Published in final edited form as:

Cytokine Growth Factor Rev. 2003 October ; 14(5): 391–407.

Transforming growth factor *beta* in cardiovascular development and function

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Abstract

Transforming growth factor betas (TGFβs) are pleiotropic cytokines involved in many biological processes. Genetic engineering and tissue explantation studies have revealed specific non-overlapping roles for TGFβ ligands and their signaling molecules in development and in normal function of the cardiovascular system in the adult. In the embryo, TGFβs appear to be involved in epithelial–mesenchymal transformations (EMT) during endocardial cushion formation, and in epicardial epithelial–mesenchymal transformations essential for coronary vasculature, ventricular myocardial development and compaction. In the adult, TGFβs are involved in cardiac hypertrophy, vascular remodeling and regulation of the renal renin–angiotensin system. The evidence for TGFβ activities during cardiovascular development and physiologic function will be given and areas which need further investigation will be discussed.

Keywords

TGFβ; Heart; Vasculature; Development; Physiology

1. Introduction

TGFβs are closely related members of a large family of structurally similar polypeptides referred to as the transforming growth factor *beta* (TGFβ) superfamily. Members of this superfamily include the activins, inhibins, bone morphogenetic proteins (BMPs), müllerian inhibiting substance, *Drosophila* decapentaplegic gene complex, and *Xenopus Vg-1* gene [1]. The TGFβs 1, 2 & 3 exhibit a variety of proliferative, inductive and regulatory functions [2] and exhibit both overlapping and distinct spatial and temporal patterns of expression throughout development and in the adult, with pronounced embryonic expression in areas undergoing morphogenesis [3,4]. Knockout (KO) mice for these ligands display dozens of non-overlapping phenotypes in most major organ systems [5,6], indicating a high degree of functional specificity.

There is considerable evidence that TGF β s can signal through multiple pathways (Fig. 1). The three TGF β cytokines are secreted as latent complexes that are activated by mechanisms only partially understood. Among the general mechanisms that can activate TGF β s are enzymatic mechanisms which include proteases such as plasmin, calpain and matrix metalloproteinases, conformation changing protein interactions with molecules such as thrombospondin and integrin $\alpha_v\beta_6$, physicochemical mechanisms such as pH, radiation and reactive oxygen species, and drugs such as antiestrogens, retinoids and glucocorticoids (reviewed in [7]). There are three classes of TGF β receptors, the transmembrane serine–threonine kinase receptors, TGF β RI and TGF β RII [1,8] and TGF β RIII receptors which include an ubiquitous extracellular β -glycan [9] and the membrane glycoprotein endoglin (CD105) [10,11]. Since TGF β 2 has a lower affinity to TGF β RII than does TGF β 1 or 3, its stronger interaction with β -glycan facilitates its interaction with the TGF β RII/I complex [9], although a shed form of β -glycan may be inhibitory [12]. In addition, a splice variant of the TGF β RII allows TGF β 2 ligand–receptor interaction in the absence of the TGF β RIII [13]. In contrast, endoglin interacts with TGF β 1 and TGF β 3, but has low affinity for TGF β 2 [10]. In several cell types endoglin is inhibitory to TGF β 1 function [14,15]. The ligand–receptor complexes signal through SMAD proteins that exhibit regulatory (SMAD2 and 3), co-regulatory (SMAD4) and inhibitory (SMAD6 and 7) activities on TGF β signaling [1,16].

As there is only partial ligand–receptor specificity, the functional specificity of TGF β s probably depends to a large degree upon extracellular ligand activation [7], differences in intracellular SMAD interactions [1,16], interactions with other signaling pathways such as the Ras/MAPK pathway [17], and interaction between SMADs and transcriptional co-activators and co-repressors [18]. TGF β signaling can also occur through SMAD-independent pathways (e.g. MAPK and Ca²⁺) [19] and through a non-transcriptional pathway (e.g. platelet aggregation) [20].

Many members of the TGF β superfamily that play important roles in the heart have been reviewed elsewhere [21–23]. This review will concentrate specifically on TGF β 1, TGF β 2 and TGF β 3 and their functions in the cardiovascular system where their importance has been evident for over a decade [24]. This review will subdivide the cardiovascular functions of the three TGF β s into two major categories: cardiovascular development and physiology. Because the TGF β literature is so vast, we will limit our review for the most part to in vivo studies carried out primarily in chicken and genetically engineered mice.

2. Cardiovascular development

The propagation of the symmetrical embryonic cardiac tube into an asymmetrical four-chambered heart with proper alignment of the atrio-ventricular (AV) segments and the great vessels with their respective chambers requires considerable morphogenetic and remodeling processes. Cardiac looping, formation of endocardial cushions, and their remodeling, in which myocardialization plays a primary role, are all required for proper inflow and outflow tract (OT) alignment, cardiac septation, and valve formation. In addition, remodeling of the pharyngeal arch artery (PAA) system leads to an asymmetrical left-sided vascular system. Defects in any of these morphogenetic and remodeling steps will lead to congenital cardiovascular defects commonly found in humans [25].

2.1. Cardiovascular Tgfb1–3 expression

Tgfb1 is expressed in the endocardium of the developing mouse heart. In vessels, *Tgfb1* is localized in the intima, whereas *Tgfb2* and *Tgfb3* are present in the media and adventitia of the great arteries (Molin et al, unpublished). Since only *Tgfb2*^{−/−} mice have obvious congenital cardiovascular defects, it is first important to review its expression in the developing heart. The most thorough study on *Tgfb2* expression during early mouse heart

development has been carried out by Dickson et al. [26]. *Tgfb2* message is found as early as embryonic day (E) 7.25 in the cardiogenic plate of the precardiac mesoderm, and it later becomes prominent in the myocardium of the aortic sac and OT regions. TGF β 2 protein is found in the entire myocardium of the heart at the time when looping occurs (E8.0–8.5).

From E8.5–9.5, when the morphogenetic process of cushion formation occurs, strong *Tgfb2* expression becomes localized to the myocardium underlying the acellular cardiac jelly of the endocardial cushions of the OT region and AV canal where it remains strong during the epithelial-to-mesenchymal cell transformation (EMT) of endocardium to cushion mesenchyme and during invasion of the transforming cells into the cardiac jelly (Fig. 2A, B, D and E). At E9.5 the looped primitive heart consists of myocardial cells and endocardial cells facing the inner lumen of the heart (Figs. 2A and D and 4A). The endocardial cushions covering the AV canal (boxed section, Fig. 2A) and OT myocardium (boxed section, Fig. 2D) are free of mesenchymal cells at this stage. *Tgfb2* is highly expressed in the myocardium of the AV canal (arrowheads, Fig. 2B and E; AVC, Fig. 2A and D) and in the OT (arrows, Fig. 2B and E; OT, Fig. 2A and D). There is no significant expression of *Tgfb3* (Fig. 2C and F) with the exception of some scattered cells in the myocardium of the OT (Fig. 2C).

After cushion formation and EMT and before myocardialization of the endocardial cushions begins (E10.5–11.5), *Tgfb2* expression is strong in the OT myocardium and in the adjacent developing cushion mesenchyme. However, as myocardialization proceeds *Tgfb2* expression is reduced in the myocardium so that from E12.5 onward it is predominantly expressed only in the mesenchyme of the cushion and OT septum, as well as in the prospective smooth muscle cells (SMC) of the great arteries [26].

During myocardialization, *Tgfb2* expression remains high in the cushion mesenchyme of the OT septum (Fig. 2G and H). At the aortic valve and condensed mesenchyme levels of the OT septum the expression of *Tgfb2* (Fig. 2H) and *Tgfb3* (Fig. 2I) partly overlap, though *Tgfb2* is more highly expressed in the area of myocardialization (arrowhead, Fig. 2H) and in the muscular ring surrounding the aortic valve (Fig. 2H). *Tgfb2* is also highly expressed in the epicardium lining the atrium (arrows, Fig. 2G and H). Both *Tgfb2* and *Tgfb3* are only weakly expressed in the distal pulmonary valve (Fig. 2K and L). Relative to the aortic valve, the muscular ring surrounding the pulmonary valve, as well as the media of the ascending aorta and pulmonary trunk, show higher *Tgfb2* than *Tgfb3* expression (compare Fig. 2H and K to I and L). By E15.5 *Tgfb1* is now the most highly expressed isoform in the endocardial cells of the myocardium, and *Tgfb2* expression is low but predominantly myocardial in nature, whereas in the epicardium *Tgfb1* and *Tgfb3* expression is higher than that of *Tgfb2* (Fig. 2M–O).

In general, the *Tgfb*s are not expressed in an overlapping fashion with the exceptions that from E12.5 onwards *Tgfb2* and *Tgfb3* are both expressed in the developing cushion mesenchyme of AV and OT cushions (Molin et al, unpublished; Fig. 2G–L), and all three are expressed in the epicardium (Fig. 2M–O). Interestingly, *Tgfb2*, and not *Tgfb3*, is expressed in the myocardium adjacent to the AV cushions and in the septal aspect which becomes myocardialized, suggesting that despite an overlap in expression they probably play non-redundant roles [27]. These observations are consistent with the fact that *Tgfb2*^{−/−} mice have abnormal pharyngeal arch artery remodeling and defective myocardialization of the OT septum [28].

2.2. Specification of early cardiac precursor cells

The formation of the early tubular heart from splanchnic mesoderm along the anterior–posterior (AP) axis of the heart fields requires cross-talk between mesoderm and underlying

endoderm. This cellular and molecular induction in the primary heart forming regions is important for the specification and differentiation of myocardial and endocardial precursor cells (reviewed in [29]). Many endoderm-derived growth factors such as BMP2, VEGF-2, FGF2 and TGF β s have been implicated in this process in the avian system [30]. TGF β 2 and TGF β receptors are expressed in the precardiac mesoderm along with BMP2, and their receptors are also localized in the endoderm adjacent to the precardiac region [26,29,31]. Consequently, members of the TGF β family can serve as putative molecular inductive signals at the heart forming fields for the formation of myocardial as well as endocardial precursor cells.

Numerous TGF β family members, such as Activin, BMP, Nodal, Lefty and others, are essential for the establishment of embryonic asymmetry (reviewed in [22]). The establishment of this asymmetry is in turn critical for heart development [21]. Since this process has been covered elsewhere, this review will limit its focus to the functional activities of the three TGF β ligands and their signaling molecules in cardiovascular development and homeostasis.

2.3. Cardiac looping

Cardiac looping is one of the important early events of cardiac morphogenesis. It is a dynamic process and can be categorized into two phases [32]. The first phase involves rightward bending of the linear heart tube at the interface between the primitive right and left ventricles into a S-shaped heart (E8.0–8.5 in mouse). In the second phase, the inner curvature tightens and the OT moves over the atrio-ventricular canal. Simultaneously, septal connections are made between the primary ring or fold of the ventricle and the atrial septation, resulting in a four-chambered heart. Although *Tgfb2* expression data would be consistent with a function in cardiac looping, analysis of heart looping in *Tgfb2*^{-/-} mice shows no gross morphological abnormalities in the initial bending of the linear heart tubes. Similarly, *Tgfb3*^{-/-} mice exhibit normal bending of early developing hearts (M. Azhar and T. Doetschman, data not shown). Therefore, the cardiovascular malformations in *Tgfb2* null mice might arise from defects in the second phase of cardiac looping which involves septation and remodeling of the developing heart [33].

2.4. Endocardial epithelial–mesenchymal transformation

As the ventricular-infundibular fold deepens during looping, the endocardial cushions begin to form at the future AV and OT regions of the heart. This involves an AV- and OT-myocyte-induced EMT of endocardial cells that break away from the endocardium, migrate into the cardiac jelly, and transform into cushion mesenchyme cells [34] (Fig. 3A). These cushions expand and fuse to eventually form aspects of the atrial, ventricular, AV and OT septa and valves. From endocardial cushion explant studies [35] there is considerable evidence that this transformation involves signals from the adjacent myocardium [36], the extracellular matrix (ECM) of the cardiac jelly [37], and TGF β s [38]. In chicken, TGF β 2 and TGF β 3 are thought to decrease cell adhesion of the transforming endocardial cells [39] and to enhance their cell migration into the cardiac jelly [40,41], though in mouse TGF β 2 is thought to carry out both of these functions [27,39]. Also, in chicken the β -glycan TGF β RIII, has been localized to the endocardium overlying both the AV and OT cushions [41]. In addition, β -glycan, but not TGF β RII, has been shown to mediate endothelial activation by triggering distinct cellular and molecular responses [42]. Together, these data imply a prominent role for TGF β 2 in the EMT of mammals.

Expression studies have shown that *Tgfb2* is localized to the cushion myocardium as well as to the invading mesenchymal cells during mouse EMT. These data also indicate that *Tgfb3* is not expressed in early cardiac endocardial cushions of the mouse (Fig. 2C and F) but only

in the established mesenchymal cells at later stages [27]. TGF β 2, and not TGF β 3, has been found to induce mouse EMT in collagen gel explant cultures [27]. The role of TGF β 1, if any, is not clear, although it is expressed in the endocardial cells of the AV canal during mouse EMT [43], where TGF β 3 is found to be expressed in avian models [39]. No cardiac developmental defects have been detected in TGF β 1-deficient mice [44,45].

In the mammalian system only *Tgfb2*^{-/-} mice have clear congenital heart defects [5]. Analysis of *Tgfb3*^{-/-} mice has revealed minor differences in position and curvature of the aortic arches and in ventricular wall thickness, though the significance of these differences from wildtype animals is not clear since they do not affect survival (compare Fig. 4G with I, and Fig. 4A and B with E and F). Interestingly, several *Tgfb2*^{-/-} fetuses exhibit early post-transformation embryonic lethality between E9.5 and E13.5, suggesting that heart malformations due to defects in EMT may occur. Also, *Tgfb2 Tgfb3* double knockout (DKO) mice do not survive beyond embryonic stage E15.0, suggesting either a redundant or an additional role for TGF β 3 in EMT or post-transformation expansion of cushion mesenchyme during valvular development (M. Azhar and T. Doetschman, unpublished data). This is consistent with the overlap in expression of *Tgfb2* and *Tgfb3* in the OT cushion mesenchyme of E13.5 mice (Fig. 2H and I; Molin et al. unpublished data). The combined functions of the three TGF β s in cardiovascular development are yet to be determined.

Gene expression profiling of TGF β -induced EMT in vitro in human keratinocytes [46] and in chick hearts [42] have revealed several common candidate genes involved in TGF β -mediated EMT. Among these are BMPs and their cognate receptors which are known to be expressed in the myocardium adjacent to the developing cushions and to regulate EMT and post-transformation cushion remodeling (reviewed in [23]). BMP2 and TGF β 3 are thought to act synergistically in avian cushion development [47]. In the mammalian system, *Bmp6 Bmp7* DKO mice display abnormal cushion development [48]. Recently Schneider and associates [49] have demonstrated that conditional ablation (CKO) of *Tgfb1a* (gene for BMP receptor ALK3) in the myocardium can result in failure of AV cushion formation, and abnormal trabeculae and ventricular myocardial compaction. Notably, no defects were found in the OT of *Tgfb1a* CKO mice. A downregulation of TGF β 2 was found, suggesting that a severe reduction in TGF β 2 in the myocardium could contribute to the defects seen in *Tgfb1a* CKO hearts. It is noteworthy that *Tgfb2*^{-/-} mice have hyperplastic AV cushions along with major defects in the OT segment, as well as spongy or less compact ventricular myocardium (Fig. 3B). In contrast, despite reduced levels of TGF β 2, *Tgfb1a* CKO hearts show reduced AV cushions and no OT defects, indicating that BMP6 and BMP7 also function independently of and antagonistically to TGF β 2 in AV cushion formation. Knockout of the inhibitory *Smad6* gene results in hyperplastic cardiac valves [50], a phenotype similar to that of *Tgfb2*^{-/-} mice and opposite to that of the *Bmp6 Bmp7* DKO mice, suggesting that during AV cushion formation the inhibitory activity of SMAD6 affects BMP and not TGF β 2 signaling.

2.5. Epicardial epithelial–mesenchymal transformation

Most studies on epicardial EMT have been done in the chicken heart. Epicardium, a third cell layer which develops over the myocardium and covers the entire mouse heart by E11–12, originates from the mesothelial cells which in turn are derived from cells migrating from the proepicardial organ (PEO) at E9.0–9.5 [51]. During heart development the epicardium transforms to mesenchyme giving rise to coronary pericytes and vascular smooth muscle cells (VSMC), invasive mesenchymal cells of the AV cushions and valves, and intermyocardial fibroblasts [52,53]. Using tissue explants it has recently been shown that treatment with FGF1, 2 and 7 promotes epicardial EMT, whereas treatment with TGF β 1, 2

and 3 inhibits epicardial EMT [54]. This is in contrast to the known functions of TGF β in avian endocardial EMT discussed above in which TGF β s play an inductive role. The expression of TGF β 2 in the PEO and the early migrating epicardial cell, and the expression of all three isoforms in later stages of the epicardium suggest a TGF β function in epicardial cardiogenesis (Fig. 2M–O; Molin et al., unpublished data).

It is possible that the cardiac phenotypes of *Tgfb2*^{-/-} mice might result, at least in part, from an epicardial EMT defect resulting in hyperplastic AV/OT cushions [33], segmentally thick epicardium (Azhar & Doetschman, unpublished data) and underdeveloped ventricular myocardium relative to lumen volume (Figs. 3B and 5). Defects at the level of the myocardium are evident in both *Tgfb2*^{-/-} and *Tgfb3*^{-/-} mice. Cross-sections of the right and left ventricle wall show a different wall thickness and compactness between wildtype (Fig. 4A and B), *Tgfb2*^{-/-} (Fig. 4C and D) and *Tgfb3*^{-/-} (Fig. 4E and F) mice, being reduced and spongier in the two knockouts. *Tgfb1*^{-/-} mice have no congenital heart defects related to epicardial EMT. However, as shown above we have preliminary indications that *Tgfb3*^{-/-} mice have minor differences in myocardial architecture (Fig. 4E and F). It remains to be determined whether multiple TGF β s synergistically affect epicardial EMT in vivo.

2.6. Myocardialization

Myocardialization is the process by which myocardial cells invade the endocardial cushions. This process is most prominent in the proximal part of the OT cushions (Fig. 3B). After fusion these cushions will form the myocardial infundibulum that lines the OT of the right ventricle. Myocardialization in the OT has been described in human embryos [55,56], chicken embryos [32,57–59] and mouse embryos [60]. The left ventral OT cushion merges with the superior AV cushion and the latter fuses with the inferior AV cushion. Also, during the formation of the atrial and ventricular septum in the AV cushion region myocardialization has been described. Notably, most cardiac malformations associated with defective myocardialization involve OT segments. Defective myocardialization can result in a thickening of the OT septum as is seen in some cases of tetralogy of Fallot, in OT obstruction in neonates with subpulmonary stenosis [55], and in diabetic rat embryos [61]. It is also possible that a lack of myocardialization could lead to a fibrotic and diminished OT septum. In neonates this leads to a double committed ventricular septal defect. It is also encountered in the trisomy 16 mouse model which lacks myocardialization of proximal cushions with the resultant failure to form a muscular OT septum [62]. A similar observation is seen in chicken embryos in which hemodynamics are altered by a venous clip procedure [63]. The cellular and molecular mechanisms underlying defective myocardialization remain to be solved, but it has been postulated that in chicken embryos TGF β 2 plays a role [58,59].

The hearts of E18.5 *Tgfb2* null fetuses have, with varying penetrance, double outlet right ventricle, double inlet left ventricle, atrial and ventral septal defects, hyperplastic inlet valves, and occasional thickened semilunar valves and persistent truncus arteriosus [5,33]. The most notable defect in the embryonic hearts of these mice is a lack of myocardialization of the OT cushions and in the portion of the AV cushions that are involved in septation (Fig. 3B). The intracardiac malformations named above result from deficient secondary looping of the heart. Whether TGF β s play a primary role in this process is unknown. It is reasonable to assume that the process of myocardialization would involve decreased myocardial cell adhesion, a more receptive (differentiated) cushion mesenchyme, or a myocardium more responsive to signals from the adjacent cushion mesenchyme. It has been noted that cushion mesenchyme close to the myocardium is more condensed and differentiated, whereas the cushion mesenchyme more luminal to the myocardium is less condensed and more highly proliferating. It is hypothesized that this gradient of proliferation to differentiation could

regulate cushion mesenchyme differentiation and induce myocardialization (reviewed in [34]).

ECM is a major component of myocardial and cushion tissues, and TGFβs are known to strongly affect ECM composition. Consequently, it is likely that ECM components such as collagens, chondroitin sulfate proteoglycan (CSPG2) and fibronectin are involved in the process of myocardialization, and they are also expressed in the cushion mesenchyme [34]. Since fibronectin can either inhibit or enhance invasion of a tissue depending on the cellularity of the invaded tissue [64], it is likely to significantly affect myocardialization because of the cell density gradient of the cushion mesenchyme cells. In addition, fibulin-2 is associated with the early, more proliferative stages of endocardial cushion development [65], whereas fibrillin-2 [34] is more highly expressed in the differentiated mesenchyme. Finally, for myocardial cells to leave myocardium they must presumably alter their differentiation state in order to weaken adhesive interactions with other myocardial cells before invading the cushion mesenchyme. Determination of alterations in these ECM components in *Tgfb2*^{-/-} mice should shed light on the mechanisms by which TGFβ2 facilitates the process of myocardialization.

2.7. Roles of neural crest cells in myocardialization

Neural crest cells (NCC) migrate into the OT cushions in the chicken embryo prior to myocardialization and cushion fusion, and are thought to be involved in the differentiation of cushion mesenchyme and myocardialization of the developing OT septum [62]. Experiments showing that quail NCC migrate into the developing chick cardiovascular system [66] and that NCC ablation leads to outlet and inlet defects [67] demonstrated that NCC migration into the aorti-copulmonary region is required for proper alignment of the OT and ventricles (reviewed in [68]). Since Pexieder's discovery that cushion mesenchymal cells of the proximal OT undergo apoptosis [69], several lines of evidence have suggested that NCC and/or their apoptosis could play a critical role in cardiovascular remodeling (reviewed in [60]). At the site of myocardialization the NCC population goes into apoptosis [59].

TGFβs are thought to be important for NCC-mediated morphogenetic processes because NCC produce TGFβ2 [70,71]. *Tgfb2* is highly expressed in the OT cushions, especially in the NCC-populated condensed mesenchyme of the OT [72] (Fig. 2G and H, and J and K). At the period of myocardialization the expression of *Tgfb2* and *Tgfb3* is restricted to the mesenchyme. The underlying myocardium, which at previous stages was the main source of their expression, ceases to express them (Fig. 2G–L). Recent unpublished data (Molin & Gittenberger-de Groot) reveal high expression levels only of *Tgfb2* and only in the septal portion that becomes myocardialized. In *Tgfb2*^{-/-} mice defective myocardialization was found to be associated with a 1-day delay in the peak of apoptosis and an additional delay in its regression. There was no subsequent decrease in the volume of total endocardial cushion, and a fibrous OT septum developed [33]. From recent data it has become clear that NCC also populate the OT septum in both *Tgfb2*^{+/+} and *Tgfb2*^{-/-} mouse embryos (Fig. 3B; Gittenberger-de Groot, unpublished observations; [72]). Hence, TGFβ2 is not required for NCC migration to the OT region, but may function in regulating NCC apoptosis. One possibility is that TGFβ2 is activated by proteolysis due to NCC apoptosis [59]. Since TGFβs are cytokines known for their involvement in apoptosis and chemotactic signaling (reviewed in [6]), it is possible that a positive feedback loop of TGFβ2 activation and apoptosis, coupled with chemotaxis, leads to myocardial cell invasion and the replacement of dying cushion mesenchyme cells.

The potential roles of NCC in pharyngeal arch remodeling will be discussed at the end of the Section 2.8.

2.8. Pharyngeal arch artery remodeling

The early embryonic mammalian pharyngeal arch artery system consists of five paired arch arteries, numbered I–VI in order of appearance from rostral to caudal, the fifth artery normally considered to be absent [73]. The remodeling of the arch artery system into the adult left-sided vascular system requires the regression of the right dorsal aorta (α -segment), the right VIth arch artery and both carotid ducts (γ -segments). When remodeling of the arch system is complete (E15.5), defects at the aortic arch level are evident in both *Tg fb2*^{-/-} and *Tg fb3*^{-/-} mice. The normally curved (dotted line) left-sided AoA in the wildtype (Fig. 4G) is regression in the *Tg fb2*^{-/-} mouse (Fig. 4H), and *Tg fb3*^{-/-} mice have minor defects in position and curvature of the aortic arches (Fig. 4I), though these do not affect embryo survival [74,75]. Molin et al. [28] have found that in normal regression, the highest degree of apoptosis shifts from the mesenchyme at the onset of remodeling towards the media and SMC at the latest stages of regression. A parallel between lumen diameter, wall volume reduction and apoptosis was found. The presence of a relatively high incidence of apoptosis at the earliest point when left/right differences become apparent underlines the relation between the early onset of remodeling and apoptosis. This increase in apoptosis might imply a relation between a reduction in lumen diameter and associated flow [76,77].

Tg fb2^{-/-} embryos exhibit decreased vascular regression in the α -segment of the right dorsal aorta [28]. This malformation is characterized by lack of a reduction in vessel lumen diameter, wall thickness and apoptosis, leading to persistence of the right dorsal aorta α -segment. On the other hand, the normally non-regressing segments of the IVth arch artery regress in *Tg fb2*^{-/-} embryos and exhibit vascular hypoplasia and interruption of the IV arch artery. Occasionally, an aberrant right subclavian artery is found due to combined interruption of the right IVth arch artery segment and concomitant persistence of the right dorsal aorta α -segment. Consequently, TGF β 2 appears to regulate apoptosis, both positively and negatively, depending upon the arch segment, during normal pharyngeal arch artery remodeling.

There is considerable in vitro evidence for TGF β effects on VSMC differentiation [78,79]. With respect to pharyngeal arch artery remodeling a difference in cellular origin of the VSMC population between segments could account for the remodeling differences which underlie formation of the asymmetric vascular system. SMC are known to originate either from ectodermally-derived neural crest or from local mesenchyme [80]. If SMC originate from ectodermally-derived cardiac neural crest, their in vitro growth response is enhanced by TGF β . However, if they are derived from local mesenchyme, TGF β inhibits their in vitro growth response [78]. Consequently, it is possible that varying compositions of SMC with different growth and apoptosis responses to TGF β 2 may play an important role in determining the persistence or regression of different arch arteries.

2.9. Calcium signaling in heart development

Morphometric analysis of the developing total ventricular myocardial volume and total ventricular luminal volume show higher total ventricular luminal volume in *Tg fb2*^{-/-} hearts (Fig. 5), such that the ventricular myocardial architecture appears to be more spongy and dilated and less compact (also see Fig. 3B) [5,33]. FKBP12 is postulated to interact with TGF β R1 [1] as well as with cardiac ryanodine and inositol 1,4,5-triphosphate receptors which regulate intracellular Ca²⁺ levels [81]. FKBP12 deficiency in mice leads to high intracellular Ca²⁺ and non-compaction of ventricular myocardium, cardiac dysfunction and dilated cardiomyopathy [82]. Intracellular Ca²⁺ is involved in controlling gene expression by activating the nuclear factor of activated T cells (NF-AT) transcription factors through dephosphorylation by calcineurin. Since both *Fkbp12*^{-/-} and *Tg fb2*^{-/-} mice have non-compaction of ventricular myocardium, and *Nfat*^{-/-} mice have reduced cardiac cushions

[83,84], it is reasonable to postulate that a TGF β 2–FKBP12–calcineurin pathway is involved in cardiovascular development. In this regard, it is of interest that a defect in such a pathway may be the cause of autoimmune disease in *Tgfb1*^{-/-} mice [85].

2.10. Congenital heart diseases associated with chromosome 22q11

Genomic disorders involving amplification (tetrasomy & trisomy) and hemizygous deletion of regions/genes present on human chromosome 22 (22q11) are often associated with human cat-eye syndrome (CES), deletion (22) syndrome and velocardiofacial/DiGeorge syndrome (VCFS)/DGS, respectively. Of these, VCFS/DGS is the most frequently found congenital disorder (1/4000 live births; reviewed in [25]). Genetically engineered mice with alterations in genes or chromosomal regions syntenic to human 22q11 (mouse 16) show promise for unraveling the complexity of these congenital disorders. Some of the features typical of disorders associated with human 22q11 or mouse 16 include abnormalities of cardiac outflow and pharyngeal arch artery system, craniofacial defects, hypoplasia of thymus and parathyroid, anomalies of the eye and inner ear, and cleft palate (reviewed in [86]). Genetic pathways involving proper NCC development and function are considered to be the major pathways leading to DGS [87,88], and trisomy 16 in mice is involved in NCC-mediated abnormalities in myocardialization of the OT septum [62]. *Tgfb2*^{-/-} mice have multiple cardiovascular defects associated with NCC [28,33] along with other multiple congenital malformations often found associated with 22q11 disorders such as cranial defects leading to cleft palate [5]. Hence, since the human and mouse genes for TGF β 2 are not linked to human 22q11 or mouse 16, it is reasonable to hypothesize that TGF β 2 modifies, or is modified by, genetic pathways on those chromosomes.

3. Cardiovascular physiology

Cell culture studies have indicated that TGF β 1 inhibits mitotic growth of cardiomyocytes [89], and stimulates hypertrophic growth [90], fibrosis [90,91] and re-expression of the fetal isoforms of myofibrillar protein genes [92]. TGF β 1 is secreted from cultured cardiomyocytes and fibroblasts during cyclic stretch [93]. In the vascular system TGF β 1 switches the response of angiotensin II (Ang II)-stimulated cultured VSMC from mitotic to hypertrophic growth [94], TGF β 1 treatment affects Ca²⁺ mobilization in VSMC from spontaneously hypertensive rats [95], vascular remodeling of coronary and aortic arteries in response to hypertension is associated with increases in TGF β 1 and TGF β 3 levels [96], and intimal thickening following shear stress is associated with increases in TGF β 1 [97]. The renin–angiotensin system plays an important role in cardiovascular homeostasis through regulation of blood pressure and extracellular fluid volume [98]. Physiological stress from salt and water imbalance can lead to hypertrophy of the juxtaglomerular apparatus and induce both TGF β 2 and renin [99], suggesting that TGF β 2 may regulate pathophysiological renal responses involving blood pressure control. Altogether, these data suggest important roles for TGF β s in several aspects of cardiovascular physiology ranging from the cardiomyocyte to vascular smooth muscle and renal control of blood pressure.

3.1. Blood pressure and cardiac contractility

Blood pressure regulation is a multiorgan-regulated process involving many signaling pathways. TGF β 1 has been implicated in several of the chronic pathologies developed from poorly controlled blood pressure, including left ventricular cardiac hypertrophy, vascular remodeling, and renal disease. There is clinical and genetic evidence demonstrating a correlation between TGF β 1 polymorphisms and hypertension [100]. One clinical study revealed that TGF β 1 mRNA and protein levels are significantly higher in hypertensive patients compared with normotensives [101]. This overexpression of TGF β 1 is more frequent in hypertensive African-Americans compared with hypertensive Caucasians and

correlates with increased frequency of the proline allele at codon 10, a signal peptide region of the *TGFβ1* gene. *Tgfb1* mRNA is elevated in aortae of hypertensive rats [102], and recent evidence demonstrates that mice deficient in SMAD6, an inhibitory protein in TGFβ signaling, has a significant elevation in mean arterial blood pressure compared to wildtype mice [50]. However, mice deficient in TGFβ1 display no difference in mean arterial blood pressure [103], and no increase in blood pressure was reported in transgenic mice with cardiac overexpression of *Tgfb1* [104]. Nonetheless, diastolic dysfunction has been found in work-performing heart preparations [105] from *Tgfb1*^{-/-} mice (Fig. 6A). These results are consistent with a TGFβ1 deficiency leading to impairment of inotropic responsiveness to β-adrenergic stimulation, but do not demonstrate causality.

The adrenergic receptor system is critical to the regulation of blood pressure and cardiac contractility/relaxation. There is evidence that the β-adrenergic system regulates *Tgfb1* and *Tgfb3* expression in cultured cardiac cells [106,107]. Similarly, in vitro studies have demonstrated that TGFβ1 can modulate α- and β-adrenergic signaling in various cell types by altering adrenergic receptor density and function [108,109]. Recent *Tgfb1* transgenic and KO mouse studies have supported these in vitro observations. In work-performing heart preparations from *Tgfb1*^{-/-} animals there is a decrease in both contraction and relaxation rates in response to the β-adrenergic agonist, isoproterenol (Fig. 6B and C). This correlates with a reduction in β-adrenergic receptor density (not shown). In addition, consistent with the diastolic dysfunction results mentioned above, only the relaxation rate is depressed in unstimulated hearts (Fig. 6C). These alterations in β-adrenergic signaling result in an enhancement of the inotropic responsiveness to β-receptor stimulation [104]. Consequently, TGFβ1 is required for full Ca²⁺ uptake in the unstimulated heart, and is required for full positive contractile and relaxation responses to β-adrenergic stimulation.

3.2. Cardiac hypertrophy

There is clinical evidence that patients with idiopathic hypertrophic obstructive cardiomyopathy have elevated cardiac *Tgfb1* mRNA and protein levels and increased TGFβ1 receptor levels on both cardiomyocytes and fibroblasts [110]. In addition, human atrial tissue stimulated with Ang II results in a significant increase in *Tgfb1* expression [111], which correlates with cardiac hypertrophy [112], fibrosis [113] and recapitulation of fetal isoforms of cardiac myofibrillar proteins [114]. However, in a survey for candidate genes related to increased susceptibility to hypertrophic cardiomyopathy, *Tgfb1* polymorphisms were not associated with the risk or severity of the disease [115]. From these data, it was unclear whether there is a causal relationship between increased levels of *Tgfb1* and hypertrophy, isoform switching and fibrosis.

The role of TGFβ1 in cardiac hypertrophy has been assessed by applying non-pressor doses of the hypertrophic stimulant Ang II to *Tgfb1*^{-/-} *Rag1*^{-/-} mice [103]. Ablation of the *Rag1* immunodeficiency gene is required [116] to circumvent the weaning-age lethality caused by a multifocal autoimmune disease [44,45]. Non-pressor doses were used to avoid compounding the Ang II stimulus with hemodynamic stimulation. Echocardiographic, gravimetric and morphometric analysis indicated that TGFβ1 is required for Ang II-induced cardiac hypertrophy since only the control (*Tgfb1*^{+/+} *Rag1*^{-/-}) mice developed hypertrophy. No difference was found in levels of fibrosis, mitotic growth or cytokine infiltration between wildtype and TGFβ1-deficient mice. TGFβ and fibrosis in the cardiovascular system will be discussed in a later section. Finally, recapitulation of the fetal myosin heavy chain (β-MHC) protein was not observed in Ang II-treated *Tgfb1*^{+/+} *Rag1*^{-/-} or *Tgfb1*^{-/-} *Rag1*^{-/-} mice, indicating that isoform switching is not obligatorily coupled with hypertrophy or TGFβ1.

Re-expression of fetal structural protein isoforms has often been used as an indicator of cardiac hypertrophy [117,118]. However, cardiac hypertrophy has been dissociated from the

re-expression of fetal isoforms under specific types of stimulation [119], suggesting that the isoform switch is dependent upon pressure overload [120,121] rather than on growth factors such as FGF2 and TGF β 1 [103,121].

3.3. Vascular smooth muscle and vessel remodeling

Several studies have implicated TGF β 1 in arterial remodeling following hypertrophic stress [94,96,122] or in hypertensive rats [95,123]. The vasoconstrictor thromboxane A₂ causes hypertrophy in cultured VSMC [124], and thromboxane A₂ and Ang II induce hypertrophy in cultured VSMC through the effects of the endogenously produced growth modifiers FGF2 and TGF β 1 [125]. However, overexpression of the human *FGF2*, especially the high molecular weight isoforms, in mouse aortic VSMC from transgenic mice enhances serum-stimulated mitotic growth [126]. Thus, it has been hypothesized that after stimulation with hypertrophic agents FGF2 functions to promote mitotic growth, while TGF β 1 prevents full cell cycle progression such that their combined activity would result in hypertrophy.

To test this hypothesis cultured *Tgfb1*^{+/+} and *Tgfb1*^{-/-} aortic VSMC were stimulated with the hypertrophic thromboxane mimetic U46619. Fig. 7 shows that in *Tgfb1*^{-/-} cells mitotic growth is more than doubled relative to vehicle alone, whereas the increase in protein synthesis over vehicle is independent of the presence or absence of TGF β 1. Addition of TGF β 1 to the U46619-treated *Tgfb1*^{-/-} cells drops mitotic growth to vehicle levels, but increases protein synthesis by nearly 50% (not shown). From this and previous studies we postulate that TGF β s may function as a general response to vasoconstricting hypertrophic agonists in several cardiovascular compartments.

Several studies have found an association of TGF β activity with SMC differentiation as it relates to intimal thickening following both vascular injury [127,128] and fluid shear stress [97], as well as in plaque development during atherogenesis in hypercholesterolemic animals [129]. These studies suggest important inhibitory roles for TGF β in all of these responses, and they are consistent with in vitro studies that suggest growth inhibitory and differentiation stimulatory roles for TGF β in VSMC [78,79,130]. One must caution, however, that an association only suggests causality. In addition, treatments that inhibit TGF β activity with antibodies or antisense RNA can result in inconsistencies, as is the case with intimal thickening following vascular injury (compare [131] (no effect) with [128] (intimal thickening)).

Since TGF β 1 is known to be a potent regulator of the immune system [85,132], immune sources of TGF β 1 may also affect the intimal thickening that occurs during cardiac transplant arteriosclerosis. Using a heterotopic mouse transplant model cardiac grafts from CBA mice were placed into *Tgfb1*^{+/+} and *Tgfb1*^{+/-} C57BL/6 recipients [133]. Arteriosclerosis was reduced in TGF β 1-deficient mice, indicating an important role for immune-cell-derived TGF β 1 in regulating intimal thickening.

From these studies it is clear that TGF β signaling plays significant roles in vascular remodeling, but the mechanisms are unclear. The application of vascular stress and injury models to genetically engineered mice will help delineate what aspect of each response requires TGF β signaling. The specific TGF β signaling mechanisms underlying these responses can then be determined.

3.4. Renal pathophysiology

When the renin-angiotensin system is stimulated by physiologic or pathologic stimuli, renin is released from intracellular vesicles of the modified SMC of the medial layer of the afferent portion of glomerular arterioles in the juxtaglomerular apparatus [134].

Hypertension [135], myocardial infarction [136] and diabetic nephropathy [137] can all result from a hyperactive renin–angiotensin system. Both co-localization and co-induction of renin and TGF β 2, as well as hypertrophy, have been observed in the juxtaglomerular apparatus after water deprivation, potassium depletion or inhibition of angiotensin I converting enzyme [99,138]. This suggests a potential role for TGF β 2 in the control of renin synthesis and/or release. However, the activities of TGF β 2 and perhaps other TGF β s would be complex, as low doses (10^{-12} M) of TGF β 1 and TGF β 2 stimulate renin secretion in rat renal cortical slices, whereas higher doses (10^{-10} M) inhibit renin levels [139].

The role of TGF β 2 in renal renin regulation under dehydration conditions has been investigated in *Tg fb2^{+/-}* mice [140]. Since *Tg fb2^{-/-}* mice die around the time of birth due to multiple developmental defects [5], the renal studies were done on *Tg fb2^{+/-}* mice whose TGF β 2 message and protein levels were approximately half those of wildtype animals. Dehydration-induced increase in plasma renin levels was not different between *Tg fb2^{+/+}* and *Tg fb2^{+/-}* mice. However, in the kidneys, in the presence of a full dose of TGF β 2, dehydration led to a large increase in renin mRNA and little increase in renin activity, whereas with a half dose of TGF β 2 there was a slight increase in renin mRNA and more than a doubling of renin activity (Fig. 8). Hence, TGF β 2 acts primarily at the transcriptional level in hydrated conditions and at the translational level in dehydrating conditions where it may act as a check on renin activity, thereby protecting the juxtaglomerular apparatus from potential pathophysiological effects of excessive renin. It is not known whether other TGF β s may have independent functions in the homeostasis and pathophysiology of the kidney. This is not unreasonable given the association of higher levels of circulating TGF β 1 in African-Americans with end-stage renal disease and a coding polymorphism in TGF β 1 that associates with hypertension in African-Americans [101].

3.5. Fibrosis

It has long been known that TGF β can induce fibrosis [141], (reviewed in [142]). With respect to cardiac fibrosis, however, it should be noted that in the autoimmune disease of *Tg fb1^{-/-}* mice there is extensive fibrosis in the heart and liver, though not in the lung, in the absence of TGF β 1 [44,143]. Furthermore, cardiac-restricted *Tg fb1* transgenic animals have atrial but not ventricular fibrosis [144]. Finally, alterations in levels of *Tg fb1* mRNA do not correlate with the increased mRNA expression of fibronectin and collagens I and III during low-dose isoprenaline treatment, again suggesting that cardiac fibrosis is not significantly mediated by TGF β 1 [145]. On the other hand, Langendorff preparations of hearts from 2-year-old *Tg fb1^{+/+}* and *Tg fb1^{+/-}* mice found greater diastolic dysfunction in the wildtype than in heterozygous mice [146]. Histological analysis of the hearts indicates that there is more fibrosis in *Tg fb1^{+/+}* hearts, suggesting that more fibrosis occurs in the presence of a full dose of TGF β 1, which in turn results in decreased left ventricle compliance. Consequently, it is the context of the heart and its environment which may determine whether and to what extent TGF β 1 induces cardiac fibrosis. Whether other TGF β s are involved has not been tested but can not be ruled out since decreased inhibitory SMAD7 in cardiac fibroblasts has been implicated in the pathogenesis of cardiac fibrosis in post-myocardial infarct rat hearts [147].

It is thought that the association between increased renin–angiotensin activity and renal fibrosis lies in the co-induction and co-localization of renin and TGF β 2 [148]. In experimental models, controlled infusion of TGF β 2 into adult mice leads to kidney fibrosis in the cortical tubular interstitium and vasculature, particularly at the cortical–medullary junction and medullary vasa recta, but not in glomeruli [149]. In a diabetic rat model TGF β 2 antibody treatment decreases glomerular and tubular fibrosis in streptozotocin-treated rats with diabetic nephropathy [150]. The extent to which Ang II-induced vasoconstriction and

tissue hypoxia and/or increased TGF β are responsible for the fibrosis is not clear, and a possible role for other TGF β s has not been ruled out. Since these results seem at odds with the renal renin study in *Tgfb2^{+/-}* mice in which less, rather than more, TGF β 2 leads to increased renin levels, we must consider the possibility that TGF β 2 affects the development of fibrosis by a renin-independent mechanism. More highly controlled dosing of TGF β 2 along with tissue and temporal specificity in its expression will be required to delineate the complex roles of TGF β in blood pressure homeostasis and renal pathophysiology.

4. Perspectives

Analysis of genetically engineered mouse models of TGF β deficiencies and overexpression have revealed numerous *in vivo* functions for TGF β s in cardiovascular development and physiology. Nonetheless, there remain many aspects of cardiovascular development for which the roles of TGF β have yet to be determined. It is not clear how TGF β s are involved in molecular specification and formation of early endocardial and myocardial precursors from the primary heart forming fields or in the recruitment of cells from the secondary heart-field to the outflow myocardium. And it is likely that TGF β s have complementary as well as redundant roles in endocardial and epicardial EMT and in NCC–SMC differentiation. Likewise, functional studies on TGF β activities in response to most forms of stress and injury to the heart, kidney and vasculature have hardly begun. Finally, since TGF β signaling can bring a multitude of pathways into play, it will be necessary to decipher what pathway networks are utilized to carry out these activities. From the practical standpoint, elucidating these cellular and molecular mechanisms will provide a greater understanding of cardiovascular diseases.

Acknowledgments

Grant support for the studies presented here: NIH grants HD26471, HL70174, HL58511, ES06096 to TD; INSERM and Association Claude Bernard to PM; VA Merit Review to GWD; NHS: ECCARD to ACG.

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Fig. 1.

TGFβ signaling can occur through multiple pathways. Many of these pathways are SMAD dependent, but there is evidence that pathways involving MAPK and FKBP12/Ca²⁺/calcineurin may be SMAD independent. The integrin pathway in platelets is independent of transcription.

Fig. 2.

Expression of *Tgfb2* and *Tgfb3* in wildtype embryonic hearts. In situ hybridization (B, C, E, F, H, I, K–O) and cardiac α -actin antibody HHF35 (A, D, G) or α -smooth muscle actin antibody 1A4 (J). A: primitive atrium; Ao: ascending aorta; AoS: aortic sac; AoV: aortic valve; AVC: AV cushion; CM: condensed mesenchyme; DAo: dorsal aorta; LA: left atrium; OT: outflow tract; PT: pulmonary trunk; PV: pulmonary valve; RV: right ventricle; V: primitive ventricle. Bars: A–L, 100 nm; M–O, 200 nm.

Fig. 3. Endocardial epithelial-mesenchymal transformation (A) to form endocardial cushions (E9.5) and myocardialization (B) to form muscularized OT/AV septum (E12.5-16.5). OTC: outflow tract cushion; AVC: atrio-ventricular cushion; IC: inner curvature; M: myocardium; IVS: interventricular septum, RV: right ventricle; LV: left ventricle.

Fig. 4. Histochemical analysis of E15.5 *Tgfb2* and *Tgfb3* KO hearts at the ventricular wall (G–I) and aortic arch artery (A–F). AoA: aortic arch; DAo: dorsal aorta; LV: left ventricle, Oe: oesophagus; RV: right ventricle. Bars: A–F, 100 nm; G–I, 200 nm.

Fig. 5. Morphometric analysis of ventricular myocardial and lumen volumes of E14.5 & E15.5 *Tgfb2*^{+/+} and *Tgfb2*^{-/-} embryos. Paired *t*-test comparison between *Tgfb2*^{+/+} and *Tgfb2*^{-/-} embryos at E14.5 and E15.5.

Fig. 6.

Diastolic and β -adrenergic deficiency in $Tgfb1^{-/-}$ hearts. Isolated, work-performing hearts from adult $Tgfb1^{-/-}$ *Scid* mice (A). β -Adrenergic-stimulated contractility (+dP/dt) and relaxation (-dP/dt) rates in LFA-1 antibody-treated adult $Tgfb1^{-/-}$ mice. Immunodeficiency (*Scid*) or immunosuppression (LFA-1 antibody treatment) is required to circumvent weaning age lethality (see Section 3.2 for explanation). IVP: interventricular pressure.

Fig. 7.

Thromboxane induces mitotic growth of *Tgfb1*^{-/-} VSMC. Quiescent VSMC derived from *Tgfb1*^{+/+} and *Tgfb1*^{-/-} mice were stimulated with vehicle or 1 μ M U44619. [³H]thymidine (A) and [³H]leucine incorporation (B) after 24 and 48 h exposure, respectively. *n* = 7, (*) *P* < 0.05 vs. *Tgfb1*^{+/+} mice).

Fig. 8.

Determination of renal renin mRNA and protein levels in the kidneys of hydrated or dehydrated *Tgfb2*^{+/+} and *Tgfb2*^{+/-} mice. The relative intensity of the renin signal was estimated as the sum of intensities for each glomerulus, divided by the number of glomeruli observed [140].