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Endocardial Brg1 Represses *ADAMTS1* to Maintain the Microenvironment for Myocardial Morphogenesis

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SUMMARY

Developing myocardial cells respond to signals from the endocardial layer to form a network of trabeculae that characterize the ventricles of the vertebrate heart. Abnormal myocardial trabeculation results in specific cardiomyopathies in humans and yet trabecular development is poorly understood. We show that trabeculation requires Brg1, a chromatin remodeling protein, to repress *ADAMTS1* expression in the endocardium that overlies the developing trabeculae. Repression of *ADAMTS1*, a secreted matrix metalloproteinase, allows the establishment of an extracellular environment in the cardiac jelly that supports trabecular growth. Later during embryogenesis, *ADAMTS1* expression initiates in the endocardium to degrade the cardiac jelly and prevent excessive trabeculation. Thus, the composition of cardiac jelly essential for myocardial morphogenesis is dynamically controlled by ADAMTS1 and its chromatin-based transcriptional regulation. Modification of the intervening microenvironment provides a mechanism by which chromatin regulation within one tissue layer coordinates the morphogenesis of an adjacent layer.

Keywords

Brg1; BAF complex; chromatin remodeling; primitive erythropoiesis; yolk sac vasculogenesis; endocardium; heart development; trabeculation; cardiac jelly; ADAMTS1; microenvironments

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INTRODUCTION

Cardiac progenitor cells of mesoderm origin differentiate into both the myocardium and the endocardium that forms the outer and inner layers of the heart, respectively. As development proceeds, these two cell layers interact with each other to form specialized structures of the heart such as heart valves and myocardial trabeculae (reviewed in (Harvey, 2002)). Myocardial trabeculation is a key morphogenetic event in heart development that begins at embryonic day E9.0 in mice, when clusters of myocardial cells in the ventricles protrude into the extracellular matrix (cardiac jelly) separating the myocardium from the endocardium. These nascent trabeculae continue to expand, forming long, thin projections. Contiguous with this expansion, ventricular endocardial cells invaginate between the myocardial projections. By E14.5, the ventricular endocardium becomes tightly associated with the myocardial cells as the cardiac jelly dissipates. Finally, the trabeculae largely collapse to thicken the compact layer of myocardium (termed compaction of myocardium). Excessive trabeculation or a failure of the trabeculae to undergo compaction causes cardiomyopathy and heart failure in humans (Jenni et al., 1999). Despite its importance, very little is known about the genetic and molecular programs that initiate and terminate trabeculation.

Several studies implicate the importance of the exchange of intercellular signals between the endo- and myocardium during trabeculation. Neuregulin is produced by endocardial cells and acts through the myocardial receptors, ErbB2 and ErbB4, to drive myocardial trabeculation (Gassmann et al., 1995; Kramer et al., 1996; Lee et al., 1995). Conversely, vascular endothelial growth factor (VEGF) and angiopoietin signal from the myocardium to the endocardium to regulate trabeculation (Ferrara et al., 1996; Sato et al., 1995; Suri et al., 1996). For these signals to be transmitted appropriately, they must traverse the cardiac jelly lying between the endo- and myocardium. Interestingly, both neuregulin and VEGF signaling are modified by extracellular matrix components, suggesting that the establishment of the correct cardiac jelly complement is critical for appropriate receipt of signals between ventricular endo- and myocardium. Consistent with this idea, mice lacking either *Hyaluronan synthase-2* (*Has-2*), an enzyme required for production of the mucopolysaccharide hyaluronan, or *Versican*, a chondroitin sulphate proteoglycan, do not develop trabeculae (Camenisch et al., 2000; Mjaatvedt et al., 1998; Yamamura et al., 1997). The decrease in ventricular cardiac jelly that is observed concomitant with the termination of trabeculation further raises the possibility that active degradation of the cardiac jelly participates in this process. This could be accomplished by a decrease in expression of cardiac jelly components or a change in expression or activity of extracellular matrix regulators. Included in the latter class are the hyaluronidases and members of the matrix metalloproteinase family, notably the aggrecanases that comprise the ADAMTS family (Reviewed in (Porter et al., 2005)). While both Has-2 and Versican, among other ECM components, and ADAMTS family members exhibit dynamic expression patterns during cardiac development (Camenisch et al., 2000; Henderson and Copp, 1998; Klewer et al., 2006; Thai and Iruela-Arispe, 2002), the mechanisms of their transcriptional regulation are unknown.

Alteration of chromatin structure provides an important source of gene regulation by determining the accessibility of DNA regulatory elements to transcription factors. Chromatin can be modified non-covalently by the rearrangement of nucleosomes catalyzed by ATP-dependent chromatin remodeling complexes to facilitate both transcriptional activation and repression (Dunaief et al., 1994). The Brg1-associated-factor (BAF) complex is a 10-subunit complex in which the ATPase component is encoded by one of two genes, *Brg1* or *Brm*. Although both genes are broadly expressed, *Brg1* null mice show peri-implantation lethality (Bultman et al., 2000) while *Brm* null mice are viable (Reyes et al., 1998). Recent reports suggest that BAF subunits are required for cardiac morphogenesis. *BAF180*, a specific subunit of the PBAF form of the BAF complex, regulates cardiac chamber maturation and is required

for survival past mid-embryogenesis (Wang et al., 2004). RNA interference of *BAF60c*, which is expressed throughout the developing heart, results in severe heart defects including a single ventricle, a poorly developed outflow tract, and no trabeculation (Lickert et al., 2004). While expression of several myocardial transcripts is reduced in *BAF60c* knockdown embryos, the molecular basis of these phenotypes is unclear. It is also unknown whether the BAF complex is required in the endocardium, myocardium, or both for cardiac morphogenesis.

We use tissue specific deletion of Brg1 in both the endocardium and myocardium to study the roles of the BAF complex in each tissue during cardiogenesis. The absence of Brg1 by embryonic day E9.0 produces trabeculation defects when it is deleted from the endocardium but not the myocardium. Neither deletion causes widespread changes in transcription of several key regulators of cardiac development. Instead, the trabeculation defects in the absence of endocardial Brg1 result from derepression of a secreted matrix metalloproteinase, ADAMTS1, which normally only increases in expression later in development to prevent excessive trabeculation. The increase in ADAMTS1 causes premature breakdown of the cardiac jelly and termination of trabeculation, demonstrating that cardiac jelly regulation by endocardial cells coordinates maturation of ventricular myocardium. Further, our data indicates the BAF complex has a surprisingly specific role in the developing heart after the basic tissue layers are formed. More broadly, we demonstrate that chromatin remodeling can influence tissue morphogenesis through regulation of the microenvironment.

RESULTS

Endothelial Brg1 is Required for Yolk Sac Vasculogenesis and Primitive Erythropoiesis

To study the function of the BAF complex in endocardial cells, we specifically deleted the core *Brg1* subunit throughout the endothelium by crossing mice carrying a *loxP*-flanked allele of *Brg1* (Sumi-Ichinose et al., 1997) with *Tie2Cre* transgenic mice (Kisanuki et al., 2001). Brg1 was normally expressed in the nuclei of both myocardial and endocardial cells, as well as all surrounding tissues (Figure 1A). In contrast, Brg1 was undetectable in the endocardial cells, as marked by expression of the transcription factor NFATc1 (de la Pompa et al., 1998; Ranger et al., 1998), of *Tie2Cre;Brg1*$^{F/F}$ embryos but remained expressed in all other non-endothelial tissues (Figure 1B and S2). This deletion was first evident at E9.0 (data not shown). At E9.5, *Tie2Cre;Brg1*$^{F/F}$ embryos were of comparable size to littermate controls (Figure 1C). However, they appeared pale due to a decrease in circulating red blood cells caused by defects in yolk sac vasculogenesis and primitive hematopoiesis (Supplemental Text and Figure S1). In contrast, the basic pattern of the embryonic vasculature was intact in E9.5 *Tie2Cre;Brg1*$^{F/F}$ embryos. By E10.5, *Tie2Cre;Brg1*$^{F/F}$ embryos were considerably smaller than their littermates (Figure 1D) and none survived to E11.5.

Brg1 is Required in the Endocardium for Trabeculation

We examined hematoxylin and eosin-stained (H&E) sections of *Tie2Cre;Brg1*$^{F/F}$ embryos between E9.5 and E10.5 and found defects in trabeculation of the heart ventricles. Normally, nascent myocardial trabeculae are present by E9.5, forming discrete outgrowths of myocardial cells migrating into the cardiac jelly. In *Tie2Cre;Brg1*$^{F/F}$ embryos, there was only limited formation of trabeculae at E9.5 (Figures 1E and 1F). By E10.5, when trabeculae were normally extensive, *Tie2Cre;Brg1*$^{F/F}$ embryos had very sparse trabeculation (Figure S2). We double stained E10.5 embryos for MF20, a myocardial marker, and PECAM, an endocardial marker, to assess the organization of the two cell layers (Figures 1G and 1H). Although both cell types were present in *Tie2Cre;Brg1*$^{F/F}$ embryos, PECAM-positive endocardial cells did not invaginate around MF20 positive cardiomyocytes outgrowing from the compact layer, consistent with histological observations.

We quantified the extent of the trabeculation defect by morphometric measurements of the thickness of individual trabeculae, the total area of trabecular myocardium, and the length of the compact wall in anti-PECAM and anti-MF20 double stained sections. The trabecular thickness was significantly reduced at both E9.75 and E10.5 in *Tie2Cre;Brg1$^{F/F}$* embryos (Figures 1I and S2). At E10.5, the compact layer was also significantly thinner than control embryos (Figure S2). We measured the total degree of trabecular atrophy by normalizing trabecular area to compact wall length. This analysis showed a significant decrease in the extent of trabeculation as well as the size of each trabecular sheet at E9.75 and E10.5 in *Tie2Cre;Brg1$^{F/F}$* embryos (Figure 1J). These results demonstrate that Brg1 has critical roles in the endocardium to regulate trabecular development.

Myocardial Brg1 is Not Required for Trabeculation between E9.5 and E10.5

To investigate the possibility that Brg1 is also required in ventricular myocardium for trabeculation, we deleted *Brg1* using the myocardial-expressed *SM22αCre* transgene (Holtwick et al., 2002; Umans et al., 2007), Figure S3). Less than 5% of ventricular myocardial cells in E9.5 *SM22αCre;Brg1$^{F/F}$* embryos expressed detectable levels of Brg1, with no evidence of deletion in endocardial cells (Figure 2A and 2B and S3). In contrast to *Tie2Cre;Brg1$^{F/F}$* embryos, *SM22αCre;Brg1$^{F/F}$* embryos had no gross defects in embryonic development up to E10.5 (Figure 2C and 2D). H&E staining of sections through the hearts of these embryos demonstrated that trabeculation was normal (Figure 2E and 2F). Therefore, myocardial BAF complexes do not regulate trabeculation between the time *Brg1* was deleted in these mice (between E8.5 and E9.5) and E10.5.

Deletion of *Brg1* After E9.0 Does Not Have Widespread Effects on Cardiac Gene Expression

To understand the molecular basis for the myocardial trabeculation defects in *Tie2Cre;Brg1$^{F/F}$* embryos, we examined the expression of myocardial genes important for this process. The expression of *Nkx2.5*, which is required for trabeculation (Lyons et al., 1995), and *NPPA* was decreased in *BAF60c* RNAi embryos (Lickert et al., 2004). However, both *Nkx2.5* and *NPPA* were expressed normally in the myocardium of both *Tie2Cre;Brg1$^{F/F}$* (Figure 3A to 3D) and *SM22αCre;Brg1$^{F/F}$* embryos (Figure S3). The defects in *Nkx2.5* and *NPPA* expression seen in the *BAF60c* RNAi mice may therefore reflect a requirement for the BAF complex prior to the onset of *Brg1* deletion using *Tie2Cre* or *SM22αCre*. *BMP10*, which is also required for trabeculation (Chen et al., 2004) and whose expression is regulated by endocardial signals (H. C and W. S. unpublished observations), was also expressed normally in *Tie2Cre;Brg1$^{F/F}$* embryos (Figure 3E and 3F). Additional myocardial proteins, such as MF-20 and Actinin (Figures 1G and 1H and S4) or transcripts, including *GATA4, ErbB2, Hey1, MEF2C, VEGF, MEST* and *Irx4* were also expressed normally in *Tie2Cre;Brg1$^{F/F}$* embryos (Figures S5 and S6). Therefore, signals originating from the endocardium required for maturation of ventricular myocardium are unaffected by the absence of endocardial Brg1.

Next, we used RNA *in situ* hybridization to survey the expression of endocardial transcripts in *Tie2Cre;Brg1$^{F/F}$* embryos to determine if Brg1 was required for their transcription. We examined *neuregulin, Tie1, Tie2*, and *VEGFR2* because each of these signaling molecules and/or their receptor/ligand partners have been shown to be required for trabeculation (Ferrara et al., 1996; Meyer and Birchmeier, 1995; Meyer et al., 1997; Sato et al., 1993; Sato et al., 1995; Suri et al., 1996). However, they were all expressed normally in E9.5 *Tie2Cre;Brg1$^{F/F}$* embryos (Figures 3G to 3L and S5). Recently, the Notch pathway has been shown to be required for trabeculation by regulating expression of *Neuregulin* (indirectly via *EphrinB2*) and *BMP10* (Grego-Bessa et al., 2007). Normal expression of these transcripts and the Notch target *Hey1* in *Tie2Cre;Brg1$^{F/F}$* embryos (Figure S5) suggests the Notch pathway is not perturbed by the absence of endocardial Brg1. These results indicate that endocardial cells maintained their differentiated state and did not have widespread changes in transcription

in the absence of Brg1. Rates of apoptosis and cellular proliferation were also not significantly changed in E9.5 *Tie2Cre;Brg1$^{F/F}$* embryos (Figure S4). Together, these results suggest that the BAF complex has a restricted role in regulating the function rather than differentiation of endocardial cells to control trabecular morphogenesis.

Endocardial Brg1 Is Required for Establishment of Ventricular Cardiac Jelly

Several extracellular matrix (ECM) components, notably hyaluronan and Versican, are required for trabeculation (Camenisch et al., 2000; Yamamura et al., 1997). Interestingly, histological analysis of *Tie2Cre;Brg1$^{F/F}$* embryos showed an apparent decrease in the amount of ECM, termed the cardiac jelly, separating the ventricular endocardium from the myocardium (Figures 1E and 1F). We stained E9.5 heart sections with Alcian blue, pH 2.5 to detect the presence of acidic mucopolysaccharides, including hyaluronan and sulfated and carboxylated proteoglycans such as Versican. In *Tie2Cre;Brg1$^{F/F}$* embryos, there was a clear decrease in the amount of Alcian-blue positive material in the ventricular cardiac jelly (Figure 4A and 4B). We examined *Tie2Cre;Brg1$^{F/F}$* embryos for the presence of hyaluronan by staining sections with biotinylated hyaluronan binding protein. Hyaluronan was found throughout the cardiac jelly, but was nearly absent in the ventricular cardiac jelly of *Tie2Cre;Brg1$^{F/F}$* embryos (Figure 4C and 4D). Similarly, Versican protein levels were reduced in the ECM of the ventricles (Figures 4E and 4F). Nevertheless, transcription of *Hyaluronan synthase-2* (*Has-2*), and *UDP-glucose-dehydrogenase* (*UGDH*), two enzymes required for hyaluronan production, and *Versican* persisted in the ventricles of *Tie2Cre;Brg1$^{F/F}$* embryos (Figures 4I to 4L and S5). *Perlecan*, a heparan sulphate proteoglycan required for heart development (Costell et al., 2002) was also transcribed normally (Figure S5), although Perlecan protein levels were also diminished in ventricular cardiac jelly by E9.75 (Figure 4G and 4H). Interestingly, and as previously reported (Henderson and Copp, 1998), *Versican* was expressed exclusively in myocardium while *Has-2* transcripts were found at higher levels in ventricular myocardium compared to the neighboring endocardium. These results are consistent with the observation that the myocardium is the primary source of cardiac jelly components (Manasek et al., 1973). The normal transcription of *Has-2*, *UGDH*, *Versican*, and *Perlecan* suggest that a change in expression of a modulator of ECM rather than the components themselves accounts for the ECM deficiency in *Tie2Cre;Brg1$^{F/F}$* embryos.

ADAMTS1 is Derepressed in the Absence of Endocardial Brg1

We employed a candidate gene approach to identify transcripts whose misregulation in *Tie2Cre;Brg1$^{F/F}$* embryos could cause the observed ECM defects (Figures S5 and S6). In our screen, we included *Hyal1*, *Hyal2*, and *Hyal3*, the three hyaluronidases whose potential upregulation could account for a decrease in HA content. We also examined members of the matrix metalloproteinase family *MMP1*, *MMP2*, *MMP3*, *MMP9*, and *MMP13*, each of which has been shown to degrade Versican or related proteoglycans (Reviewed in (Flannery, 2006)). Finally, we checked expression of *ADAMTS1*, *ADAMTS4*, *ADAMTS5*, *ADAMTS8*, *ADAMTS9*, and *ADAMTS18*, their proposed co-factor, *Fibulin-1* (Lee et al., 2005), and their inhibitor *TIMP3* (Kashiwagi et al., 2001). The *ADAMTS* family consists of 19 genes of the metalloproteinase superfamily, whose substrates include aggrecans such as Versican and Perlecan (reviewed in (Porter et al., 2005)) and which have been implicated in cardiac development (reviewed in (Manso et al., 2006)). *ADAMTS1*, *4*, *5*, *8*, *9*, and *18* were chosen for analysis because they have been described as Versicanases and/or have been shown to express in endothelial cells (A. Connolly, unpublished observations). In addition, each of ADAMTS1, ADAMTS9, and Fibulin-1 have been reported to regulate endocardial cushion development (Kern et al., 2006). Because *TIMP3* is expressed in the developing endocardium (Brauer and Cai, 2002), we also examined whether its expression was decreased in *Tie2Cre;Brg1$^{F/F}$* embryos.

Among the 19 screened ECM-related transcripts, only *ADAMTS1* exhibited a clear change in expression in *Tie2Cre;Brg1$^{F/F}$* embryos (Figures S5 and S6). In these embryos, *ADAMTS1* was expressed at high levels in ventricular endocardial cells relative to littermate controls while maintaining a low level of expression in the myocardium (Figures 5A and 5B). To confirm the observed increase in *ADAMTS1* seen by *in situ* hybridization, we performed RT-PCR analyses on mRNA from the hearts of E9.5 embryos. Using quantitative RT-PCR and normalization to *HPRT*, a ubiquitously expressed transcript, we found that *ADAMTS1* transcripts were present at 2.8x higher levels in the mutant embryos (Figure 5C). A collection of other myocardial and endocardial transcripts were not significantly changed in expression, consistent with our *in situ* hybridization experiments. An increase in ADAMTS1 expression could account for the subsequent decrease in Versican levels and, indirectly, in the HA content of the ventricular cardiac jelly as Versican contains HA-binding domains that may stabilize HA levels (LeBaron et al., 1992). Consistent with augmented ADAMTS1 activity, increased staining using an antibody that recognizes a specific cleavage product of Versican (Neo-Versican) arising from ADAMTS activity was observed in E9.25 *Tie2Cre;Brg1$^{F/F}$* embryos prior to further dissipation of Versican levels by E9.5 (Figure 5D and 5E).

We examined whether repression of *ADAMTS1* by Brg1 was a primary effect of Brg1 in endocardial cells using loss-of-function studies of *Brg1* in cultured cells. We used human umbilical vein endothelial cells (HUVECs), which express *ADAMTS1* (Norata et al., 2004), as a heterologous source of primary cells. We reduced expression of *Brg1* using RNA interference and assayed changes in *ADAMTS1* transcript levels. Lentiviral mediated expression of two separate small hairpin RNAs targeting *Brg1* produced an increase in *ADAMTS1* expression proportionate to the degree of *Brg1* transcript knockdown (Figure 5F). The most robust knockdown, to 21% of normal *Brg1* levels, resulted in a 3 fold increase in endogenous *ADAMTS1* transcripts. This result supports our *in vivo* observations that loss of endocardial Brg1 is the primary cause of the increase in endocardial *ADAMTS1* expression and demonstrates that similar repression of *ADAMTS1* by Brg1 occurs in additional endothelial cell types.

Brg1 Directly Associates With the *ADAMTS1* Locus in Endothelial Cells

The upregulation of *ADAMTS1* in HUVECs and endocardial cells in the absence of Brg1 could represent a secondary effect of misexpression of *ADAMTS1* transcriptional regulators or could be accounted for by a direct requirement for the BAF complex in *ADAMTS1* repression. To determine whether endogenous Brg1 directly binds the *ADAMTS1* locus, we performed chromatin immunoprecipitation assays and quantitative PCR using HUVECs as a source of material. We aligned the mouse, dog, rat, and human genomes to identify conserved regions in the *ADAMTS1* locus that may act as sites for Brg1 association (Figure 6A). Primer pairs within each of these regions were designed for amplification of DNA from immunoprecipitated chromatin. Antibodies directed against Brg1 precipitated more chromatin fragments from the immediate promoter area (3.7x, $p<0.002$) and a conserved stretch in the first intron (4.6x, $p<0.03$) relative to a control anti-GFP antibody (Figure 6B). Limited or no enrichment was found for an upstream candidate regulatory site (1.4x, $p<0.13$) or an unconserved region approximately 30 kb upstream from the promoter (Figure 6B and data not shown). These results support a model in which the BAF complex directly binds the *ADAMTS1* locus near the immediate promoter region to maintain its repression in developing ventricular endocardial cells.

We further tested this concept using luciferase reporter assays with regions from the *ADAMTS1* locus. We prepared four different constructs that contained either a 2.0 kb immediate promoter fragment or a longer 3.2 kb region that included the second conserved region not bound by Brg1 each with or without the conserved intron region cloned downstream

of luciferase. Importantly, the reporter constructs were cloned into Epstein-Barr Virus derived pREP4 episomal vectors that contain the virus replication origin and express nuclear antigen EBNA-1. These vectors become chromatinized when transfected into mammalian cells and therefore are informative when studying the effects of chromatin remodeling complexes (Liu et al., 2001). We transfected each of the four reporter plasmids into SW13 adenocarcinoma cells, which lack both *Brg1* and its homologue *Brm* (Muchardt and Yaniv, 1993) and therefore lack functional BAF complexes, in the presence or absence of a co-transfected Brg1-expressing plasmid. Expressing Brg1 in SW13 cells restores BAF complex function. Lysates from each of the transfection experiments contained between 2.8 and 4.5 fold lower luciferase levels when Brg1 was restored to the cells (Figure 6C). These results further support a model of direct repression of *ADAMTS1* by Brg1. The site of action is likely through the immediate promoter region (Region 2) of the *ADAMTS1* locus, as each of the reporters behaved similarly despite having only that region in common. The results also demonstrate that Brg1 activity, separate from Brm, is necessary and sufficient for repression of *ADAMTS1*.

ADAMTS1 Derepression Terminates Trabeculation

To investigate whether the increase in *ADAMTS1* expression underlies the ECM defects in *Tie2Cre;Brg1$^{F/F}$* embryos, we treated cultured embryos (Chang et al., 2004) with GM6001, a broad spectrum inhibitor of matrix metalloproteinases including ADAMTS1 (Shimada et al., 2004). Wildtype embryos harvested at E9.0 and treated with GM6001 for 36 hours developed relatively normally (Figures 7A, 7B, 7E, and 7F). Mock-treated *Tie2Cre;Brg1$^{F/F}$* embryos had severe defects in ventricle development, even more so than seen *in utero* at E10.5. They had an increase in compact layer thickness, no trabeculation, and a near complete absence of Alcian blue staining in the sub-endocardial space (Figure 7G). *Tie2Cre;Brg1$^{F/F}$* embryos treated with GM6001 remained growth-retarded and pale, being unchanged in gross appearance (Figures 7D). However, they contained Alcian blue-positive material in ventricular cardiac jelly and exhibited a significant recovery of trabeculation (Figure 7H and 7M). GM6001-treated *Tie2Cre;Brg1$^{F/F}$* embryos also recovered expression of Versican and hyaluronan in the ventricular cardiac jelly to levels seen in wildtype embryos (Figures 7I to 7L, 7N and 7O). These results indicate that the increase in ADAMTS1 in *Tie2Cre;Brg1$^{F/F}$* embryos is responsible for the collapse of the ECM through proteolysis of Versican and the subsequent failure of trabeculation. A separate broad-spectrum MMP inhibitor, minocycline (Paemen et al., 1996), which does not inhibit ADAMTS1 (Rodriguez-Manzaneque et al., 2002), did not rescue trabeculation or cardiac jelly in cultured *Tie2Cre;Brg1$^{F/F}$* embryos (Figure S7). Together with the absence of changes in expression of other matrix proteinases (Figures S5 and S6), this finding indicates that an increase in ADAMTS1 alone largely accounts for the defective trabeculation in *Tie2Cre;Brg1$^{F/F}$* embryos.

Since GM6001 treatment did not rescue the hematopoietic defects in *Tie2Cre;Brg1$^{F/F}$* embryos, as shown by the absence of red blood cells in sections of the cultured embryos (Figure 7L), *ADAMTS1* derepression does not cause all the phenotypes of *Tie2Cre;Brg1$^{F/F}$* embryos. Consistent with this observation, *ADAMTS1* was not derepressed in other endothelial cells in *Tie2Cre;Brg1$^{F/F}$* embryos (Figure S8). This result indicates that Brg1 may have an active and specific role in maintaining *ADAMTS1* repression in specific types of endothelial cells, including endocardial cells, rather than a general requirement for establishing the *ADAMTS1* transcriptional ground state. This observation raises the question why does *ADAMTS1* need to be repressed in developing ventricular endocardial cells? Interestingly, cardiac expression of *ADAMTS1* was low in wildtype embryos until E12.5, when it became highly expressed in the endocardium (marked by *Tie1* expression) and persisted through E14.5 (Figures 8A to 8F). Consistent with this observation, the amount of cardiac jelly diminished at E12.5, and was essentially absent by E14.5, when the ventricular endocardium and myocardium were in continuous, direct contact (Figures 8G to 8I). Furthermore, the ADAMTS1 substrate, Versican,

dramatically decreased in expression in the ventricular cardiac jelly at E12.5, and is essentially absent by E14.5 (Figures 8J to 8L). Since myocardial trabeculation terminates during the period from E12.5 to E14.5, the increase in ADAMTS1 expression and subsequent dissolution of the cardiac jelly might be required to prevent excessive trabeculation. We tested this idea by examining the phenotype of mice lacking *ADAMTS1*. While *ADAMTS1*$^{-/-}$ mice exhibited approximately 50% embryonic lethality, they were grossly normal at E13.5 (Figure 8M and 8N). At E13.5, the ventricles of *ADAMTS1*$^{-/-}$ embryos had increased trabecular muscle (Figure 8O and 8P). We quantitated the extent of myocardial dysmorphogenesis by morphometric measurements of the left ventricles of 3 *ADAMTS1*$^{+/-}$ and 5 *ADAMTS1*$^{-/-}$ E13.5 embryos. *ADAMTS1*$^{-/-}$ embryos had a statistically significant increase in the ratio of trabecular area to total ventricle area (0.85 +/- 0.07 to 0.58 +/- 0.11, $p<0.04$) (Figure 8Q). There was no change in the number of trabeculae normalized to the ventricle length (0.04 +/- 0.007 to 0.04 +/- 0.005, $p<0.83$), trabeculae width (14.1 +/- 1.2 to 13.7 +/- 0.6, arbitrary units, $p<0.67$), or compact layer thickness (31.7 +/- 2.8 to 33.0 +/- 3.2, arbitrary units, $p<0.57$) (Figure S9). These findings suggest that the normal upregulation of ADAMTS1 at E12.5 terminates trabeculation by reconfiguring the extracellular matrix (Figure 8R). The transition from repression to activation of *ADAMTS1* ensures the proper microenvironment is present for initial support of trabecular growth and then its termination once sufficient trabeculation has occurred.

DISCUSSION

The trabeculation defects in *Tie2Cre;Brg1*$^{F/F}$ embryos are caused by derepression of *ADAMTS1*, which encodes a matrix metalloproteinase that degrades cardiac jelly and prevents trabeculation. ADAMTS1 is a secreted matrix metalloproteinase of aggrecans, including Versican (Kuno et al., 2000; Rodriguez-Manzaneque et al., 2002). Although *ADAMTS1* deficient mice have not been previously examined for cardiac abnormalities, ADAMTS1 has been implicated in cleaving Versican within the endocardial cushion cardiac jelly to facilitate the remodeling of the cushions into atrial and ventricular septa as well as the valve leaflets (Kern et al., 2006). Versican contains hyaluronan-binding domains (LeBaron et al., 1992) which may be important for establishment of an elaborate glycosaminoglycan-containing network within the cardiac jelly. Therefore, inactivation of Versican by the observed upregulation of ADAMTS1 in *Tie2Cre;Brg1*$^{F/F}$ embryos could facilitate the degradation of hyaluronan, which then directly leads to collapse of ventricular cardiac jelly. This notion is supported by studies showing that addition of hyaluronidase to whole rat embryo cultures causes collapse of ventricular cardiac jelly and leads to trabeculation defects (Baldwin and Solursh, 1989).

There are several ways how cardiac jelly composition could influence myocardial trabeculation. During trabeculation, both myocardial and endocardial cells must undergo extensive cellular movements to form the long thin projections protruding into the ventricular cavity. Therefore, establishment of an appropriate extracellular milieu that facilitates changes in cell shape, adhesion, or migration may be critical for trabeculation. Second, ECM components could regulate trabeculation by providing a permissive environment for diffusion or post-translational modification of signaling molecules involved in trabeculation, including Notch (Grego-Bessa et al., 2007), Neuregulin (Meyer and Birchmeier, 1995; Meyer et al., 1997), EphrinB2 (Gerety and Anderson, 2002), VEGF (Ferrara et al., 1996), and angiopoietin (Sato et al., 1993; Sato et al., 1995; Suri et al., 1996). In particular, VEGFR2 signaling in embryonic stem cells is promoted by heparan sulphate proteoglycans produced by adjacent cells (Jakobsson et al., 2006) and heparan sulphate modulates VEGF gradients (Ruhrberg et al., 2002). Third, certain components of the ECM are active signaling molecules themselves or can directly modify the ability of signaling molecules to act on their receptors. For example, HA binds to the CD44 and LYVE-1 cell receptors, and activates intracellular signaling (Aruffo et al., 1990; Banerji et al., 1999). HA is also a co-factor for neuregulin signaling (Bourguignon

et al., 1997; Camenisch et al., 2002). Therefore, regulated changes in the microenvironment of the heart ventricle during development could facilitate a developmental transition to terminate cardiac trabeculation independent of the expression of the signaling factors involved.

A specific human congenital cardiomyopathy, termed spongy myocardium, hypertrabeculation, or non-compaction of the myocardium, reflects developmental defects during the trabeculation process. The genetic lesions underlying this disease remain obscure. Hearts of these patients exhibit excessive trabeculation and develop congestive heart failure with the only treatment being a heart transplant. Our observation that premature expression of ADAMTS1 in the absence of endocardial Brg1 prevents trabeculation suggests that the normal increase in endocardial ADAMTS1 expression during development is essential to prevent hypertrabeculation. Interestingly, overexpression of a non-cleavable form of Versican in embryonic chick hearts causes a thickening of the right ventricle and possibly hypertrabeculation (Kern et al., 2007). Here, we show that *ADAMTS1*$^{-/-}$ mice, a genotype which is approximately 50% embryonically lethal (Mittaz et al., 2004; Shindo et al., 2000), develop excessive trabeculation. This result supports a model in which Brg1 represses expression of *ADAMTS1* until developmental cues alleviate the repression around E12.5 to facilitate the termination of trabeculation (Figure 8R). The increase in ADAMTS1 prevents excessive trabeculation by breaking down ventricular cardiac jelly and altering the microenvironment that supports cellular movements and/or signaling events. Subsequently, the heart develops the thick compact layer that provides the extensive muscular power required in a mature heart. *ADAMTS1*$^{-/-}$ embryos that escape embryonic lethality and survive to adulthood do not have apparent non-compaction of the ventricles (data not shown). Therefore, additional cardiac jelly degrading enzymes may cooperate with ADAMTS1 in a partially redundant fashion to terminate trabeculation. Candidates include additional ADAMTS proteins or members of the MMP family, some of which are expressed in the developing heart (Figures S5 and S6 and (Ratajska and Cleutjens, 2002)) and whose substrates also include proteoglycans (Reviewed in (Flannery, 2006). A detailed understanding of the roles of cardiac jelly regulators during heart development may help identify candidate genes mutated in families with congenital cardiomyopathy and eventually lead to novel treatments for this disease.

Activities of chromatin remodeling enzymes are commonly associated with transcriptional activation given their ability to loosen chromatin to facilitate the access of transcription factors and transcriptional machinery. Therefore, although a number of genes have been described as being repressed by the BAF complex (reviewed in (de la Serna et al., 2006)), it is surprising to find that a critical function of the BAF complex in developing endocardial cells is as a site-specific transcriptional repressor. While Brg1 directly associates with the *ADAMTS1* locus, it remains to be determined how the BAF complex is recruited and how it mediates repression. Since Brg1 remains ubiquitously expressed throughout heart development (Figure S10), changes in Brg1 levels do not account for the normal derepression of *ADAMTS1* and therefore recruitment factors likely provide active regulation of the locus. Alternatively, a change in activity of the BAF complex on *ADAMTS1* may lie in a developmental switch in the combinatorial assembly of homologous BAF subunits, which can produce differential BAF complex activities in specific cell types (Lessard et al., 2007).

Our findings demonstrate an unexpectedly specific role for the BAF complex in maintaining transcriptional repression of *ADAMTS1* in developing endocardial cells to establish the appropriate microenvironment for trabeculation of the ventricles. The connection between the BAF complex and regulation of the microenvironment in the developing heart ventricle raises an intriguing possibility that chromatin-based regulation may be a common source for the establishment of extracellular environments. Recent reports indicate an analogous relationship to that between Brg1 and ADAMTS1 exists between the histone deacetylase HDAC7 and MMP10 in the developing vasculature (Chang et al., 2006) and between HDACs and ECM

components during worm development (Whetstine et al., 2005). Furthermore, Brg1 has been implicated in regulating the expression of MMP2 and MMP9 in cultured cells (Ma et al., 2004; Ma et al., 2004). Chromatin regulation may provide a source for signaling specificity by modifying the ability of intercellular signals to be transmitted in a complex environment. This premise may have broad implications given the important roles of chromatin remodeling and the microenvironment in both normal and pathologic settings (Anderson et al., 2006; Bhowmick et al., 2004; Roberts and Orkin, 2004) of tissue growth and differentiation.

EXPERIMENTAL PROCEDURES

Mouse husbandry

All mouse strains were maintained in outbred backgrounds. The targeted null allele of *Brg1*, *Brg1*$^{-}$ (Bultman et al., 2000), the *loxP* allele of *Brg1*, *Brg1*^{F} (Indra et al., 2005), *Tie2Cre* (Kisanuki et al., 2001), *Sm22αCre* (Holtwick et al., 2002), *Rosa26R*^{LacZ} (Soriano, 1999), and *ADAMTS1*$^{-}$ (Lee et al., 2006) strains are described. For tissue specific deletions, males of genotype *Tie2Cre;Brg1*$^{F/+}$ were crossed with *Brg1*$^{F/F}$ females. The date of observation of a vaginal plug was set as embryonic day E0.5, which was confirmed by ultrasonography prior to sacrificing pregnant mice (Chang et al., 2003) and examination of embryo morphology and somite number. Identical styles of crosses were used with *Sm22αCre* deletion of *Brg1*.

Immunostaining

Antibody staining of paraffin sections used standard methods, following protocols detailed in the Supplemental Experimental Procedures. The following antibodies were used: anti-Brg1 (G7) mouse monoclonal (Santa Cruz Biotechnology), anti-Brg1 rabbit polyclonal (Chemicon), anti-Brg1 (J1) rabbit polyclonal, anti-NFATc1 (7A6) (BD Biosciences), anti-smooth muscle actin (Sigma), anti-Actinin (Sigma), anti-phospho-Histone H3 (Millipore), anti-Versican (Millipore), anti-Perlecan (SPM255) (Santa Cruz Biotechnology), and anti-Neo-Versican (anti-Versican V0/V1 Neo) (Affinity Bioreagents). TUNEL staining was performed using an *in situ* cell detection kit (Roche).

Histology

Paraffin sections were prepared as described in the immunostaining section. Following re-hydration, the slides were consecutively stained with hematoxylin and eosin (Polysciences), dehydrated and mounted in Permount (Sigma) prior to imaging. Alcian blue staining was performed using a 1% solution of Alcian blue, pH 2.5 in acetic acid followed by counter-staining with Nuclear Fast Red (Vector Laboratories). Hyaluronan was stained using biotinylated-hyaluronan binding protein (Calbiochem) at 4 μg/mL, HRP-conjugated streptavidin (DAKO) and DAB chromogenic development. The sections were counterstained with hematoxylin.

Trabeculation Quantification

We used a dual fluorescence confocal image system similar to one previously described (Chen et al., 2004) to visualize, quantitate and compare the development of trabecular myocardium in *Tie2Cre;Brg1*$^{F/F}$ and littermate control embryos. Frozen sections of the embryos were stained with anti-PECAM (BD Biosciences) and anti-MF20 (Developmental Studies Hybridoma Bank) antibodies (described further in Supplemental Material). The images were captured using LaserSharp software and analyzed with Metamorph (version 6.1). We measured the thickness of trabecular ridges/sheets (μm), thickness of compact wall (μm), and area of trabecular myocardium normalized by the length of compact wall ($\mu m^2/\mu m$). For the *ADAMTS1* null and heterozygous control mice, a modification of the above protocol was used for morphometric measurements of the hearts. We performed the quantification using images

of H&E stained paraffin section analyzed with Nikon Advanced Elements software. We measured the total number of trabeculae (where the trabecular layer meets the compact layer) normalized to ventricle length, the average thickness of the trabeculae, the average thickness of the compact layer, and the total area of the trabecular sheet normalized to the area of the ventricle (in pixel units). These latter ratios do not represent absolute ratios as the normalization measurements were performed using images taken at a different magnification.

Whole mount *in situ* hybridization

In situ hybridization assays were based on the protocol described in (Wilkinson and Nieto, 1993) with details and modifications described in Supplemental Experimental Procedures. The plasmid template for the BMP10 *in situ* probe is described (Chen et al., 2004).

In situ hybridization on paraffin sections

Digoxigenin-labelled antisense transcripts were synthesized using plasmid templates (Roche). Plasmids for synthesizing the probes are from the following sources: *VEGF-A* from Douglas Hanahan (Christofori et al., 1995), *Neuregulin* from Carmen Birchmeier (Meyer et al., 1997) *Tie2* from Kari Alitalo (Korhonen et al., 1994), *Has-2* from Todd Camenisch (Camenisch et al., 2000), Tie1, VEGFR2, Nkx2.5, GATA4, ErbB2, Hey1, MEF2C, NPPA, UGDH, Versican, Perlecan, Irx4, MEST, TIMP3, Hyal1, Hyal2, Hyal3, ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, ADAMTS9, ADAMTS18, MMP1, MMP2, MMP3, MMP9, MMP13, and *Fibulin1* from Open Biosystems. All plasmids were confirmed by sequencing. Method details for probe hybridization and visualization are included in the Supplemental Experimental Procedures.

RT-PCR and quantitative RT-PCR

Whole hearts were dissected from E9.5 embryos. The hearts were disrupted in Trizol (Invitrogen) and RNA was purified using standard methods. 150 ng of total RNA from each sample was used as a template to prepare cDNA using the Superscript III reverse transcription system (Invitrogen). In the case of HUVECs, RNA was isolated using a Trizol-based method and 500 ng of RNA was used for cDNA synthesis. Quantitative PCR to measure transcript content in the individual samples was performed using primers and methods described in the Supplemental Experimental Procedures.

Lentivirus Production and RNA Interference

Lentivirus were produced in 293T cells following protocols of the Didier Trono lab by co-transfecting them with packaging and envelope plasmids and pGIPZ transfer vectors for shRNA expression (Open Biosystems). Two separate plasmids expressing shRNAs targeting human Brg1 were used while the control pGIPZ vector contains a non-silencing shRNA. Two days post-transfection of the 293T cells, the media was isolated, filtered, and concentrated using a 100k MW cutoff centrifugal filter unit (Millipore). This viral concentrate was used for transduction of HUVECs. The cells were cultured for 5 days, at which point most of the cells expressed GFP, indicating a high rate of infection. RNA was prepared using Trizol extractions and then used for cDNA synthesis. Expression levels were determined using quantitative PCR as described in the previous section.

Chromatin immunoprecipitations

Human umbilical vein cells were cultured to approximately 75% confluency on six 15 cm plates and harvested using reagents and methods from a chromatin immunoprecipitation kit (Upstate) with modifications and details described in Supplemental Experimental Procedures. Chromatin was prepared and immunoprecipitated using the J1 anti-Brg1 antibody (Wang et al., 1996) and an anti-GFP (Invitrogen) antibody as an isotype-matched negative control.

Following washes, DNA was retrieved and used for quantitative PCR using primers and methods described in Supplemental Experimental Procedures.

Reporter Assays

Reporter plasmids were constructed by cloning PCR amplified region of the mouse ADAMTS1 locus into the episomal pREP4-Luc plasmid (Liu et al., 2001). These vectors, along with an episomal renilla-luciferase plasmid (pREP7-RL) to normalize for transfection efficiency and a plasmid expressing human Brg1 or a matching empty vector plasmid were transfected into SW13 cells using lipofectamine 2000 (Invitrogen). The cells were cultured for two days and harvested for luciferase assay using the dual luciferase assay kit (Promega).

Whole embryo culture

Whole embryo cultures were performed as described (Chang et al., 2004). Embryos were isolated intact in their yolk sacs at E9.0 and cultured for 36 hours in the presence of either 20 μM GM6001 (Calbiochem), 20 μM Minocycline (Sigma), or an equivalent amount of DMSO alone. The yolk sacs were used for genotyping and the embryos harvested for histological analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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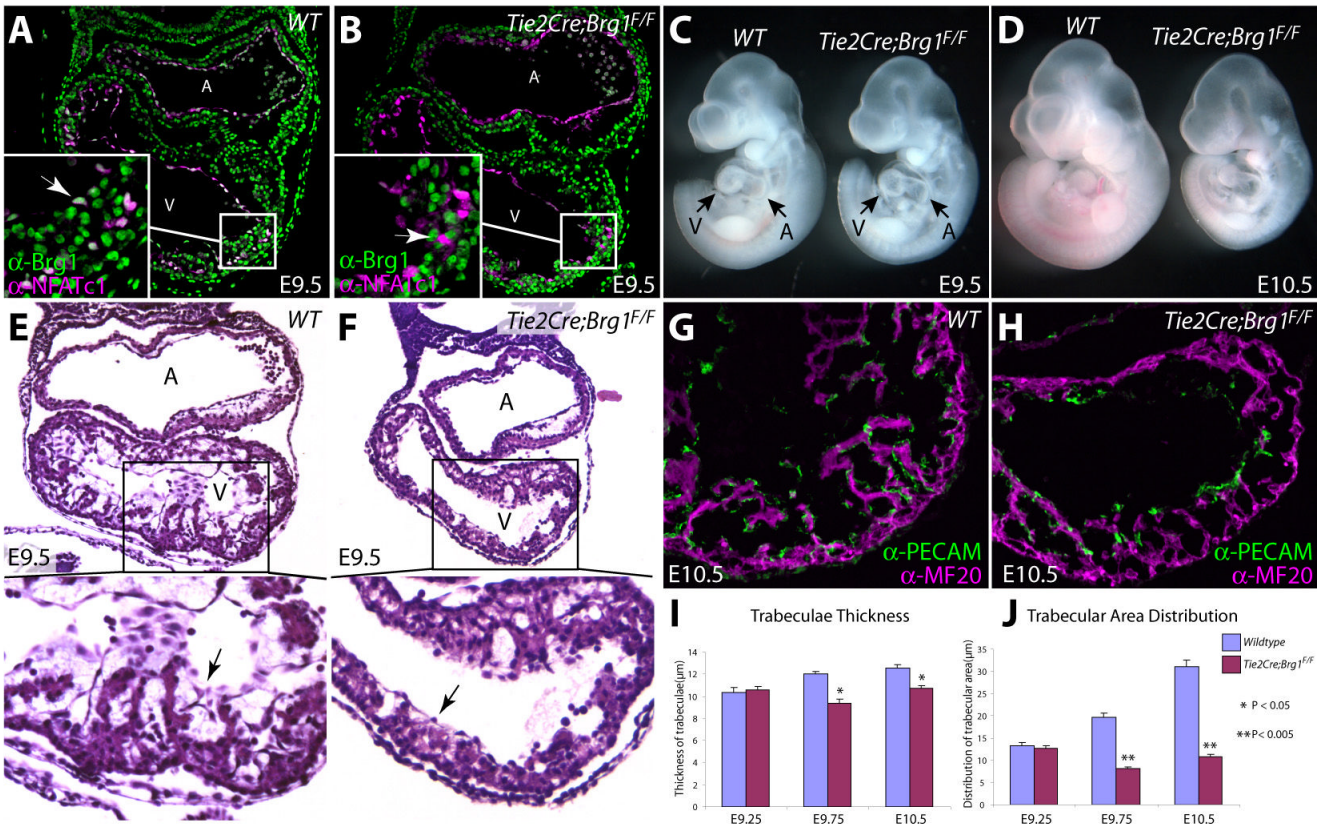

Figure 1.

Endocardial Brg1 is required for trabeculation. (**A** and **B**) Paraffin sections from wildtype (**A**) and *Tie2Cre;Brg1*$^{F/F}$ (**B**) E9.5 embryos were double stained with an anti-Brg1 antibody (green) and an anti-NFATc1 antibody (cyan) to mark endocardial cells. The arrows point at endocardial cells. (**C** and **D**) Images of E9.5 and E10.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ embryos. A: atrium. V: ventricle. (**E** and **F**) Hematoxylin and eosin (H&E) stained paraffin sections showing E9.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ heart ventricles. Arrows denote ventricular endocardial cells. (**G** and **H**) E10.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ frozen heart sections are stained with antibodies against PECAM (green) and MF20 (cyan) to mark the endocardium and myocardium, respectively. (**I** and **J**) Morphometric analysis of trabecular thickness (**I**) and the ratio of trabecular area to compact wall length (**J**) by measurement of sections stained as in (**G** and **H**) from E9.25, E9.75, and E10.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ embryos. Error bars are one standard deviation. Three hearts were measured for each group and p values were calculated using two-tailed Student's t-Tests.

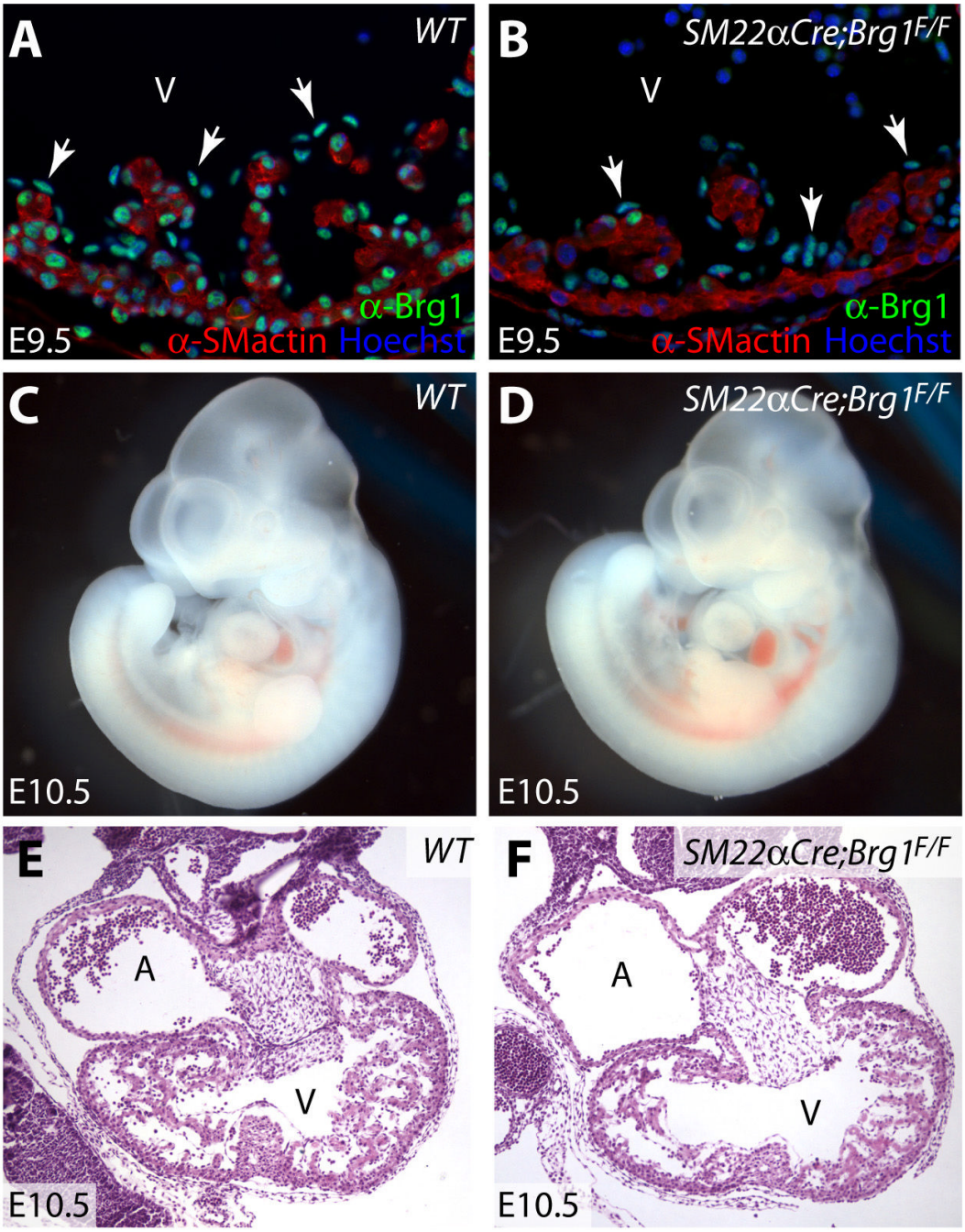

Figure 2.

Myocardial Brg1 does not have major effects on cardiac morphogenesis between E9.0 and E10.5. (**A** and **B**) E9.5 wildtype and *SM22αCre;Brg1*$^{F/F}$ heart sections are double immunostained for Brg1 (green) and smooth muscle actin (SMactin) to mark the myocardium (red). Nuclei (blue) are stained using Hoechst. Arrows indicate ventricular endocardial cells. (**C** and **D**) Gross images of E10.5 wildtype and *SM22αCre;Brg1*$^{F/F}$ embryos (**E** and **F**) H&E stained heart sections of E9.5 wildtype and *SM22αCre;Brg1*$^{F/F}$ embryos. A: atrium. V: ventricle.

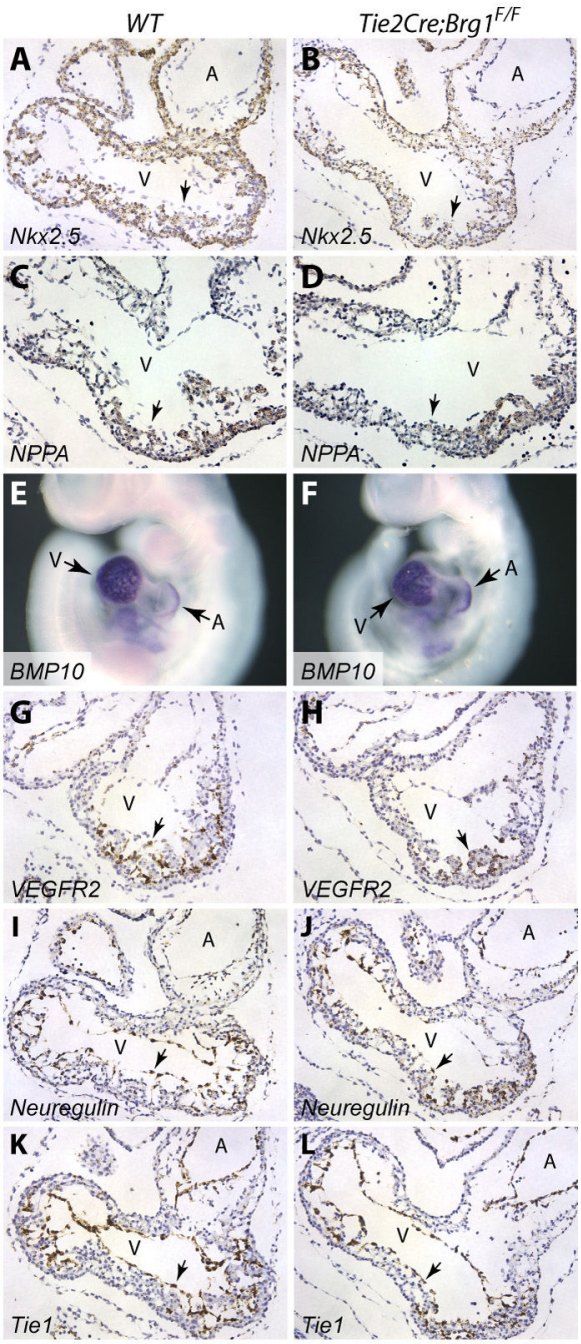

Figure 3.

Myocardial and endocardial transcripts known to regulate trabeculation are expressed normally in *Tie2Cre;Brg1*$^{F/F}$ embryos. (**A** to **D, G** to **L**) *In situ* hybridizations on paraffin sections of E9.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ embryonic hearts for the indicated transcripts (stained brown with DAB). The blue counter stain is hematoxylin. (**E** and **F**) E9.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ embryos are whole-mount stained for *BMP10* transcripts (blue). Arrows indicate ventricular endocardial cells. A: atrium. V: ventricle.

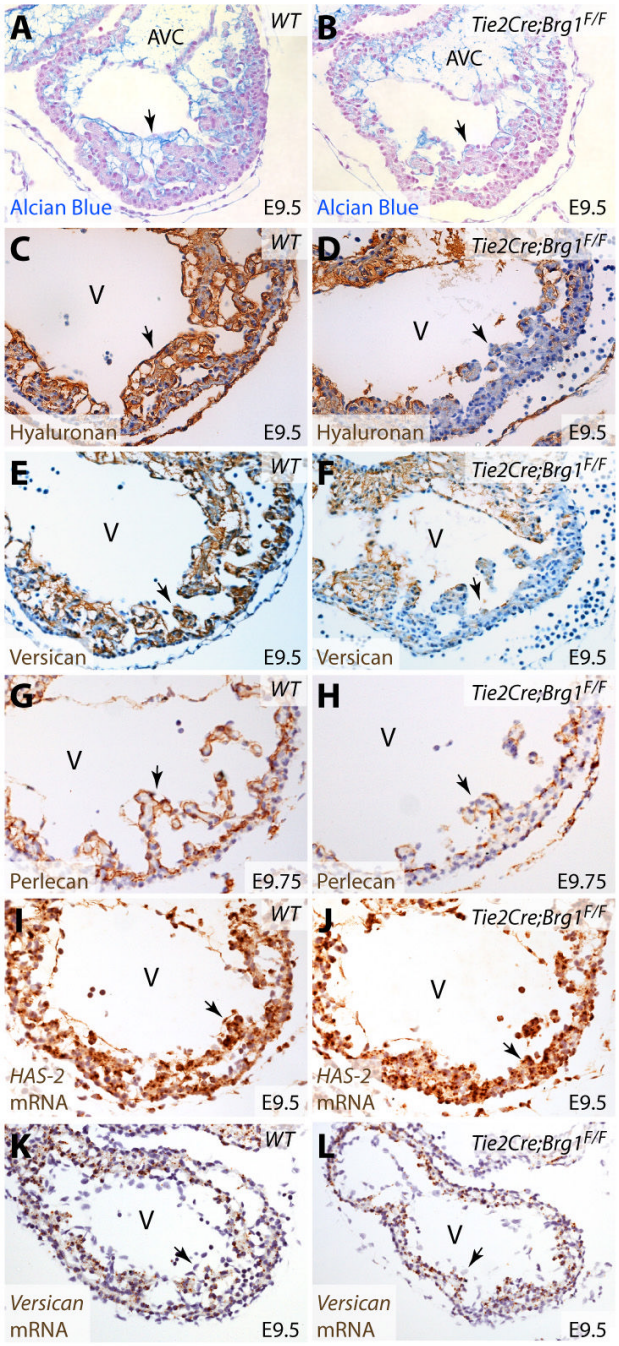

Figure 4.

Tie2Cre;Brg1$^{F/F}$ embryos have defects in ECM formation in ventricular cardiac jelly. (**A** and **B**) Alcian Blue staining of paraffin sections through ventricles of E9.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ embryos. The sections are counterstained with nuclear fast red. (**C** and **D**) Hyaluronan (HA) staining using biotinylated-HABP of wildtype and *Tie2Cre;Brg1*$^{F/F}$ ventricle sections. (**E** and **F**) Anti-Versican antibody staining of ventricle-containing sections of wildtype and *Tie2Cre;Brg1*$^{F/F}$ hearts. (**G** and **H**) Antibody staining of Perlecan in left ventricle sections of E9.75 wildtype and *Tie2Cre;Brg1*$^{F/F}$ embryos. In situ hybridizations for *Has-2* (**I** and **J**) and *Versican* (**K** and **L**) transcripts on E9.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ heart sections. The staining in (**C** to **L**) was developed with DAB (brown) and counterstained

with hematoxylin. Arrows indicate ventricular endocardial cells. V: ventricle. AVC: atrioventricular canal.

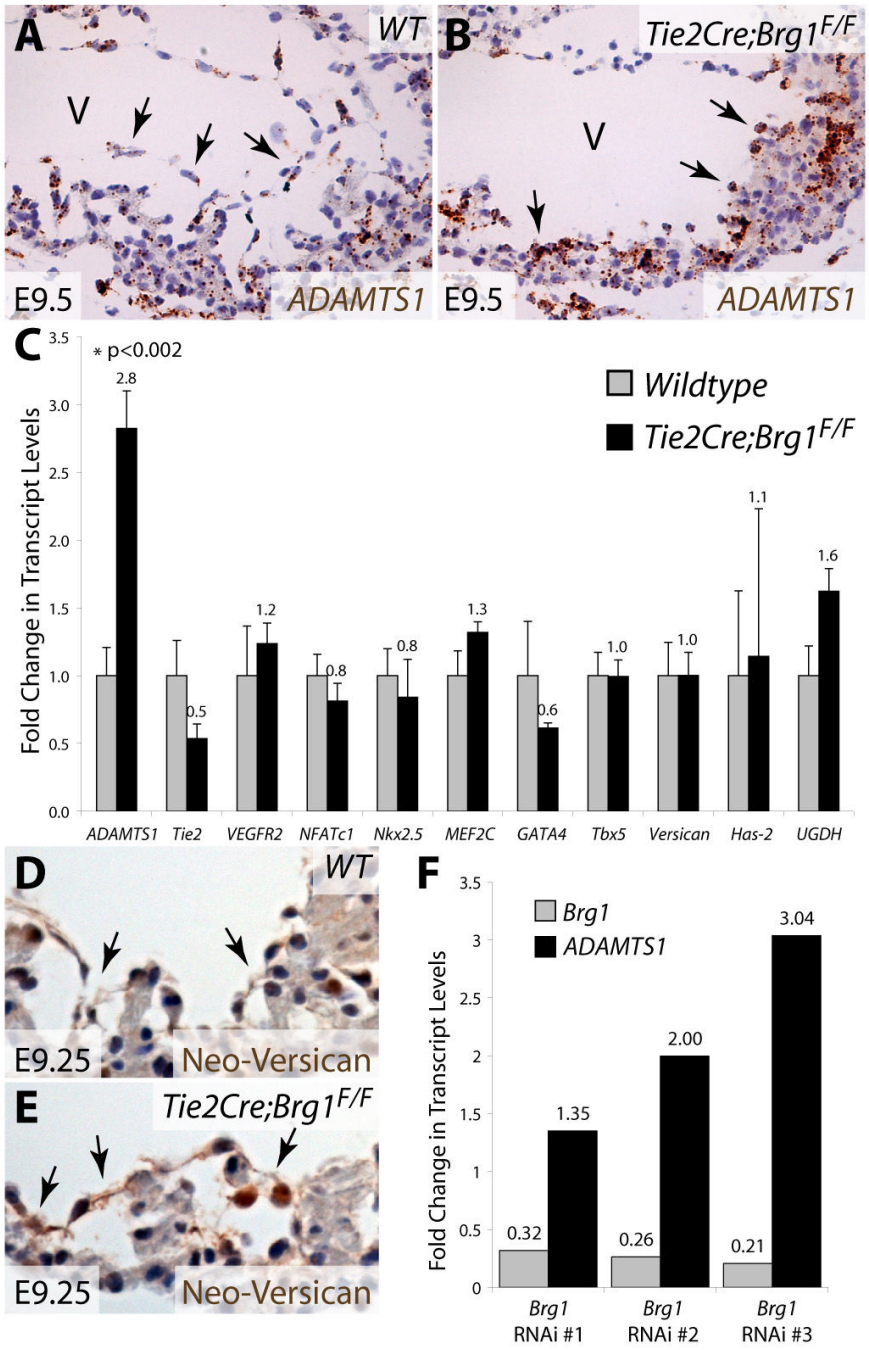

Figure 5.

ADAMTS1 is derepressed in the absence of endocardial Brg1. (**A** and **B**) RNA in situ hybridization for *ADAMTS1* transcripts (brown) on E9.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ ventricle sections. (**C**) Quantitative RT-PCR (qRT-PCR) for *ADAMTS1* and endocardial transcripts (*Tie2*, *VEGFR2*, *NFATc1*), myocardial transcripts (*Nkx2.5*, *MEF2C*, *GATA4*, *Tbx5*), or ECM-related transcripts (*Versican*, *Has-2*, *UGDH*) normalized to *HPRT* expression using RNA from dissected hearts of E9.5 wildtype and *Tie2Cre;Brg1*$^{F/F}$ embryos. Error bars are one standard deviation. P<0.002 (n=3 wildtype and mutant) of a non-significant change in *ADAMTS1* in *Tie2Cre;Brg1*$^{F/F}$ hearts by a two-tailed Student's t-Test. (**D** and **E**) Antibody staining for a Versican cleavage product (Neo-Versican) produced in ventricle sections of

E9.25 wildtype and *Tie2Cre;Brg1*$^{F/F}$ embryos. Arrows denote subendocardial cardiac jelly. (**F**) qRT-PCR for *ADAMTS1* and *Brg1* transcripts normalized to *HPRT* on RNA from cultured primary human umbilical vein endothelial cells transduced with a control lentivirus or one expressing small hairpin RNA (shRNA) directed against *Brg1* transcripts. Experiment #1 used a different shRNA sequence from Experiments #2 and #3. Each experimental set represents independent experiments that varied in their extent of RNA interference of *Brg1*.

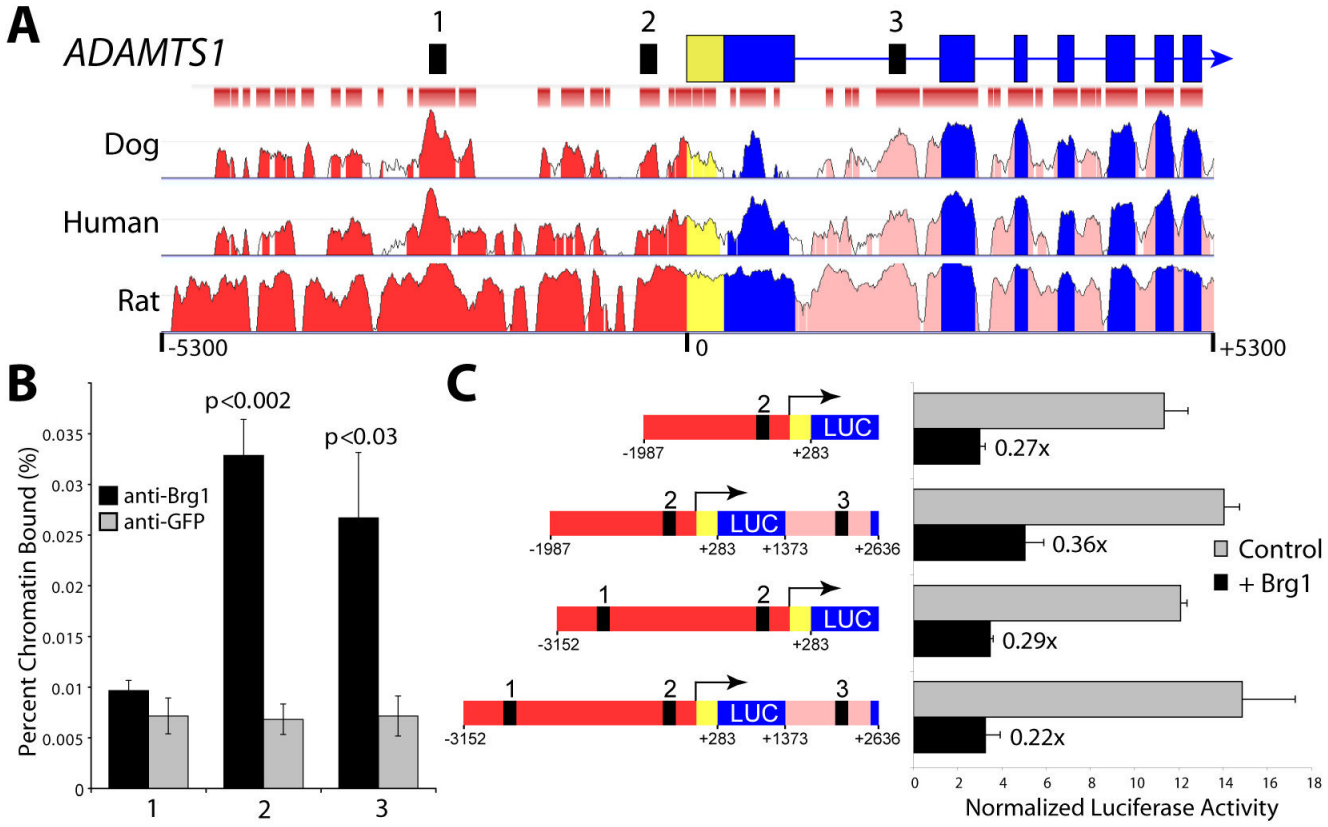

Figure 6.

The *ADAMTS1* promoter is directly repressed by Brg1. (**A**) An alignment of the *ADAMTS1* locus between the mouse and dog, human, and rat genomes, respectively. Increased conservation is shown by the height of the colored curves. Blue regions are translated portions of exons, yellow is 5′ untranslated region, and red indicates candidate regulatory regions. Three conserved regions, labeled 1, 2 and 3, were selected for further analysis. (**B**) Quantitative PCR analysis of anti-Brg1 immunoprecipitated chromatin from regions 1, 2, and 3 in human umbilical vein cells normalized to an anti-GFP control antibody. Error bars represent one standard deviation and p values were determined by two-tailed Student's t-Tests. (**C**) Episome-based reporter assays using the indicated *ADAMTS1* candidate regulatory regions cloned in luciferase expression plasmids co-transfected in SW13 cells with either a control or Brg1-expressing plasmid to restore BAF complexes to the cells. The luciferase activity is graphed relative to a co-transfected constitutive *Renilla* luciferase control and error bars represent one standard deviation between 3 independent transfections. P values were calculated using unpaired two-tailed Student's t-Tests.

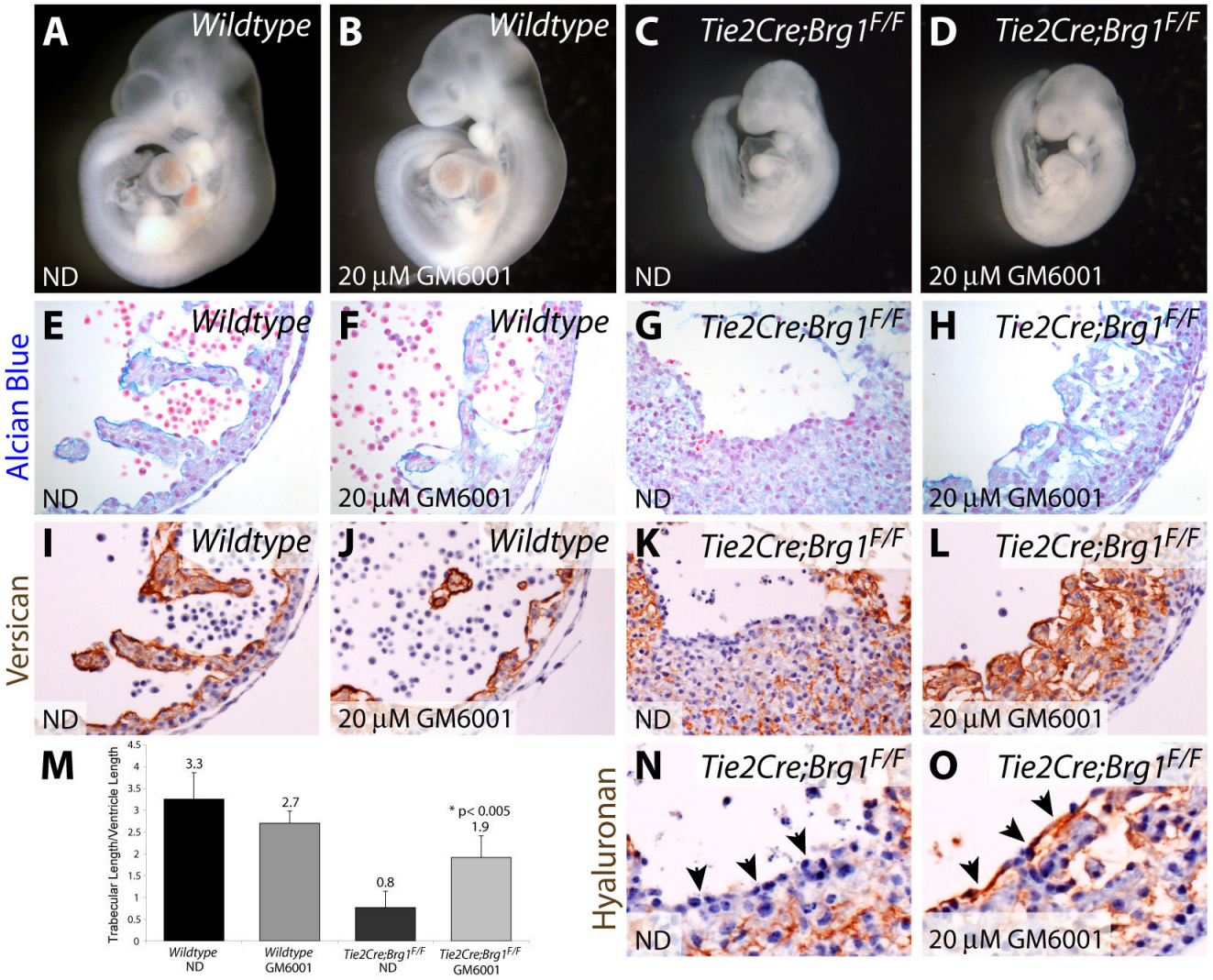

Figure 7.

Derepression of *ADAMTS1* underlies the trabeculation defects in *Tie2Cre;Brg1$^{F/F}$* embryos. (**A** to **D**) Whole mount images of wildtype and *Tie2Cre;Brg1$^{F/F}$* embryos grown in culture for 36 hours beginning at E9.0 in the presence or absence of 20 μM GM6001, an ADAMTS1 inhibitor. (**E** to **H**) Heart ventricle sections of the embryos shown in (**A** to **D**) are stained with Alcian blue to mark mucopolysaccharide components of the cardiac jelly and counterstained using nuclear fast red. (**I** to **L**) Anti-Versican antibody staining in the ventricular cardiac jelly of the same cultured wildtype and *Tie2Cre;Brg1$^{F/F}$* embryos control-treated or treated with GM6001. The sections are counterstained with hematoxylin. (**M**) The mean ratio of trabecular to outside length of the left ventricle measured on H&E stained sections is graphed for wildtype and *Tie2Cre;Brg1$^{F/F}$* embryos cultured with or without the matrix proteinase inhibitor GM6001 for 36 hours beginning at E9.0. Error bars represent one standard deviation. n=3 Wildtype/ND, Wildtype/GM6001, and *Tie2Cre;Brg1$^{F/F}$*/ND groups, n=8 *Tie2Cre;Brg1$^{F/F}$*/GM6001. P values were determined using Student's t-Tests. (**N, O**) Biotinylated-HABP stained heart sections of *Tie2Cre;Brg1$^{F/F}$* embryos cultured from E9.0 for 36 hours in the presence or absence of GM6001 to mark hyaluronan content (in brown). The sections are counterstained with hematoxylin (blue).

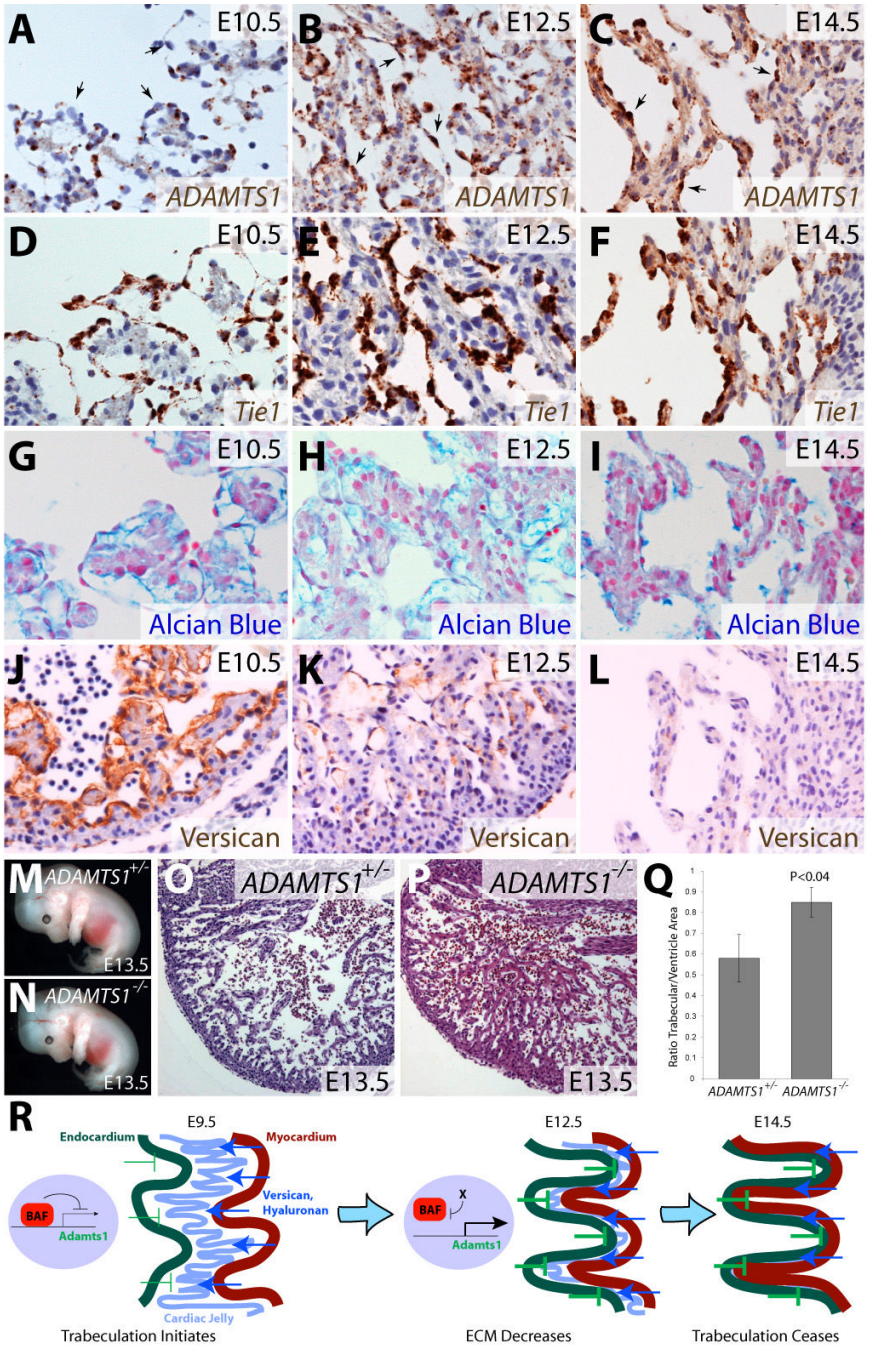

Figure 8.

An increase in expression of ADAMTS1 in ventricular endocardium terminates myocardial trabeculation by breaking down cardiac jelly. (**A** to **F**) *In situ* hybridizations on ventricle sections of wildtype embryos of the indicated stages for *ADAMTS1* (**A** to **C**) and *Tie1* (**D** to **F**) transcripts. Transcripts are stained brown and the slides are counterstained with hematoxylin. Arrows indicate ventricular endocardial cells. (**G** to **I**) Alcian blue stained sections of wildtype embryonic hearts of the indicated stages. The sections are counterstained with nuclear fast red. (**J** to **L**) Sections from E10.5, E12.5, and E14.5 wildtype ventricles are stained with anti-Versican antibodies (brown) and counterstained with hematoxylin. (**M** and **N**) Gross images of E13.5 *ADAMTS1*$^{+/-}$ and littermate *ADAMTS1*$^{-/-}$ embryos. (**O** and **P**) H&E

stained sections of the ventricles of an E13.5 *ADAMTS1*$^{+/-}$ and littermate *ADAMTS1*$^{-/-}$ embryos. (**Q**) The mean ratio of trabecular/ventricle area in E13.5 *ADAMTS1*$^{+/-}$ and littermate *ADAMTS1*$^{-/-}$ hearts from measurements of H&E stained left ventricle sections (n=3 *ADAMTS1*$^{+/-}$, n=5 *ADAMTS1*$^{-/-}$, error bars are one standard deviation, $p<0.04$ by two-sample two-tailed Student's t-Test). (**R**) A working model of myocardial trabeculation. Brg1 represses *ADAMTS1* in endocardial cells to allow myocardial cells to produce an extracellular environment that supports trabeculation. By E12.5, BAF-complex mediated repression of *ADAMTS1* is relieved (by an unknown mechanism, "X") dissipating the cardiac jelly and preventing excessive trabeculation. In *Tie2Cre;Brg1*$^{F/F}$ embryos, *ADAMTS1* is derepressed, causing the cardiac jelly to prematurely break down and disrupting myocardial morphogenesis.