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

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 flank-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)
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 1A (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 neo-intima 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. 1A, 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. 1B), quantitative RT-PCR (Fig. 1C), and Western blot analysis (Fig. 1D). 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. 2A). 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. 2B). The 100 kDa COL4A1 band is much less in cell lysate as compared with that in conditioned medium (Fig. 2B), which may be due to secretion. Strikingly, the 100 kDa band in XBP1s group existed in very
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. 2B), 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 activated proteases in conditioned medium as revealed by zymography. 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.

**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 (pXB1u) and pShuttle2-XBP1s (pXB1s) 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.

**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. 3A). 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. 3B). 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. 3B). Following 293 cells transfection with pS2-COL4A1s vector containing COL4A1s coding sequence and FLAG-tag downstream of 7S domain (Fig. 3C, upper), the conditioned medium was collected for incubation with anti-FLAG agarose beads (10 μl and 50 μl) (Fig. 3C, lower). The Western blot analysis using...
XBP1s induces soluble COL4A1s isoform

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 (p1 and p2, 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. 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. COL4A1s protein retains the signal peptide, 7S domain and NC domain with internal helix domain shortened. COL4A1s protein retains the signal peptide, 7S domain and NC domain with internal helix domain shortened.

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. 5A). These loxP-like sequences can form stem-loop structure (Fig. 5B), 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 5C, 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. 5D). 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 and lplE42 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 analysis with anti-FLAG antibodies. As shown in Figure 5E, 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. 5F, 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. 5F, 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. 5G, 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. 5G, upper). If a recombination occurs between these two elements, the primer set will amplify an 80 bp
PCR product. Indeed, nuclear extracts from Ad-XBP1s-infected HVSMC produced an 80 bp PCR product from pSI-lplE4-LacZ-lplE42 (Fig. 5G, lower) but not from pSI-lplE4-Ren-lplE42 (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 4. The full-length mRNA (A) and 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).
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 6A, 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. 6B). These results
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 6C demonstrated that pS2-COL4A1s conditioned medium increased VPC migration when compared with Mock conditioned medium. The conditioned medium was depleted with IgG or anti-FLAG agarose beads (α-FLAG), followed by transwell migration assays on VPCs, HUVECs, and HVSMSCs. 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; HVSMS, human smooth muscle cell line; VPC, vascular progenitor cell; VSMC, vascular smooth muscle cell; XBP1, X-box binding protein 1; XBP1s, spliced XBP1 protein.

**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 are involved in 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. 5A), 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 5B, 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 IRE1a-mediated unconventional splicing. Therefore, there is a possibility that COL4A1 mRNA is another unconventional splicing target of IRE1a, as we have demonstrated the binding of IRE1a to COL4A1 mRNA at exon 4 through a modified chromatin immunoprecipitation assay. Although in our artificial pSI-lplE4-Ren-lplE42 reporter system, IRE1α inhibitor 4μB8C 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 heterotrimeter. 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 cardiac cerebrovascular 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 as 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 XBP1 protein will direct COL4A1 and COL4A2 transcription via binding to enhancers within exon 4.
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, 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.

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 DSGGIDSSDSEDIC (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% CO₂. Sca-1⁺ vascular progenitor cells were isolated from an outgrowth of adventitial tissues of vessel grafts and cultured as previously described (38). For HVSMC, were isolated from an outgrowth of adventitial tissues of vessel

**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’-cca agt tgt cca gaa tgc-3’ versus 5’-tag gtt gtc cag ggt ttt cta cct ctt cct-3’; COL4A1: 5’-gcc ttc cga gag cta gct gtc-3’ versus 5’-gct gtt gtc ctt ctc gtg gag cag-3’; Col4A2: 5’-gcc agc atg aga gag cag cag-3’ versus 5’-tgt gcc cag cag cag ctc-3’; XBP1: 5’-cct tgt agt tga gaa cca gga g-3’ versus 5’-gtg cag ctt tgc cct cca gag tgg-3’. The primer set 5’-gct ttc cga gga cta gcc gtc-3’ versus 5’-gct gcc gct ggt cgg cag ctc-3’ was used to amplify the full-length COL4A1 coding sequence and identify the COL4A1s isoform. 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), IplE4-Ren-IplE42, IplE4-LacZ-IplE42 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, cysections 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.

**Plasmid construction and transfection**

The DNA fragments corresponding to FLAG-COL4A1s (COL4A1s coding sequence with FLAG inserted downstream 7S domain), IplE4-Ren-IplE42, IplE4-LacZ-IplE42 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, cysections 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 × 10⁵ 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 Untrace-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 2,000g 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 were 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 atc cag cct ggg-3’ versus 5’-ctg gtt ttc gat cca aag gag-3’” and “5’- gtt gtc gtt gct cag cag-3’ versus 5’- agg acc cat gaa ttc tgg ctc-3’” were used to amplify COL4A1 exon 4 and exon 42 from RNA samples, while the primer sets “5’- atc atc gat tgg taa acg-3” and “5’- agg acc cat gaa ttc tgg ctc-3” versus 5’- tgc gtt acc cat ccc aaa ctc-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 (bta-act gga gaa cca gga cta cct gct gaa cca aaa ggg cca) or exon 42 (bta-ccg gga gcc cag gaa ttc tgg gct gat cca aag gag acc) in 1× binding buffer (20 mmol/L Tris-Cl, 2.5 mmol/L MgCl₂, 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 × 10⁴ cells/well in triplicate 24 hr before transfection. Medium was changed to serum- and antibiotics-free DMEM. 0.1 μg/well plasmid DNA (pSI-lpLE4-4 Ren-lpLE42) plus 0.1 μg/well pShuttle2-XBP1s or 0.1 μg/well plasmid DNA (pSI-lpLE4-Renal-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-Renal-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 gta tca tta tag gtc-3’ versus 5’-gat ccc ctc tag tga aac-3’ to amplify a 80 bp fragment derived from unconventional splicing or recombine 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 0.5 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 urane 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-
XBP1s induces soluble COL4A1s isoform

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.

Funding and additional information—This study was supported by funding from British Heart Foundation project grant PG16/13/32024 (L. Z.), the Natural Science Foundation of Tianjin City No.19JCYBJC26000 (T. L.), the Tianjin Third Central Hospital National Natural Science Foundation Incubation Project of China No.2019YR01 (L. H.), the Tianjin municipal health and Health Committee Science and Technology Talent Cultivation Project of China No.KJ20008 (L. H.), and A. M. S. is supported by a British Heart Foundation Chair (CH/1999001/11735). The Tianjin Municipal Health and Health Committee Science and Technology Self Financing Project of China (No. ZD20001).

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: COLA1, type IV collagen alpha 1; ECM, extracellular matrix protein; FBS, fetal bovine serum; HUVEC, human smooth muscle cell line; HuVECs, 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.

References

1. Nguyen, A. T., Gomez, D., Bell, R. D., Campbell, J. H., Clowes, A. W., Gabbiani, G., Giachelli, C. M., Parmacek, M. S., Raines, E. W., Rusch, N. J., Speer, M. Y., Sturek, M., Thyberg, J., Towler, D. A., Weiser-Evans, M. C., et al. (2013) Smooth muscle cell plasticity: Fact or fiction? Circ. Res. 112, 17–22
2. Kaimoto, T., Yasuda, O., Ohishi, M., Mogi, M., Takemura, Y., Suhara, T., Ogihara, T., Fukuo, K., and Rakugi, H. (2010) Nifedipine inhibits vascular smooth muscle cell dedifferentiation via downregulation of Akt signaling. Hypertension 56, 247–252
3. Allahverdian, S., Chaabane, C., Boukais, K., Francis, G. A., and Bochaton-Piallat, M. L. (2018) Smooth muscle cell fate and plasticity in atherosclerosis. Cardiovasc. Res. 114, 540–550
4. Tang, Z., Wang, A., Yuan, F., Yan, Z., Liu, B., Chu, J. S., Helms, J. A., and Li, S. (2012) Differentiation of multipotent vascular stem cells contributes to vascular diseases. Nat. Commun. 3, 875
5. Hu, Y., Zhang, Z., Torsney, E., Afzal, A. R., Davison, F., Metzler, B., and Xu, Q. (2004) Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J. Clin. Invest. 113, 1258–1265
6. Paulsson, M. (1992) Basement membrane proteins: Structure, assembly, and cellular interactions. Crit. Rev. Biochem. Mol. Biol. 27, 93–127
7. Boosani, C. S., and Sudhakar, A. (2006) Cloning, purification, and characterization of a non-collagenous anti-angiogenic protein domain from human alpha 1 type IV collagen expressed in S99 cells. Protein Expr. Purif. 49, 211–218
8. Bejarano, P. A., Noelken, M. E., Suzuki, K., Hudson, B. G., and Nagase, H. (1988) Degradation of basement-membranes by human matrix metalloproteinase-3 (STROMELYSIN). Biochem. J. 256, 413–419
9. Zeng, L. F., Xiao, Q. Z., Margariti, A., Zhang, Z. Y., Zampetaki, A., Patel, S., Capogrossi, M. C., Hu, Y. H., and Xu, Q. B. (2006) HDAC3 is crucial in shear- and VEGF-induced stem cell differentiation toward endothelial cells. J. Cell Biol. 174, 1059–1069
10. Xiao, Q. Z., Zeng, L. F., Zhang, Z. Y., Margariti, A., Ali, Z. A., Channon, K. M., Xu, Q. B., and Hu, Y. H. (2006) Sca-1(+) progenitors derived from embryonic stem cells differentiate into endothelial cells capable of vascular repair after arterial injury. Arterioscler. Thromb. Vasc. Biol. 26, 2244–2251
11. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107, 881–891
12. Ron, D., and Walter, P. (2007) Signal integration in the endoplasmic reticulum unfolded protein response. Nat. Rev. Mol. Cell Biol. 8, 519–529
13. Uemura, A., Oku, M., Mori, K., and Yoshida, H. (2009) Unconventional splicing of XBP1 mRNA occurs in the cytoplasm during the mammalian unfolded protein response. J. Cell Sci. 122, 2877–2886
14. Han, D., Lerner, A. G., Vande Walle, L., Upton, J. P., Xu, W. H., Hagen, A., Backes, B. J., Oakes, S. A., and Papa, F. R. (2009) IRE1 alpha kinase activation Modes control Alternate Endoribonuclease Outputs to Determine Divergent cell Fates. Cell 138, 562–575
15. Oikawa, D., Tokuda, M., Hosoda, A., and Iwaiwaki, T. (2010) Identification of a consensus element recognized and cleaved by IRE1 alpha. Nucleic Acids Res. 38, 6265–6273
16. Yoshida, H., Nadanaka, S., Sato, R., and Mori, K. (2006) XBP1 is critical to protect cells from endoplasmic reticulum stress: Evidence from site-2 protease-deficient Chinese hamster ovary cells. Cell Struct. Funct. 31, 117–125
17. Zeng, L. F., Zampetaki, A., Margariti, A., Pepe, A. E., Alam, S., Martin, D., Xiao, Q. Z., Wang, W., Jin, Z. G., Cockerill, G., Mori, K., Li, Y. S. J., Hu, Y. H., Chien, S., and Xu, Q. B. (2009) Sustained activation of XBP1 splicing leads to endothelial apoptosis and atherosclerosis development in response to disturbed flow. Proc. Natl. Acad. Sci. U. S. A. 106, 8326–8331
18. Zeng, L. F., Li, Y., Yang, J. Y., Wang, G., Margariti, A., Xiao, Q. Z., Zampetaki, A., Yin, X. K., Mayr, M., Mori, K., Wang, W., Hu, Y. H., and Xu, Q. B. (2015) XBP1 deficiency Abrogates neointimal lesion of injured vessels via Cross Talk with the PDGF signaling. Arterioscler. Thromb. Vasc. Biol. 35, 2134–2144
19. Burboro, P. D., Martin, G. R., and Yamada, Y. (1988) Alpha 1(IV) and alpha 2(IV) collagen genes are regulated by a bidirectional promoter and a shared enhancer. Proc. Natl. Acad. Sci. U. S. A. 85, 9679–9682
20. Griffin, C. A., Emanuel, B. S., Hansen, J. R., Caveness, W. K., and Myers, J. C. (1987) Human collagen genes encoding basement membrane alpha 1 (IV) and alpha 2 (IV) chains map to the distal long arm of chromosome 13. Proc. Natl. Acad. Sci. U. S. A. 84, 512–516
21. Sawhney, R. S., Cookson, M. M., Omar, Y., Hauser, J., and Brattain, M. G. (2006) Integrin alpha2-mediated ERK and calpain activation play a critical role in cell adhesion and motility via focal adhesion kinase signaling: Identification of a novel signaling pathway. J. Biol. Chem. 281, 8497–8510
22. Rebustini, I. T., Myers, C., Lasiter, K. S., Surmak, A., Szabova, L., Holmbeck, K., Pedchenko, V., Hudson, B. G., and Hoffman, M. P. (2009) MT2-MMP-dependent release of collagen IV NC1 domains regulates submandibular gland branching morphogenesis. Dev. Cell. 17, 482–493
23. Mauldin, G. N., Foster, T. P., Waddell, C. W., and Egan, M. E. (2003) Cloning, expression and in vitro evaluation of recombinant canine Tum5, an angiostatic domain of mammalian type IV collagen. Vet. Comp. Oncol. 1, 36–47
24. Ortega, N., and Werb, Z. (2002) New functional roles for non-collagenous domains of basement membrane collagens. J. Cell. Sci. 115, 4201–4214
25. Poschl, E., Pollner, R., and Kuhn, K. (1988) The genes for the alpha 1(IV) and alpha 2(IV) chains of human basement membrane collagen type IV are arranged head-to-head and separated by a bidirectional promoter of unique structure. *EMBO J.* 7, 2687–2695

26. Sanford, J. R., and Caceres, J. F. (2004) Pre-mRNA splicing: Life at the centre of the central dogma. *J. Cell Sci.* 117, 6261–6263

27. Wu, Q., and Krainer, A. R. (1999) AT-AC pre-mRNA splicing mechanisms and conservation of minor introns in voltage-gated ion channel genes. *Mol. Cell. Biol.* 19, 3225–3236

28. Martin, D., Li, Y., Yang, J., Wang, G., Margariti, A., Jiang, Z., Yu, H., Zampetaki, A., Hu, Y., Xu, Q., and Zeng, L. (2014) Unspliced X-box-binding protein 1 (XBP1) protects endothelial cells from oxidative stress through interaction with histone deacetylase 3. *J. Biol. Chem.* 289, 30625–30634

29. Mak, K. M., and Mei, R. (2017) Basement membrane type IV collagen and Laminin: An Overview of their Biology and Value as Fibrosis Biomarkers of liver disease. *Anat Rec (Hoboken)* 300, 1371–1390

30. Gould, D. B., Phalan, F. C., van Mil, S. E., Sundberg, J. P., Vahedi, K., Massin, P., Bousser, M. G., Heutink, P., Miner, J. H., Tournier-Lasserve, E., and John, S. W. (2006) Role of COL4A1 in small-vessel disease and hemorrhagic stroke. *New Engl. J. Med.* 354, 1489–1496

31. Steffensen, L. B., and Rasmussen, L. M. (2018) A role for collagen type IV in cardiovascular disease? *Am. J. Physiol. Heart Circ. Physiol.* 315, H610–H625

32. Petitclerc, E., Boutaud, A., Prestayko, A., Xu, J., Sado, Y., Ninomiya, Y., Sarras, M. P., Jr., Hudson, B. G., and Brooks, P. C. (2000) New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth in vivo. *J. Biol. Chem.* 275, 8051–8061

33. Stanton, A. E., Tong, X., and Yang, F. (2019) Extracellular matrix type modulates mechanotransduction of stem cells. *Acta Biomater.* 96, 310–320

34. Aikio, M., Alahuhta, I., Nurmenniemi, S., Suojanen, J., Palovuori, R., Teppo, S., Sorsa, T., Lopez-Otin, C., Pihlajaniemi, T., Salo, T., Heljasvaara, R., and Nyberg, P. (2012) Arresten, a collagen-derived angiogenesis inhibitor, suppresses invasion of squamous cell carcinoma. *PloS one* 7, e51044

35. Kuo, D. S., Labelle-Dumais, C., and Gould, D. B. (2012) COL4A1 and COL4A2 mutations and disease: Insights into pathogenic mechanisms and potential therapeutic targets. *Hum. Mol. Genet.* 21, R97–R110

36. Reimold, A. M., Etkin, A., Clauss, I., Perkins, A., Friend, D. S., Zhang, J., Horton, H. F., Scott, A., Orkin, S. H., Byrne, M. C., Grusby, M. J., and Glimcher, L. H. (2000) An essential role in liver development for transcription factor XBP-1. *Genes Dev.* 14, 152–157

37. Zeng, L., Xiao, Q., Chen, M., Margariti, A., Martin, D., Ivetic, A., Xu, H., Mason, J., Wang, W., Cockerill, G., Mori, K., Li, J. Y., Chien, S., Hu, Y., and Xu, Q. (2013) Vascular endothelial cell growth-activated XBP1 splicing in endothelial cells is crucial for angiogenesis. *Circulation* 127, 1712–1722

38. Kokkinopoulos, I., Wong, M. M., Potter, C. M. F., Xie, Y., Yu, B., Warren, D. T., Nowak, W. N., Le Bras, A., Ni, Z., Zhou, C., Ruan, X., Karamariti, E., Hu, Y., Zhang, L., and Xu, Q. (2017) Adventitial SCA-1(+) progenitor cell gene sequencing Reveals the mechanisms of cell migration in response to Hyperlipidemia. *Stem Cell Rep.* 9, 681–696

39. Lee, M., Song, H., Lee, K., and Park, J. S. (1999) *In vitro* transcription assay with the purified 40kDa NF1-like protein binding to the rat p53 promoter. *Biochem. Mol. Biol. Int.* 47, 427–434

**XBP1s induces soluble COL4A1s isoform**