Metallothionein-dependent up-regulation of TGF-β2 participates in the remodelling of the myxomatous mitral valve

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Aims
Although an excessive extracellular matrix remodelling has been well described in myxomatous mitral valve (MMV), the underlying pathogenic mechanisms remain largely unknown. Our goal was to identify dysregulated genes in human MMV and then to evaluate their functional role in the progression of the disease.

Methods and results
Dysregulated genes were investigated by transcriptomic, immunohistochemistry, and western blot analyses of the P2 segment collected from human idiopathic MMV during valvuloplasty (n = 23) and from healthy control valves (n = 17). The most striking results showed a decreased expression of two families of genes: the metallothioneins-1 and -2 (MT1/2) and members of the ADAMTS. The mechanistic consequences of the reduced level of MT1/2 were evaluated by silencing their expression in normal valvular interstitial cells (VICs) cultures. The knock-down of MT1/2 resulted in the up-regulation of transforming growth factor-beta 2 (TGF-β2). Most importantly, TGF-β2 was also found significantly increased in MMV tissues. The activation of VICs in vitro by TGF-β2 induced a down-regulation of ADAMTS-1 and an accumulation of versican as observed in human MMV.

Conclusion
Our studies demonstrate for the first time that MMV are characterized by reduced levels of MT1/2 accompanied by an up-regulation of TGF-β2. In turn, increased TGF-β2 signalling induces down-regulation of aggrecanases and up-regulation of versican, two co-operating processes that potentially participate in the development of the pathology.

Keywords
Myxomatous mitral valve • Metallothionein • TGF-β • ADAMTS • Valvular interstitial cells

1. Introduction
Myxomatous mitral valve (MMV) disease is the most frequent cause of non-ischaemic mitral regurgitation in the industrialized countries.1 MMV can be sporadic or familial. Several studies have indeed shown that four loci on chromosomes 11, 13, 16, and X are associated with the disease but no specific gene has been clearly identified yet.2 MMV is characterized by an increased amount of elastin and collagen in myxomatous mitral leaflets, which is further accompanied by an altered architectural organization of their fibres.3 Another cardinal feature is an excessive deposition of proteoglycans such as decorin, biglycan, and versican.4 This extracellular matrix (ECM) remodelling progressively results in thickening of one or both leaflets, the posterior segment being the most frequently affected. This causes an abnormal displacement of mitral leaflet(s) into the left atrium during systole leading to an incomplete closure of the valve and ultimately to mitral regurgitation and cardiac insufficiency.5

Potential mechanisms have been identified to explain the excessive remodelling of the mitral valve tissue. Valvular interstitial cells (VICs) might be the key actors6 as they display features of activated myofibroblasts in MMV and seem to express increased levels of several
MMPs that are most probably responsible for the remodelling of collagen and elastic fibres. Alterations of TGF-β signalling could also be involved, since myxomatous degeneration of the mitral valve is observed in heritable connective tissue disorders such as Marfan syndrome, caused by mutations in fibrillin1 gene, and Loey-Dietz syndrome, due to inactivating mutations in TGF-βRI or II, two genetic defects characterized by an excessive TGF-β signalling. However, no study has shown so far an association between TGF-β dysregulation and idiopathic MMV in humans.

As the pathogenic mechanisms causing, or participating in, the degeneration of mitral valve are still to be clarified, the first goal of our study was to identify genes modulated in MMV compared with normal valves by a global transcriptomic analysis. The reduced expression of a series of genes [such as superoxide dismutase 2, glutaredoxin, and the metallothioneins family (MTs)] involved in the response to oxidative stress suggested that the protection against reactive oxygen species could be weakened in human MMV, as shown in some studies. Furthermore, a recent study showed a low expression of MTs in the ascending aortic aneurysm of bicuspid valve patients thought to be associated with the remodelling of ECM. As several members of the MT1 and MT2 families were down-regulated in our transcriptomic analysis, we considered this first group in priority. A second group of down-regulated genes concerned members of the ADAMTS family involved in the degradation of proteoglycans known to accumulate in MMV. The in vitro and in vivo studies reported here demonstrate a key relationship between MTs and ADAMTS through TGF-β signalling and identify mechanisms potentially participating in the progression of MMV.

2. Methods
Detailed procedures and protocols are provided in the Supplementary material online.

2.1 Tissue collection
P2 segments of posterior leaflets of MMV were obtained during elective surgery from valvuloplasty to correct severe mitral regurgitation from 23 patients. Normal P2 segments were collected from hearts of donors rejected for transplantation (n = 17). Strips of tissue, from the free edge to the annulus, were used for isolation and cultures of VICs, immunohistochemistry, transcriptomic, and proteomic analyses. The thickness of leaflets was measured on haematoxylin–eosin sections in successive fields from the annulus to the free edge. Demographics of the patients are detailed in Table 1. The study conforms to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of Liège University Hospital (B7020071262), and a written consent was obtained from the informed patients.

2.2 Valvular interstitial cells (VICs) cultures
VIC cultures were established by outgrowth from explants of human normal (n = 7) and myxomatous (n = 4) mitral valves maintained in EBM medium-10% FBS (Lonza). Cells were used at passage 4–7.

2.3 Immunocytochemistry
Characterization of VICs was achieved by immunostaining of CD31 (1/200, Dako), smooth muscle α-actin (α-SMA, 1/300, Sigma) and vimentin (1/400, Dako) on fixed subconfluent VIC cultures. Labelled and non-labeled cells were counted in six fields at a ×16 magnification using a Zeiss Axiovert25 inverted fluorescent microscope. No CD31-positive cell was detected. All VICs expressed vimentin, whereas 13 ± 6% were positive for α-SMA.

2.4 RNA extraction
Total RNA was extracted from aliquots of frozen powdered valvular tissues using the RiboPure kit (Ambion) and from VIC cultures using the High Pure RNA isolation kit (Roche Molecular Biochemical).

2.5 Microarray analysis
Transcriptomic analysis was performed by using the Affymetrix HG-U133A 2.0 chip on total RNA from MMV and control samples. Probe synthesis, hybridization, washing protocols, and signals scanning were performed at the Genomics Facility of the GIGA-Research Center, University of Liège with the GeneChip Operating Software.

2.6 RT–PCR analysis
RT–PCR analysis was performed as previously described using the set of primers and the conditions described in the Supplementary material online, Table S1.

2.7 Proteins extraction and western blotting
For MTs analyses, proteins were extracted from valvular tissues or VIC cultures using a specially designed procedure followed by stabilization with bromobimane (Sigma) as detailed in the Supplementary material online. For the other proteins, VICs were lysed in SDS–PAGE buffer, electrophoresed, and transferred to polyvinyl difluoride membranes. The electrotransfer of MTs used a protocol adapted from Mizzen. Membranes were blocked and probed with antibodies against MTs (1/200, Dako), GAPDH (1/5000, Dako), Erk1/2 (15000,
Sigma), α-SMA (1/5000, Sigma), Smad2, and phospho-Smad2 (1/500, Cell Signaling).

2.8 Immunohistochemistry

Immunohistochemistry was performed in valvular tissue sections using a primary monoclonal antibody recognizing the members of the MT1 and MT2 subfamilies (1/100, Dako), a primary monoclonal anti-versican (1/1000, Developmental Studies Hybridoma Bank) and a primary monoclonal anti-TGF-β2 (1/100, Abcam). The number of MT-positive cells was counted by two blinded observers in five microscopic fields at a x 40 magnification. TGF-β2 staining intensity in cells and in the ECM was scored by four blinded observers on a scale from 0 (no detectable labelling) to 4 (strong labelling). A mean score value was established for each tissue section and expressed in arbitrary units (a.u.). The standard deviation between scoring for the same slide by the four blinded observers was <0.6. Versican staining was quantified by image analysis using the software OlyVIA (Olympus) and Quantity One 4.6 (BioRad) and expressed in intensity/mm².

2.9 siRNA transfection

siRNA (S, 5′-GAGUGCAAAUGCACUCCU-3′ and AS, 5′-AGAGGUGCUUUGCAGUC-3′) targeting common sequences of the MT1 and MT2 subfamilies members was transfected according to a previously described procedure, using an irrelevant siRNA as control.17 Briefly, calcium-phosphate-mediated transfection was performed overnight on subconfluent VICs, from five normal mitral valves, at a final concentration of 20 nmol/L siRNA. VICs were washed twice with PBS and once with EBM with 0.2% BSA (Sigma). This last step was defined as time 0 post-transfection. Cells were lysed for western blot and RT–PCR analysis 72 h post-transfection to evaluate the silencing efficiency.

2.10 ELISA

TGF-β1 and TGF-β2 concentrations were measured in the conditioned medium of 72 h transfected VICs (DuoSet ELISA kit, R&D systems) after activation of the latent forms by HCl.

2.11 TGF-β2 treatment of VICs

After stimulation with increasing concentration (0–5 ng/mL) of human recombinant TGF-β2 (T2812, Sigma), VICs isolated from five normal mitral valves were lysed for western blot or RT–PCR analyses after 1 or 48 h.

2.12 Cadmium treatment of VICs

Subconfluent cultures from four normal mitral valves and four MMV were supplemented with increasing concentration of CdCl₂ from 0 to 5 µmol/L for 12 and 24 h. Cells were lysed for RT–PCR analysis.

2.13 Versican analyses

Conditioned mediums of VICs treated with increasing concentration of human recombinant TGF-β2 were digested at 37 °C with chondroitinase ABC (0.2 U/100 µg protein, Sigma) as described by Kischel et al.18 and analysed by western blotting using monoclonal antibody against versican (1/300, Developmental Studies Hybridoma Bank).

2.14 Statistical analyses

As the investigated variables measured in the in vivo analyses did not follow a Gaussian distribution, the significance of differences between the two groups was tested by using the Mann–Whitney U test for unpaired samples. The data are represented by box plots showing the median of the results. Upper and lower limits of boxes represent inter-quartiles (25 and 75th), whereas upper and lower bars show highest and smallest non-outlier data. The statistical analysis for in vitro experiments was performed by using a paired t-test of Student. Results were reported as mean ± SD of the indicated number of independent experiments using VICs from at least four normal and four MMV.

The correlation between age and in vivo data as well as between TGF-β2 staining scoring and valve thickness was established by using Spearman rank correlation. The proportion of female patients was not significantly different between the two groups as determined with Fisher’s exact test.

3. Results

3.1 Demographics of mitral valve tissues

The median segment P2 of the posterior leaflet was collected in 23 patients undergoing valvuloplasty to correct severe mitral regurgitation resulting from primary myxomatous degeneration. A similar segment was obtained from the heart of 17 donors unsuitable for transplantation and with no history of cardiovascular pathologies. The myxomatous leaflets were significantly thicker than normal leaflets (1.8 ± 0.4 vs. 1.0 ± 0.5 mm, respectively; P < 0.0001). The controls were significantly younger than the MMV patients as typical of the transplant donors. The proportion of female patients was slightly higher in the control group but not significantly different between the two groups (Table 1). In our study, however, none of the investigated parameters was found to be significantly correlated with age or gender (data not shown).

3.2 Transcriptomic analysis

A microarray analysis of RNA extracted from three MMV and three control samples was performed to identify potentially dysregulated genes in myxomatous segments. The expression of 414 genes was altered by, at least, a factor of 2: 158 genes being up-regulated, 256 genes down-regulated. Strikingly, several genes involved in the protection against oxidative stress, among which genes of the MT family, were downregulated (Table 2). Because of the excessive accumulation of proteoglycans consistently observed in MMV, we also noticed the down-regulated expression of aggrecanases of the ADAMTS family having probe set on the chip.

These potentially dysregulated expressions in MMV were evaluated for confirmation by RT–PCR performed on a larger number of MMV (n = 13) and control (n = 11) RNA samples. Among the investigated MT isoforms, only the MT2A, MT1E, and MT1X were expressed. As shown in Figure 1A, their expression was significantly reduced in the MMV tissues when compared with that in healthy valves, validating the microarray results. The same approach was used for measuring the expression of aggrecanases (ADAMTS-1, -4, -5, -8, -9, and -15). A significantly reduced expression was observed for ADAMTS-1, -4, -5, and -9 in the MMV samples when compared with controls (Figure 1B), the ADAMTS-8 and -15 being not expressed in any sample.
3.3 Metallothioneins expression in valvular tissue and VICs culture

MT proteins were also measured in tissue samples by western blotting (Figure 2A) using an antibody specific for a conserved epitope shared by human MT isoforms 1 and 2 (MT1/2). The mean value for MT content normalized to that of GAPDH was significantly reduced in MMV tissues when compared with controls (Figure 2B). By immunohistological staining of normal mitral valves using the same antibody, cells expressing MT 1/2 were mainly localized in the spongiosa as observed in the representative picture of Figure 2C (left panel). The staining was almost absent in MMV (Figure 2C, right panel). Quantification of positive cells in 18 MMV samples and 16 control valves (Figure 2D) showed their nearly complete absence in most of the MMV, in agreement with the western blot results.

The basal expression level of MT1/2 did not significantly differ between the VICs isolated from four MMV and four healthy valves (not shown). Exposure to 5 μmol/L CdCl\textsubscript{2} resulted in a strong induction of the three investigated MT isoforms, similar to that in control and MMV cells as shown in the Supplementary material online, Figure S1, indicating that the metal-responsive pathway was not altered in MMV interstitial cells.

3.4 Silencing of MT 1/2 up-regulates the expression and secretion of TGF-β2 by VICs

The functional consequences of MT down-regulation was evaluated in cell culture using VICs from healthy donors (n = 5) transfected with MT1/2-specific siRNA and an irrelevant siRNA as control. The effective and specific silencing of MT1/2 72 h post-transfection was demonstrated by western blotting (Figure 3A). In parallel, RT–PCR was also performed to assess the repression of individual MT1/2 isoforms. As shown in Figure 3B, the mRNA level of the three isoforms that we found down-regulated in MMV (MT2A, MT1E, and MT1X) was reduced in the siMT transfected cells. Based on the occurrence of MMV in genetic disorders associated with excessive TGF-β signalling, the levels of TGF-β1 and TFG-β2 were measured in the 72 h-conditioned medium of transfected cells. MT1/2 silencing did not modify the amount of TGF-β1 released (50 ± 14 pg/mL) but induced a significant increase in TGF-β2 production (92–263 pg/mL) when compared with the control cells (44–154 pg/mL) (Figure 3D). This siMT-induced up-regulation of TGF-β2 occurred at a pre-translational step, since TGF-β2 mRNA was significantly increased in the MT1/2 silenced VICs while TGF-β1 (Figure 3C) and TGF-β3 (not shown) mRNA level were not modified. Whether this increase depends on a transcriptional activation or a stabilization of the mRNA needs further investigation.
3.5 TGF-β2 expression is increased in MMV tissue

TGF-β1 and TGF-β2 expression was then measured by RT–PCR in individual MMV (n = 11) and normal mitral valves (n = 7). As illustrated in Figure 4A, TGF-β2 expression was significantly increased in MMV samples when compared with healthy valvular tissue, whereas TGF-β1 expression was similar in both groups. An immunohistological staining of TGF-β2 in 18 MMV and 13 control valves, as illustrated by representative samples (Figure 4B), followed by individual scoring (Figure 4C) showed a higher grade of labelling in MMV (1.5-fold) consistent with 1.6-fold increase in mRNA level (Figure 4A). Interestingly a strong staining of the surrounding ECM was observed in some parts of MMV leaflets (5/18) as shown in the lower panel of Figure 4B.

The implication of TGF-β2 in leaflet remodelling was further evaluated by analysing the potential correlation between TGF-β2 levels expressed as the score obtained from immunohistochemistry evaluations (Figure 4C) and valve thickness. Pathological valves and control valves were included in order to take into account the variability occurring in non-diseased heart valves. A positive correlation (Spearman r = 0.44, P < 0.05) was observed, further suggesting the active role of TGF-β2.

3.6 TGF-β2 activates VICs and modulates the ADAMTS-1 expression and versican production

Normal VICs were treated by recombinant TGF-β2 for increasing times. An increase in phosphorylated-smad2 was already observed after 1 h of treatment, even at a concentration as low as 0.5 ng/mL (Figure 5A). After 48 h, VICs displayed the phenotypic changes typical of myofibroblastic activation characterized notably by the induction of α-SMA (Figure 5B). Because a reduced expression of aggrecanases was observed in MMV, their expression was measured by RT–PCR in TGF-β2-treated VICs. As illustrated in Figure 5C, the addition of TGF-β2 lead to a significantly decreased expression of ADAMTS-1, the most highly expressed aggrecanase in mitral valve (Supplementary material online). It has been shown that the remodelling of ECM occurring in MMV is characterized by an excessive deposition of versican. This finding was confirmed here by using a semi-quantitative evaluation. The intensity of versican staining in MMV (n = 18) was almost two times stronger than in normal leaflets (n = 13) (Supplementary material online, Figure S2). Versican being a known substrate of ADAMTS-1, we evaluated its accumulation in the medium of TGF-β2-treated VICs. Versican can be found as four different isoforms (V0, V1, V2, and V3) resulting from alternative splicing of the primary transcript. The treatment by TGF-β2 resulted in a significant production of V0 and V1 isoforms while V2 and V3 were not detected (Figure 5D).

4. Discussion

Although MMV has been well described at clinical, functional, and histological levels, only a few studies addressed the cellular and molecular mechanisms underlying the extracellular remodelling consistently observed in this pathology. Two characteristic features of MMV are the disorganization of the collagen and elastic fibrillar structures, as well as the accumulation of material enriched in proteoglycans, leading to a thickening of the leaflets. Our starting experimental strategy was to perform a global transcriptomic analysis.
This initial characterization provided indicative data allowing to identify potential cellular processes and families of genes worth to be explored in MMV. As stated and explained in the introduction, two families of genes, down-regulated in MMV, were considered in priority: the MT involved in the protection against oxidative stress and ADAMTS, enzymes involved in the degradation of proteoglycans.

MTs are cysteine-rich small proteins that display antioxidant functions and are involved in zinc homeostasis, but the full spectrum of their biological functions is not fully elucidated yet. Under hypoxia, oxidative stress, or metal exposure, they are transcriptionally activated upon nuclear translocation of the zinc finger factor MTF1 and its binding to metal response elements (MREs) present in the MT1/2 promoters but also in other target genes. Furthermore, MTs have been recently involved in the modulation of functions of various vascular cell types and macrophages, as demonstrated in MT knockout mice. MTs seem therefore to participate in cellular processes other than metal and oxidative stress response. The pathways leading to the down-regulation of MTs in MMV are not yet known and may be multiple. To evaluate whether it could be related to intrinsic transcriptional dysregulation, we compared the MT1/2 expression in normal and MMV VICs treated with cadmium in vitro (Supplementary material online, Figure S1). The similar and strong induction observed in both types of cells suggests that the cellular machinery driving the metal-induced MT expression is not altered in MMV, at least in vitro. This contrasts with data obtained at the mRNA level with the aortic smooth muscle cells from bicuspid aortic valve patients. It has to be noticed, however, that MTs can be regulated independently of the MRE through other cis-acting elements and transcription factors in response to various other extracellular stimuli, such as VEGF or shear stress as observed in endothelial cells. In healthy valves, MTs were essentially expressed by the VIC in the spongiosa but a potential paracrine regulation by signals or soluble factors produced by endothelial cells could be considered as recently suggested. These regulations would be worth to be evaluated in co-culture of VICs and static or shear-stressed endothelial cells. Genetic acquired defects disturbing these pathways could potentially indirectly affect the MT expression in MMV. A direct modulation of VICs by mechanical stress or relaxation is another possibility. Further investigations would be required to disclose the pathways involved in the MT down-regulation in MMV.

Myxomatous degeneration of the mitral valve occurs also in patients suffering from connective-tissues disorders related to enhanced TGF-β signalling, such as Marfan and Loeys-Dietz syndromes. Moreover, mutations in ADAMTSL2 cause a rare autosomal recessive disorder called geleophysic dysplasia. Affected patients present dilatation and thickening of heart valves and are characterized by an increased production of TGF-β1 by mutated fibroblasts. Idiopathic MMV has been suspected to be associated with an altered TGF-β signalling although it was never clearly

![Figure 3](image-url)
demonstrated in humans. On the other hand, a reduced expression of MT caused by the loss of MTF1 was correlated to an increased expression and activation of TGF-β1 and to an enhanced matrix deposition in tumours.30 These evidences prompted us to investigate a possible link between MT and TGF-β signalling in cultured VICs by using a siRNA strategy. To mimic the in vivo situation, the siRNA was designed to silence all the expressed members of the MT1 and MT2 families. While this silencing did not alter TGF-β1 expression, it significantly increased the synthesis and secretion of TGF-β2. Although TGF-β1 was known to regulate MT,31,32 our data demonstrate for the first time that MTs control the expression of TGF-β2. We further showed that TGF-β2 was increased in human MMV at the mRNA and protein level. With the exception of one single study showing a high expression of TGF-β1 and TGF-β3 in canine MMV,29 the role of TGF-β, and more specifically TGF-β2, in the idiopathic form of the human disease has never been shown. The increased expression of TGF-β2 might also be involved in the hypercellularity7 and in the higher expression of type I collagen observed in

**Figure 4** TGF-β2 expression is increased in myxomatous mitral valves (MMV). (A) The relative expression of TGF-β1 and TGF-β2 mRNA was evaluated by RT–PCR. Results (in arbitrary units per unit of 28S RNA) are reported as box plots where median is indicated by horizontal bar in MMV (n = 11) and normal mitral valve (n = 7). *P < 0.05 determined with Mann–Whitney U test. (B) Representative histological sections from healthy and two MMV stained with anti-TGFβ2 antibody (bar = 100 μm). Inserts show higher magnification. (C) The TGFβ2 staining was evaluated on a scale from 0 (no detectable labelling) to four (strong labelling) by four blinded observers in sections from MMV (n = 18) and control (n = 13) mitral valves. The median is indicated by the horizontal bar. *P < 0.05 determined with Mann–Whitney U test. (D) TGF-β2 staining correlated with leaflet thickness from MMV (n = 18) and control (n = 13) mitral valves, x, y, and z representing the mitral valves shown at Figure 4.
MMV (Supplementary material online, Figure S3) and could be associated with the disease progression since its level was correlated with the leaflet thickness. The key role of this growth factor has been demonstrated during heart valve development and, more precisely, for mammalian endocardial cushion cell transformation. It also regulates the endothelial/mesenchymal plasticity of valvular progenitors. Moreover, out of the three different TGF-β isoforms deficient mice, only Tgfb2+/− mice display defects in valve remodelling during heart development.

Pathogenesis of MMV has been correlated with the emergence of myofibroblasts identified histochemically by the expression of α-SMA and thought to mediate the ECM remodelling observed in MMV. Some studies reported that TGF-β1 was able to initiate the differentiation of VICs into myofibroblasts, but nothing was known for TGF-β2. Our results showed that VICs, isolated from different donors, consistently responded strongly to TGF-β2, suggesting its active role in the dysregulated ECM remodelling in MMV. The increased production and secretion of versican in the medium of TGF-β2-treated VICs and the abundant deposits of versican in the diseased leaflets, especially in the spongiosa where cells lack MTs, further support this hypothesis. Versican is a major hyaluronan-binding proteoglycan that play a key role in vascular remodelling. Besides, its upregulation by TGF-β2 in VICs, as shown here, and in tumoural cells, the accumulation of versican observed in MMV, could also result from a repression of its degradation by aggrecanases since we found that the expression of ADAMTS-1, -4, -5.

**Figure 5** TGF-β2 activates VIC and decreases ADAMTS-1 expression. Normal VICs were treated by increasing concentrations of TGF-β2 (0 to 5 ng/mL). (A) Cells were lysed after 1 h of stimulation and analysed by western blot for phosphorylated Smad2 (P-Smad2) and total Smad2. (B) Cells were lysed after 48 h of stimulation and analysed by western blot for α-SMA. GAPDH was used as loading control. (C) ADAMTS-1 expression was measured by RT-PCR after 48 h of stimulation by TGF-β2 and expressed as the fold induction relative to the control condition (0 ng/mL) taken as 1. Results are the mean ± SD of independent experiments at least in triplicate using VIC from five normal valves. *p < 0.05 and ***p < 0.001 determined with paired t-test of Student. (D) Versicans (V0 and V1 isoforms), released in the medium of VIC after 48 h of stimulation by TGF-β2, were analysed by western blot and quantified. The results are expressed as the fold induction relative to the control condition (0 ng/mL) taken as one and are the mean ± SD of independent experiments at least in triplicate using VIC from five normal valves. *p < 0.05 and ***p < 0.001 determined with paired t-test of student.

**Figure 6** Mechanistic relationships involved in myxomatous mitral valve (MMV) suggested by our results. MT, metallothioneins; ECM, extracellular matrix; VIC, valvular interstitial cells.
and -9 was significantly reduced in MMV. To our knowledge, our study is the first to suggest the implication of an alteration of ADAMTS expression in human MMV. This hypothesis is further strengthened by recent data showing valvular and aortic developmental anomalies due to a decreased cleavage of versican in mouse model of adams-5 and adams-9 haploinsufficiency. Thus we can easily suspect that the reduced expression of ADAMTS-1, which is the most expressed aggrecanase in the human mitral valve, predominantly contributes to versican accumulation in MMV. Additionally, ADAMTS-1 was decreased by TGF-β2 in vitro. It is worth noting that altered versican content or ADAMTS level can affect the canonical TGF-β signalling pathway. These studies, including our, open new fields of investigation about the role of ADAMTS and versican not only in human idiopathic MMV but also in mitral valve disease related to various aetiological factors, genetic disorders, or ischaemic mitral regurgitation. As these conditions share common features of structural and functional alterations of the ECM, including excessive TGF-β signalling and proteoglycans accumulation, it would be worth to investigate MTs and ADAMTS in these pathologies.

At the initiation of our work, two families of proteins were found down-regulated in the MMV: the MTs and members of the ADAMTS family involved in the degradation of proteoglycans. By investigating in vitro consequences of silencing MT1/2, we highlighted an up-regulation of TGF-β2 that was remarkably also found increased in the MMV tissues. The activation in vitro of VCs by TGF-β2 resulted in a down-regulation of ADAMTS-1 and a considerable accumulation of versican, a feature of myxomatous degeneration (Figure 6). Altogether, these findings highlight new actors potentially involved in the progression of the mitral disease and propose mechanisms underlying myxomatous degeneration.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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