A spatiotemporal observation of EndMT and mesenchymal cell colonization at the onset of human cardiac valve development

Running title: Atlas of human valvulogenesis

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Summary Statement

This study provides unique insight on the process by which developing human semilunar valves become populated by endothelium undergoing EndMT, and the migration of mesenchymal cells along the elongating valve contributing toward the trilaminar valve structure.

Abstract

Elucidation of mechanisms in semilunar valve development may enable the development of new therapies. Here, we found differences in proliferation-associated genes and genes repressed by vascular endothelial growth factor between human semilunar valves from first and second trimester valve leaflets. The proliferation of valve interstitial cells and ventricular valve endothelial cells (VECs) and cellular density declined from the first to the second trimester. Cytoplasmic expression of nuclear factor of activated T-cells cytoplasmic 1 (NFATc-1) in VECs (4 weeks), and later cells in the leaflet/annulus junction mesenchyme expressing inactive NFATc-1 (5.5-9 weeks) were detected, indicative of EndMT in valvulogenesis. At this leaflet/annulus junction CD44+ cells clustered during elongation (11 weeks), extending toward the tip along the fibrosal layer in second trimester leaflets. Differing patterns of maturation in the fibrosa and ventricularis were detected via increased fibrosal periostin content, which tracked the presence of the CD44+ cells in the second trimester.

We revealed that spatiotemporal NFATc-1 expression actively regulates EndMT during human valvulogenesis, as early as 4 weeks. Additionally, CD44+ cells play a role in leaflet maturation toward the trilaminar structure, possibly via migration of VECs undergoing EndMT, which subsequently ascend from the leaflet/annulus junction.
Keywords: NFATc-1 • EndMT • heart • semilunar valves • extracellular matrix • periostin

Non-standard Abbreviations and Acronyms

AV – atrioventricular
ECM – extracellular matrix
EndMT – endocardial-to-mesenchymal transformation
HA – hyaluronic acid
NFATc-1 - nuclear factor of activated T-cells cytoplasmic 1
OFT – outflow tract
SHF – second heart field
VEC – valvular endothelial cell
VEGF - vascular endothelial growth factor
VIC – valvular interstitial cell
Introduction

Congenital heart disorders, which include aortic and pulmonary valve disease, are one of the most prevalent birth defects occurring in humans (Roger et al., 2012). Valve malformation can lead to stenosis or calcification, which can further develop into more debilitating diseases including congestive heart failure (Fedak et al., 2002). The clinical importance of understanding valve development, valvulogenesis is understood, yet the mechanisms underlying normal human fetal valvulogenesis are not fully elucidated (Lin et al., 2012). To date, most developmental studies define mechanisms of valvulogenesis in models of zebrafish, mouse or chicken (Butcher and Markwald, 2007; de Vlaming et al., 2012; Lin et al., 2012). Such investigations have identified key mechanisms and regulatory pathways; however, these data have yet to be confirmed and/or corroborated in humans.

Semilunar valvulogenesis begins within four weeks of development and originates from the endocardial cushions. Initially, these cushions derive from the cardiac jelly that is formed between the myocardial and endocardial layers within the distal aspect of the outflow tract (OFT) (Eisenberg and Markwald, 1995; Markwald et al., 1977; Srivastava, 2006). Cells contributing to semilunar valve formation originate from the second heart field (SHF) and the neural crest (Srivastava, 2006). During endocardial cushion formation, endocardial cells transition to a mesenchymal phenotype by a process called endocardial-to-mesenchymal transformation (EndMT) and migrate into the cardiac jelly (Armstrong and Bischoff, 2004). Afterwards, the thick endocardial cushions, which act as primitive valves, elongate into thin fibrous leaflets exhibiting a typical trilaminar extracellular matrix (ECM) structure (Armstrong and Bischoff, 2004; Combs and Yutzey, 2009; Lincoln et al., 2004; Markwald et al., 1977).

Little has been clarified regarding the mechanisms that lead to the elongation of the human leaflet. It is known from model systems that the spatial and temporal balance of cell proliferation and apoptosis in mesenchymal, endocardial and SHF-derived myocardial cells is pivotal for the normal remodeling, elongation and maturation processes (Rentschler et al., 2010). In the semilunar valves of mice and chickens, cell density and the proliferative capacity of endocardial cushion cells decreases during leaflet elongation (Kruithof et al., 2007). In mature human leaflets, valvular interstitial cells (VICs) are generally deemed to be quiescent and exhibit a heterogenous fibroblast-like cell phenotype (Aikawa et al., 2006). The complex phenotype of VICs connotes that they have yet to be fully characterized in detail; however, some general mesenchymal markers including the hyaluronic acid (HA) receptor...
CD44 are expressed on fibroblast-like VICs (Carthy et al., 2012; Halfon et al., 2011). This receptor is attributed to the mediation of cell motility and plays an important role in epithelial-to-mesenchymal transformation (EMT) during cancer progression, which is known to share mechanisms with EndMT during development, and could play a potential role in leaflet elongation (Kim et al., 2008; von Gise and Pu, 2012; Zoller, 2011).

The role of EndMT in leaflet development has been extensively studied in animal models such as mouse and chicken, and numerous factors involved in its regulation have been identified, including ECM proteins (Norris et al., 2009; Runyan and Markwald, 1983), growth factors (Macgrogan et al., 2011), and various transcription factors. One such transcription factor is nuclear factor of activated T-cells, cytoplasmic 1 (NFATc-1) (Chang et al., 2004; de la Pompa et al., 1998; Ranger et al., 1998). NFATc-1 is a calcium-activated transcription factor, which is reported to play an essential role in mouse semilunar valve development (de la Pompa et al., 1998; Ranger et al., 1998). Activated NFATc-1 translocates to the nucleus and has been shown to be predominately expressed in endocardial cells close to the endocardial cushions (Armstrong and Bischoff, 2004; Chang et al., 2004). Furthermore, it has been demonstrated that vascular endothelial growth factor (VEGF)-mediated, calcineurin-activated NFATc-1 regulates endothelial cell fate and maintains a valvular endothelial cell (VEC) phenotype (Johnson et al., 2003). Moreover, previous reports postulated that the spatio-temporally distinct function of NFATc-1 signaling in SHF cells and endocardial cells is necessary for semilunar cushion formation as well as leaflet elongation and maturation in rodents (Chang et al., 2004; Lin et al., 2012; Wu et al., 2011).

In this study, we performed global gene expression analyses on leaflets from first and second trimester human hearts, which revealed significant differences in the expression profiles of proliferation-associated genes and those associated with EndMT. We therefore hypothesized that human semilunar valvulogenesis is a process which actively begins within the first weeks of development and follows a spatio-temporal defined pattern in which proliferation decreases during development. We also hypothesized that replenishment of cells in the mesenchyme is contributed by VECs undergoing EndMT to migrate into the cardiac cushions. In addition, we identified expression of CD44 in second trimester valves that begins at the annulus/leaflet junction and progresses towards the leaflet tip during elongation.
Results

Cell density and proliferation in developing human semilunar valves decreases from the first to the second trimester

To identify trends in gene expression that changed between the first (9-12 weeks) and second trimesters (14-17 weeks), we employed gene set enrichment analysis (GSEA) on data previously generated by our laboratory (Votteler et al., 2013a). The GSEA revealed a significant number of proliferation associated gene sets and motifs being enriched in the first trimester when compared to the second trimester (Fig. S1, Tables S1 and S2). To confirm the observations of the GSEA, valve leaflets of first trimester (4-12 weeks, n=8) and second trimester (13-17 weeks; n=7) hearts were utilized for immunohistological staining. Thereafter, the temporal valvular cell density was quantified by counting cell nuclei in 4,6-diamidino-2-phenylindole (DAPI)-stained tissue sections. Similar to patterns in mouse and human tissues of later developmental stages (second and third trimesters) (Aikawa et al., 2006; Hinton et al., 2006; Kruithof et al., 2007), we identified a decrease in cell density during early valve development (Fig. 1A). First trimester cushions and leaflets exhibited 51.0 ± 8.0 cells per 0.01 mm², which significantly reduced to 35.3 ± 8.4 cells per 0.01 mm² in second trimester leaflets (p<0.001). Within these leaflets, the number of Ki67+ VICs and VECs (brown) on the ventricularis was significantly reduced beginning at 7-8 weeks when compared to late 4 weeks of development (Fig. 1B-E). No discrete spatial pattern of proliferative VICs was evident as Ki67+ VICs were randomly present throughout the cardiac cushion (Fig. 1B) and elongated leaflets (Fig. 1C). However, it should be noted that there were significantly fewer Ki67+ VECs on the ventricularis when compared to the fibrosa, a trend that persisted from 7-8 weeks of development (Fig. 1E).

To identify potential mechanisms driving increased proliferation in the first trimester, we screened the GSEA data for likely candidates. Interestingly, several gene sets related to MYC activity were significantly enriched in the first trimester (Fig. S2). As MYC is a known regulator of cell cycle progression (Dang, 1999), we hypothesized that MYC participates here in proliferation by up-regulating mitosis genes in first trimester leaflets. Comparison of the genes contained within the “Reactome Mitotic M-M/G1 Phases” gene set, which was significantly enriched in first trimester leaflets, with a gene set that defined direct MYC targets using ChIP-Seq (Zeller et al., 2003) demonstrated a statistically significant overlap, suggesting that MYC activity influences this enhanced proliferation. Immunohistological
staining for MYC protein on first and second trimester leaflets revealed higher levels of MYC in the first trimester, corroborating the gene expression data. Together, these data suggest that increased proliferation in first trimester leaflets is likely linked to MYC participation.

**Morphological differentiation of VECs in fetal semilunar valves**

Based on the differences in temporal proliferation detected between VECs lining the fibrosa versus the ventricularis, we sought to analyze these cells in more detail. Previous reports focusing on human postnatal valves demonstrated that VECs lining the ventricularis possess a different morphology compared to the VECs populating the fibrosa layer of the same leaflet (Armstrong and Bischoff, 2004). We established that such VEC morphological differences could be detected as early as week 4 of development (Fig. 1F). We further identified that these cell morphological features continued to exist during leaflet maturation. Immunohistological staining revealed a characteristic cuboidal morphology of the CD31+ VECs on the fibrosa layer, as opposed to the typical elongated and flattened morphology of the VECs facing the ventricles at 4 and 7 weeks of development (Fig. 1F,G). VECs of the fibrosa exhibited significantly shorter distances between junctions (length) when compared to VECs of the ventricularis (cell length: 7.7 ± 0.9 µm versus 12.6 ± 1.8 µm; p<0.001). Correspondingly, VECs of the fibrosa demonstrate a significantly higher basolateral-apical distance (height) when compared to VECs of the ventricularis (cell height: 7.3 ± 1.1 µm versus 4.7 ± 0.4 µm; p<0.001) during the first trimester of development (Fig. 1H).

**Gene and protein analyses reveal spatial and temporal changes in NFATc-1 expression**

It is established that the transdifferentiation of VECs through EndMT plays a substantial role in populating the developing cardiac cushion and subsequently elongating the valve leaflet (de la Pompa et al., 1998; Ranger et al., 1998). We observed that NFATc-1 mRNA expression significantly decreased (relative expression: 1.006 ± 0.226 versus 0.513 ± 0; p<0.006) from the first to the second trimester of human fetal development (Fig. 2A). Concurrently, we observed changes in NFATc-1 protein expression patterns. Once activated, NFATc-1 translocates to the nucleus, and cells change their polarity and morphology (Fig. 2B). As NFATc-1 is a known repressor of EndMT (Zhou et al., 2005), cells with cytoplasmic NFATc-1 could be candidates for VECs changing phenotype to populate the cushions. Notably, we identified individual cells in the endocardium of cardiac cushions at week 4 of development with cytoplasmic NFATc-1 expression that did not feature the typical morphology of
endocardial cells (Fig. 2C, green arrows). These data establish that EndMT occurs as early as week 4 in human developing cardiac valve leaflets. Comparable to reports in mice, (de la Pompa et al., 1998; Ranger et al., 1998; Wu et al., 2011) NFATc-1 was highly expressed in the nuclei of human endocardial cushion cells at 4-6 weeks of development (Fig. 2C,D), as well as in the nuclei of VECs of elongated leaflets starting as early as 7 weeks of development (Fig. 2E-G). We also detected spatial changes in NFATc-1 expression within the leaflet mesenchyme. During cushion formation and the early elongation period (weeks 4-7), we identified strong expression of NFATc-1 within the cytoplasm of VICs (Fig. 2D-F). Notably, during elongation, strong cytoplasmic NFATc-1 expression was detected in VICs of the leaflet annulus (Fig. 2K,L, white arrows). Later in development, between week 9 and early in the second trimester, NFATc-1 was also detected in the nucleus of mesenchymal cells, particularly at the leaflet annulus (Fig. 2M,N, green arrows). However, the expression intensities (grey value intensities, GVI) were significantly lower compared to NFATc-1 expression in endocardial cells (week 9: endocardium GVI = 161 ± 4.1 versus mesenchyme GVI = 111.5 ± 5.3; week 11: endocardium GVI = 139.6 ± 3.1 versus mesenchyme GVI = 60.5 ± 6.2; p<0.0001). Corresponding with reports that demonstrate NFATc-1 regulates endocardial and endothelial cell proliferation (Johnson et al., 2003; Wu et al., 2011; Zhou et al., 2005); we also detected a strong nuclear NFATc-1 expression in VECs of the elongated leaflets (Fig. 2N,O). These findings are strengthened by a reduced NFATc-1 gene expression as seen in the second trimester (Fig. 2A), as cells within the mesenchyme begin to lose NFATc-1 expression (nuclear or cytoplasmic) (Fig. 2G, H, N, O).

GSEA revealed that genes repressed by VEGF signaling were enriched in first trimester valves compared to the second trimester valves (Fig. 3A,B). In mice, VEGF has been shown to have a repressive role in regulating EndMT (Dor et al., 2001). As our gene expression data was generated from late first trimester specimens, when EndMT has begun to diminish, this would suggest that the function of VEGF in acting as a brake on EndMT is conserved. We then sought to map the expression of VEGF at the protein level in human semilunar valve leaflet development (Fig. 3C-L). We detected strong VEGF expression in the cardiac cushion VECs (weeks 4-5 of development; Fig. 3C,D), which persisted throughout the course of development. However, no statistically significant trends in protein expression were detected (Fig. S3). A weak VEGF expression was detected within the cushion and leaflet mesenchyme at all the time points investigated.
CD44 expression clusters at the leaflet/annulus junction and progresses towards the leaflet mesenchyme in second trimester human cardiac valves

Based on our data showing a significant decrease in VIC proliferation starting at week 7 (Fig. 1D), inactive NFATc-1 expression in endocardial cushion cells early in the first trimester (Fig. 2C), and the strong presence of inactive NFATc-1 expression at the annulus prior to elongation (Fig. 2L,M), it was sought to determine the contribution of CD44+ cells populating the valve leaflets during leaflet development. Real-time PCR (qPCR) analyses revealed a significant increase (relative expression: 1.374 ± 1.262 versus 19.14 ± 8.081, p<0.0003) of CD44 mRNA expression between first and second trimester leaflets (Fig. 4A). This gene expression pattern was confirmed on the protein level using immunofluorescence staining. Accordingly, we observed that CD44 was exclusively expressed on some VECs along the cardiac cushions in 4 to 7 week old hearts (Fig. 4B-D, Fig. S4). These endothelial cells appear to be assuming a mesenchymal phenotype in order to populate the mesenchyme of the developing valve leaflet. In contrast, CD44+ cells were only detectable at 4 weeks of development in the cardiac cushion mesenchyme, and were afterwards present in the myocardial wall (Fig. 4B-D).

Later in development, we detected a spatially distinct expression of CD44 in the developing semilunar leaflets. CD44 expression clustered at the junction between the valve leaflet and annulus (9 weeks, Fig. 4E). Afterwards, at 11 and 16 weeks of development, CD44+ cells were detected along the leaflet in a defined domain that progressed toward the leaflet tip over time (Fig. 4F,G). Notably, these CD44+ cells were juxtaposed along the fibrosal and spongiosal layer of the elongated leaflets, whereas fewer CD44+ cells were detected in the ventricularis. Based on this specific spatial localization, which occurred at defined time-points of leaflet development, we hypothesize that signaling through CD44 may contribute to the positioning of these cells. Mesenchymal cells expressing CD44 in other systems are known to migrate through engagement of hyaluronan receptors, and recently an induction of intracellular cross-talk between peristin and hyaluronan has been established (Ghatak et al., 2014), wherein valvular cushion cells were shown to secrete peristin into the ECM in vitro, which enhanced hyaluronan expression upon peristin/integrin/focal adhesion kinase-mediated activation of P13K and/or ERK (Norris et al., 2008; Snider et al., 2008). Although the role of peristin in fibrogenesis has yet to be fully clarified, it is agreed that this ECM protein is necessary for the initiation and regulation of collagen deposition. Its detection has been observed in mice after EndMT in the development of the atroventricular (AV) valves.
(Norris et al., 2008). Here, we report similar findings with a significant increase of the periostin content after EndMT that precedes CD44 expression (Ghatak et al., 2014). We find that this expression of periostin became extremely marked at 11 to 17 weeks of development when compared to earlier time-points (Fig. 5A-F), which is when the expression of NFATc-1 in the mesenchyme was reduced (Fig. 2G, H, N, O), and the presence of CD44+ cells became elevated. This time-dependent linkage of CD44+ cells with the secretion of periostin suggests that the elongation of the leaflet could be directed by both biochemical and biomechanical cues by either migratory or residential cells.

**Discussion**

Our data provide unique insight into the events that support human developmental valvulogenesis. Within this study, we have investigated cellular and molecular processes responsible for human valve maturation and elongation during development. We have identified that human leaflet cell density and proliferation decreased significantly from the first to the second trimester. Differential VEC proliferation patterns were identified in the ventricularis and fibrosa layers. We sought to determine the origin of cells that populate the leaflet mesenchyme during development. We detected that VECs undergo EndMT in the cardiac cushions as early as 4 weeks of development, based on inactive cytoplasmic NFATc-1 expression and CD44 expression. Once in the cushions, these cells maintain a cytoplasmic NFATc-1 expression. Between 5 and 9 weeks of development, we detected a strong expression of inactive NFATc-1 at the junction of the leaflet/annulus mesenchyme. Later in development (weeks 11 to 17) this expression pattern disappeared and active NFATc-1 was only expressed in the VECs. However, during this period (weeks 11 to 17), we saw an increased expression profile of CD44, which clustered at this leaflet/annulus mesenchyme and later appeared along the fibrosal layer of the elongating leaflet. We also identified that this was possibly linked to a periostin-mediated manner in the second trimester of human valvulogenesis.

Previous studies report that cell density and proliferation is higher in human fetal second and third trimester leaflets when compared to mature leaflets (Aikawa et al., 2006). In this present work we identified a significantly decreasing cell density from the first to the second trimester in human semilunar cushions and leaflets, which is in accordance with studies of valvulogenesis in mice and chickens (Hinton et al., 2006; Kruithof et al., 2007). However, although proliferating cells were detected randomly throughout fetal cushions and leaflets, a
specific and significant decrease in the proliferation of ventricular VECs occurred at week 7 of development, which was not detected in fibrosal VECs (Fig. 1E). The occurrence of this difference between VECs of the fibrosa and the ventricularis at 7 to 8 weeks of development is particularly significant. In the human heart, beating begins at around 4 weeks and accelerates towards a peak of 180 bpm at 7 weeks (Riem Vis et al., 2011). We have previously reported that tropoelastin/elastin deposition is first detectable in the ventricularis of human cardiac valves at this fundamental 7-week milestone (Votteler et al., 2013a). It is therefore interesting to speculate if VECs of the ventricularis have a significantly decreased proliferation at 7 weeks that could be attributed to hemodynamic differences between the ventricularis and fibrosa.

NFATc-1 has been identified as indispensable in rodent semilunar valve development (de la Pompa et al., 1998; Ranger et al., 1998). In this present study, its spatio-temporal expression pattern was analyzed in first and second trimester human cardiac valves in order to establish the contribution of VECs, undergoing EndMT, to leaflet elongation. Nuclear NFATc-1 expression was detected in all endocardial cells, with the distinct exception of some single VECs in the cardiac cushions (Fig. 2C, green arrows). These particular cells exhibited an altered cell morphology accompanied with NFATc-1 being expressed exclusively in the cytoplasm. In light of the evidence in this study, and that of previous reports (de la Pompa et al., 1998; Johnson et al., 2003; Lin et al., 2012; Ranger et al., 1998; Wu et al., 2011; Zhou et al., 2005), it can be postulated that inactivation of NFATc-1 is indicative of EndMT in human endocardial cushion cells. While nuclear NFATc-1 was highly expressed in the endocardial cells of early cardiac cushions, and also in VECs of elongated leaflets during all developmental stages, this study also identified specific developmental stages when NFATc-1 was detectable in cushion and leaflet mesenchymal cells. During semilunar cushion formation and early elongation, mesenchymal cells expressed non-activated cytoplasmic NFATc-1, particularly at the annulus of the leaflet (Fig. 2K,L). Later during leaflet elongation between week 9 and 11 of development, mesenchymal cells expressed activated nuclear NFATc-1 in the annulus of the leaflets (Fig. 2M,N). This indicates that within mesenchymal cells of the developing leaflet annulus, NFATc-1 is activated during a specific time frame to quell EndMT. This concurs with the study of Lin et al. who reported that calcineurin-activated NFATc-1 signaling acts in spatio-temporal waves in various tissues during murine semilunar valve development (Lin et al., 2012). The same study demonstrated that the role of calcineurin/NFATc-1 signaling in the SHF of E7.5 and E8.5 mouse embryos is distinct from its role in the endocardium beginning at E10.5. While calcineurin/NFATc-1 signaling in the
SHF is required for early semilunar cushion formation by preventing the regression of the cushion mesenchyme (Lin et al., 2012), NFATc-1 in the endocardium is required for cushion reorganization and leaflet elongation (Wu et al., 2011). In endocardial cells and VECs, nuclear NFATc-1 expression is required for the maintenance of the VEC phenotype and enhances their proliferation (Johnson et al., 2003), which consequently results in reduced EndMT processes. Comparable to results from mouse studies (Wu et al., 2011), this mechanism may facilitate the contribution of mesenchymal cells from the leaflet annulus to leaflet elongation. As NFATc-1 knockout mice fail to develop elongated leaflets (Lin et al., 2012; Wu et al., 2011), we hypothesize that activated NFATc-1, which we have detected in this study in the cushion mesenchymal cells during week 9 and 11 of human cardiac valve development, supports leaflet elongation. This result strongly suggests that in humans, NFATc-1 is pivotal for semilunar valve development, with distinct roles in endocardial and mesenchymal tissues.

As previously mentioned, it has been demonstrated that VEGF-mediated calcineurin-activated NFATc-1 regulates the endothelial cell fate and contributes towards maintenance of the VEC phenotype (Johnson et al., 2003). However, regulation of leaflet development by VEGF signaling is far from a simple process (Lambrechts and Carmeliet, 2004). VEGF is necessary for initial EndMT; however, it subsequently terminates this process. Initiation and termination of EndMT are both deemed to be VEGF dose-dependent and controlled within narrow spatial and temporal windows (Lambrechts and Carmeliet, 2004). In mice, VEGF levels are detectable in the myocardium and outside the AV canal at E9, which is the timeframe at which EndMT begins in mice (Dor et al., 2001). Indeed, it has been shown that lowering VEGF levels at E9.5 via hyperglycemic induction or with a soluble Flt1 chimeric protein prevents EndMT (Enciso et al., 2003). It has also been shown in mouse embryonic explants that EndMT is inhibited by VEGF, through VEGF supplementation and hypoxia-induced VEGF up-regulation (Dor et al., 2003). In mice, myocardial VEGF levels in the AV canal are elevated 5-10 fold at E10.5 (Dor et al., 2003). These previous studies have established that some VEGF expression is required for endocardial cells to undergo EndMT, but that as EndMT reaches completion, higher levels of VEGF are encountered that halt EndMT. In this study, we have detected that VEGF gene expression is significantly up-regulated in the second trimester of human cardiac valve development, which is in accordance to studies previously performed in other vertebrates, which postulate that high VEGF expression is necessary to terminate EndMT (Dor et al., 2001). Moreover, our findings fit within the timeframe of EndMT reduction and termination. VEGF protein expression was evident at the endocardial
cushions at all points of development; however, we did not detect any statistically significant patterns of this expression (Fig. S3).

One of the most significant observations in this study is the contribution of CD44+ cells towards valve elongation and colonization (Halfon et al., 2011; Hanna et al., 2007). We identified CD44+ cells clustering at the annulus of the leaflet during leaflet elongation (Fig. 4F,G), where we had detected strong expression of inactive NFATc-1 (Fig. 2K-M). With ongoing elongation, the presence of CD44+ cells extended towards the middle of the leaflet. A number of hypotheses can be put forward towards the origin of this CD44 expression. CD44 is known to mediate cell motility by HA and epidermal growth factor receptor (EGFR) interaction (Kim et al., 2008). It has been proposed that the condensation of mesenchymal cells in mouse AV valves begins at E15.5, equivalent to week 12 of human development, and expands throughout the leaflet at E18.5, which is approximately equivalent to the third human trimester (Kruithof et al., 2007). Here, in human tissue, a mesenchymal condensation of CD44+ cells was first detected at week 11 (Fig. 4F), and subsequently was predominately present in the spongiosa and fibrosa layers. One could speculate that these CD44+ cells originate from the previously NFATc-1 expressing cells at the same leaflet/annulus junction that have achieved a more mature mesenchymal cell phenotype, which then migrate towards the valve tip in a temporal spatial manner as the leaflet elongates via biochemical and biomechanical cues. Indeed, it is also possible that resident VICs, already present in the developing leaflet, begin to express CD44 in response to biophysical stimuli. We have already demonstrated that the VECs of the fibrosa and ventricularis display very different behaviors with regard to cell morphology and proliferation. The same could possibly be true for VICs neighboring these distinct locations. Previously, it has been shown in vitro that porcine fibrosal VICs exhibit much lower expression of alpha smooth muscle actin (α-SMA) when compared to ventricular VICs that were exposed to the same conditions of cyclic strain (Moraes et al., 2013). A third hypothesis could be put forward that the biomechanical forces in the developing heart elicit biochemical cues from the layer-specific VICs, which facilitate the migration of CD44-expressing cells. Post-EndMT, we identified high expression levels of periostin within the developing human valves. In postnatal valves, expression of periostin is reported to be decreased and most present at the ventricular subendothelium (Hakuno et al., 2010). Our investigation of late first trimester and second trimester human tissues does not concur with this postnatal pattern. Here, we detected from 11 weeks of development, and persisting in the second trimester at 17 weeks, high and local expression of periostin at the fibrosal layer of the leaflet. The spatial pattern of this periostin expression (Fig. 5)
Interestingly precedes the appearance of CD44+ cells (Fig. 4). This suggests that fibrillar ECM deposition begins with the occurrence of CD44+ cells at the annulus progressing towards the leaflet tip along the fibrosal leaflet side via a possible cue of periostin-binding or perhaps secretion of periostin by resident fibrosal VICs, due to other biophysical and biochemical cues. It could be, in agreement with previous reports, that this periostin expression stimulates hyaluronan expression (Ghatak et al., 2014), which could facilitate the migration of CD44+ cells. Based on published reports of postnatal tissues, it is possible that this periostin expression will extend and persist at the ventricularis (Hakuno et al., 2010).

As cell density significantly decreases during valve development, leaflet growth is primarily due to ECM synthesis and deposition (Hinton et al., 2006); however, utilizing Ki-67 staining we showed that cellular proliferation also contributes to the elongation of the leaflets, although this is significantly reduced in the second trimester. Therefore, it seems that all processes combined, the migration of VECs into the mesenchyme due to EndMT, the proliferation of VICs within the fetal leaflets, and CD44+ cells at the leaflet/annulus junction and towards the elongating leaflet tip, contribute towards leaflet development. This CD44+ population is therefore crucial for elongation and maturation towards a trilaminar semilunar leaflet in humans.

Conclusion

Taken together, our study provides unique insights into human semilunar valve development. Similar to previous studies (Chang et al., 2004) in human valvulogenesis, early cardiac cushion invasion by VECs occurs through EndMT and is dictated partially by NFATc-1-mediated endocardial and endothelial cell maintenance during valve elongation. Our findings acknowledge the involvement of NFATc-1 in early human semilunar valvulogenesis with regard to cushion formation and elongation, and a contribution of VECs towards colonization of the mesenchyme in the first trimester. Leaflet elongation in the second trimester is supported by mesenchymal proliferation and the presence of a newly identified CD44+ cell subpopulation. All these processes contribute towards normal leaflet maturation and stratification.

Due to the fact that we utilized non-diseased human tissues in this study, we were limited to descriptive analyses that do not provide functional insights. However, this advanced knowledge of early stage human semilunar valvulogenesis will impact research efforts aiming to elucidate mechanisms of congenital valve disease and bring significant insight to studies...
performed in other vertebrates and in vitro models of human development. The rapidly progressing field of tissue engineering and regenerative medicine can harness this information to create relevant disease models, identify potent beneficial pharmacological interventions and possibly create tissue-engineered constructs. Particularly for pediatric valve surgery, the in vitro recapitulation of developmental processes will contribute towards the future generation of functional tissue-engineered heart valves, which ideally possess the ability to grow and remodel in vivo.

**Materials and methods**

**Tissue procurement and processing**

This study was performed in accordance with institutional guidelines and was approved by the local research ethics committees (UCLA IRB #05-10-093; University Tübingen IRB #356/2008BO2 and #406/2011BO2). Human first trimester (n=8; 4-12 weeks of gestation) and second trimester (n=7; 13-18 weeks of gestation) hearts were obtained from electively aborted fetuses following informed consent and de-identification. After procurement, all tissues were immediately washed in sterile Dulbecco’s phosphate buffered saline. Tissues were then fixed in either 10% phosphate-buffered formalin and embedded in paraffin or directly used for RNA extraction.

**Gene Expression Analyses**

Laser capture microdissection was employed to isolate pure populations of valve leaflet cells and total RNA was extracted using a special isolation kit for formalin fixed paraffin embedded samples, as previously described in detail (Votteler et al., 2013b). Microarray data previously generated by our laboratory (Votteler et al., 2013a) was evaluated at the level of gene sets to define and quantitate trends in gene expression. Ranked gene lists were created and submitted to the online public repository provided by the BROAD Institute for GSEA (Mootha et al., 2003; Subramanian et al., 2005).

qPCR was performed using the QuantiFast Probe one-step assay from Qiagen (Hs_NFATc1_1_FAM QuantiFast Probe Assay, Hs_CD44_1_FAM QuantiFast Probe Assay). We employed 10 ng of total RNA using the manufacturer’s recommended cycling conditions (95°C for 3 minutes followed by 45 cycles at 95°C for 3 seconds, 60°C for 30 seconds).
Immunohistological analyses, semi-quantification and microscopic imaging

Tissue sections were deparaffinized and all slides were processed as previously described (Monaghan et al., 2014). The following antibodies were used for immunofluorescence staining: c-Myc (ab32072, 1:100, Abcam, Cambridge, UK), NFATc-1 (sc-7294, 1:1000, Santa Cruz, Heidelberg, Germany), PECAM-1 (CD31) (sc-71872; 1:1500, Santa Cruz), VEGF (RB-9031-P, 1:4000, Thermo Fisher Scientific Inc., Waltham, USA) and the Prestige® antibody CD44 (HPA005785; 1:3500, Sigma Aldrich, Munich, Germany). For NFATc-1, CD31, VEGF and CD44 detection, we performed amplified immunofluorescence staining using Tyramide Signal Amplification kits (T20911 and T20915) (Life Technologies, Darmstadt, Germany). After incubation with a primary antibody detection procedure, all slides were exposed to a DAPI solution for 10 minutes followed by mounting using ProLong Gold antifade mounting medium (Molecular Probes, Life Technologies). Fluorescence images were acquired using an Axio Observer Z1 (Carl Zeiss, Jena, Germany) or a LSM 710 confocal microscope (Carl Zeiss). Images were processed with Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, CA). Immunohistochemical staining of the proliferation marker Ki67 (antibody: MIB I, KI67-MM1-L-CE; 1:100, Leica Biosystems GmbH, Wetzlar, Germany) was kindly performed by the pathology laboratory of Prof. Dr. Burkhard (Reutlingen, Germany). The staining procedure was conducted automatically using the staining machine BOND-MAX according to the manufacturer’s suggested protocol (Leica, Biosystems GmbH). For semi-quantification of NFATc-1 protein expression levels, GVIs were measured by densitometry and analyzed using ImageJ software as described before (Schesny et al., 2014). All data are displayed as means ± standard deviations of results obtained from twenty cells, for each cell phenotype in each sample.

Assessment of cell density

Cell density was calculated as the mean number of cells from a minimum of 20 DAPI-stained heart valve cushion and leaflet sections using a high-power magnification (400x). The cell number was reported as cells per 0.01 mm² within the cushion and leaflet tissue section.

Analysis of statistical significance

Statistical significance was determined by one way ANOVA followed by Tukey’s multiple comparison tests and student’s t-test using GraphPad Prism 5 software (GraphPad Software,
Inc., La Jolla, CA, USA). $p$-values detected less than 0.05 were defined as statistically significant.
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Disclosures

No conflicts of interest have to be declared

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Author Contributions


Supplementary Material

Supplementary material is available online at:
References


Figures

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<th>A</th>
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**D**

**VICs**

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<td>30 ± 10</td>
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* indicates a significant difference.

**E**

**VECs**

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<td>15 ± 5</td>
<td>10 ± 5</td>
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* indicates a significant difference.

**F**

**G**

**H**

- **fibrosa**: 7.3 ± 1.1 μm, 7.7 ± 0.9 μm
- **ventricularis**: 4.7 ± 0.4 μm, 12.6 ± 1.8 μm
Figure 1: Cell density and proliferation significantly decrease from the first to the second trimester of leaflet development and defined VEC morphologies are visible as early as 4 weeks. (A) DAPI staining of developing cardiac valves shows the different cell densities of the leaflets. Scale bars equal 200 µm. The red square indicates an area of 0.01 mm². * indicates statistical significance \( p < 0.001 \) (B, C) Proliferating Ki67+ VICs (brown) are randomly distributed throughout the fetal cushions and leaflets during developmental stages. (B) Fetal semilunar valve cushions at late 4 weeks, and (C) leaflets at 7 weeks of development. Red lines highlight the semilunar cushions. Scale bar equals 100 µm. (D, E) Proliferation rate of VICs and VECs on the ventricularis is significantly decreased beginning with 7-8 weeks of development when compared to late 4 weeks, *\( p < 0.05 \). (F, G) CD31+ VECs in developing valves (green) show typical morphological differences: VECs with a cuboidal morphology line the fibrosa layer, whereas VECs facing the ventricle appear elongated and flattened. Scale bars equal 100 µm. (H) Schematic of cell morphologies: VECs of the fibrosa exhibit significantly shorter lengths when compared to VECs of the ventricularis.
Figure 2: Overview of gene and protein expression of NFATc-1 reveals a spatio-temporal expression. (A) Gene expression of NFATc-1 in first and second trimester semilunar valve leaflets (*p<0.006, n = 4). (B) Schematic of spatial NFATc-1 expression during EndMT. Active NFATc-1 (red), maintaining the endothelium phenotype, is located in the cell nucleus. While inactive, NFATc-1 is expressed in the cytosol. (C-O) Immunofluorescence analyses of NFATc-1 protein expression patterns. NFATc-1 is depicted in red; DAPI in white. (C) The green arrow indicates cells appearing to undergo EndMT. (G) White arrow point to cells in the mesenchyme, where NFATc-1 expression intensities in the nucleus are significantly decreased compared to endocardial cells. (I-O) During a specific time window between weeks 4 to 8, NFATc-1 is also present in the cytosol (white arrows) of
cushion mesenchymal cells (J-L). (M-N) Between week 9 and 11, NFATc-1 is expressed in the nucleus (green arrows) of mesenchymal cells at the leaflet annulus (an). (O) In second trimester leaflets, nuclear NFATc-1 is predominately found in VECs. Scale bars equal 50 µm (C-H) and 200 µm (I-O). OFT − outflow tract; * = erythrocytes; f = fibrosa; v = ventricularis; CC = cardiac cushion
**Figure 3: Expression of VEGF targets in the first trimester is increased.** (A) Heat map representing the relative expression and fold enrichment of genes repressed by VEGF-A in the first trimester (n=4) and second trimester (n=3) in outflow tract leaflets (created using Molecular Signatures Database v5.0). (B) GSEA of enrichment of VEGF-A repression from leaflets obtained from the first and compared to the second trimester. The false discovery rate (FDR) q value and normalized enrichment score (NES) are shown. (C-L) Immunofluorescence imaging of NFATc-1 (red) and VEGF (green) protein expression patterns during early endocardial cushions and elongated leaflets. DAPI is shown in white. Scale bars equal 50 µm. (C, D) At the early cushion stage, fibrosa and ventricularis are not yet identifiable. Images E-H depict the view of the fibrosa layer, and I-L shows the ventricularis.
Figure 4: Overview of CD44 gene and protein expression reveals an increased gene expression from the first to the second trimester and CD44 positive cells in a spatiotemporal pattern of expression. (A) CD44 gene and (B-G) protein expression analyses reveals the presence of temporally and spatially distinct CD44+ cells (red) during human semilunar valvulogenesis. DAPI is shown in white. Scale bars equal 200 µm (B-E) and 400 µm (F-G). The green lines highlight the semilunar cushions. OFT - outflow tract; * = erythrocytes; f = fibrosa; v = ventricular side; CC = cardiac cushion; an = leaflet annulus.
Figure 5: Periostin expression accumulates strongly at the fibrosal layer of the developing leaflet at the second trimester. Temporal and spatial distribution of periostin (red) in human (A-E) first trimester and (F) second trimester semilunar leaflets. Nuclei are visualized with DAPI (white). Scale bars equal 200 µm (A-D) and 400 µm (E-F). The green lines highlight the semilunar cushions. OFT - outflow tract; * = erythrocytes; f = fibrosa; v = ventricular side; CC = cardiac cushion; MW = myocardial wall