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Developmental basis for filamin-A-associated myxomatous mitral valve disease

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Aims We hypothesized that the structure and function of the mature valves is largely dependent upon how these tissues are built during development, and defects in how the valves are built can lead to the pathological progression of a disease phenotype. Thus, we sought to uncover potential developmental origins and mechanistic underpinnings causal to myxomatous mitral valve disease. We focus on how filamin-A, a cytoskeletal binding protein with strong links to human myxomatous valve disease, can function as a regulatory interface to control proper mitral valve development.

Methods and results Filamin-A-deficient mice exhibit abnormally enlarged mitral valves during foetal life, which progresses to a myxomatous phenotype by 2 months of age. Through expression studies, *in silico* modelling, 3D morphometry, biochemical studies, and 3D matrix assays, we demonstrate that the inception of the valve disease occurs during foetal life and can be attributed, in part, to a deficiency of interstitial cells to efficiently organize the extracellular matrix (ECM). This ECM organization during foetal valve gestation is due, in part, to molecular interactions between filamin-A, serotonin, and the cross-linking enzyme, transglutaminase-2 (TG2). Pharmacological and genetic perturbations that inhibit serotonin-TG2-filamin-A interactions lead to impaired ECM remodelling and engender progression to a myxomatous valve phenotype.

Conclusions These findings illustrate a molecular mechanism by which valve interstitial cells, through a serotonin, TG, and filamin-A pathway, regulate matrix organization during foetal valve development. Additionally, these data indicate that disrupting key regulatory interactions during valve development can set the stage for the generation of postnatal myxomatous valve disease.

Keywords Filamin-A • Serotonin • Myxomatous valve disease • Transglutaminase-2 • Valve maturation

1. Introduction

Mutations in the *filamin-A* gene have recently been identified in multiple families with an X-linked form of myxomatous valvular dystrophy (MVD).[1,2] Defined as a heterogeneous group of disorders including Marfan syndrome and isolated valvular diseases (e.g. mitral valve prolapse), MVD is phenotypically characterized by the loss of normal matrix patterning and zonal interfaces coupled with fragmented

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collagen and excess proteoglycan production. These alterations compromise structural integrity of the valve leading to billowing of the leaflets and can cause functional regurgitation. Thus, we sought to determine how the intracellular cytoskeletal protein filamin-A can cause human defects largely attributed to disrupted extracellular matrix (ECM) patterning.

Filamins are large cytoplasmic proteins that can function as molecular tethers by interacting with both ECM-bound cell-surface integrins and the actin cytoskeleton.[3,4] Through these interactions, filamin-A serves as a mechanosensor and can relay extracellular signals to the cytoskeleton.[5] These proteins not only function as structural units, but also serve as docking platforms for second messengers important in signal transduction.[6] The Filamin group of proteins contains three members: A, B, and C. Filamins-A and B are widely expressed, whereas Filamin-C expression is restricted to cardiac and skeletal muscle. The filamins are present as homo or heterodimeric Y-shaped proteins with each chain consisting of actin and integrin-binding regions at the amino and carboxyl termini, respectively.[7] Gene knockout studies have indicated the importance of these proteins in diverse developmental processes, and filamin-A appears to be the major family member utilized in cardiac and vascular development. To date, two filamin-A mutant mice have been generated and evaluated for various defects.[8,9] Both studies report that filamin-A null mice exhibit embryonic lethality and a wide range of cardiovascular malformations including incomplete septation of the outflow tract, atrial and ventricular septal defects, type B interruption of the aortic arch, abnormal vascular permeability, and valve defects.[8,9]

To uncover mechanisms by which filamin-A may lead to postnatal myxomatous valve disease, we conditionally removed filamin-A from the atrioventricular (AV) valves during development. In doing so, we observe a 100% penetrant postnatal MVD in addition to defects in the number and branching pattern of chordae tendineae. We were able to define the inception of the defect to an initial error in foetal valve development at E15.5–E17.5, whereby enlargement and alteration in valve structure were readily apparent. This quantifiable change in the valve size and form is likely due to alterations in interstitial fibroblast function/activity, since cell proliferation, cell number, or increased matrix production is not altered during this foetal timepoint. By using the human filamin-A point mutations as a guide, we observed interstitial cells during foetal valve development require a unique molecular interaction between filamin-A, the primary amine 5-hydroxytryptamine (a.k.a. serotonin) and the cross-linking enzyme, transglutaminase (TG2). Our studies define that serotonin physically interacts with filamin-A and is dependent on the activity of TG *in vivo* and *in vitro*. The consequence of this interaction is an enhancement in interstitial cell contractility resulting in proper extracellular matrix condensation and organization.

Little is known regarding how the valve leaflets form after the initial EMT event. Histological assessments have indicated that the matrix becomes compacted during foetal life, but the mechanism by which this occurs has remained elusive. Our data demonstrate the identification of a novel molecular mechanism by which valve interstitial cells actively remodel the foetal leaflet. These findings illustrate an important, previously unrecognized mechanism to explain how extracellular matrix is organized into specific histological lamina (zones) during development. Our findings further define that postnatal MVD can emanate from a disruption in these regulatory interactions during foetal valve development. Only by understanding the molecular mechanisms by which clinically expressed valve disease originates can we begin developing targeted therapeutics for the treatment of valvular heart disease.

2. Methods

Detailed methodologies are described in the Supplementary material online.

2.1 Gene-targeted mice

All mouse experiments were performed under protocols previously approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina. Prior to cardiac resection, mice were anaesthetized by halothane overexposure by inhalation followed by cervical dislocation in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Histology, immunohistochemistry, and immunocytochemistry

Standard histological and immunochemical procedures were used as previously described.[10] For all immunohistochemistry (IHC) experiments, 5-min antigen retrieval was performed with VectaStain and Pressure Cooker (Cuisinart). Antibodies used for immunological experiments were: Serotonin Transporter (Abcam), TG2 (Neomarkers), Filamin-A (Epitomics), Tph1 (Epitomics), Collagen I (Rockland), Rhodamine Phalloidin (Invitrogen), Ki67 (Dako), PCNA (Abcam). Primary antibodies were used for IHC or immunocytochemistry (ICC) at a 1:100 dilution. Appropriate secondary antibodies were used for detection; $n > 5$ for each experiment.

2.3 Volumetric quantification by reconstruction

Three-dimensional reconstructions were performed using Amira 5.3.3 software (Visage Imaging, Andover, MA, USA). Eighty to one hundred 5 μm thick sections were used to generate each E17.5 reconstruction. Haematoxylin and Eosin stained slides were used in the analyses. Volumetric measurements were generated from three filamin-A conditional knockout (cKO) male hearts and three WT male littermate hearts. Final data are presented as average volumes obtained from Amira 3D reconstructions of the cKO valves compared with WT.

2.4 TG2/serotonylation assays

Assays were performed as previously described.[11] Briefly, mitral valves were obtained from foetal chick (HH40) and solubilized using 1X RIPA buffer. Samples were incubated in the presence or absence of the pan TG antagonist, Cystamine (10 mM) in a calcium-rich buffer (250 mM Tris–Cl, pH 7.4; 33 mM NaCl, 250 mM $CaCl_2$, 1X protease inhibitor-Sigma), for 30 min at 37°C. Co-IP reactions were performed using a serotonin antibody (Pierce, 1:100). Immunocomplexes were recovered using protein-A/G agarose beads (Roche) and solubilized in 2X SDS–PAGE buffer followed by Western analyses for Filamin-A.

2.5 Matrix compaction assays

Compaction assays were performed as previously described.[12,13] Data represent a minimum of five experiments from four cKO and five WT littermate cells and were repeated in quadruplicate.

2.6 Statistical consideration

Statistical significance was determined using Student's *t*-test (two-tailed, type 2), with significance ($P < 0.05$). Statistical data are presented as the mean $\pm$ 1 SD from the mean.

3. Results

3.1 Expression of filamin-A during cardiac development

Immunohistochemical analyses demonstrate filamin-A expression in non-myocyte cells during embryonic, foetal, and neonatal life (*Figure 1*). Expression is observed in endocardium, epicardium, and interstitial cells within the valves and myocardial wall. Expression in the adult is significantly reduced (data not shown and [14]) suggesting filamin-A may play a more influential role during developmental processes.

3.2 Filamin-A-deficient mice exhibit myxomatous mitral valvular dystrophy that emanates from faulty development

To interrogate the functional significance of filamin-A in regulating valve biology, filamin-A floxed mice were bred onto the Tie2-Cre background to generate cKO mice. Since the AV leaflets are largely endothelial derived, this mating strategy permits investigation into the functional consequence of filamin-A loss in AV valve biology. Filamin-A-deficient mice from this inter-cross are viable and present in appropriate Mendelian ratios as expected (Supplementary material online, *Table S1*). Whereas no cardiac defect was detected in female heterozygote animals at the timepoints examined, male cKO mice exhibit a 100% penetrant phenotype as described below. As seen in *Figure 2A*, 2-month-old filamin-A cKO male mice exhibit significant anterior and posterior leaflet thickening with excess/redundant tissue present. Using Movat's Pentachrome and Mason's stainings, filamin-A cKO mitral leaflets exhibit a disruption of the normal collagen/proteoglycan boundary interfaces observed in the wild-type animals. Additional IHC analyses further demonstrate a complete loss of these boundaries in the cKO valves with a significant increase in collagen and proteoglycan (hyaluronan) production throughout the leaflet (*Figure 2A*). These data suggest that the Filamin-A cKO mitral leaflets exhibit a myxomatous phenotype.

As chordae tendineae are reported to be primarily endothelial derived, we additionally examined whether loss of filamin-A in these tissues resulted in disrupted chordae formation. Gross, whole mount views of the mitral chordae tendineae demonstrate thickening of primary chordae and altered and/or loss of normal chordal branching pattern in the anterior and posterior mitral leaflets of the filamin-A-deficient mice (*Figure 2B*). This phenotype, in many cases, results in near fusion of the tip of the leaflet with the papillary muscle. Quantification of the branching order defects are presented in *Figure 2B*.

To define the temporal onset of the disease, filamin-A cKO mice were examined during developmental timepoints. Whereas no appreciable morphological alterations were evident at E13.5, significant valve enlargement is evident by E17.5 and continues during neonatal timepoints (*Figure 3A–N* and Supplementary material online, *Figure S1*). Volumetric measurements using AMIRA 3D reconstructions confirmed that filamin-A deficiency leads to a significant increase in the

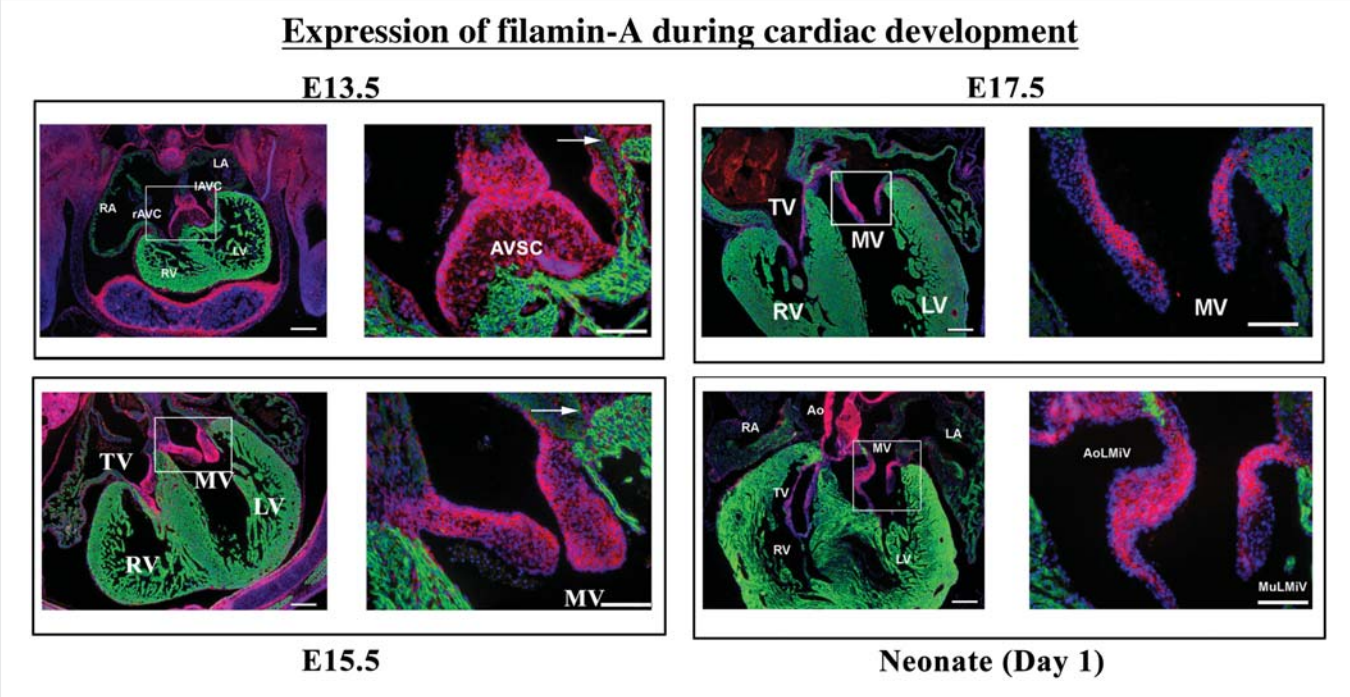

Figure 1 Protein expression of filamin-A during cardiogenesis. Immunohistochemistry (IHC) was performed on E13.5, E15.5, E17.5, and neonatal Day 1 hearts for filamin-A (red) and MF20 (myocytes-green). Each timepoint shows a low magnification and higher magnification of boxed region, primarily focusing on the development of the mitral valve leaflets. Filamin-A is robustly expressed throughout cardiac development, being expressed in interstitial cells of the atrioventricular septal complex (AVSC), the mitral and tricuspid valves (MV and TV), the epicardium, AV sulcus (arrow), endocardial cells, developing blood vessels including coronaries and aortae (Ao). Expression is not detected in cardiomyocytes. Scale bars: low magnification = 200 μm, high magnification = 100 μm. RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; AoLMiV, aortic leaflet of the mitral valve; MuLMiV, mural leaflet of the mitral valve; iAVC, inferior AV cushion; rAVC, right AV cushion.

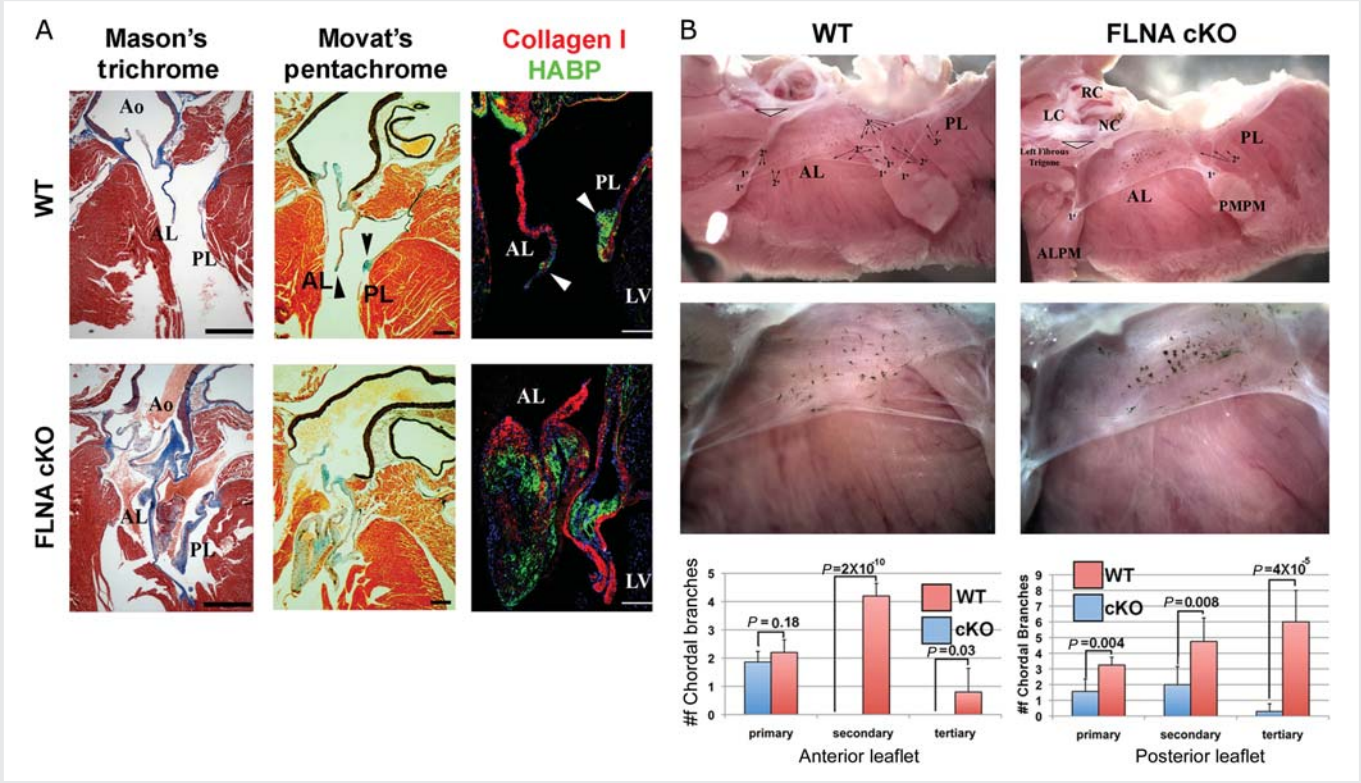

Figure 2 Filamin-A cKO mice develop myxomatous valves. (*A*) Mason's trichrome, Movat's pentachrome stains showing filamin-A cKO mice have enlarged, excess tissue at 2 months of age. Scale bars: 1 mM. IHC indicate profound disorganization of the collagen (red)/hyaluronan (green) matrix indicative of a myxomatous phenotype. Scale bars = 200 μm. AL, PL, anterior and posterior mitral leaflets. (*B*) Gross low and high magnification whole mount views of 2-month-old mitral leaflets showing chordae tendineae branching patterns connecting the anterior (AL) and posterior (PL) leaflets to the papillary muscles. Whereas WT leaflets exhibit ordered branching patterns (primary, secondary, and tertiary), the filamin-A cKO mice have a reduction and/or loss in branched chordae. Branching pattern is quantified in the graph. PMPM and ALPM, posterio medial and anterior lateral papillary muscles; LC, RC, NC, left, right and non-coronary aortic leaflets. *P*-values are denoted. $n = 5$ for WT, $n = 7$ for cKO.

mitral valve size, in addition to the leaflets appearing misshapen (*Figure 3*). To ensure that this defect was not due to secondary effects of genetically removing filamin-A from all endothelium and endothelial-derived cells throughout the animal, the filamin-A floxed mouse was bred onto an NfatC1-Cre background, which is only expressed in endocardial cells within the developing heart (Bin Zhou and Scott Baldwin, personal communication). Filamin-A/NfatC1-Cre mice exhibited a similar phenotype observed for the Tie2-Cre matings (Supplementary material online, *Figure S2*). Thus, removal of filamin-A using either of these two Cre lines causes a primary defect in the proper regulation of valve shape and size. Mice from either NfatC1-Cre or Tie2-Cre X filamin-A matings were viable and present in appropriate Mendelian ratios as expected (Supplementary material online, *Table S1*).

Potential mechanisms contributing to the valve enlargement were next interrogated. There were no appreciable differences in proliferation (assayed by Ki67 and PCNA stainings), apoptosis, or total cell number throughout the valve (Supplementary material online, *Figure S3*). The proliferating cells present in the valve were largely confined to the endocardium and sub-endocardial mesenchyme and did not show significant overlap with filamin-A-deficient cells. We next examined whether increased production of matrix components could be causal to the valve enlargement phenotype in the filamin-A cKO mice. Based on IHC analyses of E17.5 AV valves, we detected no significant change in collagen, versican and/or hyaluronan, suggesting that matrix production was not significantly increased in the filamin-A cKO compared with wild-type at this time-point, and as such, likely not a contributing factor in the valve enlargement phenotype (Supplementary material online, *Figure S4*). As cell proliferation and matrix production do not appear to be directly involved in the valve enlargement phenotype at E17.5, we turned our attention back to the patient with X-linked myxomatous valvular dystrophy (XMVD). We hypothesized that analysis of the mutations may provide insight into biological activities by which the native protein functions.

Three-dimensional *in silico* models of the amino end of the protein were generated (Supplementary material online, *Figure S5A and B*) and indicate that each of the mutations in XMVD patients are inwardly oriented on filamin-A repeats, suggesting these residues may be involved in the formation of a protein-docking interface. A closer examination of amino acid sequences in these regions identified potential recognition sites for the enzyme TG2. The sequences around the P637Q point mutation (WPQEA) that cause MVD in humans is nearly identical to defined TG2 interacting sequences (APQQEA) found in fibrillin-1.[15] Thus, we examined potential interactions between TG2 and filamin-A.

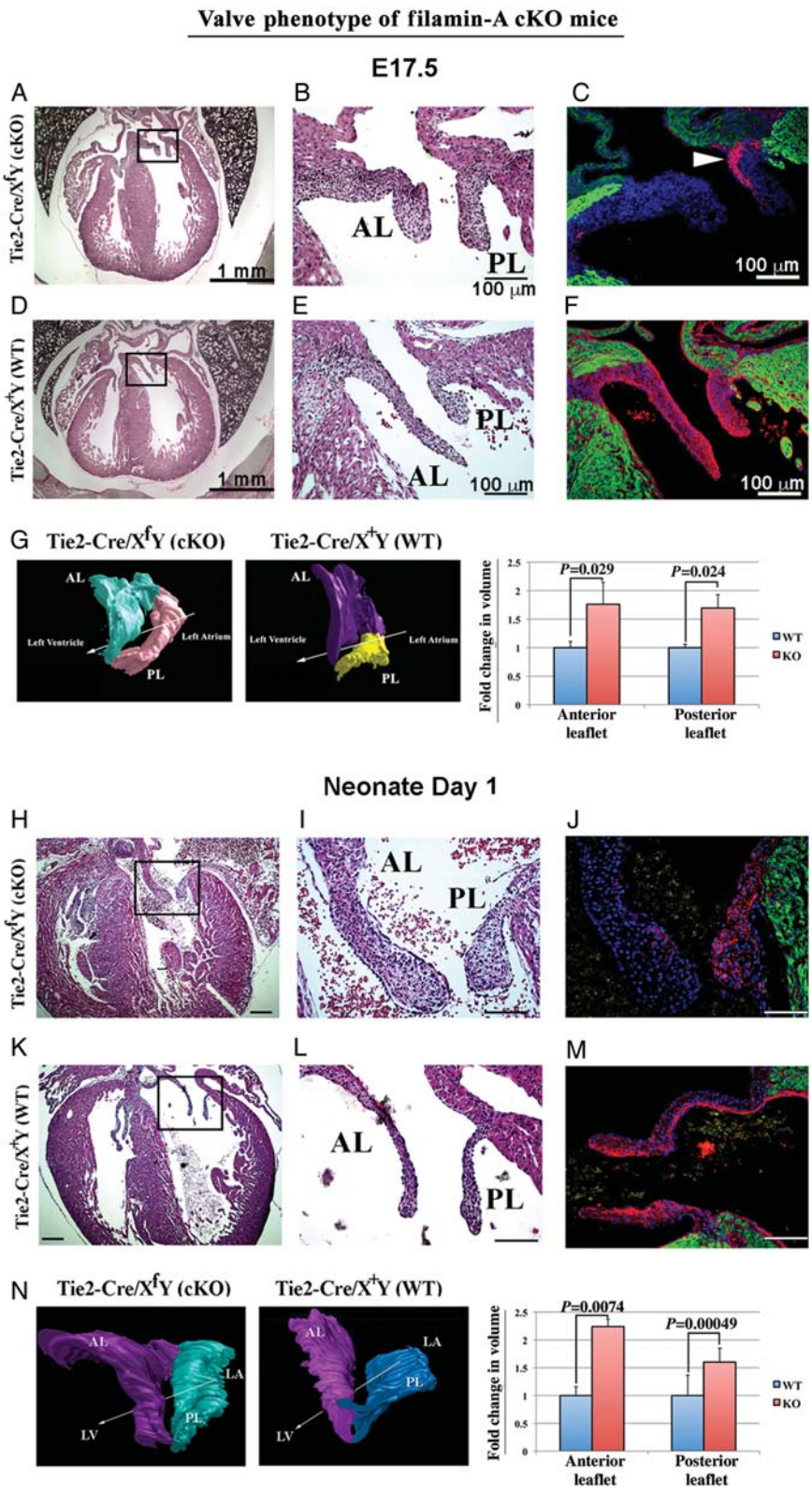

Figure 3 Filamin-A cKO mice exhibit enlarged AV leaflets during foetal and neonatal life. (*A*, *B*, *D*, *E*, *H*, *I*, *K*, and *L*) The histological assessment of filamin-A conditional KO mice (cKO; Tie2-Cre/$X^{f}Y$) compared with littermate control animals (Tie2-Cre/$X^{+}Y$) at E17.5 and neonatal Day 2 using H&E stains. Mitral leaflets of the cKO mice exhibit significant changes in the valve width, length, and shape. (*C*, *F*, *J*, and *M*) IHC to confirm filamin-A is genetically removed using the Tie2-Cre recombinase mouse line. Little staining of filamin-A (red) is seen in the anterior leaflet of the cKO mouse (AL), whereas significant filamin-A positivity is observed in the posterior leaflet (PL, arrowhead). Green staining is MF20. (*G* and *N*) AMIRA 3D reconstructions were performed on the entire mitral leaflet and show shape modifications in the cKO coincident with a significant increase (*P*-values noted) in volume. Arrows in G and N indicate direction of blood flow. $n = 3$ for each genotype. All samples were littermates, sex, and age matched. Magnification bars: *A, D, H, K* = 1 mm; *B, C, E, F, I, J, L, M* = 100 μm.

3.3 Expression of filamin-A, TG2, tph-1, and SERT during cardiac development

TG2 has recently been shown to post-translationally modify intracellular and extracellular proteins (e.g. fibrillin, collagen, smooth muscle actin, and filamin-A) through the covalent attachment of primary bioamines (e.g. serotonin).[11,16] The functional consequence of this modification is largely unknown, but recent reports suggest this modification may confer changes in cytoskeletal activity.[11] Thus, we hypothesized that intracellular interactions between filamin-A, TG2 and internalized serotonin in interstitial cells may be important in regulating the valve tissue size by stimulating contractile-dependent matrix compaction. To test this hypothesis, expression patterns of filamin-A, TG2, tryptophan hydroxylase-1 (tph-1; rate limiting enzyme in serotonin biosynthesis), and the serotonin transporter (SERT; main mechanism for internalizing serotonin) were analysed to determine potential overlap in protein expression during valve development. As shown in *Figure 4A*, filamin-A protein is robustly expressed at E13.5 in all non-myocyte cells throughout the heart including the developing prevalvular leaflets. Whereas the expression of TG2 protein at this timepoint is absent, SERT is found in a punctate pattern in the AV septal complex and tph1 is primarily found in endocardial and subendocardial mesenchyme of the developing leaflets.

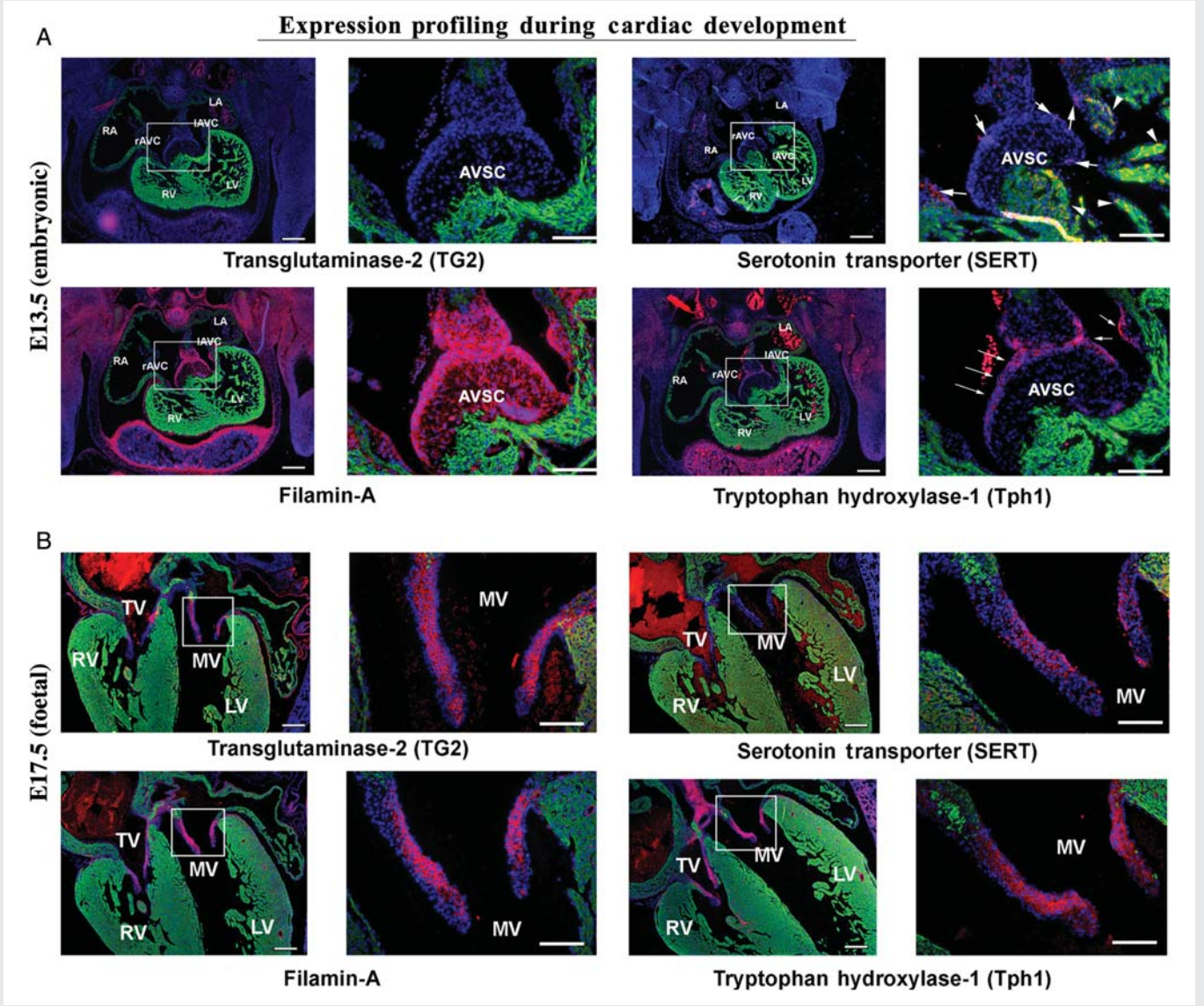

Figure 4 Co-expression of filamin-A, TG2, SERT, and Tph1 during foetal cardiac valve maturation. IHC's were performed to determine overlap of the expression of filamin-A, TG2, SERT, and tph1 during cardiac development. For each immunostain, MF20 is green, nuclei-blue, and specific protein is in red. (*A*) At E13.5, no significant overlap of expression is observed between each of the proteins. No detectable expression of TG2 is seen. Only punctate expression of SERT is observed in the developing valves (arrows), as well as expression in the endocardium and myocytes (arrow heads). Tph1 is expressed in the developing cushions but is restricted to the endocardium and subendocardial mesenchyme (arrows) filamin-A is expressed in all non-myocyte cells at this stage. (*B*) By E17.5, each protein shows significant overlap in expression patterns being primarily restricted to the interstitial valve fibroblast. Scale bars: low magnification = 200 μm, high magnification = 100 μm.

Thus, overlap of expression of these proteins at E13.5 is not apparent. Co-expression of these proteins begins around E15.5 and by E17.5, filamin-A, TG2, tph1, and SERT are each expressed in the developing mitral and tricuspid leaflets, being primarily restricted to interstitial valve cells (*Figure 4B* and Supplementary material online, *Figure S6*). No appreciable overlap in expression of these proteins during adulthood is observed (data not shown). Western analyses demonstrate that each of these proteins are expressed by cultured primary foetal mitral valve fibroblasts and are not secreted (Supplementary material online, *Figure S7*).

3.4 Serotonin is attached to filamin-A and is dependent on TG2 activity

ICC was performed to examine whether intracellular serotonin co-localizes with the filamin-A/Actin cytoskeleton during valve development and whether this localization is dependent on TG and/or SERT activity. Primary foetal mitral interstitial valve cells were incubated with biotinylated serotonin. Serotonin signal is detected in an overlapping pattern with both filamin-A (*Figure 5A*) and Actin (data not shown). To demonstrate that this overlap in expression patterns is dependent on serotonin transporter and TG activity, experiments were performed in the presence of either clomipramine (SERT antagonist) or cystamine (pan TG antagonist). As shown in *Figure 5A*, SERT antagonism resulted in loss of co-localization of serotonin with filamin-A in addition to disruption of the filamin-A cytoskeletal network as observed by lack of stress fibres and diffuse filamin-A staining. This depolymerization of the filamin-A/Actin network was also observed when using an additional SERT inhibitor, fluoxetine (data not shown). Perturbing TG activity using cystamine also disrupted the interaction between serotonin and filamin-A (*Figure 5A*). These findings demonstrate that intracellular serotonin and TG activity are required for serotonin co-localization with filamin-A/actin as well as the importance of this interaction in the maintenance and/or formation of stress fibres.

In order to validate a direct interaction between filamin-A and serotonin, co-immunoprecipitation experiments were performed either in the presence or absence of the TG inhibitor, cystamine. As demonstrated in *Figure 5B*, filamin-A immunoprecipitates with serotonin *in vivo* (lane1), which is blunted (~50%) by the addition of cystamine. We were surprised to observe such a modest inhibition of filamin-A/serotonin interactions in the presence of cystamine. One possibility is that this covalent interaction has already occurred *in vivo*, thus precluding a more significant effect of the TG inhibitor. To test this potential and to verify the filamin-A–serotonin interaction is dependent on TG activity, reciprocal co-IP reactions using a chemically synthesized biotinylated-serotonin were performed (*Figure 5C*). During the reaction, all samples received biotinylated serotonin either in the presence or absence of cystamine. Samples were co-immunoprecipitated with a filamin-A antibody, run on a Western and detected with a streptavidin-HRP antibody. Two bands at the corresponding molecular weight of filamin-A are detected only in the absence of cystamine, demonstrating TG activity is required for this reaction to occur. These data demonstrate a TG-mediated interaction between intracellular serotonin and filamin-A in mitral valve fibroblasts *in vitro* and *in vivo* and suggest their functional importance in forming and/or maintaining stress fibres.

3.5 Functional consequences of serotonin/filamin-A perturbations on matrix organization

Our approach has defined a novel interaction between filamin-A and serotonin that is specific for foetal valve development *in vivo*. Perturbing these interactions appears to result in failed co-localization of filamin-A and serotonin and causes a loss of stress fibres. Owing to the importance of stress fibres in promoting cellular-dependent contractility, we next examined whether the serotonin–filamin-A interaction is required for retaining contractile function of valve interstitial cells. This was tested in the context of matrix remodelling/gel compaction assays, which are accepted tools for monitoring cytoskeletal-dependent matrix condensation/organization. Foetal mitral valve fibroblasts were entombed within collagen or fibrin gels and allowed to freely compact over time. We initially tested the serotonin pathway and its role in promoting matrix compaction (*Figure 6A*). Following polymerization of the gels at Day 0, the gels were released from the wells to be free-floating and stimulated with increasing doses of serotonin. After Day 1, valve fibroblasts receiving a dose of 10 μM serotonin showed a significant increase in matrix compaction (*Figure 6A*). The maximal effect of serotonin was observed at the 10 μM dose. A statistical increase in serotonin-mediated compaction was observed from Days 1 to 3, demonstrating serotonin is required for promoting foetal valve cell contractility and extracellular matrix compaction. Whether intracellular serotonin was required for this cellular behaviour was interrogated by blocking serotonin transporter function using two highly specific inhibitors: clomipramine and fluoxetine (data not shown). Addition of either drug plus 100 μM of exogenous serotonin resulted in a significant inhibition of compaction (*Figure 6B* and data not shown), demonstrating a requirement for intracellular serotonin in matrix compaction and not a result of serotonin receptor engagement. In our three-dimensional matrix system, we detect no changes in proliferation and/or apoptosis (consistent with pervious reports of this assay system[17] and data not shown) indicating the changes observed are not due to differences in cell numbers (data not shown). Although we cannot rule out cell surface serotonin receptor (e.g. 5-HT_1, 5-HT_2) engagement as having a contribution, our data demonstrate that intracellular serotonin is the primary and major mode for promoting matrix compaction.

As serotonylation of filamin-A requires intracellular TG activity, we hypothesized that blocking this activity should result in failed matrix compaction. To test this hypothesis, cells/gels were incubated in the presence of 100 μM serotonin and increasing doses of the TG inhibitor, cystamine. Blockade of TG activity resulted in attenuation of matrix compaction (*Figure 6C*), consistent with our observations that intracellular serotonin/filamin-A interactions provide a primary means for remodelling the extracellular matrix. To further validate the interdependency of filamin-A/serotonin/TG interactions, we performed matrix compaction assays using freshly isolated E17.5 filamin-A cKO mitral valve interstitial cells in the presence of serotonin. As shown in *Figure 6D*, filamin-A-deficient cells are unable to effectively respond to serotonin treatment and result in the abrogation of matrix compaction.

4. Discussion

Studies have defined myxomatous valves as having a disorganized extracellular matrix, blurring of the matrix boundary interfaces, and

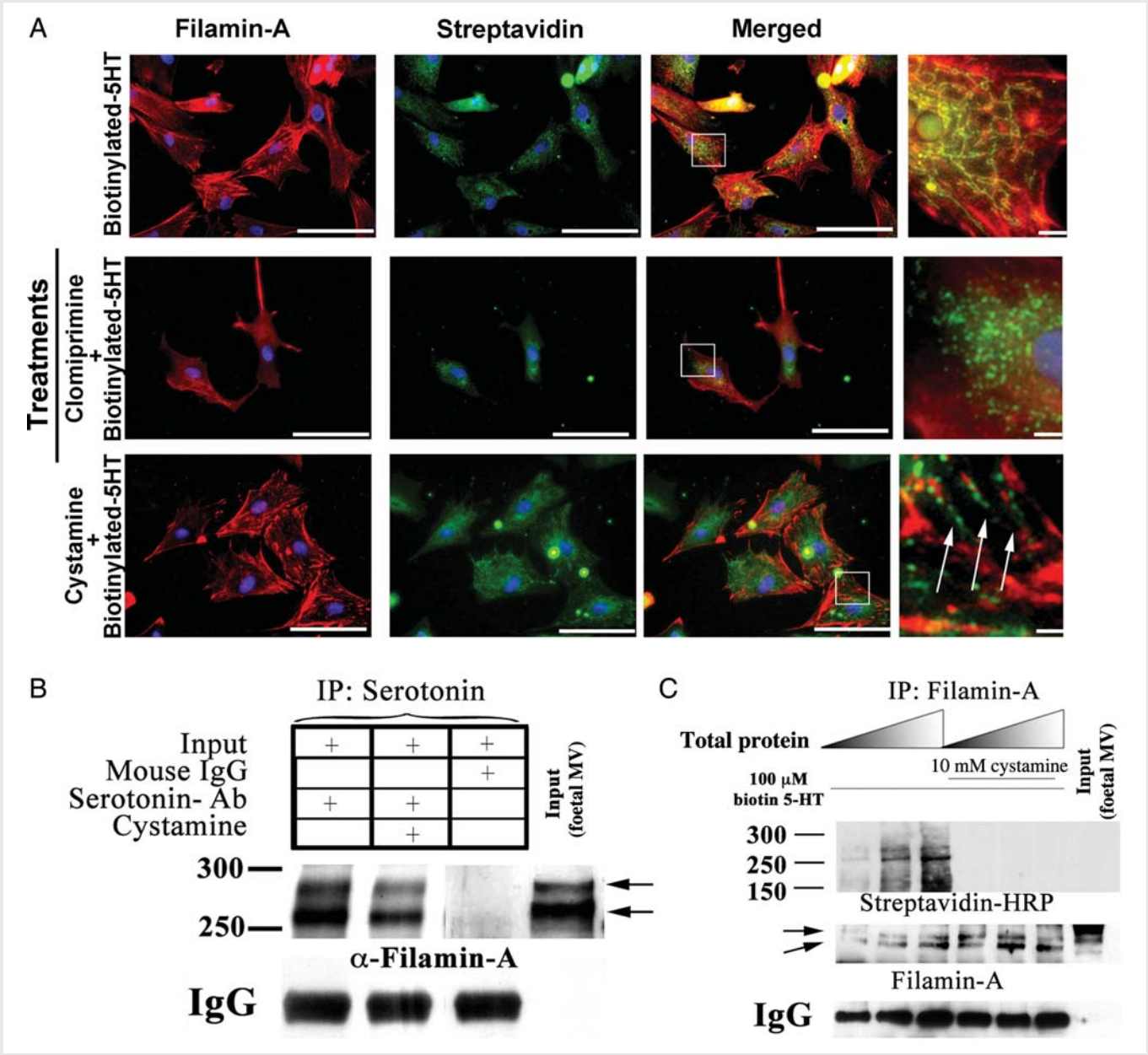

Figure 5 Internalized serotonin interacts with filamin-A and promotes stress fibre formation. (*A*) Primary foetal mitral valve fibroblasts were supplemented with biotinylated serotonin (biotinylated-5HT) either in the presence of a SERT inhibitor (clomiprimine) or a TG inhibitor (cystamine), or no inhibitor. Cells were fixed and stained for filamin-A, and/or streptavidin (to detect biotinylated-5HT). In controls (biotinylated-5HT treatment only), serotonin appears to decorate the filamin-A cytoskeletal networks (yellow staining in the merge samples). Blocking internal transport (Clomiprimine) or TG2 activity (cystamine) resulted in a disruption of this interaction suggesting internalization and/or TG2 activity are required for serotonin incorporation into the cytoskeleton. Additionally, loss or depolymerization of stress fibres (arrows) is apparent in clomipramine- or cystamine-treated cells, respectively. Blue-Nuclei. (*B*) Foetal mitral valve lysate was used in TG2 enzymatic assays (in the presence or absence of cystamine). Co-IP's demonstrate physical interaction between serotonin and filamin-A with a reduction in binding upon TG2 inhibition. (*C*) Serotonin is incorporated into filamin-A in cultured foetal mitral valve fibroblasts and is dependent on TG2. Assays were performed as described in (*B*) except filamin-A antibody was used to IP filamin-A and α-streptavidin-HRP was used to detect biotinylated serotonin. Input represents starting material. Two forms of filamin-A are present in the valves (FLNA-1/2).

the expansion of the proteoglycan-rich spongiosa layer. As a functional consequence, these diseased valves are unable to properly open and close during the cardiac cycle and can result in prolapse and/or regurgitation. Although the characterization of the myxomatous phenotype is well known, relatively little is understood about the origination of the defects and, more importantly, the mechanistic underpinnings contributing to the disease phenotype. Data presented in this report indicate that myxomatous valve disease can have its roots in development during a defined temporal window of foetal life. These findings stem from our studies on the function of filamin-A, the only known gene to date that causes human non-syndromic myxomatous valve disease. We show that filamin-A-deficient adult mice

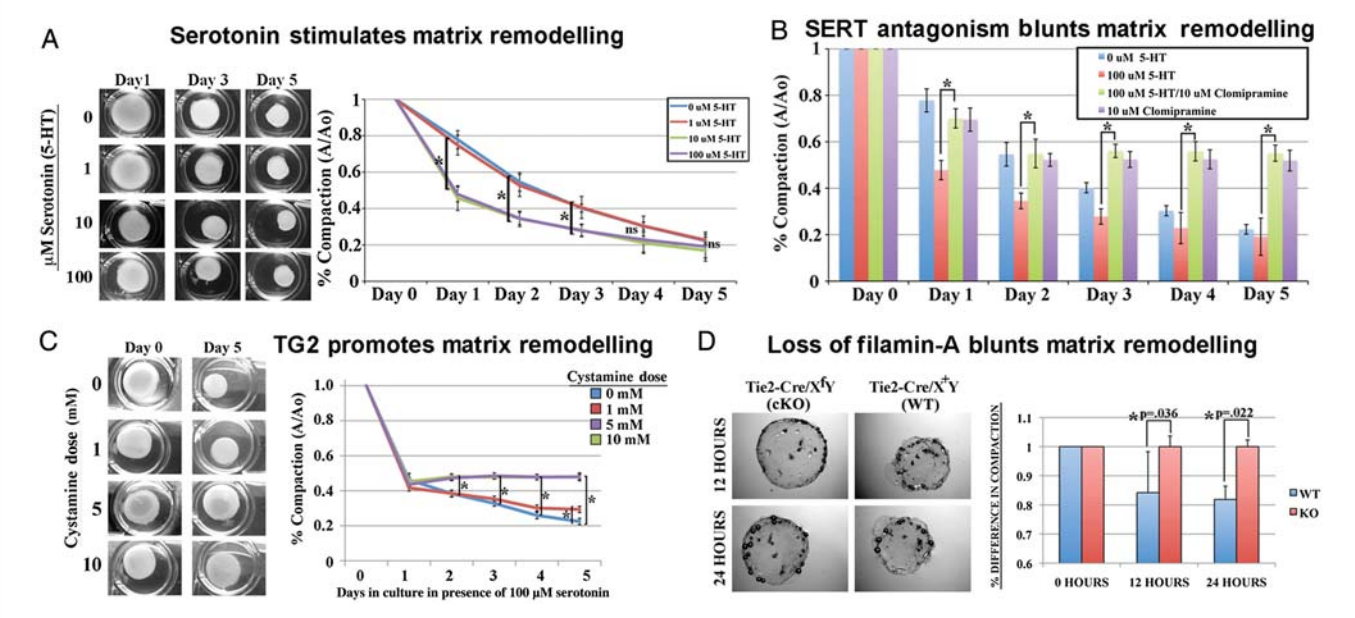

Figure 6 Perturbation of filamin-A–serotonin interactions blunt matrix remodelling. (*A*) Serotonin significantly increased the ability for valve fibroblasts to condense collagen over a 5-day period. Actual gels are represented in (*A*) to demonstrate effect of exogenous addition of Serotonin. (*B* and *C*) Serotonin-induced compaction (in the presence of 100 μM serotonin) was blunted using a SERT inhibitor (clomiprimine) and a TG inhibitor (cystamine), demonstrating internalized serotonin and TG activity are required for matrix condensation. (A/Ao, final area of gel divided by initial area). *Statistical significance: $P < 0.05$. ns, not significant. $n > 12$ for each condition. (*D*) E17.5 anterior mitral leaflets were removed from cKO and littermate WT mice placed in fibrin compaction assays in the presence of serotonin. Filamin-A cKO cells exhibit ~20% reduced ability to efficiently compact the matrix (compare left panels to right panels) in the presence of 100 μM serotonin with *P*-values denoted. $n = 20$ constructs analysed for each genotype.

exhibit a myxomatous valve phenotype by 2 months of age. Of clinical relevance, the observed phenotypes (foetal and postnatal) in filamin-A-deficient mice are consistent with reports from the filamin-A mutation patients, whereby antenatal and adult ultrasound diagnoses demonstrated significant mitral valve defects initially detected during foetal life.[1,2,18] As recent reports demonstrated that these patients with filamin-A mutations also exhibit defects in chordae tendineae,[2] we further examined chordae formation in the filamin-A-deficient mouse. Consistent with the human data, the filamin-A-deficient mice exhibit defects in the number of chordae tendineae and their branching pattern, defining the importance of filamin-A in the formation of the chordae tendineae. Our data presented here as well as previous reports define that filamin-A as well as the matricellular protein, periostin are both critical players in how chordae tendineae are formed.[19] However, defining a link between periostin and filamin-A in the regulation of valve development and chordae tendineae remains to be determined.

We were able to define that the postnatal MVD observed in the filamin-A cKO mice is likely the result of a developmental error in how extracellular matrix is assembled and organized. These mice exhibit profound defects in the regulation of valve shape and size during foetal life, which can be attributed to a failure in how the ECM normally becomes compacted. To provide insight into the molecular mechanisms by which filamin-A functions in regulating valvulogenesis, we initially performed three-dimensional *in silico* modelling and database analyses of myxomatous valve disease causing filamin-A point mutations. All causal mutations to the disease are located in the amino-end of the filamin-A protein, a region harbouring consensus motifs for the enzyme, TG2. TG2 is a member of the TG family that catalyzes thiol and calcium-dependent transamidation reactions. The transamidation reaction catalyzed by this family of proteins results in the formation of a covalent bond between the γ-carboxyamide group of a peptide bound glutamine and a primary amine group.[20] Recent reports have demonstrated that intracellular TG2 catalyzes a reaction between the primary amine, serotonin, and various cytoskeletal proteins, including filamin-A.[11,21] Based on the importance of serotonin in contributing to cellular contractility as well as valvular pathologies[22–27] and the presence of TG2 consensus interacting motifs in a region spanning mutations in filamin-A that cause human valvular disease, we hypothesized that co-operative interactions between serotonin, filamin-A and TG2 are important for cell contractility-dependent matrix organization. We demonstrate that the components of this molecular complex are temporally and spatially co-restricted to interstitial cells during foetal valve morphogenesis, and perturbation of these molecular interactions causes functional deficits in the cells ability to remodel the matrix both *in vivo* and *in vitro*. The precise mechanism by which these molecular components functionally integrate to promote matrix remodelling is not yet clear. However, our *in vitro* data are supportive of filamin-A–serotonin interactions being required for the formation of stress fibres. As these cytoskeletal structures are required for generating traction forces required for ECM remodelling/compaction, it is tantalizing to propose that the valve defects observed in both the filamin-A cKO mice and the human patients may be attributed to deficiencies in stress fibre formation and/or stability.

Although valve defects in the TG2 KO mice have not been rigorously investigated, mice deficient in the serotonin transporter display a valvulopathy consistent with filamin-A patients.[2,28] The defect in the SERT KO is hypothesized to result in a failure of serotonin clearance from the circulation resulting in the hyperstimulation of the serotonin receptors. Serotonin receptor engagement is thought to promote hyperactivity of TGF-β pathways and may contribute to the pathology observed in the mice.[28–30] This murine phenotype is similar to individuals suffering from carcinoid valve disease due to elevated peripheral serotonin levels as well as those having received the ergot derivative, FenPhen, which functions as a SERT inhibitor and a serotonin receptor agonist.[23,24,31,32] Additionally, recent data suggests that myxomatous valves from canines exhibit higher levels of serotonin receptors and down-regulate the serotonin transporter, which may contribute to the valvulopathy observed in this large animal model.[33] Whether the filamin-A mice down-regulate SERT expression or promote a downstream elevation in serotonin-induced TGF-β signalling is tantalizing to propose since the valve phenotype of the patients with filamin-A mutations appears similar to the disease models described above. In addition, the contribution of SSRIs (selective serotonin reuptake inhibitors) has been associated with a variety of development defects including atrial septal defects indicating a potential role for the serotonergic system during cardiac development.[34,35] It is surprising to note that besides these retrospective clinical studies on SSRIs, and the well-known role for serotonergic contribution to valvular heart disease, no studies have evaluated the mechanistic role of serotonin during cardiac development. Thus, our study provides a unique first-examination of the role of serotonin in regulating valve development, and a mechanistic underpinning of its function.

Foetal valve development is a critical developmental timepoint during which cardiac valves undergo a remodelling or maturation phase necessary for the organization and alignment of cells with the extracellular matrix.[13,36] Defects during this phase of development have been hypothesized to be compatible with life, but can lead to long-term detrimental effects often not realized until much later after birth.[37] Outside of descriptive studies, little is known about matrix remodelling/maturation mechanisms driving this critical timepoint of development. Data by us and others have demonstrated that the actin and microtubule cytoskeleton are required for matrix remodelling to occur.[12,38,39] The mechanism by which this occurs appears to minimally require linkage of the actin network to integrin receptors. These receptors, especially integrin β1, are bound to the fibronectin and collagen matrix meshwork and can provide mechanical cues back to the cell.[5,40,41] One of the main linker proteins to establish this actin–integrin bridge is filamin-A. In this study, we focus on filamin-A-mediated intracellular interactions and demonstrate that filamin-A-deficient cells display an impaired contractile phenotype *in vitro* and *in vivo* resulting in failed compaction and tissue remodelling of both fibrin gels (*in vitro*) and valve leaflets (*in vivo*). Interrogation of the inter-relationship between integrin β1, filamin-A, the serotonergic system, and formation of stress fibres to promote contractility is a logical extension of these studies and will likely provide new and exciting findings pertinent to not only valve development but also disease pathogenesis.

5. Concluding remarks

Previous studies have demonstrated that valve development proceeds through two main phases: EMT and post-EMT maturation. Only during the maturation phase is the matrix actively remodelled and organized into a structure that will allow the valves to adapt to the increase in haemodynamic forces during cardiac growth. In this study, we show for the first time that the organization of the valve matrix during the maturation phase is dependent on cooperative cytoskeletal interactions between serotonin, TG2, and filamin-A. More importantly, our data indicate that human filamin-A-mediated valve disease likely has a developmental origin and may be linked to perturbation in cytoskeletal-dependent matrix remodelling events that gradually progress to clinically relevant secondary pathologies during postnatal life.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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