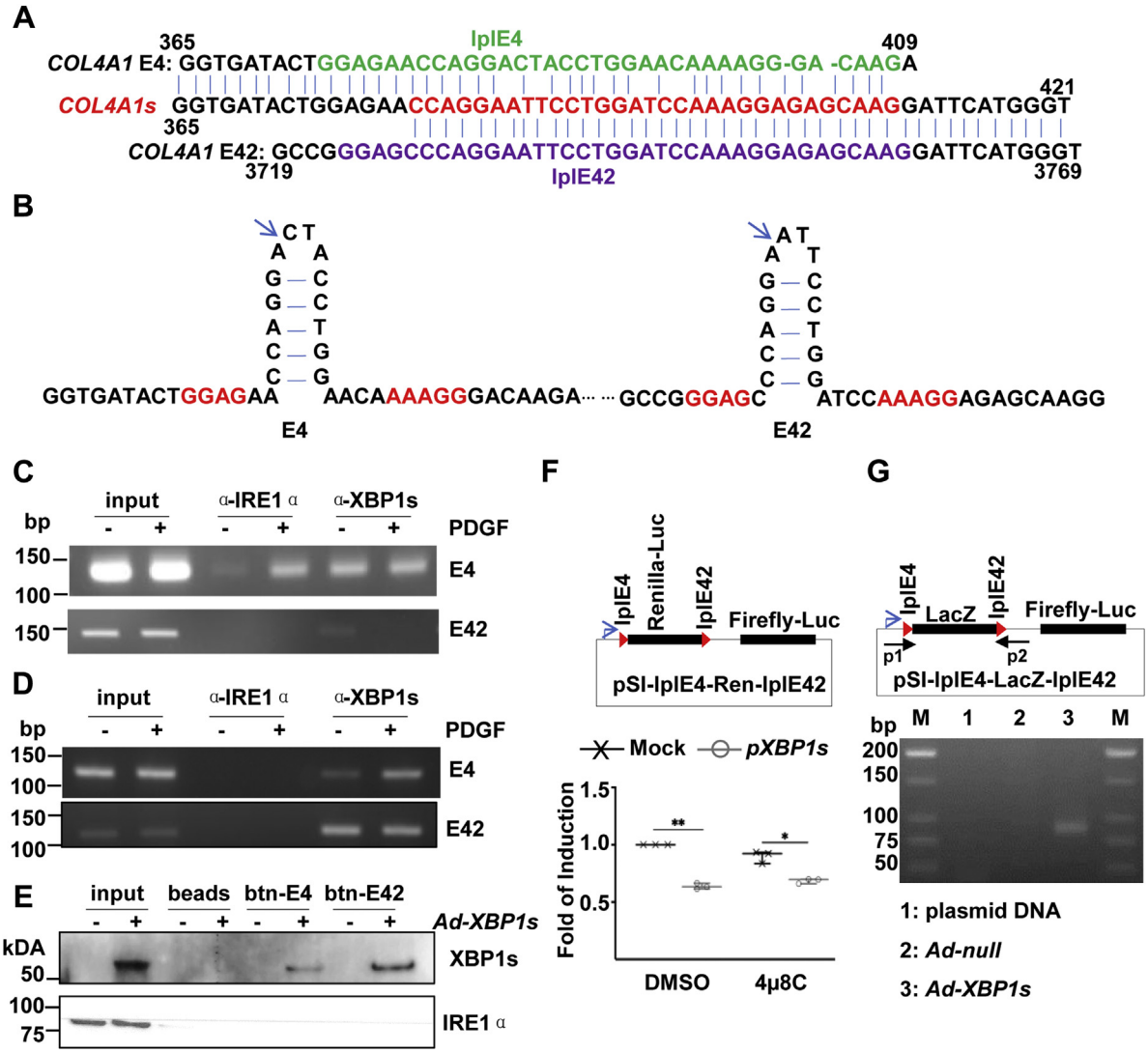

Figure 5. XBP1s might induce COL4A1s *via* IRE1α-mediated unconventional splicing or loxP-like element-mediated intron-bypass transcription. *A*, a schematic illustration of the potential loxP-like element in exon 4 (IplE4, in *green*) and exon 42 (IplE42, in *purple*) with the joint sequence in COL4A1s in *red*. Numbers show the nucleotide positions. *B*, a schematic illustration of the stem-loop structures from exon 4 and exon 42 respectively. *Blue arrows* indicate the potential cleavage sites. *C*, IRE1α and XBP1s could physically associate with exon 4 but not exon 42 region of COL4A1 mRNA as revealed by modified chromatin-immunoprecipitation assay and RT-PCR. *D* and *E*, XBP1s but not IRE1α bound to exon 4 and exon 42 DNA as revealed by routine chromatin-immunoprecipitation assay (*D*) and DNA-protein pulldown assay (*E*). btn-E4 and btn-E42 were biotin-labeled IplE4 and IplE42 double stranded DNA probes respectively. *F*, IRE1α inhibitor 4μ8C had no effect on XBP1s-induced pSI-IplE4-Ren-IplE42 reporter expression decrease. HVSMCs were transfected with pSI-IplE4-Ren-IplE42 reporter together with *pShuttle-XBP1s* (*pXBP1s*) in the presence of 10 μmol/L 4μ8C, followed by dual luciferase assays. *pShuttle-GFP* and dimethyl sulfoxide were included as mock and vehicle control. Fold of induction was defined as the ratio of Renilla-Luc to firefly-Luc with that of Mock/dimethyl sulfoxide group set as 1.0. *G*, XBP1s might induce COL4A1s *via* IplE4 and IplE42-mediated recombination. *In vitro* transcription was performed with linearized pSI-IplE4-LacZ-IplE42 and nuclear extracts from *Ad-null* or *Ad-XBP1s* infected HVSMCs. The linearized pSI-IplE4-LacZ-IplE42 plasmid was included as PCR control. Data presented as box plot and whiskers or representative images shown from three independent experiments. *: $p < 0.05$; **: $p < 0.01$. COLA1, type IV collagen alpha 1; HVSMC, human smooth muscle cell line; *IRE1α,* inositol-requiring enzyme 1 alpha; VSMC, Vascular smooth muscle cell; XBP1, X-box binding protein 1; XBP1s, spliced XBP1 protein.

COL4A1s protein increased VPC migration

As described above, the XBP1 gene deficiency in VSMCs reduced stem cell antigen 1-positive cell recruitment in the injured vessels, suggesting that XBP1 activation in VSMC may produce paracrine factors to mobilize VPCs. To test this, we collected the conditioned medium from *Ad-XBP1s*-infected HVSMCs and transferred it to transwell migration assay with murine VPCs. As shown in Figure 6*A*, the conditioned medium from the *Ad-XBP1s*-infected HVSMCs significantly increased VPC migration as compared with *Ad-null*-infected HVSMCs. It has been reported that the NC domains derived from collagen IV, XV, XVIII exert versatile roles in cell migration (7, 23, 24). To understand the effect of these collagens, the conditioned medium was precleared with IgG (control), anti-COL4A1, anti-Col15A1, and anti-Col18A1 antibodies before VPC treatment. Only anti-COL4A1 antibody significantly attenuated XBP1s-HVSMC medium–induced VPC migration as compared with IgG control, while this was not observed in Col15A1 or Col18A1 (Fig. 6*B*). These results

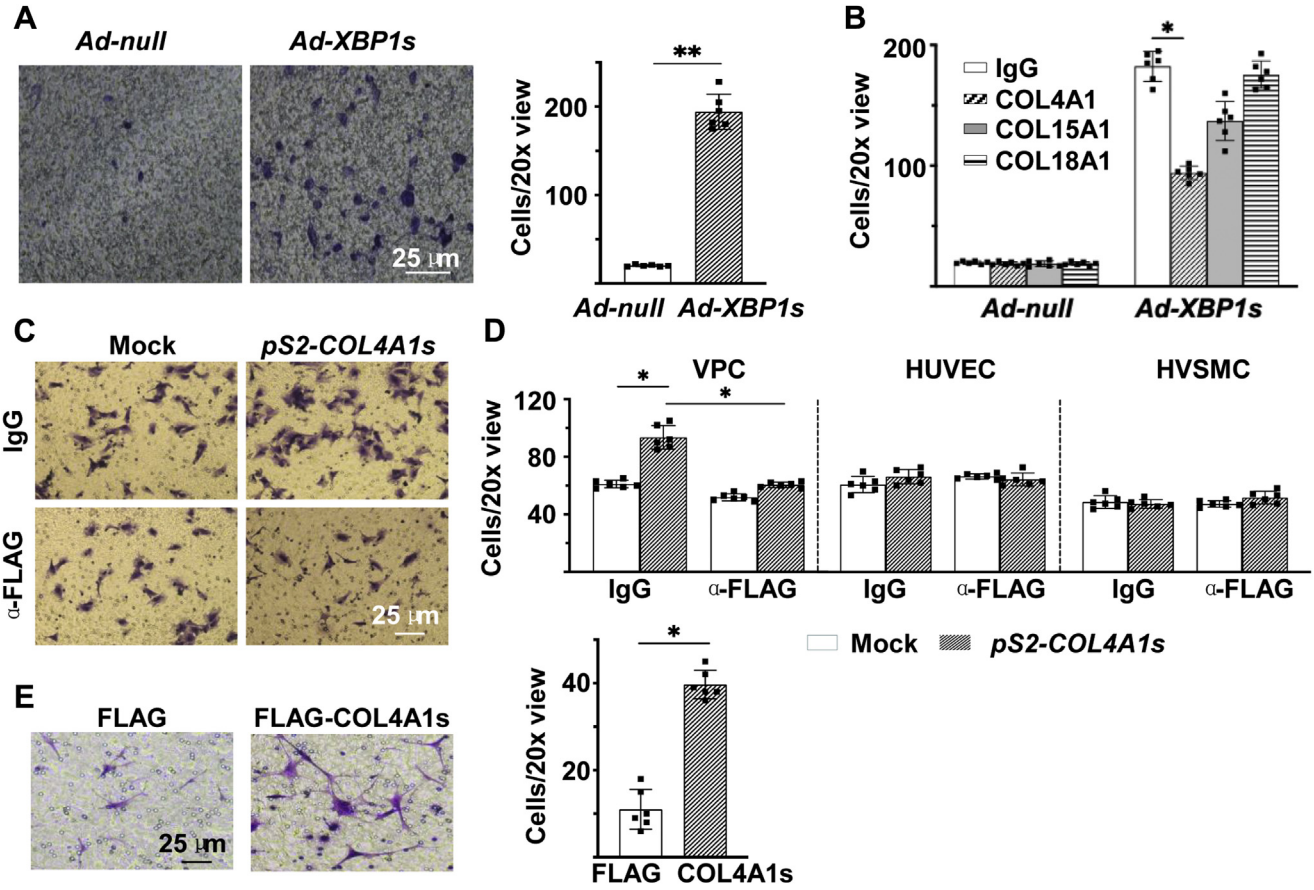

Figure 6. COL4A1s induced VPC migration. *A* and *B*, overexpression of XBP1s in HVSMC induced conditioned medium promoted VPC migration. HVSMCs were infected with *Ad-null* or *Ad-XBP1s* for 24 h. Conditioned medium was then collected in the following 48 h. Transwell migration assays were performed on VPCs using the condition medium. A, representative images of migrated cells (*Left panel*) and VPC migration data shown as a histogram (*Right panel*). *B*, the conditioned medium was depleted with anti-COL4A1, anti-Col15A1, and anti-Col18A1 antibodies before transwell migration assays on VPCs. IgG was included as control. *C* and *D*, Exogenous COL4A1s increased VPC migration. No change observed in HUVEC and HVSMC migration. Two hundred ninety-three cells were transfected with either *pS2-COL4A1s* vector or *pShuttle2-GFP* (Mock) as control. The conditioned medium was depleted with IgG or anti-FLAG agarose beads (α-FLAG), followed by transwell migration assays on VPCs, HUVECs, and HVSMCs. *C*, show the representative images of migrated VPC cells. *D*, show histogram representation of migrated VPCs, HUVECs and HVSMCs. *E*, the purified COL4A1s promote VPC migration. COL4A1s was purified using anti-FLAG-agarose beads incubation with *pS2-COL4A1s*-transfected 293 cells conditioned medium and subsequent FLAG peptide mediated elution. This was followed by transwell migration assay on VPCs. FLAG peptide was included as control. *Left panel* shows the representative images, and the right panel shows migrated VPCs as histogram representation. Data are presented as mean ± SD from six independent experiments or representative images of six views from 20x microscope. *: $p < 0.05$; **: $p < 0.01$. COLA1, type IV collagen alpha 1; HUVEC, human umbilical vein EC; HVSMC, human smooth muscle cell line; VPC, vascular progenitor cell; VSMC, vascular smooth muscle cell; XBP1, X-box binding protein 1; XBP1s, spliced XBP1 protein.

suggest that XBP1 splicing in VSMC can modulate VPC migration in a paracrine manner at least partly through type IV collagen.

To further examine whether this effect on VPC migration was because of COL4A1s isoform, we transfected the *pS2-COL4A1s* vector into 293 cells and collected the conditioned medium containing FLAG-COL4A1s. The results shown in Figure 6*C* demonstrated that *pS2-COL4A1s* conditioned medium increased VPC migration when compared with Mock conditioned medium. This effect on VPC migration by COL4A1s was attenuated by anti-FLAG agarose beads mediated FLAG-COL4A1s depletion. By contrast, the *pS2-COL4A1s* conditioned medium did not promote cell migration in human umbilical vein ECs (HUVECs) and HVSMCs (Fig. 6*D*). Additionally, to confirm this stimulating effect of COL4A1s on cell migration, COL4A1s was purified from the *pS2-COL4A1s*-transfected 293 cells conditioned medium *via* affinity column using anti-FLAG agarose-beads and subsequent FLAG peptide elution. As shown in Figure 6*E*, the purified COL4A1s also significantly increased VPC migration.

Discussion

Type IV collagen is one of the major components of the basement membrane and extracellular matrix in multiple tissues and organs. The type IV collagen family consist of six distinct genes that are organized into three sets: COL4A1/Col4A2, Col4A3/Col4A4, and Col4A5/Col4A6 genes, which locate within the different chromosome and possess independent regulation mechanism in each set. The two genes of each set are located head-to-head, sharing the same promoter. The COL4A1 and Col4A2 genes are in chromosome 13, sharing a 127 bp promoter. In this study, we demonstrate that XBP1 regulates COL4A1/Col4A2 gene transcription and induces a novel soluble isoform (COL4A1s) which is involved in VSMC and VPC intercellular communication, working as a paracrine cytokine.

XBP1s induces soluble COL4A1s isoform

The 127 bp promoter of COL4A1/Col4A2 genes contains the CCAAT and GC islet elements for transcription factors CEBPs, NF-Y, and Sp1 binding (25). In our study, the global knockout of *XBP1* gene decreased COL4A1 expression in the whole embryo in a gene dose-dependent manner. In VSMC-specific knockout mice, decreased COL4A1 in the vessel wall was observed. These observations indicate that XBP1 is an essential transcription factor for the COL4A1 gene transcription. XBP1 does not seem to directly bind to the promoter region, as the overexpression of XBP1s had no effect on pGL3-127bp-luc reporter gene expression (data not shown). Thus, XBP1 may bind to a distal enhancer element. Indeed, in this study, we have identified such an enhancer element (Fig. 5*A*), which exists as nearly identical sequences in both exon 4 and exon 42. However, the core consensus element needs to be defined by further detailed investigation.

Another important and interesting finding of this study is that the overexpression of XBP1s induced a novel COL4A1s isoform, in which the front part of exon 4 and rear part of exon 42 are joined together. The joint site seems different to the conventional GT-AG splicing rule (26, 27). As illustrated in Figure 5*B*, the elements in exon 4 and exon 42 can form a pair of stem-loop structure as observed in *XBP1* mRNA which is needed for IRE1α-mediated unconventional splicing. Therefore, there is a possibility that *COL4A1* mRNA is another unconventional splicing target of IRE1α, as we have demonstrated the binding of IRE1α to COL4A1 mRNA at exon 4 through a modified chromatin immunoprecipitation assay. Although in our artificial pSI-lplE4-Ren-lplE42 reporter system, IRE1α inhibitor 4μ8C could not rescue XBP1s-induced reporter gene expression decrease, we cannot exclude this possibility of COL4A1 splicing in living cells. This is because the observed decrease in reporter gene expression might be because of physical blockade caused by XBP1s binding to lplE4 element in front of Renilla, thus preventing its translation.

The lplE4 and lplE42 elements are nearly identical, in a similar way to LoxP. As XBP1s can form homodimer *via* its N terminal (28), it is plausible that XBP1s can bind to these elements. This brings both exon 4 and exon 42 together, directing RNA polymerase to transcribe RNA from exon 4 directly to exon 42 bypassing the internal part. This mechanism is termed as intron-bypass transcription (Fig. 7). The preliminary data from the pSI-lplE4-LacZ-lplE42 reporter system support this hypothesis. As the relatively smaller pSI-lplE4-Ren-lplE42 reporter system did not give the same 80 bp PCR product observed in pSI-lplE4-lplE42-LacZ reporter system, therefore, the intron-bypass transcription mechanism may only apply to the transcription of larger introns. We hypothesize that the main advantage of this transcription mechanism is energy saving by avoiding the unnecessary unwinding of internal chromatin, transcription, and subsequent degradation of the unwanted internal RNA fragment. In COL4A1s case, the internal ∼33kb fragment was bypassed. As to the exact mechanisms involved in this intron-bypass model, a further detailed investigation is required.

All type IV collagen proteins have similar domain structures that include a signal peptide for secretion, a short N-terminal NC 7S domain (7S), a long internal triple helical collagenous domain, and a C-terminal NC domain. In basement membrane, two COL4A1 subunits and one Col4A2 subunit form a heterotrimer. Using the NC domain heads, two heterotrimers are dimerized, and these dimers form a superstructure *via* their 7S domains resulting in the collagen IV network (29). Mutations in COL4A1 gene cause basement membrane structural defect, leading to cardiocerebral vascular disease, such as coronary heart disease and hemorrhagic stroke (30, 31). The C-terminal NC domain can bind to integrins on target cells, modulating multiple cellular functions including cell migration (32, 33). In this study, we found that XBP1s could induce a novel COL4A1s isoform, which retains the signal peptide, 7S and C-terminal NC domain with the internal helix domain largely shortened. This isoform is secreted and exists as a soluble form. It can function as a paracrine cytokine but different from the NC domain fragments such as arresten that is derived from the protease-mediated degradation. It has been shown that arresten suppresses cell migration *via* integrins (34). In contrast, we found that COL4A1s isoform promoted stem/progenitor cell migration. It may bind to integrins *via* NC-domain, but the longer N-terminal may be involved in interaction with other cell surface components. Further detailed investigation will be required to decipher COL4A1s cellular functions.

Endothelial dysfunction or endothelium denudation is the initial step of multiple vascular diseases. Beyond the endothelium, the basement membrane functions a dynamic scaffold, maintaining the vessel wall homeostasis including mechanical support, permeability, and interaction with other cellular components. COL4A1s retains the main structural features of canonical COL4A1 protein, except the internal helix domain is shortened. It is possible that COL4A1s can still be incorporated into collagen IV superstructure. On this occasion, owing to the shortened internal helix domain within COL4A1s, the unbound helix domains in the other COL4A1 and Col4A2 of the heterotrimer will protrude out. The resulting superstructure may be organized more densely with plenty of protruding loops, increasing mechanical support forces, and decreasing permeability. Further studies will be required to confirm the incorporation of COL4A1s into the superstructure.

It has been shown that mutations in COL4A1 and Col4A2 cause multiple diseases in humans, whereas COL4A1 and Col4A2 deficiency (*COL4A1*$^{-/-}$ *and Col4A2*$^{-/-}$) induce mouse embryonic lethality at mid-gestation exhibiting various defects (35). It was reported that *XBP1* deficiency induced embryonic lethality at mid-gestation with defects in liver and cardiovascular system (36, 37). In this study, we found that XBP1 deficiency dramatically decreased COL4A1 expression in mouse embryos, suggesting that the deficiency in COL4A1 expression may contribute to the XBP1 deficiency–induced embryonic lethality.

In summary, vascular injury by endothelium denudation triggers thrombosis, releasing PDGF. PDGF may trigger *XBP1* splicing in VSMCs. The XBP1s protein will direct *COL4A1* and *COL4A2* transcription *via* binding to enhancers within exon 4

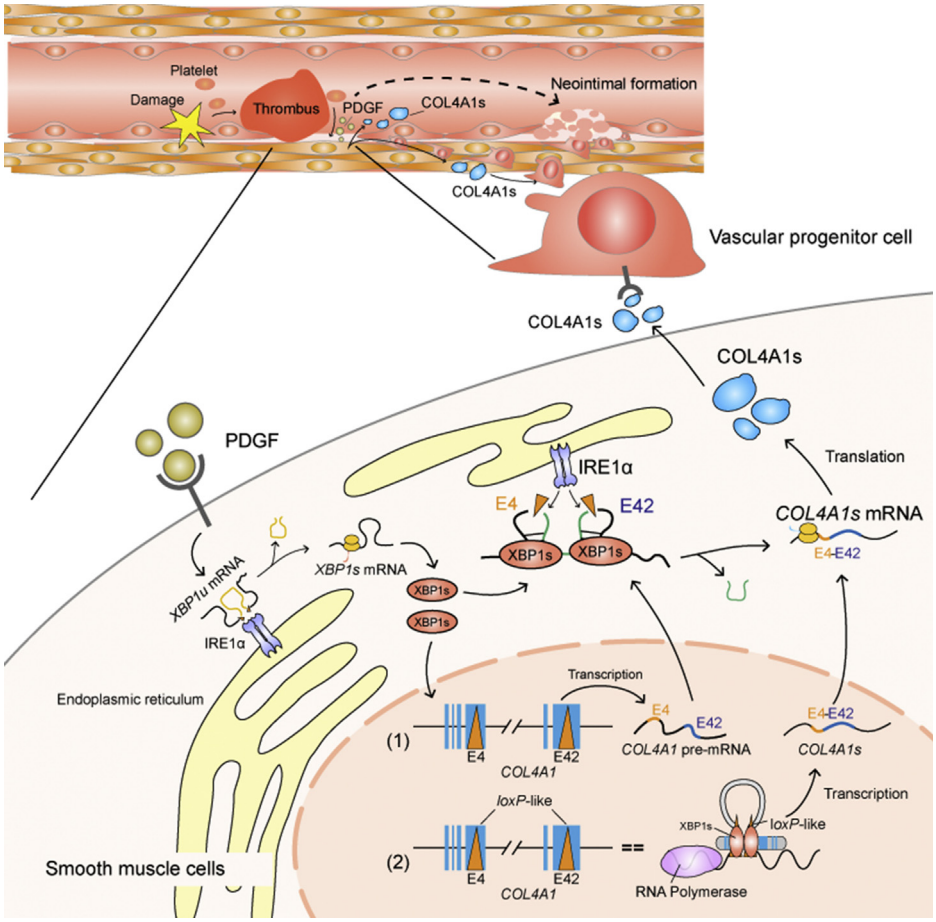

Figure 7. The schematic diagram of mechanisms on XBP1s-mediated COL4A1s production in VSMC and promoting VPC migration in response to vascular injury. The denudation of endothelium triggers thrombosis, releasing platelet derived growth factor PDGF. PDGF activates *XBP1* mRNA unconventional splicing to produce the transcription active isoform XBP1s. On one hand, XBP1s binds to remote enhancer element in exon 4 and exon 42, increasing COL4A1 transcription. The XBP1s protein may bind to COL4A1 mRNA and direct it to IRE1α, which induces an unconventional splicing between exon 4 and 42, leading to the novel spliced isoform *COL4A1s*. On the other hand, Exon 4 and 42-bound XBP1s dimerizes and directs RNA polymerase transcription from exon 4 to exon 42 bypassing the internal sequences, generating a short transcript variant, *COL4A1s*. The translation of the COL4A1s mRNA produces a soluble COL4A1s protein, which is secreted into extracellular environment. The COL4A1s protein may function as a chemoattractant to mobilize vessel wall resident stem/progenitor cells, which in turn are involved in injury repair or neointima formation. COLA1, type IV collagen alpha 1; PDGF, platelet growth factor; VPC, vascular progenitor cell; VSMC, vascular smooth muscle cell; XBP1s, spliced XBP1 protein; XBP1, X-box binding protein 1.

and exon 42 of COL4A1 gene on one hand. The resulting *COL4A1* mRNA may undergo unconventional splicing between exon 4 and exon 42 by IRE1α to remove the internal sequence with the facilitation of XBP1s protein. On the other hand, the exon 4 and exon 42-bound XBP1s are dimerized to bring exon 4 and exon 42 together and direct RNA polymerase transcription from exon 4 to exon 42 bypassing the internal parts. Either mechanism leads to the production of a shortened soluble COL4A1s isoform, which acts as paracrine cytokine to recruit VPCs to injured site, contributing to injury repair or disease development (Fig. 7). This study provides a novel insight into the regulation of type IV collagen transcription and XBP1 functions.

Experimental procedures

Materials

All cell culture media and sera were purchased from Thermo Fisher Scientific, whereas cell culture supplements, growth factors, and inhibitors were purchased from Sigma. The XBP1u and XBP1s antibodies were raised by GenScript using peptide CRSSQRTQKDPVPY (XBP1u) and DSGGIDSSDSESDIC (XBP1s). The antibodies against Sca-1 (ab51317), IRE1α (ab37073), COL4A1 (ab6586), Col15A1 (ab58717), and Col18A1 (ab52022) were purchased from Abcam; antibody for GAPDH (sc-25778) was from Santa Cruz Biotechnology; antibody for FLAG (A4596 and F1804), alpha SMA (A5228), and all other chemicals from Sigma; all secondary antibodies were purchased from Dakocytomation.

Cell culture

HVSMCs, HUVECs, and HEK293 cells were purchased from ATCC. HVSMCs and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HUVECs were maintained in M199 medium supplemented with 1 ng/ml β-EC growth factor, 3 μg/ml EC growth supplement from bovine neural tissue, 10 μg/ml heparin, 1.25 μg/ml thymidine, 10% FBS, 100 U/ml penicillin, and streptomycin in humidified incubator

supplemented with 5% CO_2. Sca-1$^+$ vascular progenitor cells were isolated from an outgrowth of adventitial tissues of vessel grafts and cultured as previously described (38). For HVSMC, HUVEC and Sca-1$^+$ VPC culture, the flasks were coated with 0.04% gelatin, and experiment passages between 5 and 10 were used in this study.

RNA extraction and RT-PCR

Total RNAs were extracted using RNeasy Mini kit (Qiagen) according to the protocol provided. 1 μg of RNA with its genomic DNA ablated was converted into cDNA as instructed by the QIAGEN OneStep RT-PCR Kit (Qiagen). 20 ng cDNA (relative to RNA amount) was subjected to quantitative PCR with a SYBR green dye–based PCR amplification and detection master mix (ThermoFisher Scientific) in Eppendorf Master Cycler gradient S machine (Eppendorf). The primers were designed using *ncbi primer pickup* software and synthesized by ThermoFisher. The primer sequences include: *Gapdh*: 5'-cga ctt caa cag caa ctc cca ctc ttc-3' versus 5'-tgg gtg gtc cag ggt ttc tta ctc ctt-3'; *COL4A1*: 5'-gcc ttc cga gag cta gct gtc-3' versus 5'-gct gtg ctc ctc gtg gag cag-3'; *Col4A2*: 5'-gcc agc atg ggg aga gac cag-3' versus 5'-ctg gcc cag gct gac cac gtc-3'; *XBP1*:5'-cct tgt agt tga gaa cca gga g-3' versus 5'-ggt cca agt tgt cca gaa tgc-3'. The primer set 5'-gcc t tc cga gag cta gct gtc-3' versus 5'-gct gac gtg cgt gcg cag ctc-3' was used to amplify the full-length COL4A1 coding sequence and identify the *COL4A1s* transcript variant. The fold of induction was defined as the ratio of the difference between target gene and *Gapdh* (internal control) with that of control group set at 1.0.

Plasmid construction and transfection

The DNA fragments corresponding to FLAG-COL4A1s (COL4A1s coding sequence with FLAG inserted downstream 7S domain), lplE4-Ren-lplE42, lplE4-LacZ-lplE42 were synthesized by GenScript. The DNA fragments were then subcloned into pShuttle2 and pSI-Check2 vectors respectively and verified by DNA sequencing. For transient transfection, plasmid was introduced into target cells with Lipofectamine 2000 reagents (ThermoFisher Scientific) according to protocol provided.

Immunostaining

For immunostaining, cryosections were air dried at room temperature and fixed with cold methanol (4 °C) for 15 min. The fixed sections were permeabilized with 0.1% Triton X-100 at RT for 15 min, followed by blocking with diluted swine serum (1:20) for 1 hr, incubation with diluted primary antibodies (1 μg/ml in diluted swine serum) for 1 hr and with secondary antibodies (Alexa Fluor-488 or 594 for immunofluorescence staining or horseradish peroxidase-conjugated for immunohistological staining combined with diaminobezidin) for 45 min. Nucleus was counterstained with 4',6-diamidino-2-phenylindole for immunofluorescence staining or with Harris hematoxylin for immunohistological staining. Images were taken by using SP5 confocal microscope (Leica) and processed by Adobe Photoshop software.

Transwell migration assay

A 100 μl of 5 × 10^5 cells/ml cell suspension (VSMCs, HUVECs or VPCs) was added into the insert and 600 μl of media containing 0.5% FBS or conditioned medium were added into the holder of the transwell (8 μm pore size), followed by incubation for 5 hr. Purified COL4A1s was added into the holder. The cells were then fixed with methanol/acetic acid (3:1) for 15 minutes and stained with crystal violet for 15 minutes. Migrated cells were observed under Nikon Eclipse TS100 microscope, and images were taken by Nikon Digital Sight system. The average cells per 20x lens view from six views of each group were presented.

Western blot analysis

Cells were lysed with IP-A buffer (10 mmol/L Tris-HCl, pH7.5, 120 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100 plus protease inhibitor cocktail tablets), and the protein concentration was measured by BioRad protein assay (BioRad). Lysate (50 μg) was applied to SDS-PAGE, polyvinylidene fluoride membrane transfer, blocking with 5% milk powder in TBST buffer (10 mmol/L Tris-HCl, pH7.5, 120 mmol/L NaCl, 1 mmol/L EDTA, 0.05% Tween-20). Primary antibodies goat anti-COL4A1, rabbit anti-XBP1 and mouse anti-Flag, and secondary antibodies conjugated to horseradish peroxidase were used. Visualization was performed using ECL chemiluminescence development and X-ray film exposure.

Zymography assays

HVSMCs were infected with *Ad-null* or *Ad-XBP1s* viruses at 10 multiplicity of infection for 24 h. The cells were then incubated in serum-free medium for 24 h. The conditioned medium was collected as infection 48 h group, and the cells were further incubated in serum-free medium for another 24 h, and the medium was collected as infection 72 h group. The conditioned medium was spun at 10,000 rpm at 4°C for 5 min to remove cell debris. The supernatant was then concentrated with Amicon Untracel-10 K column (Millipore) by 20-fold, *i.e.*, 2 ml conditioned medium was concentrated into 100 μl. The concentrated conditioned medium was subjected to Western blot or zymography with Novex 10% Zymogram Plus (Gelatin) Protein Gels (ThermoFisher Scientific) according to the manufacturer's protocol.

Chromatin immunoprecipitation assay

The standard chromatin immunoprecipitation was slightly modified. Briefly, the proteins and RNA or chromatin DNA were crosslinked by incubation of the cells with 1% formaldehyde at 37°C for 15 min. Following the removal of DMEM media containing formaldehyde, the cells were incubated with DMEM media containing 10% FBS and 125 mmol/L glycine at 37°C for 15 min. Afterwards, cells were detached, washed, and resuspended in IP-A buffer and sonicated. After centrifuging at 2,000×*g* at 4°C for 5 min, the supernatant was recovered and precleared with 2 μg antibody per 1 mg cell lysate with rabbit IgG and 10 μl Protein G-magnetic beads for 1 h. Afterward, 1 mg cell lysate was

incubated with 2 μg rabbit anti-XBP1s with rabbit IgG included as control at 4°C for 2 h and then 10 μl protein G-magnetic beads was added and incubated for further 2 h. The beads were collected using a magnetic stand, followed by five times of washing with TBST buffer. The beads was incubated with Proteinase K buffer (20 mmol/L Tris-HCl, PH7.5, 1% SDS, 10 mmol/L EDTA, 0.6 mol/L NaCl, 1 mg/ml Proteinase K) at 60 °C for 8∼16 h, followed by either RNA extraction (using Phenol/Chloroform pH 4.5) or DNA extraction (with Phenol/Chloroform pH 8).Cell lysate (50 μg) was directly digested with the Proteinase K buffer for input control. All extracted RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen) as per manufacturer instructions. Then 2 μl of cDNA or DNA were used for PCR reactions. The primer sets "5'-cct gga atg caa gga cct gag-3' versus 5'-ctg ggt ttc cag ggt agc cag-3'" and "5'- gtg ttg gct atc cag gaa gtc-3' versus 5'- agg acc cat gaa tcc ttg ctc-3'" were used to amplify COL4A1 exon 4 and exon 42 from RNA samples, while the primer sets "5'- atc atc gat tgt gag tag caa ctg-3' versus 5'- ctt gag ata gcg gtg atc aat gac-3'" and "5'- agg acc cat gaa tcc ttg ctc-3' versus 5'- tgc gtt acc cat cac ctc aaa ctg-3'" were used to amplify COL4A1 exon 4 and exon 42 from DNA samples.

DNA-protein pulldown assay

Briefly, 500 μg protein lysates from the *Ad-null* or *Ad-XBP1s*-infected HVSMCs were incubated with 100 μmol/L biotin labeled double stranded DNA probes of exon 4 (btn-act gga gaa cca gga cta cct gga aca aaa ggg aca) or exon 42 (btn-ccg gga gcc cag gaa ttc ctg gat cca aag gag agc) in 1× binding buffer (20 mmol/L Tris-Cl, 2.5 mmol/L $MgCl_2$, 150 mmol/L NaCl and 1 mmol/L EDTA) for 45 min. Then, 15 μl streptavidin agarose beads were added and incubated at 4°C for 1 h. Afterward, the beads were collected and washed several times with 1× binding buffer. The bound proteins were eluted with 1× SDS loading solution (10 mmol/L Tris-HCL, pH8.8, 2% SDS, 30% glycerol and 0.025% bromide blue phenol) and subjected to Western blot analysis.

Plasmid transfection and luciferase assay

HVSMCs were seeded in 12-well plates at 5×10^4 cells/well in triplicate 24 hr before transfection. Medium was changed to serum- and antibiotics-free DMEM. 0.1 μg/well plasmid DNA (pSI-lplE4-Ren-lplE42) plus 0.1 μg/well *pShuttle2-XBP1s* or 0.1 μg/well plasmid DNA (pSI-lplE4-Ren-lplE42) plus 0.1 μg/well *pShuttle2-GFP* were transfected with FugeneHD (Promega). Five hours later, the transfection solution was removed, and fresh complete growth medium was added, and the cells were incubated for 24 hr, followed by luciferase activity assay with the dual reporter system (Promega) according to the manufacturer's protocol. The relative luciferase activity was defined as the ratio of readout for Renilla luciferase to that for Firefly luciferase with that of control group set as 1.0.

In vitro *transcription*

In vitro transcription was performed based as reported previously (39). Briefly, 5 μg linearized plasmid DNA (pSI-lplE4-Ren-lplE42 or pSI-lplE4-LacZ-lplE42) was incubated with nuclear fraction from HVSMCs cultured with either *Ad-null* or *Ad-XBP1s*, NEB 10× transcription buffer, and 100 mM rNTPs (Promega) at 37°C for 2 h and then digested with DNase I at 37 °C for 30 min. RNA extraction was performed using Phenol/Chloroform (pH 4.5), followed by RT-PCR with primer set of 5'-tac gac tca cta tag gct agc-3' versus 5'-gat ccg ctc tag gtt taa acg-3' to amplify a 80 bp fragment derived from unconventional splicing or recombinate between the two loxP-like elements.

COL4A1s protein concentration from conditioned medium

HEK293 cells were transfected with pS2-COL4A1s-FLAG plasmid for 24 h, then subjected to serum-free medium incubation for another 24 h. The conditioned medium was collected and centrifuged at 2000×*g* at 4°C for 5 min to remove cell debris. The supernatant was recovered and subjected to Ultracel-10K (Amicon) column or anti-FLAG antibody–mediated concentration. For the Ultracel-10K column concentration, 5 ml conditioned medium was applied to 15 ml Ultracel-10K column and centrifuged at 20,000×*g* at 4°C for 15 min. The concentrated medium was transferred to a O.5 ml Ultracel-10K column to further concentrate with a final volume of 80 μl. 5x SDS loading buffer (20 μl) was added to denature the concentrated proteins, and 20 μl of the lysate was applied as input for Western blot. For anti-FLAG antibody mediated concentration, 10 μl anti-FLAG-magnetic beads was mixed with 5 ml conditioned medium and incubated on a rotator at 4°C overnight. The antibody bound COL4A1s-FLAG proteins were collected by magnetic bar and either resuspended in 100 μl 1xSDS loading buffer with 20 μl applied to Western blot or eluted with 1 μg FLAG peptide.

Animal study

All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals, and all procedures conformed to the guidelines from Directive 2010/63/EU of the European Parliament. The Global knockout heterozygous of *Xbp1* (*Xbp1*$^{+/-}$) and SMC conditional knockout (*Xbp1smcko*) C57bl/6 mouse line are both available in our group and maintained in King's College London animal facility according to standard procedures (18, 37). The *Xbp1*$^{+/-}$ mouse was crossbred and embryos isolated at E10.5 stage. Twelve weeks WT and XBP1smcko mice were subjected to femoral artery injury with platinum wire to scratch off endothelial cells under 2% isoflurane anesthesia as described previously (18). The injured vessels were collected 2 weeks postsurgery. Immunohistochemical and immunofluorescence staining was performed on these tissues according to the purpose of the study.

Statistical analysis

Data are expressed as box (first and third quartiles) and whiskers (maximum and minimum). The middle line in the box represents the median. Mean ± SD was used. All data are analyzed using GraphPad Prism 7 software with *t* test for pair-

wise comparisons or analysis of variance, and significance was depicted by asterisks, $^{*}p < 0.05$, $^{**}p < 0.01$.

Data availability

Data available on request from the authors.

Author contributions—A. A. and L. H. experimental design, performance, data analysis, and paper writing; Y. Z., J. M., and P. L., experimental performance; Y. G., and A. M. data analysis; T. L., W. K., and L. Z. experimental design, data analysis, and manuscript writing. All authors were involved in critical evaluation and intellectual contribution to the manuscript.

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Abbreviations—The abbreviations used are: COLA1, type IV collagen alpha 1; ECM, extracellular matrix protein; FBS, fetal bovine serum; HVSMC, human smooth muscle cell line; HUVEC, human umbilical vein EC; IRE1α, inositol-requiring enzyme 1 alpha; MMP, matrix metalloproteinase; NC, noncollagenous; PDGF, platelet growth factor; Sca1+, stem cell antigen 1-positive; VPC, vascular progenitor cell; VSMC, Vascular smooth muscle cell; XBP1, X-box binding protein 1; XBP1s, spliced XBP1 protein.

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