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PhD Thesis

INVOLVEMENT OF THE "TRACTION MODEL"
AS TGF-β1 ACTIVATION MECHANISM
IN THE DEVELOPMENT OF REACTIVE FIBROSIS
IN SPONTANEOUSLY HYPERTENSIVE RATS CARDIAC FIBROBLASTS

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Summary

Abstract

Cardiac reactive fibrosis is a well-known pathological effect derived from arterial hypertension. TGF-β plays a key role in the progression from the inflammation state to the fibrosis process establishment. Nonetheless, only the active form of TGF-β is effective, and this occurs if it is unbound to the latency complex proteins (i.e. Latency Associated Protein, LAP and Latent TGF-β Binding Protein, LTBP.

In vivo and *in vitro* models of different fibrosis-based pathologies (e.g. pulmonary and dermal fibrosis), have highlighted a novelty in the activation of TGF–β, which occurs not only by proteolysis on latent proteins, which involves enzymes as plasmin, thrombospondin and matrix metalloproteases, but also through a non-proteolytic mechanism, defined by Keski-Oja in 2004 "Traction model". This mechanism consists in specific binding of several integrins (αVβ3, αVβ6 and αVβ5), expressed by myofibroblasts, with LAP and LTBP; contraction forces exerted by myofibroblasts are able to active latent TGF-β.

On the basis of the “traction model” are an initial production of TGF-β, the conversion of fibroblast into myofibroblast, the production of α-SMA by myofibroblast and their subsequent contractile activity, the increase in ECM production and, therefore, further TGF-β production, in a positive feedback mechanism, able to amplify the fibrotic process progression.

Aims of this study are 1) the development of a more fast and efficient protocol to cardiac fibroblast extraction, 2) the evaluation of possible involvement of LTBP-1 and αVβ5 integrin in development of hypertensive-induced cardiac fibrosis and so 3)

the speculation about TGF-β1 mechanical activation by traction in primary cardiac fibroblasts isolated in hypertensive rats.

Aims of study

This study were aimed to:

- develop a standard protocol, more fast, simple and efficient then commonly used, for isolation of rat cardiac fibroblasts;
- investigate on cardiac tissue of two rats strains, normotensive Wistar Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR) the hypertension-induced fibrosis;
- deepen key mediators of TGF-β1 activation by non-proteolytic pathway or "traction model", such as Latent TGF-β Binding Protein -1 (LTBP-1), αvβ5 integrin and TGF-β1 itself, both on cardiac tissue and on isolated cardiac fibroblasts;
- hypothesize the involvement of "traction model" in hypertensive-induced cardiac fibrosis.

1. Introduction

1.1 Cardiac reactive fibrosis and hypertension

With terms “cardiac remodeling” or “ventricular remodeling” are described a series of alterations in size, shape and function of the left ventricle in response to changes in hemodynamic loading conditions, neuro-hormonal activation, or induction of local mediators that alter the structural features of the myocardium. Remodeling is a dynamic and complex process, resulting from activation of cellular and molecular pathways involving cardiomyocytes, fibroblasts and extracellular matrix (ECM). Cardiac remodeling can be physiologic (described in elite athletes) or pathologic. Pathologic remodeling occurs in three major patterns: a) concentric remodelling, when pressure overload causes growth in cardiomyocyte thickness and ECM proteins deposition, b) eccentric remodelling, resulting from a volume load that produces cardiomyocyte lengthening and c) post-infarction remodeling, which involves a combined pressure and volume load on the non-infarcted area [1]. One of the major consequence due to first mentioned remodeling type, i.e. concentric remodeling, is cardiac hypertrophy. Even if both ventricles may be involved in the development of cardiac hypertrophy, the most common feature is left ventricular hypertrophy (LVH), which includes arterial hypertension and heart valve stenosis among its causes **(Figure 1.1)**.

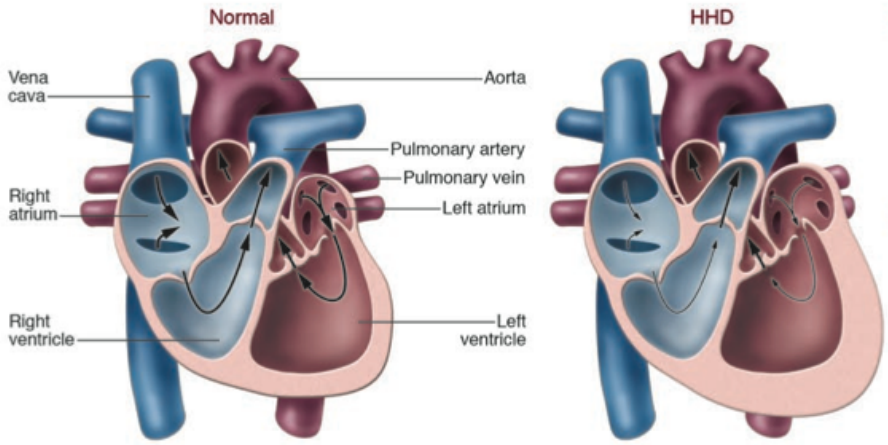

Figure 1.1 | Differences between normal heart tissue (left) **and hypertrophic heart disease (HHD) phenotypes** (right).

LVH is developed by 15% to 20% of hypertensive patients [2]. Different studies have shown that hemodynamic burden accounts for only 10% to 30% of the left ventricular mass variability in hypertensive individuals [3, 4]. Myocardial structure in hypertensive patients with LVH is affected by two key pathological processes: myocytes hypertrophy and a progressive accumulation of fibrous tissue within the cardiac interstitium [5].

Pressure overload, induced by arterial hypertension or aortic stenosis, is associated initially with increased stiffness and diastolic dysfunction, which frequently progresses to ventricular dilation and heart failure and results in extensive cardiac fibrosis [6, 7]. This type of fibrosis is named *reactive fibrosis*, to distinguish it from *reparative fibrosis*, which is a process of wound healing after myocardial damage due to cardiomyocyte necrosis [8, 9]. The main difference between reparative and reactive fibrosis is the different localization of the new collagen fibers: if in reparative fibrosis the deposition of new ECM take the place of necrotic cells (e.g. cardiomyocytes after infarction), on the contrary, in reactive fibrosis the fibrotic tissue is morphologically placed perivascularly and involves the intramural coronary

arterial vasculature. Thus, the latter form of fibrosis is an interstitial fibrosis **(Figure 1.2)**, accumulated in the perimysium [10].

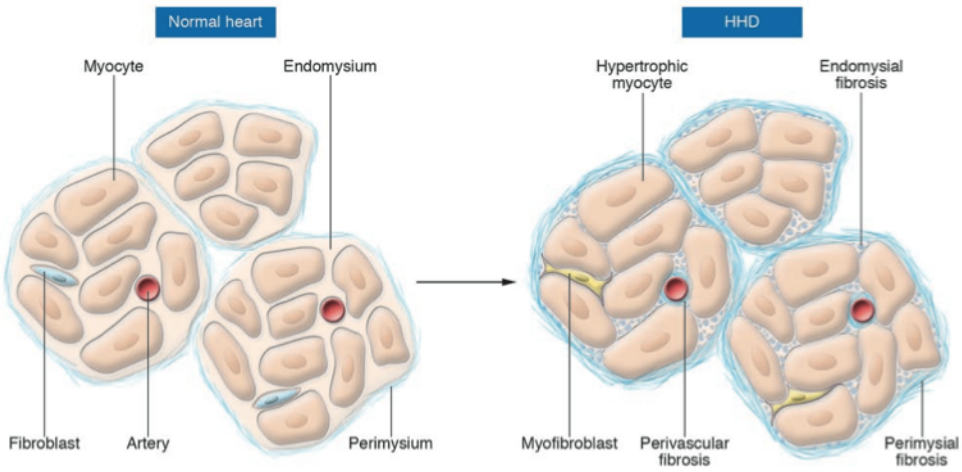

Figure 1.2 | Interstitial hypertrophy. HHD: hypertrophic heart disease. In blue are indicated fibrotic fibers.

Reactive fibrosis is a key pathological process in LVH [11]: both post-mortem and endomyocardial biopsy studies have shown that along with a variable increase in left ventricular mass, the collagen volume fraction of the myocardium is increased in hypertensive patients compared with their normotensive counterparts; moreover, histologic evidence of fibrosis, such as interstitial fibrosis has been found during the early phases of hypertension in patients with a mild degree of LVH [10, 12].

1.2 TGF-β

Recent studies have shown the important roles played by both molecular (i.e. chemokines, cytokines) and cellular (i.e. macrophages, fibroblasts, myofibroblasts) inflammation mediators in pro-fibrotic processes; although the initial trigger leading to fibrous tissue development is different between reparative and reactive fibrosis (i.e. necrosis vs. hemodynamic changes), in both cases the molecular events leads to an increased production of the pro-fibrotic transforming growth factor–β (TGF–β), which then acts locally, in an autocrine or paracrine fashion, to promote cardiac remodelling [1].

TGF-β is a pleiotropic cytokine, which is implicated in a wide variety of cell functions, critically regulating cell proliferation, differentiation and growth, inflammation and ECM deposition. Three structurally similar isoforms of TGF-β (TGF-β1, 2 and 3), encoded by three distinct genes, have been identified in mammalian species [13]. TGF-β1 is the prevalent isoform and is found almost ubiquitously, whereas the other isoforms are expressed in a more limited spectrum of cells and tissues.

TGF-β is produced by many cell types and is secreted as a latent complex, unable to associate with its receptors. Activation of TGF-β signaling pathways is primarily regulated by release of the active TGF-β from the latent complex, which is stored in significant amounts in most tissues. The latent dimeric complex contains the C-terminal mature TGF-β and its N-terminal pro-domain, the latency-associated peptide (LAP) [14], that prevents TGF-β from interacting with its receptors, functioning as an inhibitor due a non-covalent high affinity association with TGF-β. Release of bioactive TGF-β requires separation of LAP from TGF-β, a step that involves processing of the pro-TGF-β complex by several extracellular mediators,

such as plasminogen activator inhibitor-1 (PAI-1) [15], thrombospondin (TSP-1) [16] and matrix-metalloproteinase (MMP)-2 and MMP-9 [17] **(Figure 1.3)**. After its release, TGF-β binds to the constitutively active TGF-β type II receptor (TβRII) at the cell surface. The complex, subsequently, recruits and trans-phosphorylates the type I receptor of TGF-β (TβRI), also known as ALK5. Apart from the well-characterized ALK5, which is expressed by many different cell types, endothelial cells express a second TβRI, termed ALK1. Both TβRI types activation propagates downstream intracellular signals through the Smad proteins, but while Smad2 and Smad3 are activated through phosphorylation by ALK5, on the otherside, Smad1, Smad5 and Smad8 are activated by ALK1 [18].

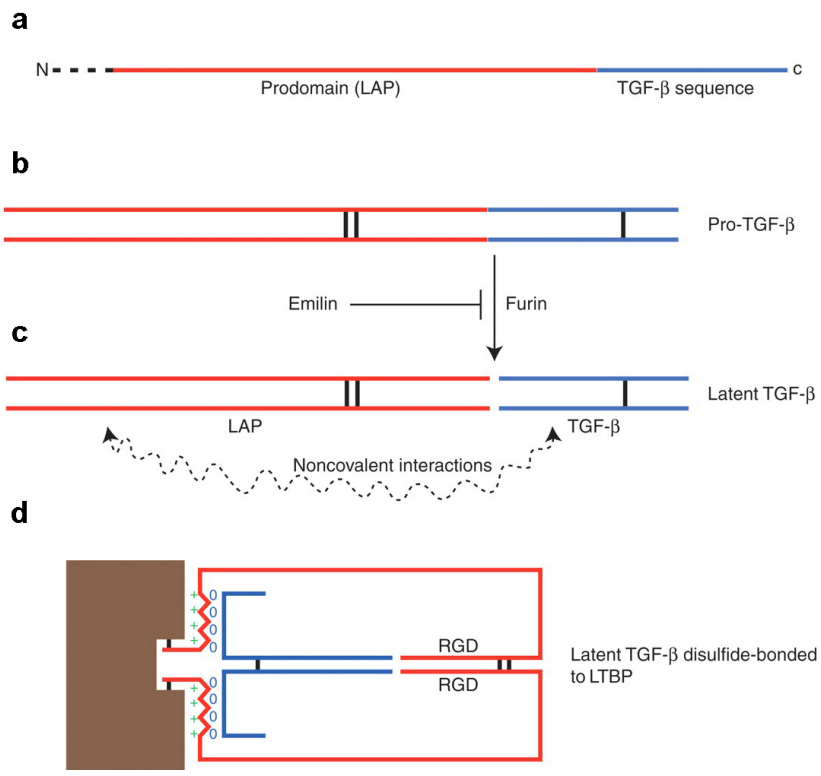

Figure 1.3 | TGF-β latency complex. a | Monomeric pro-TGF-β peptide. **b** | Dimeric pro-TGF-β. **c** | Small latency complex unfolded. **d** | Schematization of large latency complex. In brown is indicated LTBP protein. | LAP: latency associated protein; RGD: tripeptide Arg-Gly-Asp; LTBP: latent TGF-β binding protein.

Numerous studies have established the importance of the renin-angiotensin system (RAS) in cardiac remodeling. The RAS is markedly activated in response to pressure overload and local generation of angiotensin II (AngII) directly induces cellular responses in both cardiomyocytes and interstitial cells, such as fibroblasts. AngII stimulates fibroblast proliferation and expression of ECM proteins. Extensive evidence suggests a direct functional association between the RAS system and the TGF-pathway, indicating that TGF-β1 acts downstream of AngII [19]. AngII stimulation induces TGF-β1 mRNA and protein expression by cardiomyocytes and cardiac fibroblasts [20, 21]. Treatment with angiotensin converting enzyme inhibitors or angiotensin receptor type 1 blockers markedly decreased TGF-β1 levels in hypertrophic [22] hearts, suggesting that TGF-β induction in the myocardium remodeling is at least in part mediated by AngII-mediated signaling.

To date, it is well know the key role of TGF–β1 in mediating cardiac hypertrophy [23]: it is, indeed, able to stimulate cardiomyocytes hypertrophy, fibroblast activation and proliferation, and synthesis of collagen and other ECM proteins in cardiac tissue [1, 7]. Activation of a small fraction of latent TGF-β1 is capable to generate maximal cellular response [17].

In animal models of pressure overload, myocardial TGF–β1 mRNA expression is upregulated in parallel with increasing levels of left ventricular mass and ECM proteins [24, 25]. In human studies, analysis of ventricular tissue from pressure-overloaded hearts showed that myocardial TGF–β1 overexpression correlates with the degree of fibrosis [23, 26] **(Figure 1.4)**.

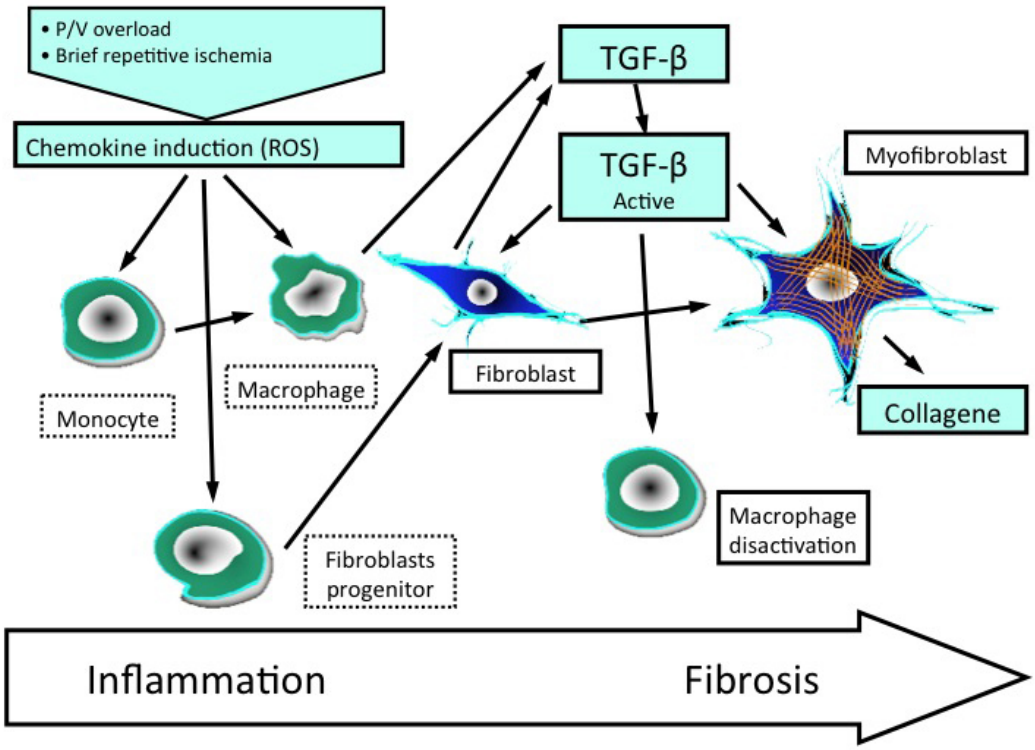

Figure 1.4 | The key pleiotropic role of TGF-β in fibrosis process. After different stimuli (e.g. pressure/volume overload or brief repetitive ischemic events), subsequent ROS production involves macrophagic and fibroblastic progenitor maturation. Once mature, these cell types produce TGF-β, which active form is able to stop inflammation process and induce fibrotic process progression (i.e. differentiation of fibroblast into myofibroblast and collagene deposition).

1.3 Myofibroblasts

An important effect of TGF-β1 is found on the most represented cell type of the connective tissue: the fibroblast. Since 1993, indeed, it is well known that TGF-β1 plays a key role in modulating phenotype switching of fibroblasts into myofibroblast [27]; cells become able to acquire contractile features, given by expression of tipical stress fibers containing α-smooth muscle actin (α-SMA) expression [28]. This is the reason of myofibroblast definition as "specialized contractile fibroblast": modified fibroblasts with SM-like features were first observed in granulation tissue of healing wounds. These findings led to the suggestion that these cells have a role in the production of the contractile force that is involved in this process of wound healing [29].

Morphologically, myofibroblasts are characterized by the presence of a contractile apparatus that contains bundles of actin microfilaments with associated contractile proteins such as non-muscle myosin, and which is analogous to stress fibers that have been described in cultured fibroblasts. These actin bundles terminate at the myofibroblast surface in the fibronexus — a specialized adhesion complex that uses transmembrane integrins to link intracellular actin with extracellular fibrobectin fibers [30, 31].

Functionally, this provides a mechano-transduction system, in which the force that is generated by stress fibers can be transmitted to the surrounding ECMand then transduced into intracellular signals [31].

To further produce necessary tension, myofibroblast ECM synthesis and processing activity are enhanced in the process of remodeling. The most prominent myofibroblast ECM products are collagens of type I, III, IV, and V [32], which are produced by a variety of cells. However, the most reliable marker of the

myofibroblast ECM to date is the fibronectin (FN) splice variant ED-A FN [33]. ED-A FN is also expressed in low amounts by fibroblastic cells in culture [31, 34] and by vascular SMC *in vivo* and *in vitro* [35].

Recently, collagen type VI attracted attention as it is upregulated during myocardial interstitial fibrosis [36], as well as in other fibrotic tissues. It is important to point out that myofibroblasts are not found in healthy myocardium, they only appear following cardiac injury [37]. The main mechanism that mediates the migration of fibroblasts to the site of injury involves the release of chemokines. In addition, myofibroblast themselves produce and secrete a number of cytokines (for example, IL-1α, IL-1β, IL-6, IL-10 and TNF-α), which in turn help to maintain the inflammatory response to injury [38].

1.4 “αv” integrins

TGF-β controls the transcription of several genes, including those encoding for integrins **(Table 1.1)**, in several cell types and tissues [39]. Intriguingly, the induction of integrin expression by TGF-β can be driven by cooperative signalling between the integrin and TGF-β, thereby creating a feedforward loop [40].

Integrins are heterodimeric transmembrane receptors **(Figure 1.5)**, composed by an “α” and a “β” subunit [41, 42]. They are composed by a large extracellular domain (700–1100 amino acids), a single transmembrane segment and a short cytoplasmic tail, ranging from 20–60 amino acids. Ligand binding occurs to the extracellular domain of the integrin heterodimer, a process that is modified by range of amino acids spread throughout both the extracellular and transmembrane domains. The cytoplasmic domain of many of the subunits is highly homologous, while the subunits sequences are significantly different. It is through the cytoplasmic tail that the integrins bind both a range of cytoskeletal linker molecules and also signal.

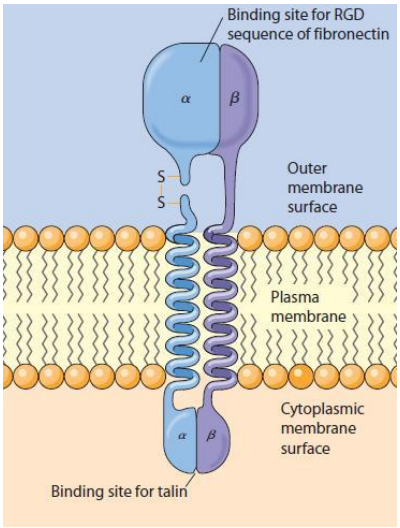

Figure 1.5 | Generic integrin structure. Three main domains compose the single integrin monomer: a cytoplasmic tail (indicated as the binding site for talin), a transmembrane α-elix domain and an extracellular domain, responsible for RGD sequence of LAP binding.

Table 1.1 – Integrins and their main features.

Integrin	Main ligand	Effect of TGF-β	Cell type	Integrin Regulation of TGF-β activation or signalling	Context
α1β1	Collagens	Upregulation	Fibroblasts	?	Collagen remodelling and contraction, myofibroblast differentiation during wound healing and fibrosis
α2β1	Collagens	Upregulation, downregulation	Keratinocytes, fibroblasts	?	Collagen remodelling and contraction, myofibroblast differentiation during wound healing and fibrosis, re-epithelialization during wound healing
α3β1	Laminins	Upregulation, downregulation	Keratinocytes, fibroblasts, carcinoma cells, lung alveolar epithelial cells	α3β1 Modulation of TGF-β signalling by enabling formation of a β-catenin–Smad2 complex, or by repressing Smad7 expression	Re-epithelialization during wound healing, EMT, cancer cell migration and invasion; EMT during IPF, re-epithelialization during wound healing?
α5β1	Fibronectin	Upregulation	Keratinocytes, fibroblasts, carcinoma cells, endothelial cells	α5β1 Control of TGF-βRII expression. NA, binding and activation of LAP	Re-epithelialization during wound healing, EMT, cancer cell migration and invasion, endothelial cell migration and tube formation
α6β1	Laminins	Upregulation	Carcinoma cells, lung alveolar epithelial cells, promonocytic leukaemia cells	?	Macrophage maturation, cancer cell migration and invasion
α8β1	RGD	Upregulation	Fibroblasts, vascular smooth muscle cells	α8β1 Binding of LAP1 and LAP3, but no activation of TGF-β	Myofibroblast differentiation, vascular smooth muscle cell contraction
α6β4	Laminins	Upregulation, downregulation	Keratinocytes, carcinoma cells		Re-epithelialization during wound healing, EMT, cancer cell migration and invasion
αvβ1	?	?	?	αvβ1 Binding of LAP1 and LAP3, activation of TGF-β is unclear	?

Integrin	Main ligand	Effect of TGF-β	Cell type	Integrin Regulation of TGF-β activation or signalling	Context
αvβ3	RGD	Upregulation	Fibroblasts, carcinoma cells, endothelial cells	αvβ3 TGF-β activation *in vitro*, modulation of TGF-β signalling by physical association with TGF-βRII, control of expression of TGF-βRI and II	Myofibroblast differentiation during wound healing and fibrosis, angiogenesis, carcinoma cell migration and invasion; Regulation of granulation tissue during wound healing, carcinoma cell migration and invasion, possible role in SS/scleroderma
αvβ5	RGD	Upregulation	Keratinocytes, fibroblasts	αvβ5 TGF-β activation in vitro and *in vivo*, enhancement of TGF-β signalling by physical association with TGF-βRII	Myofibroblast differentiation during fibrosis, re-epithelialization during wound healing, EMT, cancer cell migration and invasion; Pulmonary fibrosis, possible role in SS/scleroderma
αvβ6	RGD	Upregulation	Keratinocytes, fibroblasts, carcinoma cells,	αvβ6 TGF-β activation *in vitro* and *in vivo*	Myofibroblast differentiation during fibrosis and in tumours, re-epithelialization during wound healing, EMT, cancer cell migration and invasion; Development, IPF, kidney and renal fibrosis, SS, wound healing, EMT, carcinoma migration and invasion
αvβ8	?	?	?	?	Development, suppression of T-cell-mediated immunity, possible role in COPD or wound healing
αLβ2	ICAM1	Upregulation	Promonocytic leukaemia cells	αvβ8 TGF-β activation in vitro and *in vivo*	Macrophage maturation
αEβ7	E-cadherin	Upregulation	T lymphocytes	?	T-lymphocyte infiltration into epithelia

EMT, epithelial-to-mesenchymal transition; ICAM1, intercellular adhesion molecule-1; RGD, arginine–glycine–aspartate; TGF-β, transforming growth factor-β.
COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; LAP, latency-associated protein; NA, not assessed; SS, systemic sclerosis; TGF-βR, TGF-β receptor.

Modified from [39].

TGF-β regulates also the expression of integrin ligands, including tenascin, vitronectin, fibronectin, and several members of the laminin and collagen families. In addition also stimulates the expression of integrin-associated proteins, which could increase integrin activation. Therefore, the transcriptional control exerted by TGF-β can strongly affect integrin-mediated processes.

TGF-β activation requires its own dissociation from the latent complex, which occurs at low pH (e.g. action of ROS, by acidification of extracellular milieu) or through the mechanisms previously described (i.e. proteases, TSP-1. plasmin). The LAPs of TGF-β1 and TGF-β3 —but not of TGF-β2— contain RGD motif (Arginine-Glycine-Aspartate), which can be potentially bound by the αv-containing integrins and αIIbβ3, α5β1 and α8β1 [39].

Integrin-mediated TGF-β activation seems to be possible in a protease-dependent or -independent manner: the former has only been demonstrated for αvβ8 and depends on simultaneous binding of αvβ8 to the RGD site in LAP and recruitment of MMP-14, which then releases TGF-β by proteolytic cleavage [43]. This mode of activation does not require the proximity of activating and target cells **(Figure 1.6)**. Physiological evidences of the mutual interrelation between TGF-β and this integrin was found in the differentiation induction of airway fibroblasts into myofibroblasts carried out by integrin-mediated active TGF-β; the expression of αvβ8 is increased in the airways of chronic obstructive pulmonary disease patients, correlating with the severity of the obstruction [44, 45].

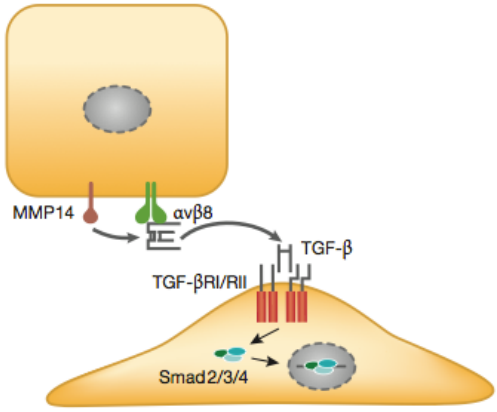

Figure 1.6 | The proteolytic activation pathway of TGF-β involves the synergic action of αvβ8 integrin and MMP-14. The selective binding between αvβ8 integrin and latent TGF-β lead to presentation of TGF-β complex to matrix-metalloprotease 14 (MMP-14), which activate TGF-β by proteolysis.

The non-proteolytic TGF-β activation occurs through cell traction forces exerted by the actin cytoskeleton. These forces are translated by integrins into a conformational change of the TGF-β–LAP–LTBP complex, leading to the exposure and consequent activation of TGF-β [46-49]. This mechanism has been defined only in the 2004 by Keski-Oja as "traction model" [50] **(Figure 1.7 a)**.

Non-proteolytic activation of latent TGF-β has been demonstrated *in vitro* for αvβ3, αvβ5 and αvβ6, as well as for β1-containing subunit with a still unidentified α-subunit integrin [49]; whether or not the activation of TGF-β by a β1-integrin is relevant physiologically remains controversial.

1.4.1 Integrins αvβ6 and αvβ8

The importance of integrin-mediated activation of TGF-β *in vivo* is evident, as mutation of the RGD site of LAP leads to defects similar to those observed in TGF-β1-null mice [51]. In addition, genetic ablation of the β6-subunit, or conditional deletion of αv or β8 from dendritic cells, causes exaggerated inflammation as a result of impaired TGF-β signalling [52, 53]. The phenotype of mice lacking both the

αvβ6 and αvβ8 integrins recapitulates the abnormalities observed in TGF-β1 and TGF-β3 —but not in TGF-β2— knockout mice, indicating that the integrins αvβ6 and αvβ8 can account for the full activation of TGF-β1 and TGF-β3 in vivo.

The first clue that the integrin–TGF-β interplay is important in fibrosis came from the observation that mice lacking the β6-subunit are protected from bleomycin-induced pulmonary fibrosis [54] and β6 knockout mice are partly or completely protected from pulmonary fibrosis induced by radiation [55]. In addition, low doses of antibodies against αvβ6 prevent radiation-induced or bleomycin-induced pulmonary fibrosis in mice, without causing inflammation [56, 57]. Furthermore, it has been shown that constitutive expression of αvβ6 in the basal layer of the epidermis leads to elevated TGF-β1 activation and the development of spontaneous chronic ulcers with severe fibrosis [58]. In wild-type mice, fibrosis can be equally inhibited by treatment with antagonists of TGF-β signalling or by using a blocking antibody against αvβ6 [59, 60]. The importance of αvβ6 in fibrogenesis has been demonstrated subsequently in several models; αvβ6 is not normally expressed in healthy epithelia but its expression is induced in many human fibrotic disorders involving the kidney, liver and lung (e.g. sclerosis and idiopathic pulmonary fibrosis). The specific inhibition of αvβ6-induced TGF-β activation at sites of injury is a promising therapeutic tool to combat TGF-β-mediated fibrosis.

1.4.2 Integrins αvβ3 and αvβ5

Mice lacking β3, β5, or both do not develop abnormalities similar to those due to deficient TGF-β signalling [61-63]. Nevertheless, αvβ3-mediated or αvβ5-mediated TGF-β activation could be important in pathological conditions. In fact increased expression of both of these integrins, has been observed in the dermis of

scleroderma patients (a chronic disease, involving cutaneous manifestations of fibrosis), and these integrins elicit autocrine TGF-β signalling in fibroblasts of patients *in vitro* [64-67]. In addition, TGF-β activation by αvβ5 is important in pulmonary fibrosis; however, a causal effect of αvβ3-mediated TGF-β activation in human pathology has not yet been established.

Observations suggest that the integrins αvβ3 and αvβ5 provide additional therapeutic targets for this pathology, because of their possible contribution to the pathogenesis of systemic sclerosis and scleroderma by TGF-β1 activation [65, 66]. In human fibrotic lungs, epithelial cells expressing αvβ5 and PAR1 co-localize with myofibroblasts, and TGF-β-mediated pulmonary fibrosis is reduced by the blockade of αvβ5 in a mouse model [68].

Recently, Henderson et al. have published on Nature Medicine a paper where show a system developed to allow gene manipulation in myofibroblasts in multiple tissues and where used this system to demonstrate that αv-containing integrins on myofibroblasts are components of a core cellular and molecular pathway that contributes to pathologic fibrosis in multiple solid organs, suggesting that targeting this core pathway could have clinical utility in the treatment of patients with a broad range of fibrotic diseases [69].

1.5 The “traction model” in cardiac tissue

The mechanism proposed by Keski-Oja with name of “traction model” [50] **(Figure 1.7 a)** has been expanded by Wipff *et al.* in 2007 **(Figure 1.7 b)**, with the introduction of key components involved in this model: 1) cytoskeletal integrity of myofibroblasts (and, overall, presence of α-SMA), 2) presence of integrin, connected by their cytoplasmic β-tail to the cytoskeleton structure, 3) a mechanically resistant ECM, containing all TGF-β latent complex proteins components [49].

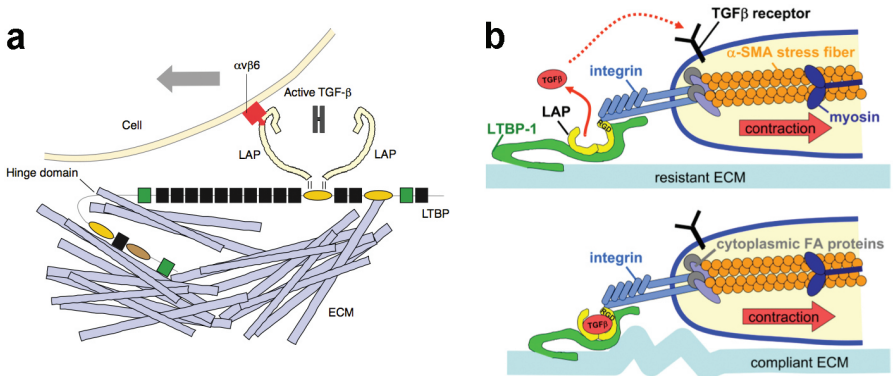

Figure 1.7 | The evolution of TGF-β1 activation by traction model. a | The traction model depicted by Keski-Oja, 2004. An integrin-expressing cell recognizes the RGD domain in latency-associated protein (LAP). In addition to the other extracellular matrix (ECM)-binding sites, the large latent complex associates with the ECM. Both interactions are essential for integrin-mediated TGF-b activation. Cell movement (gray arrow) distorts the structure of the small latent complex. Because the integrin is connected to the cytoskeleton, the retraction of membrane protrusions pulls LAP from the mature TGF-b to release the active cytokine from the protein complex. **b |** The traction model depicted by Wipff, 2007. The high contractile activity generated by α-SMA in stress fibers is transmitted at sites of integrins binding to RGD sites in the LAP protein, which also includes TGF-β1 and LTBP-1. In the upper cartoon, when the latent complex is anchored in a comparably stiff ECM, cell-mediated stress can induce allosteric changes in LTBP-1 and/or LAP conformation, leading to liberation of TGF-β1; such activated TGF-β1 possibly feeds back by binding to its receptor, which is located close by in the activating cell. In the lower figure, in the context of compliant ECM, the latent complex is dragged toward the pulling cell but because of the lack of mechanical resistance, no conformation change occurs and TGF-β1 remains latent. Likewise, inhibition of high cell contraction and interaction of integrins with the latent complex block mechanical activation of latent TGF-β1.

Although several studies have shown the presence in cardiac tissue of most of the "traction model” components, it is not clear yet whether this pattern of activation of the TGF-β may be involved in the development of cardiac fibrosis and whether a hypertensive context may be a trigger stimulus for its activation.

TGF-β mRNA is abundantly expressed in hearts from patients with dilated and hypertrophic cardiomyopathy and is associated with increased collagen deposition [70]. Furthermore, two different mouse models with cardiac overexpression of TGF-β1 suggested a pro-fibrotic effects of TGF-β in the heart. First, Rosenkranz *et al.* demonstrated that cardiac TGF-β1 overexpression resulted in ventricular fibrosis associated with increased collagen deposition and inhibition of interstitial collagenases [19]. Second, a study of Nakashima *et al.* showed that transgenic mice with a blocking mutation of covalent tethering of the TGF-β1 latent complex to the extracellular matrix had a large proportion of constitutively active TGF-β1 in heart tissue and the fibrosis [71]. Interestingly, despite showing similar levels of TGF-β in both atria and ventricles, these animals exhibited only atrial and not ventricular fibrosis. Other investigations demonstrated that decreased TGF-β activity prevented fibrotic remodeling of the ventricle in several distinct experimental models of cardiac fibrosis. TGF-βeta blockade prevented myocardial fibrosis in pressure overloaded rats, decreasing fibroblast-to-myofibroblast phenotype switching, limiting collagen deposition and lowering diastolic dysfunction [7, 72].

Myofibroblasts are not founded in normal healthy adult myocardium and appearing after cardiac injury [38]. In particular, in pressure overload-induced cardiac hypertrophy, myofibroblasts increase in number and induce ECM proteins synthesis [73]. It has been reported that myofibroblasts derive from transdifferentiation of resident cardiac fibroblasts [74] or endothelial cells [75, 76]. Endothelial-to-mesenchymal transformation can be induced by TGFβ in a Smad-dependent fashion during cardiac fibrosis, while bone morphogenic protein (BMP)-7 blocks this process and could serve as an antifibrotic factor [75].

Myofibroblasts response to TGF-β1 not only results in phenotype switching, but also in expression of proteins involved in the “traction model”, such as TGF-β1 latent complex proteins and integrins. Two important studies published on the "Hypertension" journal show how integrin αv, β1, β3 and β5 regulate the activation of angiotensin receptor type 1 (AT1) and are upregulated in the hypertrophic ventricles of spontaneously hypertensive rats (SHR). Moreover, Kawano *et al.* show that selective blocking of AT1 is associated with downregulation of the same previously mentioned integrins [77]. Furthermore, Graf *et al.* showed how AngII activity increases the expression of integrin αvβ3, TGF-β1 and PDGF in neonatal rat cardiac fibroblasts [78].

This study was aimed 1) to develop a more fast and efficient protocol to cardiac fibroblast extraction, 2) to evaluate a possible involvement of LTBP-1 and αVβ5 integrin in development of cardiac fibrosis induced by hypertensive stimulus, and 3) to speculate about TGF-β1 mechanical activation through “traction model” in primary cardiac fibroblasts isolated in SHR.

2. Material and Methods

2.1 Reagents

- LOADING 5X: 8% SDS, 20% β-mercaptoethanol, 80 mM TRIS, 10% Blu di Bromofenolo, pH=6.8;

- RUNNING BUFFER: 25 mM TRIS, 250 mM Glicina, 0.1% SDS;

- TRANSFERT BUFFER: 250 mM Glicina, 25 mM TRIS;

- T-PBS: 20 mM TRIS, 130 mM NaCl, 0.05% Tween 20%

- DIGESTION BUFFER: PBS with 1% Penicillin/Streptomycin, 1% Fungizone and Liberase TH Research Grade Blendzyme (Roche)

- FIBROBLASTS MEDIUM CULTURE (FMC): Dulbecco's Modified Eagle Medium, High Glucose (Euro-clone), 15% Fetal Bovine Serum (Euro-clone), 2 mM L-glutammine, 200 U/ml penicillin, 200 µg/ml streptomycin, 0.5% Amphotericin B;

2.2 Cell isolation and culture

Primary rat cardiac fibroblasts were isolated from the whole heart of 10 Wistar Kyoto rats (WKY) and 10 Spontaneously Hypertensive rats (SHR) (Charles River), by using a novel protocol strategy. Rats, 5 male and 5 female for each strain, were all 5 weeks old. Briefly, the rats were sacrificed, dissected **(Figure 2.1 a)**, whole hearts extracted with sterile pliers **(Figure 2.1 b)** and then placed in 50 mL

tubes **(Figure 2.1 c)** with sterile DMEM High Glucose, with 1% Penicillin/Streptomycine, 1:500 Gentamicin and 1% Fungizone (Euroclone). Cardiac tissue was transferred into a previously sterilized 100 mm tissue culture dish of glass and minced using disposable sterile scalpels **(Figure 2.1 d)**. The tissue fragments were incubated at 37°C for 15 minutes in 10 mL of Digestion Buffer **(Figure 2.1 e)**. After washing 3 times, digested tissue fragments were cultured at 37°C, 5%CO_2 in FMC medium **(Figure 2.1 f)**. Fibroblasts were treated with 5 ng/ml TGF-β1 (Sigma-Aldrich) for 72 hours.

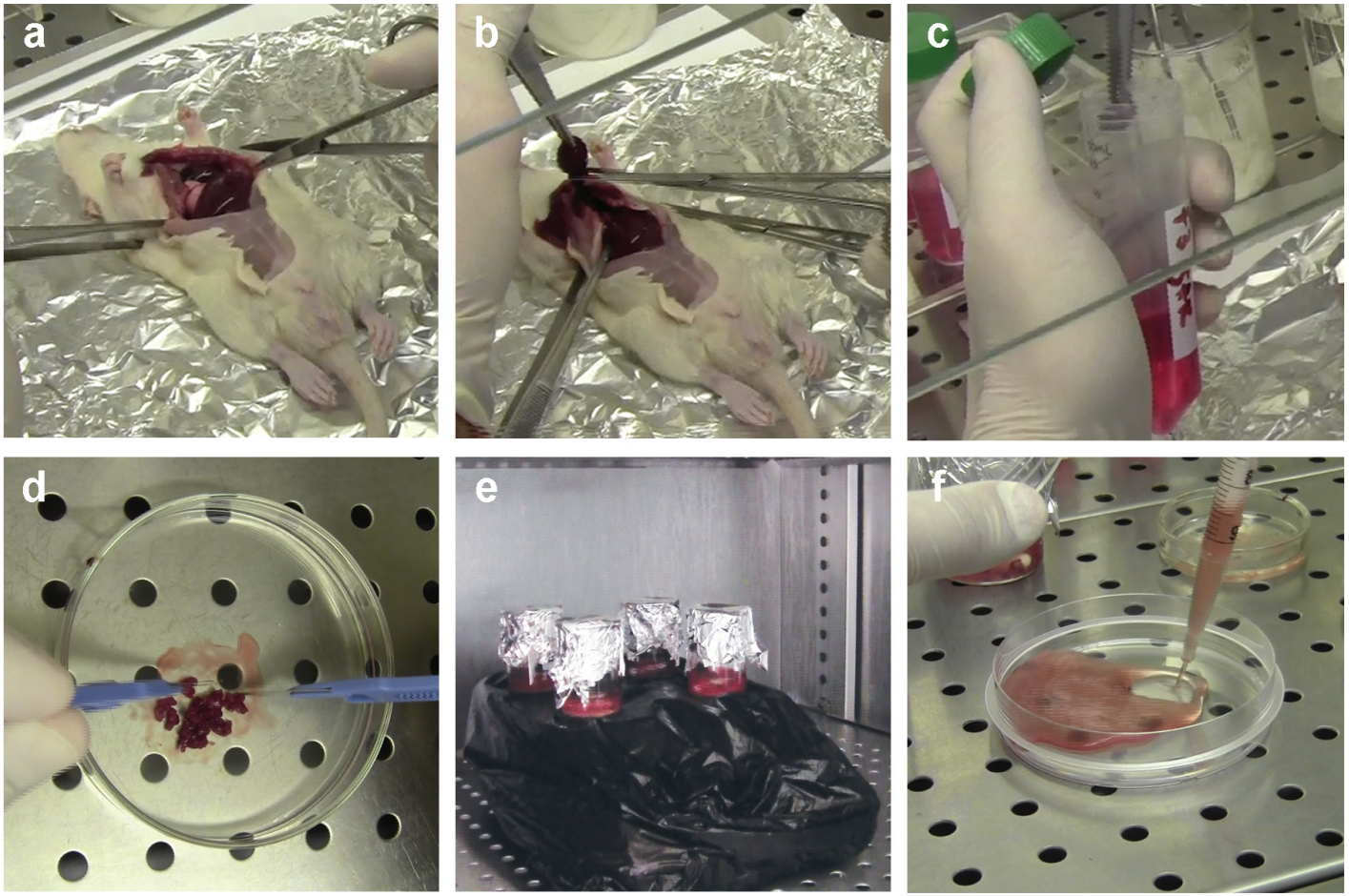

Figure 2.1 | Isolation of whole rat heart primary fibroblasts protocol.
a | Rats chest dissection. **b |** Rats heart extraction. **c |** Rats heart washing. **d |** Cardiac tissue mincing. **e |** Minced cardiac tissue digestion. **f |** Digested cardiac tissue plating.

2.2 Antibodies

Following primary antibodies were used: anti-vimentin (Mouse monoclonal [V9] against vimentin, Dako), anti-α-SMA (Mouse monoclonal [1A4] against α-SMA, Dako) anti-TGF-β1 (Mouse monoclonal [2Ar2] against TGF-β1, AbCAm), anti-LTBP-1 (Rabbit polyclonal against LTBP-1, AbCAm) and anti-integrin αvβ6 (Mouse monoclonal [P1F6] against Integrin αV+β5, AbCAm).

For Western blotting, we used HRP-conjugated secondary antibodies goat anti–mouse and goat anti–rabbit (AbCAm).

2.3 Histology, immunohistochemistry and microscopy

Histological and immunohistochemical studies were carried out on 4 μm thicktissue sections using a Novolink Polymer Detection System (Novocastra Laboratories) with the previously described primary antibodies at following concentration and solution for antigen retrieval: anti-LTBP-1, 1:400 dilution (citrate), anti-αvβ5, 1:400 (EDTA), anti-TGF-β1, 1:300 (EDTA), anti-vimentin, 1:5000 (citrate) and anti-α-SMA, 1:50 (citrate).

Cell cultures on glass slides were obtained according to the following protocol: fibroblasts isolated from rat heart and frozen at the third passage were thawed, plated, brought to confluence, detached and plated on poly-L-lysine coated slides at a concentration of 1.5 x 10^5 cells/mL. On the slide, placed in Petri dish, were seeded 500μL of cell suspension and then placed in growth for 48 hours with 95% humidity, 5% CO_2. The slides were then rinsed with PBS solution and soaked for about 2 minutes in a solution of 4% formalin.

Sections were deparaffinized in BioClear (Bio Optica) for 20 minutes, washed twice in ethanol; after this step, both cell cultures and sections underwent to the same procedure. Culture and sections were kept 35 min at 97.5°C in 9 mM sodium citrate pH 6.0, EDTA pH 8.0. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 minutes; incubation of primary antibodies was performed for 1 hour. Staining was performed with 3,3-diaminobenzidine (DAB) as a chromogen. Slides were immunostained in the samebatch, including negative controls lacking the primary antibody.

The light microscopy used for photos acquisition was AxioSkop 2 Plus (Zeiss); quantification analysis has been performed with a dedicated software (AxioVision Rel 4.7).

2.4 Western blotting

Fibroblasts were washed with cold PBS and lysed in a lysis buffer, containing a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich). The lysates were centrifuged at 14000 rpm for 10 minutes to remove any cell debris. Protein concentration was measured using the Bradford Assay (Bio-Rad Laboratories Ltd, UK). Equal amounts of proteins were electrophoresed on SDS-Polyacrylamide Gels (SDS-PAGE) and electro-transferred into nitrocellulose membranes (Amersham, UK) for 90 minutes at 4°C. Then, membranes were blocked for 1 hour in PBS containing 0.05% Tween 20 (PBS-T) and 5% of nonfat dry milk. Membranes were incubated overnight at 4°C in 5% milk in PBS-T solution containing primary antibody, with constant agitation. At the end of the overnight incubation, membranes were washed three times for 10 minutes with PBS-T and incubated in HRP-conjugated secondary antibody (AbCam) in 5% milk in PBS-T solution for 1 hour at room temperature. After washing (as previously), positive staining was detected using enhanced chemiluminescence (ECL; Amersham, UK). Quantitative antibody staining was measured by densitometric analysis (Alliance, UviTech, Cambridge).

3. Results

3.1 Novel approach for isolation of rat cardiac fibroblasts

The isolation protocol of cardiac fibroblast used in this study introduces a novelty in the field of rat primary fibroblast culture, because of speed, efficiency and simplicity of the used equipments and procedures. Whole hearts explanted from WKY and SHR rats were excised in the cell culture hood to avoid any type of bacterial or fungal contamination and immediately placed in sterile DMEM, supplemented with 1% penicillin/streptomycin, 1% fungizone and gentamicin 1:500. Whole heart were minced and digested with Liberase TH Research Grade for 15 minutes at 37°C, 95%O_2, 5% CO_2. This ready-to-use mixture containing thermolysin at high concentration and several collagenases shows pretty efficacy in limited time (performing entire protocol takes approximately 90 minutes). The whole suspension of minced tissue was eluted with DMEM (high glucose), supplemented with 15% fetal bovine serum (FBS), 2% L-glutamine, 1%penicillin/streptomycin and 1% fungizone to stop both thermolysin and collagenases activity. After centrifugation, pellet was re-suspended in the same complete medium. The resulting cell suspension was seeded into 100 mm culture dishes. After incubation for 6 days at 37°C, non-adherent cells were removed by rinsing with sterile PBS, and the still present tissue pieces were plated on new dishes with complete medium **(Figure 3.1 a,b)**. Cells were cultured in DMEM (high glucose concentration), supplemented with 15% FBS, 2% L-glutamine, 1% penicillin/streptomycin and 1% fungizone **(Figure 3.1 c,d)**.

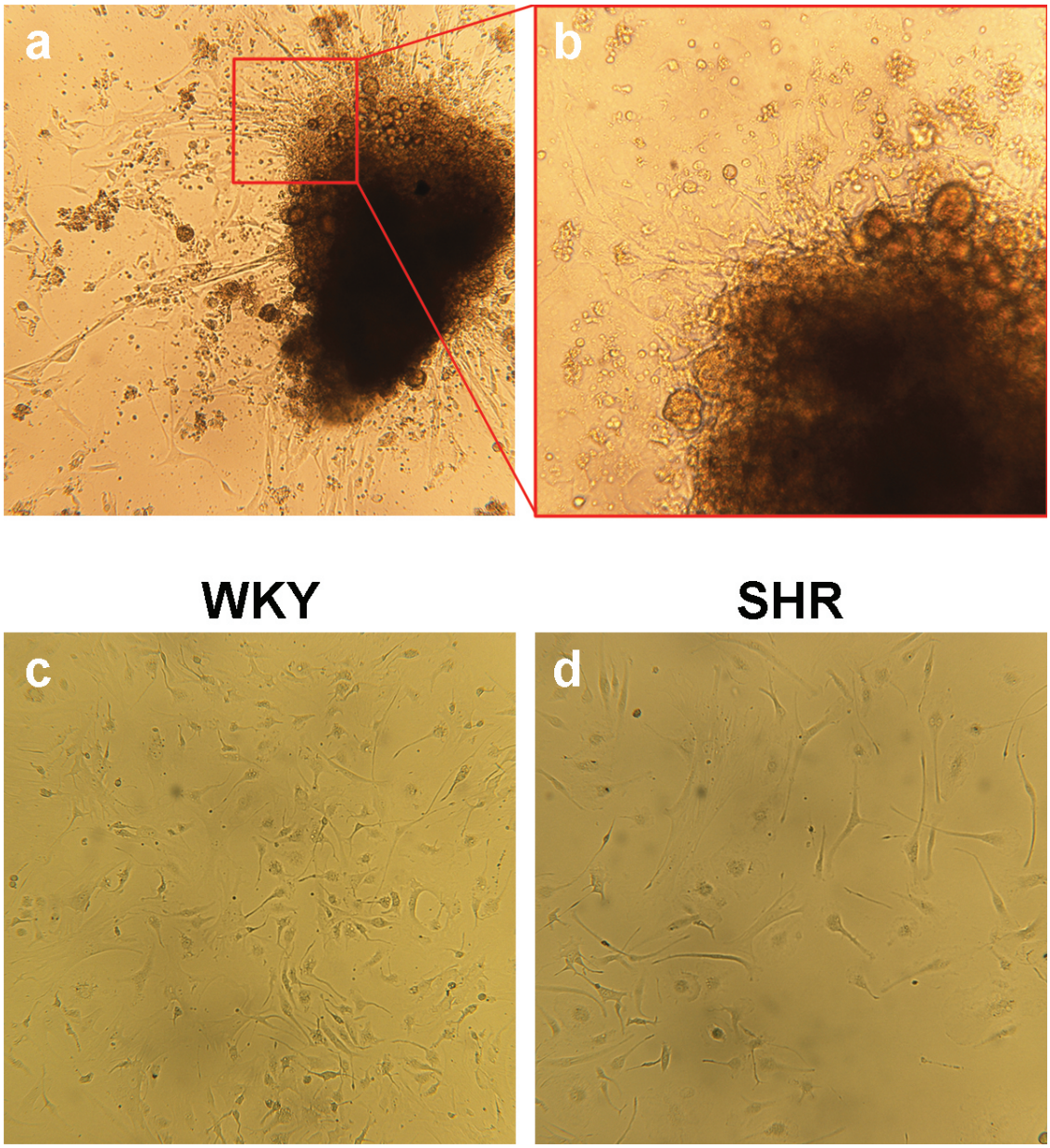

Figure 3.1 | Phases of fibroblast isolation protocol. a | Fibroblast migration out of heart tissue pieces after cardiac tissue treatment in six days of culture. **b |** Higher magnification (20X) shows tipical fibroblast shape exiting from tissue biopsy. **c,d |** Fibroblast cultures. | WKY = Wistar Kyoto; SHR = Spontaneous Hypertensive Rats.

After 2 weeks, cells were trypsinized and splitted into new plates, to allow amplification of the cultures. At this culture passage, vimentin immunohistochemistry

was performed (Figure 3.2) to characterize fibroblast cell type in culture and also to assess the final fibroblast yield in both rat strains. Cell purity was 95–97%, small rate of contaminating cells were vascular smooth muscle cells (VSMC) and pericytes. So, results of immunohistochemistry for vimentin confirm the efficiency of isolation protocol.

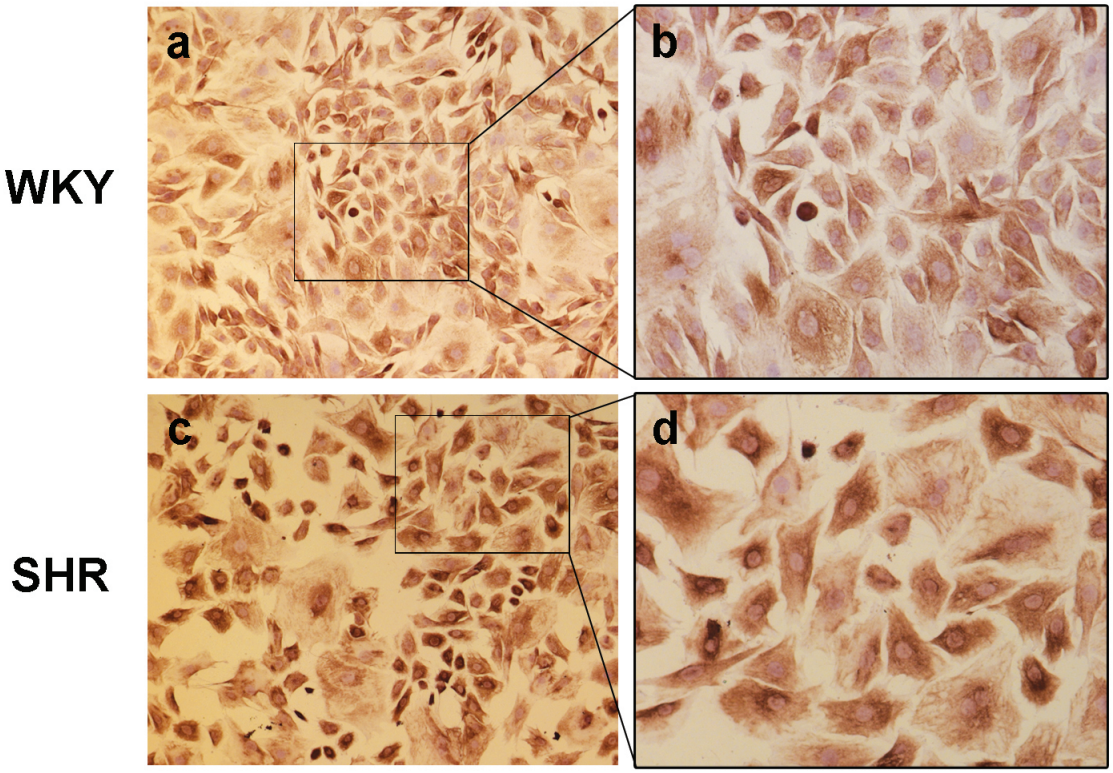

Figure 3.2 | Vimentin immunostaining on isolated primary cardiac fibroblasts. a,b | Vimentin immunostaining on fibroblast culture from normotensive rat heart (WKY). b panel is higher magnification (20X) of black squared area in panel a. **c,d |** Vimentin immunostaining on fibroblast culture from hypertensive rat heart (SHR). d panel is higher magnification (20X) of black squared area in panel c.

3.2 SHR heart show a higher amount of collagen deposition

Since pressure overload is one of upstream causes of cardiac hypertrophy and LVH pathology, a Masson staining, specific for extracellular collagen fibers, has been performed on SHR and WKY tissues **(Figure 3.3)**. Masson staining is also able to detect and highlight, with shades of blue, the older fibers (dark blue) and collagen fibers of more recent deposition (light blue). Twenty high magnificated fields for each rat were chosen in blind to perform quantification analyses of connective tissue deposition. Results demonstrated a higher degree of fibrosis in SHR cardiac tissue in comparison with WKY; moreover, as visible in **Figure 3.3 b** and **Figure 3.3 e** and in their higher magnification **(Figure 3.3 c,f)**, in SHR tissue most of deposited fibers occur in perivascular region in comparison with normotensive rats tissue.

These findings suggest that SHR rats are more prone to fibrosis compared to the WKY ones.

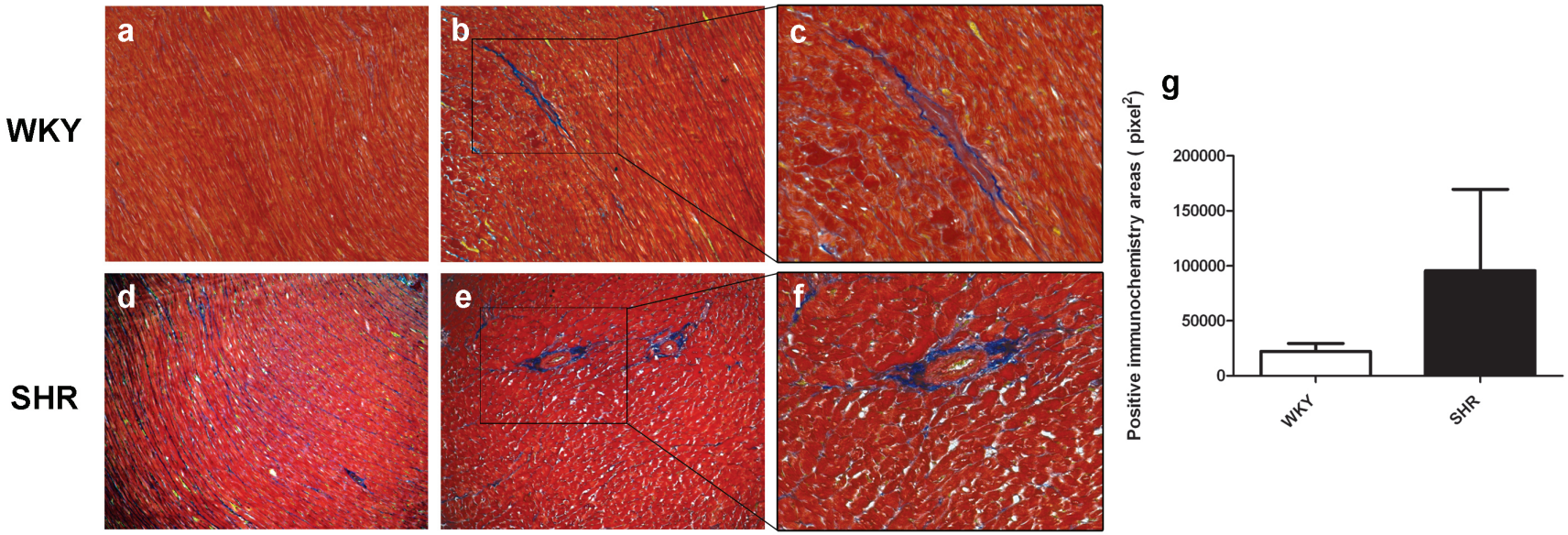

Figure 3.3 | Masson staining on heart tissue of both rat strains reveals massive collagen deposition in SHR cardiac tissue. a,d | Localization of connective tissue (blue staining) in interstitial region of WKY and SHR cardiac tissue. **b,e |** Localization of connective tissue (blue staining) in perivascular region of WKY and SHR cardiac tissue. **c,f |** 20x magnification of boxed region, rispectively of panels b,e. **g |** Quantification of positive areas for Masson staining (blue areas), expressed as pixel^2.

3.3 SHR cardiac tissue overexpresses TGF-β1 and LTBP-1

To assess whether higher cardiac hypertrophy levels were associated with increased expression of the latent form of TGF-β1 **(Figure 3.4 a-d)** and LTBP-1 **(Figure 3.5 a-d)**, immunohistochemistry of these two markers has been performed in both SHR and WKY heart tissues. As previously seen with Masson staining, results of this analysis demonstrated a higher signal rate (brown dots) of both molecules in SHR cardiac tissue, in comparison with WKY **(Figure 3.4 e; Figure 3.5 e)**. Moreover, TGF-β1 pattern in SHR cardiac tissue clearly follows a spot distribution **(Figure 3.4 d)**, consistently with its autocrine/paracrine activation.

These results suggested that the increase in TGF-β1 and LTBP-1 expression is related to ECM deposition in cardiac fibrosis observed in SHR.

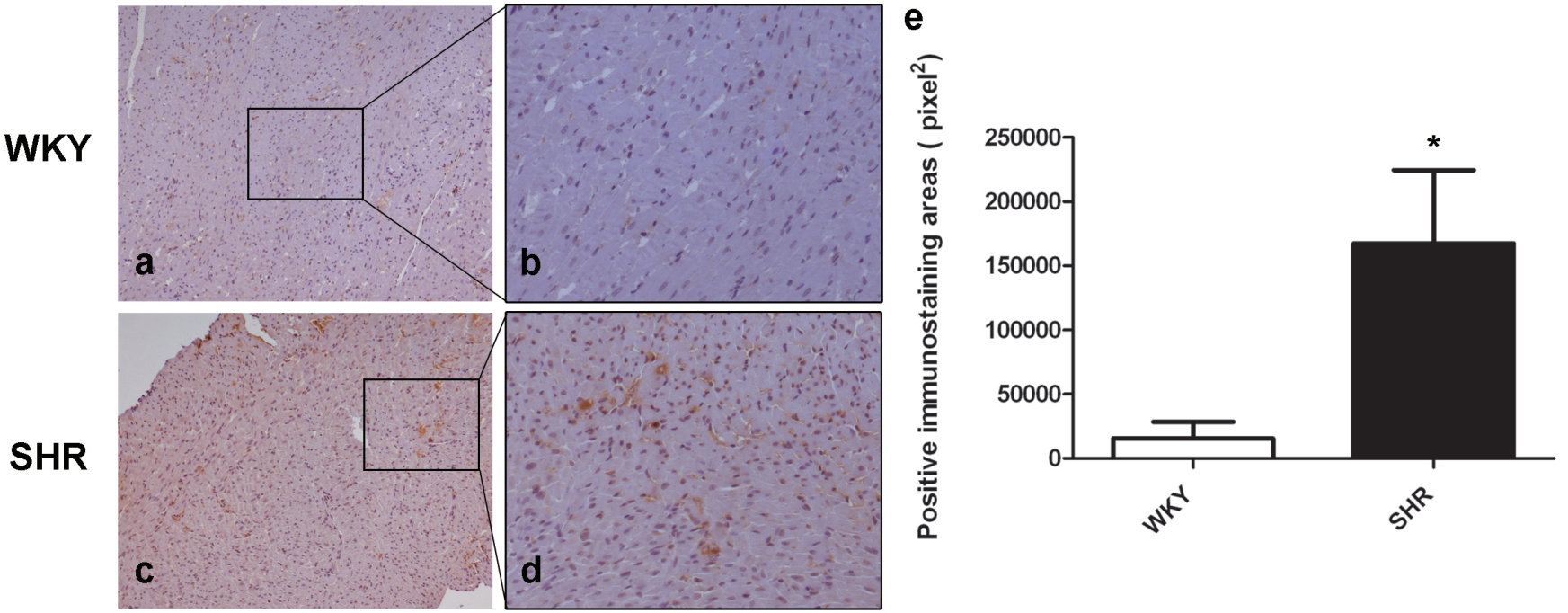

Figure 3.4 | TGF-β1 immunostaining on rat heart tissue. a,b | TGF-β1 immunostaining on normotensive WKY cardiac tissue. b panel is a higher magnification (20X) of black squared area in panel a. **c,d |** TGF-β1 immunostaining on hypertensive SHR cardiac tissue. d panel is higher magnification (20X) of black squared area in panel c. **e |** Quantification of positive areas for TGF-β1 (brown dots), expressed as pixel^2.

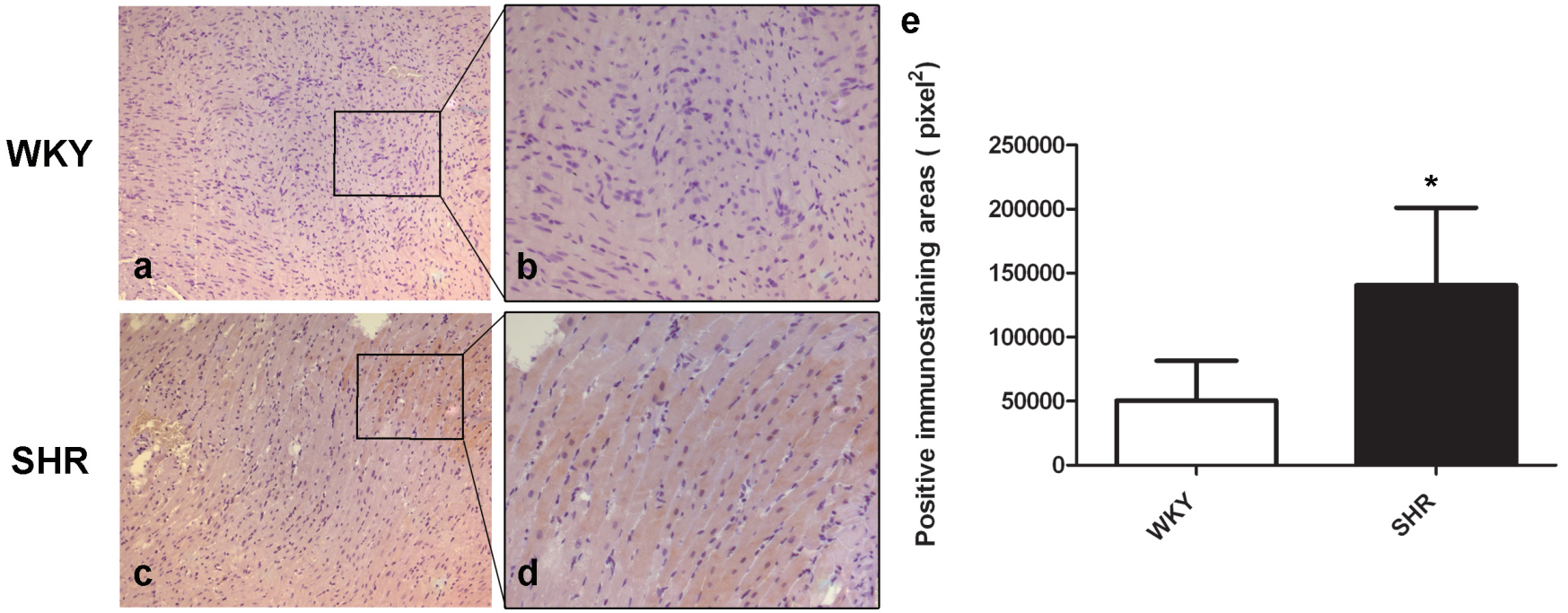

Figure 3.5 | LTBP-1 immunostaining on rat heart tissue. a,b | LTBP-1 immunostaining on normotensive WKY cardiac tissue. b panel is a higher magnification (20X) of black squared area in panel a. **c,d |** LTBP-1 immunostaining on hypertensive SHR cardiac tissue. d panel is a higher magnification (20X) of black squared area in panel c. **e |** Quantification of positive areas for LTBP-1 (brown dots), expressed as pixel^2.

3.4 SHR cardiac tissue overexpresses αvβ5 integrin

To verify whether cardiac hypertrophy and higher levels of both TGF-β1 and LTBP-1 were associated with an enhancing of the third key player of "traction model", such as αvβ5 integrin, immunohistochemistry also of this marker has been performed, in both SHR and WKY heart tissues **(Figure 3.6 a-d)**. This analysis showed higher signal rate of αvβ5 integrin in SHR cardiac tissue **(Figure 3.6 e)** and suggest a possible involvement of αvβ5 integrin in development of fibrotic process in hypertensive rats.

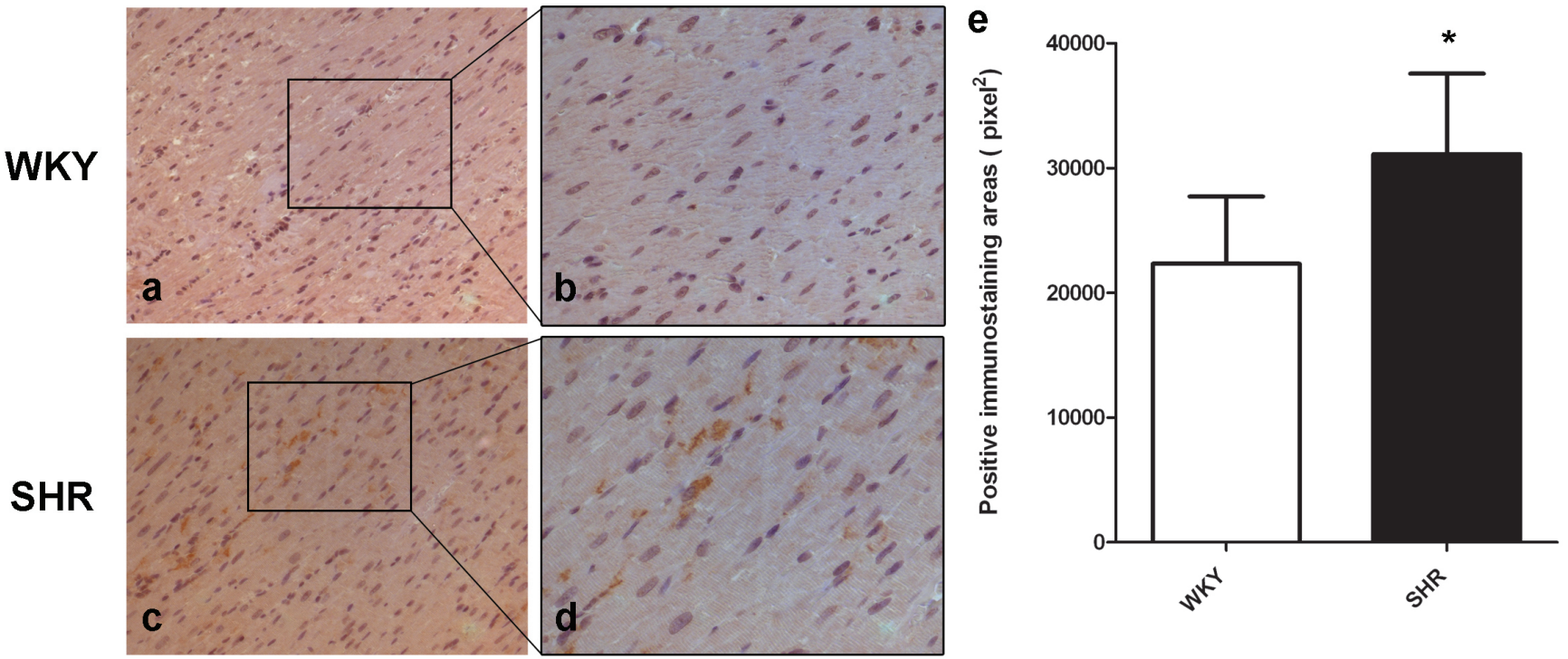

Figure 3.6 | αvβ5 immunostaining on rat heart tissue. a,b | αvβ5 immunostaining on normotensive WKY cardiac tissue. b panel is a higher magnification (20X) of black squared area in panel a. **c,d |** αvβ5 immunostaining on hypertensive SHR cardiac tissue. d panel is a higher magnification (20X) of black squared area in panel c. **e |** Quantification of positive areas for αvβ5 (brown dots), expressed as pixel^2.

3.5 SHR cardiac fibroblast switch into myofibroblast

To evaluate whether fibroblasts in culture transdifferentiate into myofibroblast cell type, an immunohistochemistry for α-SMA has been performed on both WKY and SHR cell at third passage of culture **(Figure 3.7)**.

This analysis has been performed on WKY and SHR fibroblasts at basal condition to better investigate if hypertension has *per se* a direct effect on cell features.

WKY fibroblasts show a negative result for α-SMA and seems to maintain a fibroblastic cell phenotype **(Figure 3.7 a,b)**, on the contrary, in SHR fibroblasts the α-SMA signal is enhanced and so cellular phenotype appears to differentiate in myofibroblastic sense. Nonetheless, same SHR cultures express both vimentin and α-SMA, maintaining also fibroblast features.

Results suggest that hypertensive stimulus *per se* elicit a phenotype transition of fibroblast into pathologic myofibroblasts.

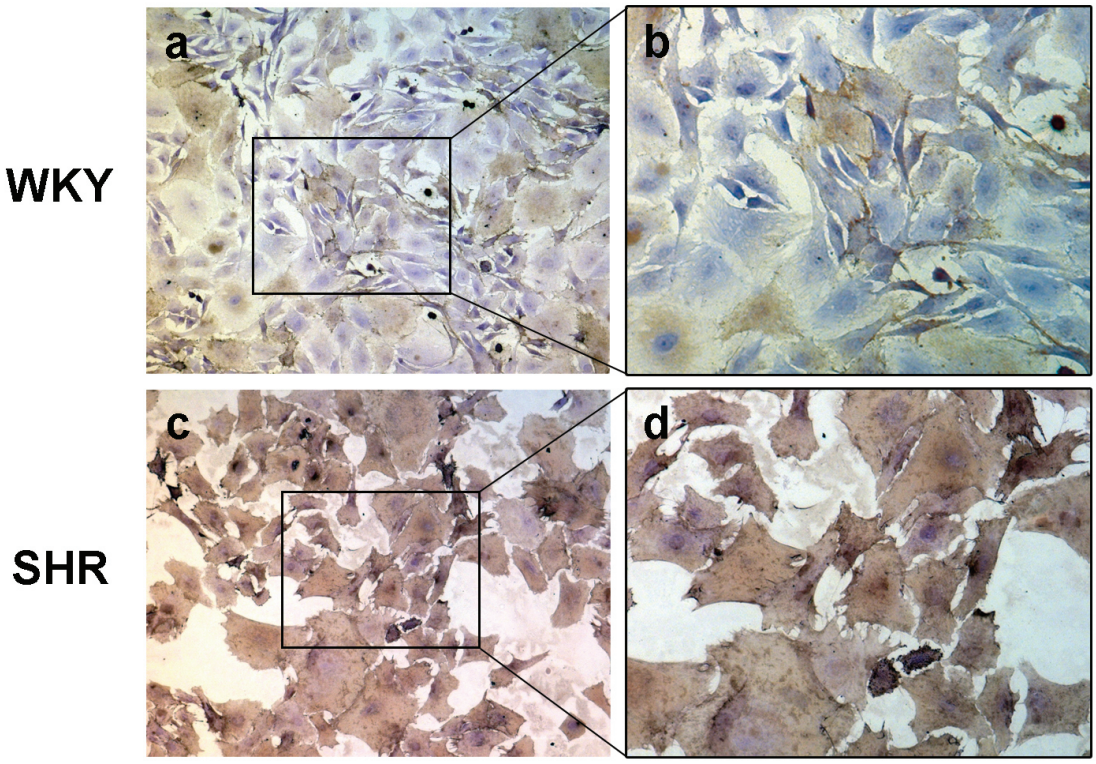

Figure 3.7 | α-SMA immunostaining on isolated primary cardiac fibroblasts. a,b | α-SMA immunostaining on fibroblast culture from normotensive rat heart (WKY). b panel is a higher magnification (40X) of black squared area in panel a. **c,h |** α-SMA immunostaining on fibroblast culture from hypertensive rat heart (SHR). d panel is a higher magnification (40X) of black squared area in panel c.

3.6 SHR cardiac fibroblasts *in vitro* overexpresses LTBP-1 and αvβ5 integrin

To better understand the effect of TGF-β1 and its relation with "traction model" markers *in vitro*, LTBP-1 and αvβ5 integrin were investigated by using two approaches: a western blot assay, to display total protein levels and an immunohistochemical assay, performed directly on cells grown on glass slides.

Western blotting analyses were performed on cells in both basal conditions and under treatment with TGF-β1. Results of both western blot analysis for LTBP-1 and αvβ5 integrin in basal conditions **(Figure 3.8 and 3.9 e**, white bar graph**)** did not show substantial differences between the two rats strains. However, cells under treatment **(Figure 3.8 and 3.9 e**, black bar graph**)** reveal an increase in both markers, more evident and statistically significant in SHR then WKY.

Immunohistochemistry were performed on cells under basal conditions. Results of immunohistochemistry highlighted the different distribution pattern of both molecules between the two rats strains: LTBP-1 and αvβ5 integrin in WKY cells showed only a cytosolic signal **(Figure 3.8 b, 3.9 b)**. On the contrary, in SHR cells, LTBP-1 signal is in extracellular matrix **(Figure 3.8 d**, red arrows**)** and αvβ5 signal is more evident along the cell membrane **(Figure 3.8 d**, red arrows**)**.

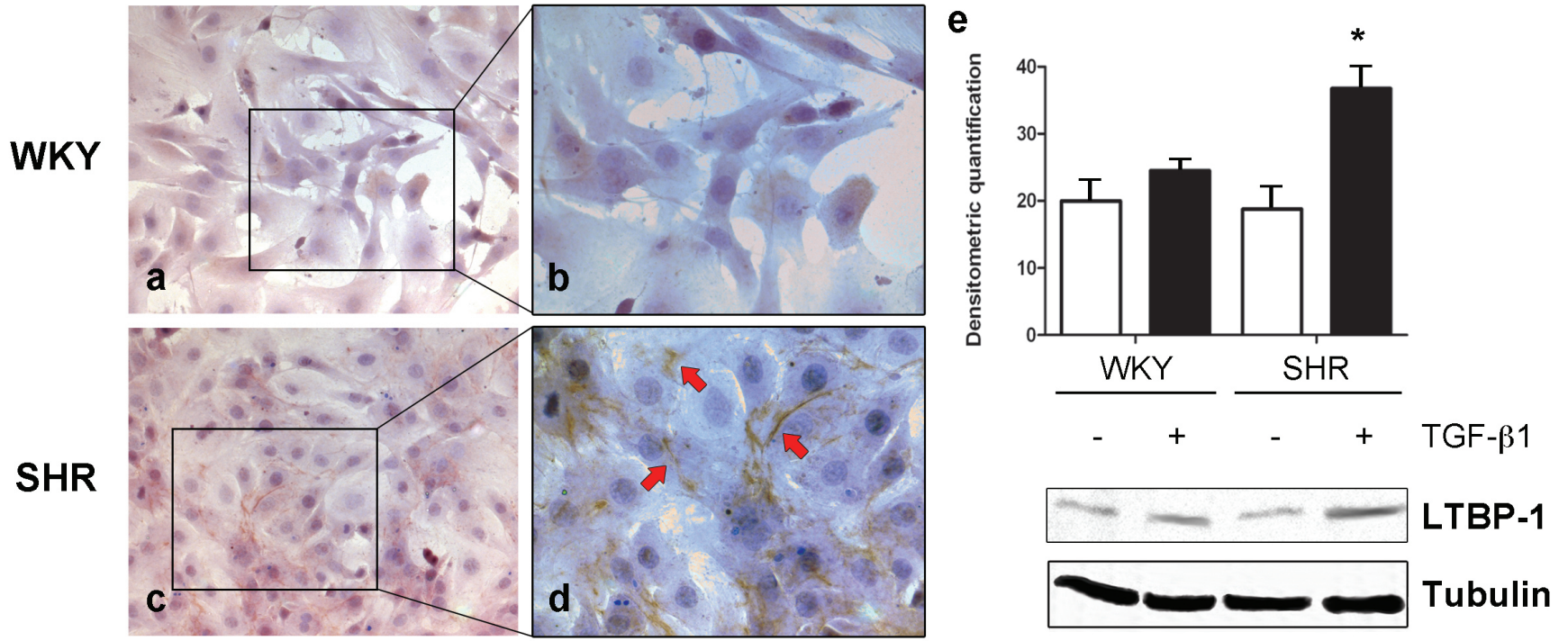

Figure 3.8 | LTBP-1 immunostaining and western blotting analysis on isolated primary cardiac fibroblasts. a,b | LTBP-1 immunostaining on fibroblast culture from normotensive rat heart (WKY). b panel is a higher magnification (40X) of black squared area in panel a. **c,d |** LTBP-1 immunostaining on fibroblast culture from hypertensive rat heart (SHR). d panel is a higher magnification (40X) of black squared area in panel c. Red arrows indicate extracellular pattern distribution of LTBP-1 in hypertensive fibroblasts. **e |** Western blot analysis of LTBP-1 in fibroblast protein extracts of both rat strains, under basal condition (white bar graph) and after TGF-β1 treatment (black bar graph). Tubulin was used as loading control.

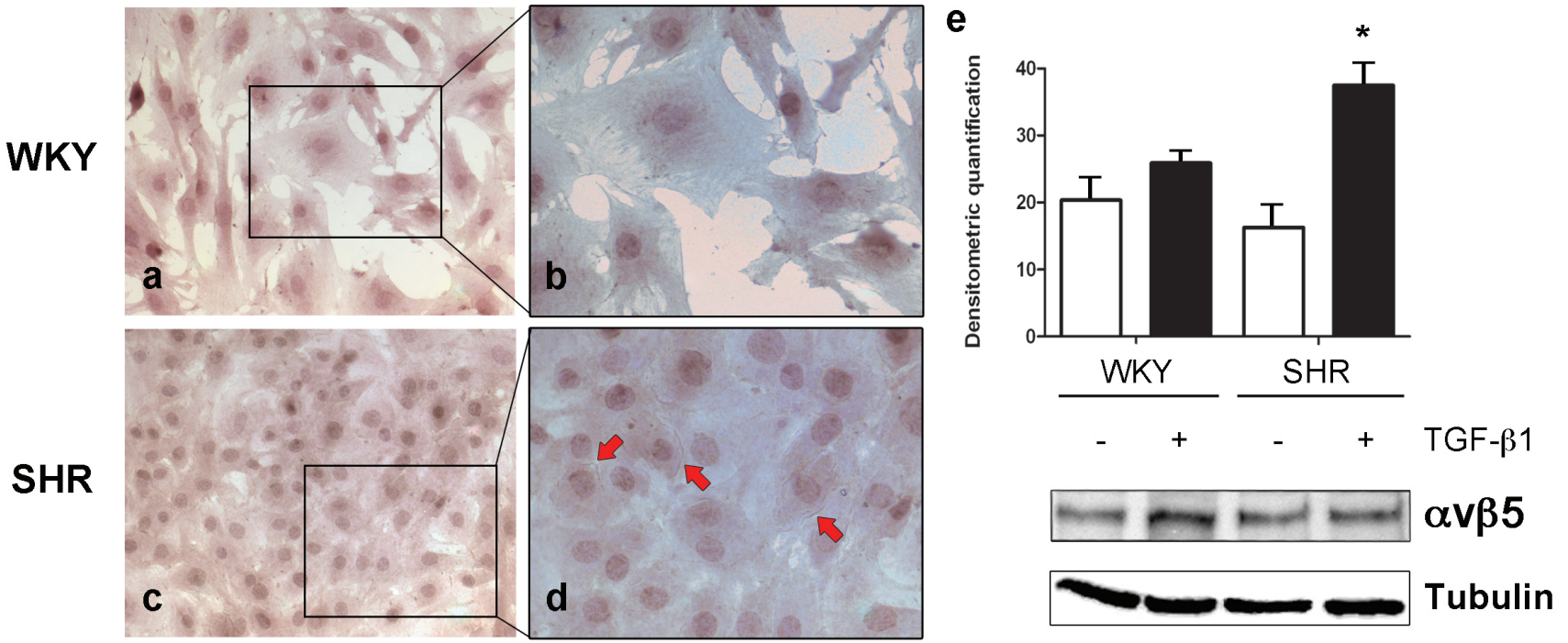

Figure 3.9 | αvβ5 immunostaining and western blotting analysis on isolated primary cardiac fibroblasts. a,b | αvβ5 immunostaining on fibroblast culture from normotensive rat heart (WKY). b panel is a higher magnification (40X) of black squared area in panel a. **c,d |** αvβ5 immunostaining on fibroblast culture from hypertensive rat heart (SHR). d panel is a higher magnification (40X) of black squared area in panel c. Red arrows indicate pattern distribution of αvβ5, alonh cell membrane of hypertensive fibroblasts. **e |** Western blot analysis of αvβ5 in fibroblast protein extracts of both rat strains, under basal condition (white bar graph) and after TGF-β1 treatment (black bar graph). Tubulin was used as loading control.

4. Discussion

The innovations introduced in this study concern two different aspects.

The first novelty regards the description of an alternative protocol for cardiac fibroblasts extraction from tissue samples. The isolation protocol is simpler, faster and more efficient then those commonly used in research (e.g. cardiac perfusion in Langerdorff system). Nevertheless, comparing more similar protocols, i.e. protocols which involve the use of different mixtures of collagenases, the employment of Liberase with high concentration of Thermolysin allows to further shorten the digestion time (only 15 minutes), with a good fibroblast yield **(Figure 3.2)**.

The second novelty regards the identification of the "traction model" in the heart as key mediator of fibrosis. Although results of this study on cardiac tissue from normotensive (WKY) and hypertensive rats (SHR) reveal and confirm previously shown data about association between fibrotic phenotype in the cardiac tissue and pressure-overload hypertrophy [73], for the first time are provided new insights into the mechanism of TGF-β activation by the "traction model". This mechanism involves 1) initial production of TGF-β; 2) fibroblast conversion into myofibroblast; 3) subsequent myofibroblast contractile activity; 4) increase in ECM deposition; 5) further TGF-β activation by a selective binding between integrins (αvβ3, αvβ5) and proteins of TGF-β latency complex (LAP and LTBP-1).

Several new findings establish myofibroblast contraction as a novel mechanism to directly activate latent TGF-β1 in the ECM. Indeed, it has been reported that fibroblasts, as well as myofibroblasts, which in α-SMA expression was downregulated, were less efficient in TGF-β1 activation in comparison with with α-

SMA–positive counterparts [49], because of their low contractile capability, So, mechanical tension is an essential prerequisite for TGF-β1 activation. Additionally, it was reported that stimulation of myofibroblast contraction with AngII, endothelin-1, and thrombin increases the level of active TGF-β1 independently from protease activity [49]. However, this particular mechanism has not been investigated in a pathological context, such as hypertension. The gap was filled in this project. In particular, in this study both cardiac tissue and isolated fibroblasts of normotensive WKY rats (as control) and SHR (as pathologic model) were compared.

As expected, only hypertensive stimulus has effects on collagen deposition **(Figure 3.3)** and TGF-β1 **(Figure 3.4)** expression, but, surprisingly, also on proteins involved in "traction" model, i.e. LTBP-1 **(Figure 3.5)** and αvβ5 integrin **(Figure 3.6)**.

Cells isolated from the two rat strains also have shown different behaviour. Cells isolated from WKY heart were visibly more similar to the classic fibroblasts (quickly growing in plate, rapidly reaching 100% of confluence, and forming monolayer). On the contrary, SHR fibroblasts presented a slow growth, as slowly reached the confluence, that is a typical aspect of differentiating cells. Moreover, SHR fibroblasts have not shown the typical spindle cell shape of fibroblast, but morphologic features more similar to myofibroblast, such as stellate, oblong or triangular, shape [79, 80]. This morphological analysis has been confirmed by the higher α-SMA positivity in fibroblasts derived from hypertensive rat hearts, compared to WKY **(Figure 3.7)**.

On the basis of results of this study, LTBP-1 was more expressed by SHR cardiac tissue and fibroblasts; moreover, treatment with TGF-β1 enhances LTBP-1 protein expression. However, one of most important result was revealed by immunohistochemical analysis: LTBP-1 localizes in the extracellular space only in

SHR fibroblasts **(Figure 3.8 d)**, while in WKY fibroblasts LTBP-1 is not only produced in smaller quantities **(Figure 3.7 e)**, but also not secreted **(Figure 3.7 b)**.

In a similar manner, immunohistochemistry analysis for αvβ5 integrin on cell culture has a peculiarity: the protein is clearly visible along the membrane of SHR fibroblasts **(Figure 3.9 d)**. On the contrary, in WKY fibroblasts, αvβ5 integrin protein is only spread within the cytosol **(Figure 3.9 b)**. In light of these findings, it can be speculated that the hypertensive stimulus *per se* is sufficient 1) to induce the expression of "traction model" components; 2) to lead LTBP-1 and αvβ5 integrin in the correct localization (LTBP-1 in ECM and αvβ5 along cell membranes) and 3) to elicit a pro-fibrotic state.

Until now, activation of latent TGF-β1 by cell traction has been suggested exclusively for the epithelial αvβ6 integrin [46], which is involved in the initiation of lung fibrosis [54]. During fibrosis of epithelialized tissues, such as kidney and lung, myofibroblasts are partly recruited through mesenchymal transition of epithelial cells [75] involving αvβ6 integrin– mediated latent TGF-β1 activation. On the basis of results collected in this study, hypothesis of epithelial-to-mesenchimal transition in rat heart should be further deepen, starting from the visible difference in perivascular fibrotic tissue thickening found in SHR in comparison with WKY **(Figure 3.9 c,f)**. It should be possible, indeed, that endothelial cells, subjected to hypertensive stimulus, undergo to switch into mesenchimal cells, as previously described in 2007 by Zeisberg *et al.* In the study authors proposed that hemodynamic stress could trigger a pro-inflammatory process in the vicinity of the intracardiac vasculature, leading to the activation of endothelial cells; these cells, in turn, can produce fibrogenic mediators and induce fibroblast activation and myocardial fibrosis. Moreover, Zeisberg *et al.* speculate on further activities of TGF-β, not only involved

in activation of endothelial cells to produce fibrogenic mediators, but also to allow exactly this endothelial-to-mesenchimal transition [75].

This present study provides, for the first time, evidences on involvement both *in vivo* and *in vitro* of “traction model” in cardiac tissue. Altogether, it is now clear that during hypertension 1) cardiac fibrosis has TGF-β as initial trigger and key mediator; 2) cardiac fibroblasts switch into myofibroblasts; 2) the expression and the activity of all the components of the “traction model” are exarcebated. Interstitial and perivascular deposition of collagen and spotted distribution pattern of TGF-β expression in cardiac tissue supports the hypothesis for an autocrine/paracrine positive feedback loop in the production and activation of TGF-β, initially borne by the fibroblasts and subsequently carried out by differentiated myofibroblasts.

In conclusion, results of this study support the involvement of the “traction model” in cardiac fibrosis development, but further aspects must be investigated in future.

Future prospects regard the validation of the data presented to fully provide new insight into the mechanisms by which TGF-β, through the “traction model”, orchestrate the fibrotic process during hypertension. Elucidation of this mechanism may offer new therapeutic targets for the treatment of targets for the treatment of many disease characterised by fibrosis.

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