Antagonistic relationship between the unfolded protein response and myocardin-driven transcription in smooth muscle

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Abstract
Smooth muscle cells (SMCs) are characterized by a high degree of phenotypic plasticity. Contractile differentiation is governed by myocardin-related transcription factors (MRTFs), in particular myocardin (MYOCD), and when their drive is lost, the cells become proliferative and synthetic with an expanded endoplasmic reticulum (ER). ER is responsible for assembly and folding of secreted proteins. When the load on the ER surpasses its capacity, three stress sensors (activating transcription factor 6 [ATF6], inositol-requiring enzyme 1α [IRE1α]/X-box binding protein 1 [XBP1], and PERK/ATF4) are activated to expand the ER and increase its folding capacity. This is referred to as the unfolded protein response (UPR). Here, we hypothesized that there is a reciprocal relationship between SMC differentiation and the UPR. Tight negative correlations between SMC markers (MYH11, MYOCD, KCNMB1, SYNPO2) and UPR markers (SDF2L1, CALR, MANF, PDIA4) were seen in microarray data sets from carotid arterial injury, partial bladder outlet obstruction, and bladder denervation, respectively. The UPR activators dithiothreitol (DTT) and tunicamycin (TN) activated the UPR and reduced MYOCD along with SMC markers in vitro. The IRE1α inhibitor 4μ8C counteracted the effect of DTT and TN on SMC markers and MYOCD expression. Transfection of active XBP1s was sufficient to reduce both MYOCD and the SMC markers. MRTFs also antagonized the UPR as indicated by reduced TN and DTT-mediated induction of CRELD2, MANF, PDIA4, and SDF2L1 following overexpression of MRTFs. The latter effect did not involve the newly identified MYOCD/ SRF target MSRB3, or reduced production of either XBP1s or cleaved ATF6. The UPR thus counteracts SMC differentiation via the IRE1α/XBP1 arm of the UPR and MYOCD repression.

Keywords
CNN1, ER stress, smooth muscle differentiation, TAGLN
1 | INTRODUCTION

Phenotypic modulation of smooth muscle cells (SMCs) involves loss of contractile differentiation and gain of proliferative and synthetic properties or vice versa (Miano, 2015). This cellular plasticity not only contributes to atherosclerosis and neointima formation (Bennett, Sinha, & Owens, 2016; Owens, Kumar, & Wamhoff, 2004) but is also seen in gastrointestinal (MacDonald, 2008) and urogenital conditions (Krawczyk et al., 2016). The SMC gene program is controlled by myocardin-related transcription factors (MRTFs). These powerful coactivators (myocardin/MYOCID, MRTF-A/MKL1, and MRTF-B/MKL2) drive SMC differentiation by binding to the serum response factor (SRF; Miano, 2003; Wang et al., 2001). The resultant complex activates genes important for contraction and shortening, and the targets include smooth muscle myosin and actin (MYH11 and ACTA2), ion channels (e.g. KCNMB1), and cytoskeletal proteins (e.g. SYNPO2). MRTF-driven transcription is controlled by actin dynamics (Miralles, Posern, Zaromytidou, & Treisman, 2003; Olson & Nordheim, 2010) and binding to SRF occurs in competition with ternary complex factors (TCFs; Z. Wang et al., 2004). TCFs are activated by mitogen-activated protein kinases (e.g. ERK1/2; Marais, Wynne, & Treisman, 1993), and TCFs favor distinct SRF-dependent target genes important for growth (Miano, 2003).

Changes in protein synthesis and folding activate a gene program referred to as the unfolded protein response (UPR; Hetz & Papa, 2018; Walter & Ron, 2011). The UPR is a homeostatic control mechanism that matches the folding capacity of the endoplasmic reticulum (ER) to the cellular demand by recruiting chaperone genes and by expanding the ER volume. The UPR also halts protein translation and promotes protein degradation to reduce the load of unfolded proteins. The UPR has three main sensors in the ER membrane namely inositol-requiring enzyme 1α (IRE1α), activating transcription factor 6 (ATF6), and PERK. IRE1α is an endoribonuclease that splices X-box binding protein 1 (XBP1) to yield an active transcription factor (XBP1s). In response to ER stress, ATF6 travels to the Golgi apparatus where it is cleaved and activated. Finally, PERK phosphorylates the eukaryotic translation initiation factor-2α and this shuts down the protein synthesis and activates ATF4. If these attempts to restore protein folding fail, the cells undergo caspase-dependent apoptosis (Hetz & Papa, 2018; Lerner et al., 2012; Walter & Ron, 2011). The UPR is often studied in vitro by treatment with chemical ER stressors. These include tunicamycin (TN), which blocks formation of N-glycans to cause unfolding stress and dithiothreitol (DTT), which breaks disulfide bonds that are important for protein 3D structure.

Examples of UPR activation in association with repression of the SMC gene program are prevalent (Furmanik & Shanahan, 2017). In atherosclerosis for example, the UPR is activated (Tabas, 2010) and SMC markers are reduced (Bennett et al., 2016; Perisic Matic et al., 2016). Arterial neointima formation similarly leads to UPR activation (Ishimura et al., 2014) and repression of SMC-specific genes (Regan, Adam, Madsen, & Owens, 2000; Talasila et al., 2013). Transient UPR activation via ATF6 and XBP1 with a concordant reduction of SMC marker expression was also seen in bladder outlet obstruction (Krawczyk et al., 2016). Inducible knockout of MYOCID, finally, reduces SMC marker expression and causes UPR activation (Huang et al., 2015). From these observations, made in different studies and at different times, it is evident that the SMC and UPR gene programs often change in opposite directions. Direct evidence that chemical ER stressors reduce SMC marker expression via the UPR has yet to be provided however.

In the present work we tested the hypothesis that there is an antagonism between the UPR and the SMC gene programs, and sought to pinpoint the major molecular mechanism involved. To test our hypothesis we examined the experimental models of SMC phenotypic modulation in vivo and used chemical UPR activation in vitro. Our findings argue that the UPR and SMC gene programs are reciprocally coupled via multiple mechanisms involving XBP1 and MYOCID.

2 | MATERIALS AND METHODS

2.1 | Ethics

Bladder muscle tissue from four individuals was excised during cystectomies with written informed consent, and in keeping with procedures approved by the Ethical Review Board (http://www.epn.se, approval number 2008-4). Human coronary artery SMCs were acquired from Gibco (Life Technologies). Consent was again obtained (https://tools.lifetechnologies.com/content/sfs/COAPDFs/2012/1130140_C0175C.pdf).

2.2 | Correlations of UPR and SMC markers in data sets from models of phenotypic modulation

Microarray data sets from rat bladder denervation (Zhu, Ekman, et al., 2018), partial bladder outlet obstruction (Ekman et al., 2013), and carotid artery balloon injury (Perisic Matic et al., 2016) were used to examine anti-correlations between UPR and SMC transcripts using Spearman correlation analyses in GraphPad Prism. Data sets are accessible via the Gene Expression Omnibus using accession numbers GSE47080 and GSE104540.

2.3 | Cell culture and treatment with chemicals

As described before (Zhu, Ekman, et al., 2018; Zhu et al., 2017), human bladder smooth muscle cells (HBSMCs) were isolated from detrusor strips by cutting them into fine pieces followed by incubation in serum-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco; 11966-025) containing 0.2 mg/ml elastase (Sigma-Aldrich; E7885) and 2 mg/ml collagenase type-2 (Worthington Biochemical Corporation; LS004176) at 37°C in a humidified atmosphere of 95% air/5% CO₂ for 3 hr with gentle swirling every 30 min. After sedimentation of debris, the
suspended cells were transferred to a sterile 15 ml tube and centrifuged (1,000 rpm; 3 min). The cell pellet was washed using phosphate buffered saline (PBS; VWR; L1825) and re-suspended in DMEM/Ham’s F-12 medium with glutamine (Biochrom; FG4815), 10% fetal bovine serum (FBS; Biochrom; S0115), and 50U/50 µg/ml penicillin/streptomycin (Biochrom; A2212). The human coronary artery SMCs (HCASMCs) from Gibco (Life Technologies; 1130140) were cultured in Medium 231 (Life Technologies; M231500) with 5% growth supplement (SMGS; Life Technologies; S-007-25) and 50U/50 µg/ml penicillin/streptomycin. Both types of cells were cultured in a water-jacketed culture incubator and the media was refreshed every 48 hr. Cells were passaged by treating with trypsin (Gibco; 25200056) and cells between passages 3 and 8 were used.

DTT (Sigma; D9163) and TN (Biotechnie/Tocris; 3516) were used as ER stress inducers. In the experiments with TN, untransfected cells or cells transfected with recombinant adenoviruses were grown in complete media with 10% FBS and treated with TN (1.0 µg/ml) diluted in dimethyl sulfoxide (DMSO; Sigma Aldrich; D5879) for 24 hr. In the experiments with DTT, untransfected cells or cells transfected with recombinant adenoviruses were grown in complete media with 10% FBS. DTT (1 mM) diluted in sterilized Milli-Q water was added for 24 hr. In the time course experiments with TN or DTT, untransfected HBSCMs (or HCASMCs) were grown with 10% FBS (or 5% SMGS) and treated with TN or DTT for 1, 2, 6, 12, 24, and 48 hr. For determination of cell viability, HBSCMs were seeded and treated with different concentration of TN as indicated. After 24 hr, Cell Counting Kit-8 (CCK-8; dojindo, CK04-11) was added to the wells (1:10) for 2 hours. Absorbance was measured using a 450 nm filter.

Small-molecule UPR inhibitors included an inhibitor of PERK (GSK2606414; Tocris; 5197), an inhibitor of IRE1 (4µ8C; Sigma; SML0949), and an inhibitor of ATF6 (ceapin-7A). The latter compound was a gift from Dr. Walter at Howard Hughes Medical Institute. In the experiments with UPR inhibitors, HBSCMs (or HCASMCs) were grown in media with 10% FBS (or 5% SMGS) until 80–90% confluence and then the serum (or SMGS) was removed. The cells were subsequently treated with 1.0 µg/ml of TN diluted in DMSO (or 1 mM DTT diluted in Milli-Q water) combined with 4µB (100 µM), GSK2606414 (1 µM), and ceapin-7A (10 µM) for 24 hr before harvesting. DMSO was balanced when the cells were treated with more than one compound diluted in DMSO, and an equal volume of DMSO was added to vehicle controls.

Latrunculin B (LatB) is a compound that induces depolymerization of actin. In the experiments with LatB, HBSCMs were grown in complete media with 10% FBS until 80–90% confluence and then the serum was removed by washing twice with PBS. The cells were maintained in 0% FBS DMEM/Ham’s F-12 medium for 24 hr, and then 100 nM of LatB was added for 24 hr.

2.4 siRNA-mediated knockdown of SRF

The Silencer® Select Pre-designed siRNAs for SRF (s13427) and the negative control (NC) siRNA (AM4635) were purchased from Ambion Thermo Fisher Scientific. HBSCMs were cultured as described above. 24 hr after seeding, the cells were transfected with 50 nM SRF siRNA or 50 nM negative control siRNA using oligofectamine transfection reagent (Invitrogen; 12252-011) and Opti-MEM in reduced serum medium ( Gibco; 11058021). The cells were harvested 72 hr after transfection. SRF was silenced by ≥76% after siRNA transfection compared with the negative control cells (Zhu, Rippe, et al., 2018).

2.5 Transduction of recombinant adenoviruses

Adenoviruses for overexpression of MRTF-A (MKL1, Ad-h-MKL1/eGFP, ADV-215499), MRTF-B (MKL2, Ad-h-MKL2, ADV-215500), and MYOCD (Ad-h-MYOCD, ADV-216227) were obtained from Vector Biolabs. Empty vector (Ad-CMV-Null; Vector Biolabs; 1300) at the same multiplicity of infection (MOI) was used as control. For overexpression of MKL1, MKL2, and MYOCD, HBSCMs were transduced with 100 MOI 24 hr after seeding. The cells were maintained in a virus-containing medium for 24 hr, and for another 72 hr in fresh complete media with 10% FBS before harvest or treatment with TN (1.0 µg/ml diluted in DMSO) or DTT (1 mM diluted in Milli-Q water) as described above.

For knockdown of methionine sulfoxide reductase B3 (MSRB3), we used a short hairpin construct (Ad-GFP-U6-hr-MSRB3-shRNA, shADV-215932) and compared it with a null construct (Ad-GFP-U6-shRNA, Vector Biolabs, 1122). 24 hr after seeding, HBSCMs were transduced with 300 MOI of Ad-GFP-U6-hr-MSRB3-shRNA or control virus, respectively. The cells were maintained in a virus-containing medium for 24 hr, and for another 72 hr in a fresh medium before harvest or treatment with TN (1.0 µg/ml) or DTT (1 mM) as described above. For the combination of overexpression of MKL1 (or MYOCD) and knockdown of MSRB3, 100 MOI of MKL1 (or MYOCD) plus 300 MOI of Ad-GFP-U6-hr-MSRB3-shRNA were transduced, and 100 MOI of Ad-CMV-Null plus 300 MOI of Ad-GFP-U6-shRNA were transduced as control. Moreover, 300 MOI of Ad-GFP-U6-shRNA was also used together with MKL1 (or MYOCD). After transduction, the cells were treated with TN (1.0 µg/ml) as described above.

2.6 Transfection of XBP1s

For plasmid transfection, the HCASMCs were transfected with pCMV5-Flag-XBP1s plasmid (Addgene plasmid # 63680) 24 hr after seeding using FuGENE® 6 Transfection Reagent (Promega, E2691). They were then kept in the media for 48 hr and then collected in Qiazole.

2.7 Luciferase assay

HCASMCs were seeded in six-well plates and transduced with adenovirus as described above. After 24 hr of transduction, the
cells were transfected with promoter reporter plasmid for MSRB3 (GeneCopée), containing both Gaussia Luciferase and secreted alkaline phosphatase using FuGENE® 6 Transfection Reagent (Promega, E2691). The 100 μl medium was collected 72 hr after transfection and stored at −20°C. The promoter reporter activity was measured using a dual luciferase kit and high-sensitivity GL-H buffer (Secrete-Pair Dual Luminescence Assay Kit, GeneCopée) according to the manufacturer’s instructions. In brief, 10 μl medium was mixed with substrate and incubated for 30 s before measurement using a Glomax Luminometer (Promega). The readings were normalized to secreted alkaline phosphatase.

2.8 | RNA isolation and RT-qPCR

HBSMCs and HCASMCs were washed twice with cool PBS and lysed in Qiazol (Qiagen; Cat. 79306). The total RNA was isolated with the Qiagen miRNeasy mini kit (Qiagen; 217004), and the concentration and purity of the RNA was determined using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). Reverse transcription and qPCR reactions were prepared using the Quantifast SYBR Green RT-PCR kit (Qiagen; 204156) with Quantitect (Qiagen) primer assays for ACTA2 (QT00088102), CALR (QT00089215), CNN1 (QT00067718), CRELD2 (QT00034398), CYR61 (QT00003451), MANF (QT00050659), MSRB3 (QT00197162), MYH11 (QT00069391), MYOC (QT00072884), PDI A4 (QT0015883), SDF2L1 (QT00027769), SRF (QT00084063), SYNPO2 (QT00075614), TAGLN (QT00072247), and 18S (QT00199367). The 18S was used as a reference gene throughout. RT-qPCR reactions were run using the StepOnePlus qPCR cycler (Applied Biosystems).

2.9 | Correlation analyses using human RNA-Seq data

The GTExPortal (GTExPortal.org) is a resource enabling study of tissue-specific gene expression (Consortium, 2015), which includes RNA-Seq data from over 50 tissues and 700 donors. For the present study, we downloaded data in 2016 with normalization as described earlier (Krawczyk et al., 2015). We used 10 tissues with the highest expression of MSRB3 at that point of time, and these were the tibial artery (n = 332), aorta (n = 224), coronary artery (n = 133), the muscular layer of the esophagus (n = 247), the sigmoid colon (n = 149), the gastro-esophageal junction (n = 153), the uterus (n = 83), the urinary bladder (n = 11), the fallopian tube (n = 6), and the lung (n = 320). MSRB3 was correlated versus all other transcripts using the Pearson method in Excel. The sum of correlation coefficients across tissues was then calculated (Rsum) and sorted in descending order. Examples from the Rsum extreme were tested using Spearman analysis in GraphPad Prism.

2.10 | Western blot analysis

Cells were harvested after the indicated treatments and isolated proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels followed by transfer to nitrocellulose membranes as described earlier (Krawczyk et al., 2015). The following primary antibodies were used at recommended dilutions: XBP1s (279015; Cell Signaling Technology), ATF6 (65880S; Cell Signaling Technology), and GAPDH (MAB374; Sigma Aldrich) together with the appropriate HRP-linked anti-mouse or anti-rabbit secondary antibodies (Krawczyk et al., 2015).

2.11 | Statistics

Statistical calculations were made in Graph Pad Prism 7. Means ± S.E.M. are plotted in all graphs. Single comparisons were made using a two-tailed student’s t test. Multiple comparisons were made using one-way ANOVA followed by Bonferroni’s post-hoc test. Individual correlations were tested using the Spearman method. p < .05 was considered significant. *p < .05, **p < .01, ***p < .001, and ****p < .0001.

3 | RESULTS

3.1 | UPR and SMC markers are anti-correlated in the models of phenotypic modulation in vivo

To examine whether the UPR and SMC gene programs are inversely coupled we examined four UPR markers (the chaperones Sdf2l1, Calr, Manf, and Pdia4; Krawczyk et al., 2016) and three SMC markers (Myh11 and Synpo2, which are cytoskeletal proteins and Kcnmb1, an ion channel) plus myocardin (Myocd) in correlation analyses using three expression data sets involving insults to SMCs. All of the SMC markers chosen are target genes of Myocd (Long et al., 2009; Turczynska et al., 2015; Yoshida et al., 2003). Using the Spearman method we then tested the correlations between UPR and SMC markers. In rat bladder denervation, a model that associates with growth and reduction of SMC markers (Zhu, Ekman et al., 2009), reaching a significance in 9 of 16 analyses. Myocd and Kcnmb1 were underperformed in this data set (Figure 1b), and were exceptions. The last data set was from the rat carotid artery balloon injury (Perisic Matic et al., 2016), where numerous SMC markers are transiently reduced following insult. Highly significant negative correlations between UPR and SMC markers were seen throughout, with the singular exception for Calr versus Kcnmb1 (Figure 1c).
concluded that significant reciprocal relationships between the UPR and SMC gene programs are seen in pathological in vivo models that involve SMC phenotypic modulation.

### 3.2 | ER stressors TN and DTT repress SMC markers in vitro

Having found reciprocal relationships between the UPR and SMC programs in three in vivo data sets we next asked if loss of SMC markers can be elicited by chemical ER stressors in vitro. In the first round of experiments, we generated time-curves using human bladder SMCs stimulated for various times with DTT and TN. As shown in Figure 2a,b, both substances increased UPR markers (blue symbols) and reduced SMC markers (red symbols). UPR activation preceded the loss of SMC markers, and myocardin (MYOCD) was among the more strongly affected SMC transcripts.

Similar experiments were also made using human coronary artery SMCs (Figure 2c). TN again induced the UPR markers, and, with a delay of about 6-18 hr, repressed the two SMC markers examined, and, with a delay of about 6-18 hr, repressed the two SMC markers examined. UPR activation using DTT moreover associated with less forceful SMC marker repression in the presence of 4μ8C compared with vehicle (Figure 3d). 4μ8C only partially restored MYOCD, while some of the SMC markers were more completely restored. This may suggest of differentiation markers in primary human SMCs from two anatomical origins, and that this effect correlates poorly with effects on cell viability.

### 3.3 | Inhibition and activation of IRE1α/XBP1 in vitro

The UPR has three major sensors in the ER membrane designated as IRE1α, ATF6, and PERK (graphically depicted in Figure 3a). 4μ8C, Ceapin-A7, and GSK2606414 are small molecule inhibitors of the respective branches of the UPR. In pilot experiments, Ceapin-A7 (targeting ATF6) and GSK2606414 (targeting PERK) appeared to be without effects (ACTA2: 1.0 ± 0.09 for vehicle; 0.49 ± 0.11 for 1μg/ml TN; 0.55 ± 0.04 for 1μg/ml TN plus 1μM GSK2606414; and 0.40 ± 0.2 for 1μg/ml TN plus 10μM Ceapin A7, n = 3-5). 4μ8C tended to be effective, and we therefore designed a more definitive experiment using a higher concentration than initially used. 4μ8C at 100μM effectively blocked induction of XBP1s at the protein level (Figure 3b, DTT: 1 ± 0.16 vs. DTT + 4μ8C: 0.14 ± 0.06, p < .001, n = 6, and TN: 1 ± 0.11 vs. TN + 4μ8C: 0.09±0.04, p < .001, n = 6). In human bladder SMCs treated with TN, the induction of the UPR marker PDIA4 was somewhat curtailed by this concentration of 4μ8C, and the repression of SMC markers were antagonized (Figure 3c). UPR activation using DTT moreover associated with less forceful SMC marker repression in the presence of 4μ8C compared with vehicle (Figure 3d). 4μ8C only partially restored MYOCD, while some of the SMC markers were more completely restored. This may suggest...
the effects of UPR activation beyond repression of MYOCD in bladder SMCs. 4μ8C counteracted TN-driven repression of differentiation markers also in human coronary artery SMCs (Figure 3c), and in this case, recovery of MYOCD was more complete. Taken together, these studies implicate IRE1α/XBP1s in UPR-driven loss of SMC differentiation via MYOCD.

We next examined whether spliced and active XBP1 is sufficient for SMC marker repression. For this, we overexpressed spliced XBP1 (XBP1s). This should bypass the ER stress caused by TN/DTT and act independently of unfolded proteins in the ER. The overexpression of XBP1s in human coronary artery SMCs increased XBP1s and the UPR marker SDF2L1 as expected (Figure 3f). To our surprise, PDIA4, which responded promptly to both TN and DTT (see above), was reduced by XBP1s. The SMC markers TAGLN and MYOCD were reduced as predicted. ACTA2 repression did not quite reach the level of significance in this experiment, but ACTA2 was significantly repressed when we included only the samples with the highest SDF2L1 induction (not depicted), suggesting perhaps a threshold effect. Taken together, both gain and loss of function approaches therefore implicate XBP1s in UPR-dependent repression of MYOCD and loss of SMC differentiation.

3.4 | Reciprocal antagonism

Our findings so far argued that activation of the UPR in vivo and in vitro reduces the expression of SMC differentiation markers. However, the tight anti-correlations between SMC transcripts and UPR transcripts observed in vivo may arise in part because MRTFs antagonize the UPR. To address this possibility we overexpressed MYOCD and MRTF-A (MKL1) and examined UPR markers by RT-qPCR in control conditions and after stimulation with TN and DTT. The basal levels of the UPR markers CRELD2, MANF, PDIA4, and SDF2L1 were all reduced by MYOCD and MRTF-A/MKL1 (Figure 4). The levels of these transcripts were also reduced by MYOCD/MRTF-A after treatment with the ER stressors TN (Figure 4a–d) and DTT (Figure 4e–h). SMC differentiation and UPR activation therefore appear to be mutually antagonistic.

3.5 | Identification of MSRB3 as an MRTF target gene possibly involved in UPR moderation

We reasoned that MRTF-dependent repression of the UPR may depend on an MRTF target gene important for protection against ER stress. To identify such a gene we overlapped a data set of genes that are induced by Jasplakinolide (Jasp; Turczynska et al., 2015), which activates MRTFs, with a set of genes that have CArG boxes in their promoters (Benson, Zhou, Long, & Miano, 2011). Fifty-eight genes were found in the overlap between these data sets (Figure 5a), and when individually scrutinized, the gene MSRB3 captured our attention. MSRB3 encodes a methionine sulfoxide reductase...
that resides in the ER and that was shown to antagonize ER stress and UPR activation (Kim, Kim, Kwak, Oh, & Kim, 2014; Kwak & Kim, 2017). In keeping with the view that MSRB3 is a novel SMC marker, we found that it correlated tightly with other SMC markers, such as MYH11, and with MYOCD and SRF across human tissues (Figure 5b–d). MSRB3 staining in the Human Protein Atlas (https://www.proteinatlas.org; Uhlen et al., 2015) also supported SMC–enrichment at the protein level (Figure 5e). Viral overexpression of MRTFs increased MSRB3 in bladder SMCs by 2–3-fold (Figure 5f), and silencing of SRF reduced MSRB3 expression (Figure 5g). The proximal MSRB3 promoter contained two CArG boxes, and was directly regulated in a reporter assay (Figure 5h). Latrunculin B (LatB), which depolymerizes actin, moreover decreased MSRB3 (Figure 5i). We thus concluded that MSRB3 is a direct target of MRTF/SRF signaling.

To address if MSRB3 is involved in MRTF-dependent UPR antagonism we next silenced MSRB3 using an adenovirus. The MSRB3 mRNA level was reduced by >50% (not depicted, and Figure 6c), and repression of ACTA2 by DTT under basal conditions tended to be greater (Figure 6a, bladder SMCs). Repression similarly tended to be more exaggerated with TN after MSRB3 silencing (shMSRB3 + TN) compared with control (U6 + TN, Figure 6a). However, induction of the UPR marker PDIA4 was comparable with and without MSRB3 silencing (Figure 6b), suggesting an unaltered UPR response. We next performed an experiment where we combined MSRB3 silencing with MYOCD/MRTF-A overexpression.

**FIGURE 3** IRE1α/XBP1 contributes to repression of SMC markers in response to ER stress in primary human SMCs. (a) Carton of the three major branches of the UPR localized in the ER. Inhibitors for the respective branch are shown in red lettering to the right. This image was created with Biorender.com. Pilot experiments suggested partial rescue of SMC markers by 4μ8C. 4μ8C at 100 μM effectively blocked induction of XBP1s as shown using western blot analysis (b). (c) The human bladder SMCs were treated with TN in the presence and absence of 100 μM 4μ8C for 48 hr (n = 6 throughout). The UPR marker PDIA4 and five SMC markers were assayed using RT-qPCR. The design of the experiments in (d) was similar to that in (c) but using DTT instead of TN (n = 6 throughout). (e) The effect of 4μ8C in human coronary artery SMCs (n = 6 throughout). (f) XBP1s overexpressed for 48 hr. The transcript levels were assayed using RT-qPCR (n = 9 throughout). DTT, dithiothreitol; ER, endoplasmic reticulum; IRE1α, inositol-requiring enzyme 1α; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SMC, smooth muscle cell; TN, tunicamycin; UPR, unfolded protein response; XBP1, X-box binding protein 1.
MRTF-A/MKL1 and MYOCD increased MSRB3, and MSRB3 silencing eliminated these effects (Figure 6c) as expected. Despite effective MSRB3 silencing, MRTF-A and MYOCD were equally effective antagonists of UPR marker induction (CALR, Figure 6d, bracketed bars). Taken together, this argued against involvement of MSRB3 in MRTF-driven repression of the UPR.

To address if MRTFs may interfere with generation of XBP1s and cleaved ATF6, we next examined induction of XBP1s and cleavage of ATF6 by western blot analysis (Figure 6e). The levels of XBP1s (Figure 6e,f) and cleaved ATF6 (lower band in Figure 6e, and summarized data in G) increased promptly with TN as expected, but their levels were unchanged with MRTF-A/MKL1 overexpression, both under basal conditions and in the presence of TN. This appears to rule out a generalized impairment of stress sensing at the level of the ER.

4 | DISCUSSION

The present work highlights a model for phenotypic modulation of SMCs that involves crosstalk between the UPR and SMC gene programs. This may account for a signature feature of the synthetic SMC phenotype, namely the dramatically expanded ER (Krawczyk et al., 2016). This follows from the fact that the UPR drives ER growth by targeting enzymes that synthesize ER lipids (Bommiasamy et al., 2009; Krawczyk et al., 2016; Sriburi, Jackowski, Mori, & Brewer, 2004) in addition to chaperones. By repressing SMC markers, the UPR would synchronize loss of contractile properties with gain of synthetic properties. Our analyses suggest that UPR-mediated repression of the SMC gene program involves IRE1α/XBP1 and repression of MYOCD. IRE1α is responsible for generation of XBP1s, and for a process known as regulated IRE1-dependent decay (RIDD). RIDD speeds up breakdown of numerous mRNAs and thus alleviates ER stress, but RIDD also acts on transcripts of non-ER-resident proteins. We cannot rule out that MYOCD is a substrate of RIDD, which would explain the effect of the IRE1α inhibition using 4μ8C. However, our XBP1s overexpression data, showing clear-cut repression of MYOCD and TAGLN, argues that RIDD alone does not explain the repression MYOCD and SMC markers during ER stress. Similarly, general translational repression via PERK is not likely to play a role for the ER-stress-driven repression of SMC markers because effects were manifested at the mRNA level, and PERK inhibition using a specific inhibitor was ineffective. Our overall conclusion therefore is that XBP1s induction and pursuant MYOCD repression are at least partly responsible for the reciprocal regulation of the UPR and the SMC markers, but the exact mechanisms involved remain to be precisely defined.
FIGURE 5  MSRB3, which mitigates ER stress in cancer cells, is an MRTF target gene. To identify target genes of MRTFs that could contribute to moderation of the UPR we overlapped genes induced by actin polymerization, which activates MRTFs, with CArG-box containing genes (a). One of the genes in this overlap, MSRB3, was shown to mitigate ER stress in prior work (Kwak & Kim, 2017). (b) MSRB3 was correlated against all transcripts (RNA-Seq data from the GTExPortal; Consortium, 2015) in 10 human tissues with highest MSRB3 levels. The sum of correlation coefficients across tissues were calculated and sorted in descending order and the top 300 (~5%) were plotted. Numerous SMC transcripts were present in the extreme including MYH11 and ACTA2. ZEB1, previously shown to regulate MSRB3, was also represented (purple), as was SRF (pink) and MYOCD (blue). Examples from the extreme are highlighted with colored symbols. (c,d) Examples of correlations between SRF and MSRB3 and MYOCD and MSRB3 in the human coronary artery (N = 133). (e) MSRB3 staining from the Human Protein Atlas (Uhlen et al., 2017). SMCs in the walls of small arteries (arrowheads) in the rectum, hippocampus, and a lymph node are all positive (brown). SMCs in the urinary bladder, gall bladder, and prostate are similarly positive. (f) Human bladder SMCs were transduced null adenovirus or with viruses encoding MRTF-A/MKL1, MRTF-B/MKL2, or MYOCD. The MSRB3 transcript was next assayed using RT-qPCR (n = 7-9). (g) Reduction of the MSRB3 transcript following silencing of SRF (by 76%, n = 6). (h) A promoter reporter assay for MSRB3 in control conditions and after overexpression of MRTFs (n = 6). (i) MSRB3 transcript level is reduced by depolymerization of actin using LatB (n = 9). CYR61 was used as appositive control in these experiments. ER, endoplasmic reticulum; MRTF, myocardin-related transcription factor; MSRB3, methionine sulfoxide reductase B3; RT-qPCR, reverse transcription quantitative polymerase chain reaction; UPR, unfolded protein response.
The concept that the UPR influences cellular differentiation has been documented in other cell types. In osteoblasts, for example, PERK signaling promotes BMP2-driven differentiation (Saito et al., 2011), and PERK-deficient mice have reduced bone mass (Saito et al., 2011). This is partly mirrored by human mutations in PERK (Wolcott–Rallison syndrome), giving rise to dysplastic bone growth (Delepine et al., 2000). Epithelial to mesenchymal transition in the lung is similarly driven by the UPR (Tanjore et al., 2011), and this promotes lung fibrosis (Lawson et al., 2011) and expression of smooth muscle α-actin (ACTA2; Tanjore et al., 2011). Differentiation of myofibroblast was moreover found to be promoted by UPR activation (Heindryckx et al., 2016; Matsuoka et al., 2015). Induction of ACTA2 in epithelial cells and fibroblasts by the UPR is at odds with our findings in SMCs where UPR activation by chemical ER stressors reduced ACTA2. The opposite influences on ACTA2 in different cell types could perhaps be explained by different inherent levels of expression (high in SMCs). Different levels

**FIGURE 6** MSRB3 does not appear to be responsible for MRTF-driven moderation of the UPR. Human bladder SMCs were treated with control adenovirus (U6) or with adenovirus encoding a short hairpin targeting MSRB3 (shMSRB3) causing reduction of the MSRB3 transcript (c.f. panel c). Control and silenced cells were then treated with vehicle, DTT or TN, and the levels of ACTA2 (a, n = 2) and PDIA4 (b, n = 4) were assayed using RT-qPCR. The relative and absolute PDIA4 increases were not different in control and silenced cells (b, p > .05). Next, to test the role of MSRB3 in MRTF modulation of the UPR we combined overexpression with MRTF-A/MKL1 and MYOCD with MSRB3 silencing during treatment with tunicamycin (c, d, n = 2 for both). (c) The levels of MSRB3 in the different treatment groups. MRTF-A and MYOCD induced MSRB3, and MSRB3 was reduced to basal levels by silencing (shMSRB3) as expected. (d) MRTF-A/MKL1 and MYOCD reduced the UPR marker CALR similarly in control and MSRB3-silenced cells (compare bars highlighted with brackets). This argues that MSRB3 did not mediate the MRTF-driven reduction of CALR. (e) Western blot analysis for XBP1s and ATF6, respectively, at 24 hr of TN treatment (1 µg/ml). MRTF-A/MKL1 and Null viruses were added 72 hr before TN addition. (f,g) Summarized data on XBP1s and cleaved ATF6 (ATF6c, the faster migrating band appearing with TN) from the experiments in (e) (n = 6). DTT, dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MRTF, myocardin-related transcription factor; MSRB3, methionine sulfoxide reductase B3; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SMC, smooth muscle cell; TN, tunicamycin; UPR, unfolded protein response.
of miR-150, proposed to underlie ACTA2 induction by the UPR in the skin (Heindryckx et al., 2016), may also contribute. Whatever the reason, it is clear that the influence of the UPR on ACTA2 is cell type-dependent. We studied numerous SMC markers in the present work, and for the majority, expression levels clearly go down with UPR activation. Our overall conclusion that the UPR represses SMC markers therefore stands firm.

The coupling between the SMC and UPR gene programs may be reciprocal because we find that forced SMC differentiation by overexpression of MYOCD/MRTF-A also mitigates UPR activation. Using bioinformatics we identified MSRB3 as a possible mediator of this effect (Kim et al., 2014; Kwak & Kim, 2017), and we could demonstrate that MSRB3 is a direct target gene of MYOCD/MRTF-A. Silencing of MSRB3 did however not mitigate the anti-UPR effect of MYOCD/MRTF-A (reduced CALR induction), leaving this effect unexplained. Possibly, the mechanism could involve the gene CALU, also identified in our analyses as a tentative MRTF target gene, but this possibility would need to be addressed in separate work. Importantly, inducible knockout of MYOCD in SMCs associates with late stage UPR activation (Huang et al., 2015), suggesting repression of the UPR by MYOCD also in vivo. Similarly, and as shown here, UPR-driven inhibition of SMC differentiation occurs both in vivo and in vitro and can be initiated by ER stress, which is the physiological activation mechanism, clearly warranting further studies of these phenomena.

Our gain and loss of function experiments implicate IRE1α/XBP1 in repression of MYOCD and SMC markers by the UPR. Activation of XBP1 is unusual and involves splicing of an intron from the mRNA. This changes the reading frame to yield a 54 kDa active transcription factor (Calfon et al., 2002). The endonuclease activity of IRE1α is increased by accumulation of unfolded proteins in the ER, and can be inhibited by 4μC8C. Two prior studies have examined the aspects of XBP1 function in SMCs in the context of vascular disease (Zeng et al., 2015; Zhao et al., 2017). The first of these studies showed that vascular injury increased splicing of XBP1, and that XBP1 deficiency in SMCs abrogates subsequent neointima formation (Zeng et al., 2015). It was also found that treatment with PDGF increases XBP1 splicing, and that this contributes to proliferation and migration of SMCs. In agreement with the current work, XBP1s was moreover found to reduce calponin (CNN1), supporting modulation to the synthetic phenotype. Another study found that unspliced XBP1 (XBP1u) is lost in aortic aneurysms in parallel with loss of contractile differentiation. Unspliced XBP1 promoted CARG- and MYOCDE-dependent promotor activation (Zhao et al., 2017). It follows that XBP1u and XBP1s could be antagonistic such that XBP1u promotes contractile differentiation under homeostatic conditions whereas XBP1s modulates SMCs toward the synthetic phenotype upon unfolding stress. Our findings are consistent with such a balance, but generation of XBP1s appears sufficient to explain the loss of MYOCD that we see with chemical ER-stressors.

5 | CONCLUSION

The present study has strengthened the view that the UPR and SMC programs are directly and reciprocally coupled using three in vivo models of phenotypic modulation. We also find that chemical ER stressors reduce SMC markers in primary human SMCs in vitro, and this appears to involve IRE1α/XBP1s. The transcription factors that drive SMC differentiation (MYOCD/MRTF-A) moreover dampen the UPR response. This effect does not involve MSRB3 (Kim et al., 2014; Kwak & Kim, 2017), identified here as a novel and direct MRTF target gene, or reduced generation of either XBP1s or cleaved ATF6.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Designing of the study and critical revisions: all authors. Data collection and analysis: all authors. Manuscript drafting: B. Z., F. D., K. S.

DATA AVAILABILITY STATEMENT

Data sets are accessible via the Gene Expression Omnibus using the accession numbers GSE47080, GSE104540, and via the GTExPortal.org. The experimental data will be made available on request.

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