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Treatment with integrin α_v inhibitor abolishes compensatory cardiac hypertrophy due to altered signal transduction and ECM gene expression

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Die Promovendin ist Ärztin

Für meine geliebten Eltern

Content

1. INTRODUCTION

1.1. Hypertrophy in cardiac remodelling

1.1.1. Definition of cardiac hypertrophy

Cardiac hypertrophy is the cellular response to an increase in biomechanical stress, such as arterial hypertension or alveolar heart disease. The essential cardiac response to a fixed increase in hemodynamic load is an increase in cardiac mass. This frequently results in cardiac hypertrophy, which is induced by the mechanical stress on the cardiomyocytes and the activation of neuroendocrine mechanisms, particularly the renin-angiotensin-aldosterone system and the sympathetic nervous system. The defining features of hypertrophy are an increase in cardiomyocyte size, enhanced protein synthesis, and a higher organization of the sarcomere. The complex molecular processes that lead to cardiomyocyte growth involve membrane receptors, second messengers, and transcription factors. The common final step of all these intracellular pathways is gene expression, whose variations are being revealed in increasing detail. Myocardial hypertrophy represents an independent risk factor for cardiovascular events such as ischemic heart disease, arrhythmias, and sudden death and is a powerful predictor for the development of heart failure. (Levy, Garrison et al. 1990).

A. Model of normal heart B. Model of hypertrophic heart

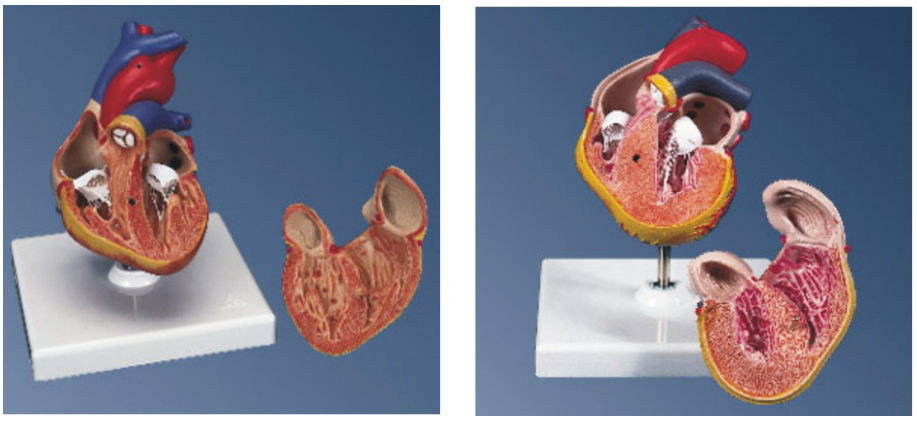

Figure 1.1. Artificial model of cardiac hypertrophy. A: It shows a normal structure of heart, The front heart wall is detached to reveal the chambers and valves inside. B: It shows the long-term effects of increased heart activity due to high blood pressure. The muscular wall of the left hear ventricle is considerably thickened and the tip of the heart is visibly rounded.

1.1.2. Cardiac hypertrophy: compensatory response versus maladaptation

Development of cardiac hypertrophy is initially beneficial since it augments the number of contractile units and reduces ventricular wall stress to normal levels according to the law of Laplace (Basford 2002). It is generally accepted that cardiac hypertrophy can be adaptive in some situations, specifically in athletes. Howerer, it is not clear if a hypertrophic response to pathological situations, such as chronic arterial hypertension or a myocardial infarction, is initially a compensatory response (that only later becomes mal-adaptive) or if this type of myocardial growth is detrimental from the beginning. It has been reported that different types of cardiac hypertrophy differ both on the morphological as well as the molecular level: Exercise-induced hypertrophy is typically not accompanied by an accumulation of collagen in the myocardium (Medugorac 1980), the hypertrophic response usually shows a modest increase in ventricular wall thickness. However, some hypertrophic pathways, such as calcineruin-dependent signalling, appear to be activated in both pathological and physiological exercise-induced hypertrophy (Ritter, Hack et al. 2002; Vega, Yang et al. 2002).

A long-standing hypothesis has been that hypertrophy is compensatory by normalizing wall stress and acts to maintain normal cardiac function. Epidemiological data, however, have shown that cardiac hypertrophy is associated with increased mortality.The beneficial effect of myocyte hypertrophy as a response to increased work is limited. In pathologic states, at some point in time, the hypertrophy itself contributes to a decline in function and the development of heart failure. The enlarged myocytes have increased metabolic requirements with inadequate vasculature. When the heart can no longer keep up with the demand, it begins to dilate. Dilatation with stretching of the myocytes, while initially improving contractility (Frank-Starling mechanism), becomes detrimental at a certain point. Contractility sharply decreases with the onset of failure. Although hypertrophy in response to pathologic signalling has traditionally been considered an adaptive response required to sustain cardiac output upon stress, prolonged hypertrophy is associated with a significant increase in the risk for sudden death or progression to heart failure, independent of the underlying cause of hypertrophy (Levy, Garrison et al. 1990; Vakili, Okin et al. 2001),suggesting that the hypertrophic process is not entirely beneficial. The view is further supported by using some inhibitor of hypertrophy in clinic trials, such as a calcium antagonist, nicardipine SR, and an angiotensin-

converting enzyme (ACE) inhibitor, both reduced left ventricular hypertrophy and improved left ventricular systolic and diastolic function (Sumimoto, Ochi et al. 1997), reduced the risk for several endpoints like death and progression to heart failure, whereas persistence of hypertrophy revealed adverse results (Mathew, Sleight et al. 2001).

The resulting hypertrophy, as described before is the product of the thickening and shortening of the muscle fibers of the heart. It becomes, thereafter, more difficult to relax and go through the normal cycle of contraction and relaxation. It appears that in the myocardium actual changes in the collagen content result in increased stiffness. There is an impaired diastolic relaxation, but also heightened vulnerability to ischemic events. The end result is decreased cardiac output, and inability to meet the circulatory needs of the body. The eventual inability to perfuse the body is called heart failure. Although clinically distinct, hypertrophy and heart failure have many molecular characteristics in common. In addition, hypertrophy of cardiac myocytes and fibrosis are almost universal prerequisites for heart failure. (Hunter and Chien 1999; Schnee and Hsueh 2000; Ritter and Neyses 2003). Therefore, the molecular pathways that lead to hypertrophy can be considered as the initial steps of heart failure. If hypertrophy in response to stress is entirely maladaptive, it is important to reveal the underling molecular events and eventually development strategies to prevent or reverse the hypertrophic response. Thus, the subsequent development of heart failure could be circumvented at the initial stage. To understand the molecular mechanisms that underlie adaptive and maladaptive cardiac hypertrophy, investigation has centered around a characterization of intracellular signal transduction pathways that promote cardiac myocyte growth.

1.1.3. Inhibition of cardiac hypertrophy

As we mentioned above, some of the genetic and exogenous inhibitors of cardiac hypertrophy have been identified previously. Initiating hypertrophy as well as inhibiting hypertrophy involves multiple signalling pathways. Considering that good control of hypertension leads to reduced mortality, then intervention at any regulatory molecular events related to hyperthrophy would also end in a benefitial cardiac phenotype? The available data suggest that this is not the case. Studies showed that the treatment with different inhibitors of cardiac hypertrophy can have different functional consequences, although they may have a similar degree of inhibition of

cardiac hypertrophy. (Frey and Olson 2003) For example, it was reported that a genetically altered mouse model with an attenuated hypertrophic response to 8 weeks of pressure overload prevent cardiac dysfunction despite increased wall stress. (Esposito, Rapacciuolo et al. 2002). In contrast, overexpression of RGS4, a GTPase-activating protein for G proteins, which reduced cardiac hypertrophy to a similar degree as the inhibition mentioned above, led to rapid decompensation in most animals characterized by left ventricular dilatation, depressed systolic function, and increased postoperative mortality. (Rogers, Tamirisa et al. 1999). These results suggested that hypertrophic response is not always associated with contractile (dys-) function.

Research of inhibition of cardiac hypertrophy may eventually make it possible to exploit therapeutically desired aspects of hypertrophy (i.e., induction of certain gene, increased sarcomere organization or improvement of contractility) while inhibiting others (i.e., increase of heart wall, which associated with adverse events such as arrhythmia). In this regard, there have been several reports indicating beneficial effects of matrix metalloproteinase (MMP) inhibitors on heart failure(Spinale, Coker et al. 1999).

1.1.4. Alterations in gene expression of cardiac hypertrophy

Recent technological advances in the production of cDNA micro arrays have made it possible to profile gene expression of tens of thousands of genes simultaneously (Brown and Botstein 1999). Over the past decade, the importance of inherited gene defects in the pathogenesis of primary cardiomyopathy has been recognized, with mutations in 18 genes now known to cause either HCM and /or dilated cardiomyopathy (DCM) (Fatkin and Graham 2002). Cardiac hypertrophy is accompanied by distinct qualitative and quantitative changes in gene expression. Specific changes have been observed in cardiomyocytes during the development of cardiac hypertrophy (Cooper 1987; Ruwhof and van der Laarse 2000) e.g. quantitative and qualitative changes in gene expression, rapid induction of proto-oncogenes (such as c-fos. C-jun, and c-myc) and heat shock protein genes (such as hsp 70). which are the first response to hemodynamic overload (Izumo, Nadal-Ginard et al. 1988). As a later event, the expression of several genes that encode sarcomeric proteins is switched to expression of fetal isoforms, for example transition from cardiac α-actin to skeletal α-actin. Hypertrophy is characterized by an increase

in cell size in the absence of cell division, and a switch from the α-form of myosin heavy chain (MHC) to the β-MHC form in rodents (Schwartz, de la Bastie et al. 1986). In addition, several changes in isogene expression of proteins involved in energy metabolism have been described (Revis, Thomson et al. 1977; Meerson and Javich 1982). Furthermore, the expression of atrial natriuretic peptide (ANP) that is restricted to the atria shortly after birth, is reappearing in the ventricles upon hemodynamic overload (Mercadier, Samuel et al. 1989). Besides the qualitative changes in gene expression described above, there are also quantitative changes in constitutive expression of genes, i.e. stimulation of gene expression which contributes to hypertrophy, and down-regulation of genes. Several genes that encode membrane proteins are down-regulated in hypertrophied hearts, for example, the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) gene (Anger, Lompre et al. 1998). The rate of protein synthesis is probably increased by a different mechanism although its involvement in the development of cardiac hypertrophy has to be proven.

1.2. Mechanisms and signal transduction pathways of cardiac hypertrophy

Cardiac hypertrophy is a well known response to increase hemodynamic load. Mechanical stress is considered to trigger a growth response in the overloaded myocardium. Furthermore, the mechanical stress induces the release of growth-promoting factors, such as angiotensin II, endothelin-1, and transforming growth factor-β (Zolk and Bohm 2000; Ritter, Schuh et al. 2003; Wolf, Jablonski et al. 2003), which promote a second line of growth induction.

1.2.1. Stimuli inducing cardiac hypertrophy

1.2.1.1. Mechanical stress

Mechanical stress or an accompanying increase in neural or humoral factors have been considered the primary stimulus for cardiac hypertrophy. However, cardiac hypertrophy can be induced by hemodynamic overload even after adrenoreceptor blockade (humoral) or sympathectomy (neural)(Cooper, Kent et al. 1985), suggesting that mechanical stress itself is the primary factor for cardiac hypertrophy in response to hemodynamic overload. The view was supported by several ex vivo and in vitro studies. Kiral et al demonstrated that in isolated hearts, an increased cardiac load stimulated protein synthesis (Kira, Kochel et al. 1984). Furthermore, alterations in gene expression in stretching cultured cardiomyocytes were observed without involvement of neural or humoral factors (Komuro, Kaida et al. 1990; Sadoshima, Jahn et al. 1992; Kira, Nakaoka et al. 1994; Vandenburgh, Solerssi et al. 1995). Moreover, the alterations in vitro experiments using stretched cardiomyocytes have been demonstrated to be similar to those in vivo in hearts in response to hemodynamic overload, i.e. re-expression of fetal genes and increased protein synthesis do also occur in isolated cells in vitro. These findings demonstrated that mechanical stress is possibly the main contributor to the hypertrophy.

1.2.1.2. Growth factors and hormones

Besides mechanical loading, a range of substances, such as growth factors, cytokines, catecholamines, vasoactive peptides and hormones are involved in

mediating cardiac myocyte hypertrophy (Hefti, Harder et al. 1997).(Sacca, Napoli et al. 2003) The expression and /or release of these factors have been reported in hearts that hypertrophied due to hemodynamic overload, and in cardiomyocytes that were hypertrophied due to stretch. The classic peptide growth factors can be divided into five families: epidermal growth factor (EGFs,) Fibroblast growth factor (FGFs), Insulin-like growth fachor (IGF), platelet derived growth factor (PDGFs) and transforming growth factor (TGFs). TGFβ and PDGF have been shown to cause cardiomyocyte hypertrophy in vitro systems. In the heart, PDGF is produced by non-myocytes. FGF and TGFβ can be produced by cardiac myocytes and non-myocytes (Brand and Schneider 1995; Seger and Krebs 1995), therefore, these two factors may act in a paracrine or autocrine manner in the heart. Smad proteins have been identified as the first family of putative TGF-β signal transducers (Kretzschmar, Liu et al. 1997). Upon phosphorylation by activated receptors, Smads associate with DNA binding proteins and activate gene transcription. Thus, cardiac myocytes and other cell types, such as cardiac fibroblasts, endothelial cells and vascular smooth muscle cells, may secrete growth promoting factors after a mechanical stress stimulus, which induce hypertrophy of cardiomyocytes in an autocrine/paracrine way.

1.2.2. Signal tansducton of the stretch stimulus

The signal transduction pathways that may be involved in mechanical stress-induced hypertrophy belong to two groups: (1) the mitogen-activated protein kinases (MAPK) pathway; and (2) the janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway.

1.2.2.1. The mitogen-activated protein kinase (MAPK) pathway

The MAPK superfamily is a widely distributed group of enzymes that can be divided in several subfamilies. The three characterized MAPK cascades are: 1) the extra cellular-regulated kinases (ERKs) 2) the c-Jun N-terminal kinases (JNKs); and 3) the p38 MAPKs cascade. The latter two belong to the group of stress-activated protein kinases (SAPKs) (Ruwhof and van der Laarse 2000).

The extracellular-regulated kinase (ERK) pathway.

The best characterized are: the 44-kDa MAPK (ERK1), the 42-kDa MAPK (ERK2), and the 63-kDa MAPK (ERK3). In the heart, ERK1 is the most highly expressed

ERK. The expression of ERK3 is hardly detectable in adult heart. Substrates for ERKs are transcription factors, such as c-*jun*, and $p62^{TCF}$ (Elk-1), and the 90-kDa S6 kinase ($p90^{RSK}$)(Boulton, Nye et al. 1991). Mechanical stress has been reported to stimulate this pathway by activation of Ras, ERK1 and ERK2, and $p90^{RSK}$ (Sadoshima and Izumo 1993).The precise mechanism of the stimulation of this pathway is still unknown. Moreover, stretch of cardiomyocytes caused activation of ERKs and resulted in increased expression of c-*fos* and skeletal α-actin, indicating that ERKs and mechanical stress-induced hypertrophy may be linked (Sadoshima and Izumo 1993) Taken together, these results indicate that the ERKs may partly participate in the mechanisms of mechanical stress-induced hypertrophy.

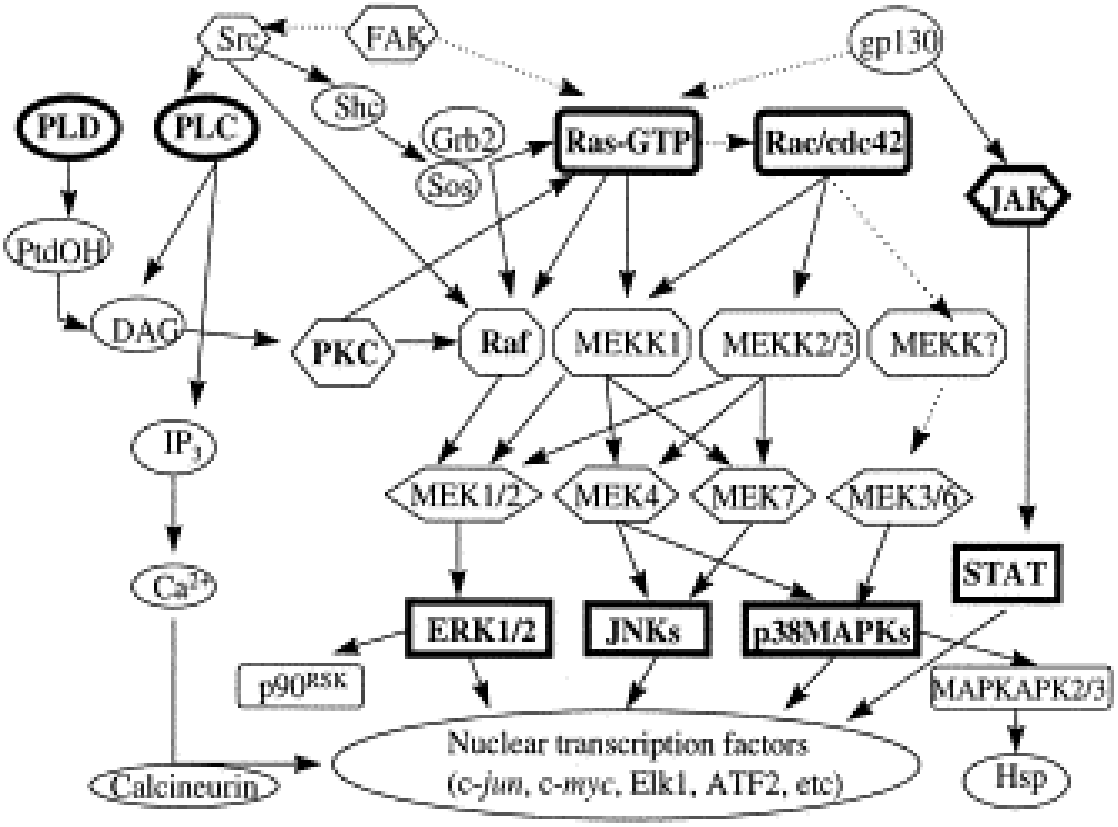

Fig 1.2. Signal transduction pathways possibly involved in mechanical stress-induced hypertrophy. These include two major pathways: the MAPK pathway and the JAK/STAT pathway. The MAKP pathway is a three-module cascade of phosphorylating kinases: MEKK→MEK→MAPK. This pathway consists of several subfamilies among which the ERK pathway, the JNK pathway and the p38 MAPK pathway. They are activated by heterotrimeric G proteins coupled to membrane receptors, by small G proteins (such as Ras, cdc42, Rac), by protein kinases (such as Src and FAK), by PKC via activation of PLC or/and PLD, or by JAKs via gp130. Their downstream targets are cytosolic kinases (such as $p90^{RSK}$, and MAPKAPK2/3) and nuclear transcription factors (such as c-jun, c-myc, and Elk1). The JAK/STAT pathway is directly activated probably via gp130. Upon activation of STATs by JAKs, STATs translocate to the nucleus and induce gene transcription. In addition, a direct pathway linking mechanical stress to gene expression has been considered to be operative via the cytoskeleton. The involvement of calcineurin in the development of hypertrophy is still controversial. The dashed lines refer to poorly understood mechanisms. (Ruwhof and van der Laarse 2000)

The c-Jun N-terminal protein kinase (JNK) pathway

The JNK pathway may be involved in mechanical stress-induced hypertrophy via phosphorylation of the transcription factors c-Jun, and ATF2 (Clerk and Sugden 1997). This activation of JNKs was independent of secreted Ang II, extracellular Ca^{2+}, and PKC. Others have found that cardiomyocytes submitted to cyclic stretch had a maximal activation of JNKs at about 5 min. Using MEKK1-transfected

cardiomyocytes, Thorburn et al. (Thorburn, Xu et al. 1997) showed that over expression of MEKK1 induced ANP expression (a marker of hypertrophy). Furthermore, these authors found that the small G protein Rho was also required for MEKK1-induced ANP expression (Thorburn, Xu et al. 1997). Controversially, Nemoto et al. found that activation of JNKs inhibited MEKK 1-induced ANP expression via a feedback loop of c-jun. They reported that ANP expression is activated by p38 MAPKs. Taken together, it seems that the induction of ANP expression is biphasic, first a short-living response upon JNK activation followed by a prolonged response upon p38 MAPK activation

The p38 MAPK pathway

The function of the p38 pathway in myocardial hypertrophy has recently been studied. It has been observed that p38 and p38β activity were increased in a mouse model of pressure overloading and that the p38 and p38 β activity were well correlated with phenotypic ventricular hypertrophy (Wang, Harkins et al. 1997). In these experiments, by introducing constitutively active MKK6 or MKK3 into cardiac myocytes they elicit characteristic hypertrophic responses, including an increase in cell size, enhanced sarcomeric organization, and elevated atrial natriuretic factor expression. The two isoforms of p38 group kinase, p38 and p38β, appear to have different effects on cardiomyocyte hypertrophy: p38β seems to be more potent in inducing hypertrophy, whereas p38 appears to be more important in other function such as apoptosis. Interestingly, the hypertrophic response induced by p38 MAPKβ included several markers of hypertrophy, i.e. an increase in cell surface area, enhanced organization of sarcomeric proteins, and induction of ANP expression (Wang, Huang et al. 1998).

1.2.2.2. The janus kinase/signal transducers and activators of transcription

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway

Janus –associated kinases are named after their dual functions in signal transduction in the cytoplasm and activation of transcription in the nucleus. Binding of ligands to their cytokine receptors, such as dardiotriphin-1 (CT-1), leads to phosphorylation and activation of the receptor-JAK complex with subsequent recruitment of STATs and

activation of STATs by phosphorylation. The JAK/ STAT pathway was found to be activated in rat hearts with pressure overload-induced hypertrophy (Pan, Fukuda et al. 1997). Mechanical stretch induced rapid phosphorylation of JAK1, JAK2, Tyk2, STAT1, STAT3, and glycoprotein 130 was observed as early as 2 minutes and peaked at 5 to 15 minutes after strech. (Tian, Cui et al. 2004).

1.2.2.3. Calcineurin-dependent pathway

Recently, a new novel hypertrophic signaling pathway has been revealed that cardiac hypertrophy can be induced by the Ca^2 /calmodulin-dependent phosphatase calcineurin. Constitutive activation of calcineurin in transgenic mouse heart is sufficient to induce massive cardiac hypertrophy and eventually heart failure that could be prevented by cyclosporine, an inhibitor of calcuineurin (Molkentin, Lu et al. 1998). A similar¸ but less dramatic, response was obtained by overexpression of a constitutively nuclear NFAT3 mutant, suggesting that NFAT is also a principal target of calcineurin- dependent signalling in cardiomyocytes. This involvement of calcineurin in development of cardiac hypertrophy was conformed by Ritter et al that hypertrophy in HOCM and AS without heart failure is characterized by a significant increase in calcineurin activity, suggesting an involvement of this pathway in the pathogenesis of human cardiac hypertrophy.(Ritter, Hack et al. 2002). Although these studies demonstrated that activation of the calcineurin/NFAT pathway is sufficient for the development of cardiac hypertrophy, whether calcineurin is also necessary in this process has been unclear. Confusion surrounding this issue has arisen largely from conflicting results of treatment of calcineurin inhibitors cyclosporine A and FK 506 in several models of hypertrophy. (Molkentin 2000; Olson and Williams 2000; Leinwand 2001) i.e., CsA showed to prevent hypertrophy of neonatal rat cardiomyocytes in response to stimulation with angiotension II (AngII) in vivo. Moreover, Ritter et al found that the C-terminus of calcineurin A protein containing the autoinhibitory domain was less abundant in myocardial hypertrophy than in normal heart (Ritter, Hack et al. 2002). Whether calcineurin activation plays a crucial role in the development of overload –induced cardiac hypertrophy, remains uncertain at this time

1.3. Implication of integrins in cardiac hypertrophy.

During the development of hypertrophy in response to increased hemodynamic overload, transduction of mechanical stress into biochemical signals is at least in part mediated by a group of cell-surface receptors called integrins, which links extracelluar matrix to the cellular cytoskeleton (Li, McTiernan et al. 2000).

1.3.1. Integrin structure and expression in cardiac hypertrophy

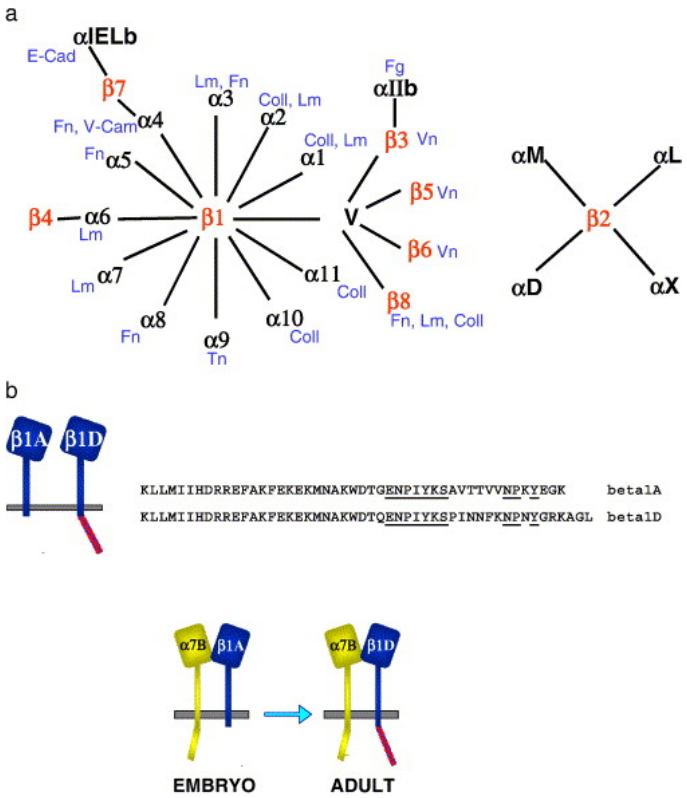

Fig 1.3 The integrin family of adhesion receptors and their main ligands. The integrin family and muscle specific isoforms. a) Mammalian integrins comprise 8 β and 18 α subunits. Based on their association in heterodimers, the integrin family can be clustered in the β1 and αV subgroups that are ubiquitously expressed and the β2 subgroup selectively expressed in leukocytes. The bars connecting different α and β subunits indicate all known heterodimers. Each integrin heterodimer binds to specific extracellular matrix proteins or cell surface counter receptors (ligands for the β2 subgroup are omitted). Adult cardiomyocytes, in particular, express the laminin binding α7β1 as the major integrin heterodimer. b) Muscle cells express β1D, an isoform of β1 integrin subunit generated by alternative splicing and characterized by specific amino acid sequences (indicated by the one letter amino acid code) at the cytoplasmic domain (slashed area). β1D is expressed in newborn and adult cardiomyocytes while embryonic cardiomyocytes express mainly the ubiquitous form β1A. The short horizontal gray bar represents the plasma membrane. Coll: Collagen; E-Cad: E-cadherin; Fg: fibrinogen; Fn: fibronectin; Lm: laminin; Tn: tenascin; V-Cam: vascular cell adhesion molecule; Vn: vitronectin. (Brancaccio, Hirsch et al.2006)

Integrins are transmembrane receptor composed of noncovalently associated α and β subunits, with alpha subunits ranging in size from 120 to 180 kDa whereas β subunits are 90 to 110 kDa.(Ross 2002). The ingegrin family comprises more than 18α and 8β subunits in mammals. More than 24 pairs of integrins have been identified. The subunits of α and β integrin are single transmembrane spanning proteins with the bulk of the molecule in the extracellular domain and generally short cytoplasmic domains. After ligand binding the integrin conformation is altered and subsequently the heterodimer can participate in events critical for organization of the cytoskeleton and other intercellular signalling events that might be important for cell survival or initiation of cardiac myocyte hypertrophic events.

The relative number of specific α and β integrin subunits expressed in the myocardium is small. In particular, α1, α2, α5, α7, α10 and α11 are detected in cardiomyocytes (Ross 2002). These alpha integrin subunits appear to be only associated with splice-variants of β1 integrin, including the splice variant β1D, which is dominantly expressed in striated muscle and is the prime β1 integrin isoform expressed in postnatal heart. Adult rat cardiac fibroblasts express a wide range of integrins including α1β1(most predominant) α2β1, α5β1, αvβ3, αvβ1 and α4β1, αvβ5, αvβ6 (MacKenna, Dolfi et al. 1998). In the adult heart, integrins are the principal receptors for ECM, thus their appropriate expression and function is necessary for the normal cardiovascular function. (Keller, Shai et al. 2001). Disruption of integrin function in the murine myocardium leads to perinatal lethality, fibrosis, and abnormal cardiac performance (Keller, Shai et al. 2001). In vitro studies have shown that disruption of α1β1 function by antibodies or adenovirus mediated inhibition resulted in altered phenotype of cultured myocytes and altered patterning of their myofibrils (Simpson, Terracio et al. 1994).

Integrins belong to the important factors, which are responsible for the process of cardiac hypertrophy. Studies in vitro have directly linked β1 integrin (both isoforms A and D) to the hypertrophic response of neonatal ventricular myocytes. The hypertrophy response caused by α1 adrenergic agents was induced by the overexpression of these integrins.(Pham, Harpf et al. 2000). Overexpression of β1 integrin in the cardiomyocyte was found to increase protein synthesis and ANP expression without affection DNA synthesis. In contrast, inhibition of β1 function and signalling reduced the adrenergically mediated hypertrophy. (Ross, Pham et al. 1998; Pham, Harpf et al. 2000). Studies of integrins function in vivo have begun to be

assessed in the myocardium of both rats and mice provoked to undergo morphologic hypertrophy or myocardial infarction have demonstrated the importance of integrins. In these rodent models, increased expression of β1A and β1D, α3, α7B were detected. α1and α5 integrin subunits behave like many embryonic genes in the ventricle. They are expressed in the embryonic heart, become down-regulated postnatally, and can be re-induced after mechanical loading of the heart through aortic constriction. Pressure overload of the right ventricle in a cat model has demonstrated the mobilization of β3 integrin to the cytoskeletal fraction of lysed myocardial tissue 4 hours after banding. Integrin β3 is present in both the cytoskeletal and membrane bound fractions by 48 hours after pressure overload. However, the levels returned to baseline by one week. (Kuppuswamy, Kerr et al. 1997).

Cardiac fibroblasts express a repertoire of α subunits like that of the cardiac myocyte, but they do not express α_6 and α_7 as these cells have no laminin-containing basement membrane. In contrast, α_v and the collagen-specific α_2 subunit appear to be uniquely expressed by the cardiac fibroblasts but not by cardiac myocytes (Terracio, Rubin et al. 1991; Maitra, Flink et al. 2000).

1.3.2. Integrin-mediate signalling in cardiac hypertrophy

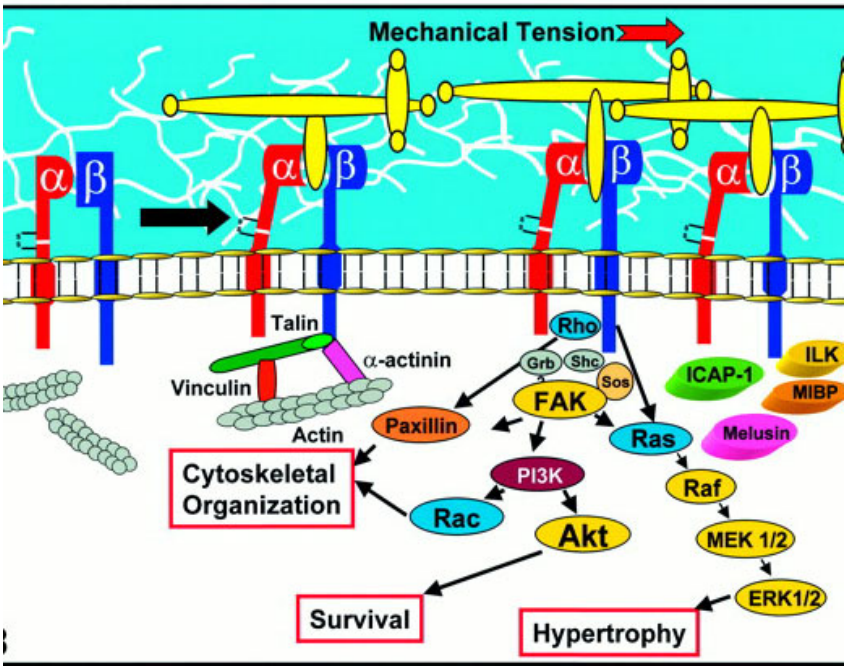

Fig 1.4.Diagrammatic representation of outside-in signaling of integrins. The inactive integrin heterodimer is depicted on the left-most portion of the Figure. After ligand binding (eg, of laminin, shown in yellow), the integrin conformation is altered and subsequently the heterodimer can participate in

events critical for organization of the cytoskeleton and other intracellular signaling events that might be important for cell survival or initiation/propagation of cardiac myocyte hypertrophic events. Thus, mechanical tension outside the cell could be converted to intracellular biochemical signals through the integrins. For this process, the cytoplasmic domain of the integrin subunits signals through a host of molecules such as kinases (FAK, Akt, Raf, MEK, ERK [shown in olive]), cytoskeletal organizers (eg, paxillin), small GTPases (eg, Rho, Rac, Ras [shown in blue]), and other molecules. Cytoplasmic domain–binding proteins have also been identified, some of which are dominantly expressed in striated muscle (eg, MIBP or melusin) but whose function is still incompletely understood. (Ross and Borg 2001).

The role of integrin signalling in myocardium is still incompletely understood. Integrins may be implicated in cardiac hypertrophy as mechanosensors that sense increased work load of the heart muscle. In an integrin (β1)-dependent and matrix-specific way, integrins have been shown as mechanotransducers in cardiac cells. The integrin-dependent pathways involving FAK and Src-family kinases have been studied in some detail. There are two views on the mechanism of integrin mediated signalling, which may be complementary.

Some studies support the first view that integrins transmit signals by organizing the cytoskeleton (actin filaments) through intermediary molecules including α-actinin, talin, vinculin, paxillin, and tensin, thereby stabilizing cell adhesion and regulation cell shape, morphology, and motility. (Ingber 1991; Zhang, Weinheimer et al. 2003). A large body of evidence suggests that mechanical stress is first received by integrins, and that next interlinked actin microfilaments transduce mechanical stress in concert with microtubules and intermediate filaments. Moreover, Bloom (Bloom, Lockard et al. 1996) suggested that this mechanism even could modulate gene expression. They showed that intermediate filaments transmit mechanical stress to the chromatin and hypothesized that alterations in the chromatin induce modulation of gene expression(Bloom, Lockard et al. 1996).

The second view is that integrins are regarded as true receptors capable of inducing biochemical signals within the cell that regulate gene expression and cellular growth, which based on the co-localization of signalling molecules in focal adhesion complexes (FACs). Upon clustering of integrins at focal adhesion sites they recruit the non-receptor kinases FAK and Src. This recruitment is followed by the association and activation of additional cytoskeletal proteins as well as signal-transducing molecules (such as Grb2, Sos, Ras, Raf, PLCγ, ERKs, and SAPKs) in the forming FACs (Miyamoto, Teramoto et al. 1995) In these FACs signalling proteins and their substrates are brought into close proximity, thereby facilitating signal

transduction. In fact, integrins may induce activation of FAK with the help of Src (Parsons and Parsons 1997), which may lead to activation of the ERK pathway through Grb2-Sos-Ras or through activation of PLCγ. Integrins can also collaborate with growth factor receptors and their substrates to phosphorylate their receptor kinases and to activate ERKs and JNKs upon ligand binding (Parsons and Parsons 1997). Thus, integrins may integrate a variety of different signalling pathways that are activated by both the ECM and growth factors to establish a well-coordinated response.

1.3.3. Function of Integrin αv β3 molecule in heart

Increasing evidence suggests that integrins play an important role in cardiovascular remodelling. Integrins that bind to the RGD motif, such as α_v $ß_3$ are important in the angiogenic process. αvβ3 integrin binds a variety of ECM proteins containing the peptide sequence arginine–glycine–aspartate (RGD sequence) including collagen, vitronectin, fibronectin, thrombin, fibrinogen and thrombospondin (Schnee and Hsueh 2000; Kumar 2003).

The primary function of the αvβ3 protein is to bind with fibrinogen for platelet aggregation in blood clots. Mutations in αvβ3 are associated with a variety of diseases incuding Glanzmann's Thrombosthenia and other clotting disorders. αvβ3 integrin can also be implicated in heart attacks and strokes. Inhibitors of these integrins inhibit angiogenesis and may limit tumor growth by thus limiting their blood supply (Brooks, Montgomery et al. 1994). In addition, the $\alpha_{11b}ß_3$ antagonists, which inhibit platelet aggregation, also inhibit the $\alpha_v ß_3$ integrins (Hammes, Brownlee et al. 1996). This effect is thought to play a role in the ability of $\alpha_{11b}ß_3$ antagonists to inhibit restenosis after vascular injury, thus implying a role for α_v $ß_3$ in vascular remodelling (Choi, Engel et al. 1994). Study demonstrated that the expression of $\alpha_v ß_3$ integrin is augmented by Ang II, PDGF, and TGF-$ß_1$ in neonatal cardiac fibroblasts (CFBs). Furthermore, this integrin is involved in the chemotaxis, motility, and adhesion of CFBs. The present findings support the current concept that integrins participate in the control of fibroblast behaviour during cardiac remodelling mechanisms.(Graf, Neuss et al. 2000). RGD-dependent integrins also appear to play a role in cardiac fibroblasts actions that contribute to remodelling in the heart. Our previous studies have demonstrated that integrin αv subunit is required during the wound healing following cardiac infarction and that it does so by modulating the expression of a

matrix protein: SPARC (secreted protein, acidic and rich in cysteine), and eventually effecting migration of fibroblasts (Wu, Laser et al. 2006). However, little is known about the contribution of integrin αv-subunit in the development of hypertrophy. Knowledge of integrin αv mediated signal transduction and ECM gene expression in pressure overloaded heart is unclear.

1.4. Extracellular matrix in heart hypertrophy and failure

The development of cardiac hypertrophy and the progression of the disease towards heart failure include hypertrophy of cardiomyocytes, hyperplasia of fibroblasts, changes in the composition of interstitial tissue or ECM and ventricular remodelling. The cardiac ECM is defined as a network surrounding and supporting the cells which make up the myocardium (Bendall, Heymes et al. 2002). Cardiac remodelling upon pressure overload is generally associated with changes in the expression in extracellular matrix (ECM) proteins as well as their transmembrane receptors, the integrins. It is well known now that the ECM provides a structural, chemical, and mechanical substrate that is essential in cardiac function and responses to pathophysiological signals. Modifications of various elements of the ECM are associated with cardiac hypertrophy and heart failure, and the molecular events bringing a better insight into the dynamics of the ECM.

1.4.1. Extracelluar matrix and cardiac fibrosis

The accumulation of proteins of the ECM leads to fibrosis, Myocardial infarction, hypertrophy, hypertension and heart failure are well known to be associated with progressive cardiac fibrosis. The accumulation of collagen I-III, which occurs in the hypertrophied heart and /or in the heart failure, is the major constituent of fibrosis. Myocardial fibrosis due to maladaptive extracellular matrix remodelling contributes to dysfunction of the failing heart. Interstitial fibrosis contributes to ventricular wall stiffness and consequently impairs cardiac compliance, resulting impaired diastolic function (Doering, Jalil et al. 1988; Jalil, Janicki et al. 1989). Neither the ECM nor the fibroblasts contribute to systolic contraction, increased ECM and fibroblast number means that systolic work is being performed by a smaller proportion of the cardiac mass, contributing to systolic dysfunction. Interstitial fibrosis leads to increased distance that oxygen must diffuse and therefore potentially lowers

Partial Pressure of Oxygen in Arterial Blood (PaO_2) for the working myocytes (Sabbah, Sharov et al. 1995). Moreover, electrical coupling of the cardiomyocytes may be impaired by the accumulation of ECM proteins and fibroblasts since such accumulation causes morphologic separation of myocytes (Weber and Brilla 1991). Taken together, changes in the ECM profoundly affect myocyte metabolism and performance, and ultimately, ventricular function. Investigations should address whether approaches that attenuate fibrosis can improve ventricular function and remodelling

When cardiac mass and shape are altered in response to increased workload or stress, two major cell types are involved: myocytes and interstitial fibroblasts. In pathologic hypertrophy, such as occurs in the setting of systemic hypertension, extracellular connective tissue increases relative to myocytes, and in addition there is a change in the type of collagen that is produced. This may contribute to decreased chamber compliance, with an adverse effect on left ventricular filling. Capillary growth does not keep up with myocyte growth. This results in an increase in intercapillary distances, a factor which contributes to impaired oxygen diffusing capacity and an increased susceptibility of the hypertrophied myocardium to ischemia. The stimulated myofibroblast proliferates and increases its production of ECM proteins, including fibronectin, laminin, and collagen I and III. As the process continues, there is progressive fibrosis, which is a major component of the remodelling process(Weber, Brilla et al. 1992). The volume of the heart that is fibrotic is increased relative to the volume that is muscle compared to these volumes in normal heart (Weber, Brilla et al. 1992). The fibrosis impairs myocyte contractility, oxygenation, and metabolism, thus contributing to ventricular dysfunction. Ultimately, compensated hypertrophy becomes decompensated, resulting in ventricular failure, although events that trigger decompensation are unknown. It is likely that fibrosis contributes to the decompensation. Boluyt et al (Boluyt, O'Neill et al. 1994). demonstrated that hearts from spontaneous hypertensive rats with failure expressed increased TGF-ß, fibronectin, and collagen compared to hearts from spontaneous hypertensive rats without failure. Senescence

1.4.2. Functions and modulation of different component of ECM

The cardiac ECM is defined as a network surrounding and supporting the cells which make up the myocardium. The main components of the ECM include. Structural proteins, such as collagen types 1 and 111 and elastin; adhesive proteins, such as laminin, fibronectin and collagen types 1v and vi; anti-adhesive proteins, such as SPARC, tenascin, thrombospondin and thrombospondin; proteoglycans and enymes, such as metalloproteinases, which regulate the organisation and composition of ECM. The structural proteins of the ECM interact at the cellular level with integrins, the transmembrane receptors of the ECM.

1.4.3. Different elements of cardiac ECM and their functions

Collagen

Collagens, the major components of extracellular matrix, are a tightly regulated family of proteins that determine the structural and functional integrity of heart. It is one of the long, fibrous structural proteins whose functions are quite different from those of globular proteins such as enzymes. It is tough and inextensible, with great tensile strength Among the 5 types of collagen (I, III, IV, V and VI) identified in the heart. Collagens I and III represent more than 90% of the total collagen and support the myocardial structure. In the cares of dilated cardiomyopathies, the network of collagen is degraded by proteases, such as the metalloproteinases (MMP), resulting in alterations of myocyte adhesion to the basement membrane and leading to a focal loss of myocyte continuity with the ECM and a deceased capacity of sarcomere shortening to be efficiently translated into muscle contraction. (Spinale 2002).

Excessive collagen deposition or pathological fibrosis is an important contributor to left ventricular (LV) dysfunction and poor outcome in hypertension, MI and heart failure. It is also an important problem in the aging heart. Anti fibrotic agents that target steps in the collagen synthesis and degradation pathways therefore represent promising strategies for these diseases. Because reparative fibrosis is an essential component of healing of the infarct zone (IZ) after MI, loss of collagen fibrils and struts lead to myocyte slippage, ventricular dilation, and progressive contractile dysfunction. (D'Armiento 2002). Failed human hearts examined either at autopsy or explantation invariably exhibit alterations of the ECM primarily due to changes in collagen. The ability of the heart to respond to

mechanical and chemical stimuli by modulating the delicate balance between synthesis and degradation of structural components of ECM has a significant impact on cardiac function.

Fibronectin

Fibronectin is an extracellular adhesion molecule. It exists in two main forms: 1) as an insoluble glycoprotein dimer that serves as a linker in the ECM (extracellular matrix), and; 2) as a soluble disulphide linked dimer found in the plasma (plasma FN). The plasma form is synthesized by hepatocytes, and the ECM form is made by fibroblasts, chondrocytes, endothelial cells, macrophages, as well as certain epithelial cells. (Schwarzbauer 1991). This glycoprotein contains domains able to bind to the cell (the RGD motif), to other ECM proteins and to proteoglycans. *In vitro,* FN is crucial to the three-dimensional organisation of the embryonic heart (Main, Harvey et al. 1992; George, Baldwin et al. 1997)

Fibronectin (FN) is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion. Fibronectin sometimes serves as a general cell adhesion molecule by anchoring cells to collagen or proteoglycan substrates. FN also can serve to organize cellular interaction with the ECM by binding to different components of the extracellular matrix and to membrane-bound FN receptors on cell surfaces. The importance of fibronectin in cell migration events during embryogenesis has been documented in several contexts, e.g.: 1) mesodermal cell migration during gastrulation can be blocked by injection of Arg-Gly-Asp (RGD) tri peptides that block cellular FN receptors (integrins); 2) injection of anti-FN antibodies into chick embryos blocks migration of precardiac cells to the embryonic midline, and; 3) the patterns of FN deposition in developing vertebrate limbs determines the patterns of precartilage cell adhesion to the ECM, thereby specifying limb-specific patterns of chondrogenesis.

Vitronectin

Vitronectin, also known as S-protein, serum spreading factor and epibolin, is a mixture of two monomeric glycoproteins (65 and 75 kD) present in blood and the extracellular matrix (ECM) of many tissues. Vitronectin and fibronectin are the two major adhesive proteins in plasma and serum. Like many other adhesion molecules, vitronectin binds to cells through an interation of the Arg-Gly-Asp (RGD) sequence in

its cell binding domain with vitronectin-specific cell surface receptors, such as integrin αvβ3 and integrin αvβ5.(Nemerow and Stewart 1999).

Interactions with ECM components are mediated principally through the collagen-binding domain of vitronectin. By binding to plasminogen activator inhibitor type I, matrix-bound vitronectin plays a role in regulating pericellular proteolysis. When used as a thin coating on tissue-culture surfaces, vitronectin is useful to promote cell attachment, spreading, proliferation, and differentiation of many normal and neoplastic cells, and to study cell migration (Serini, Valdembri et al. 2006).

SPARC

Osteonectin/SPARC (secreted protein, acidic and rich in cysteine) is a secreted protein and belongs to a group of glycoproteins which includes thrombospondin 1 and 2, tenascins C and X, and osteopontin (Schellings, Pinto et al. 2004). SPARC has been shown to be important in ECM remodelling In monocytic cells, SPARC stimulates production of interstitial collagenase (MMP-1) and gelatinase B (MMP-9) through prostaglandin synthase-dependent signalling (Sage, Decker et al. 1989). In invasive human breast cancer cell lines (MDA-MB-231 and BT549) SPARC induces matrix metalloproteinase 2 (MMP-2) activation, possibly through decreasing levels of tissue inhibitor of MMPs (TIMP-2) (Gilles, Bassuk et al. 1998). SPARC mRNA has been reported to be present in primary human trabecular meshwork cell cultures and explant organ cultures. since ECM remodelling is thought to be important in the regulation of IOP and SPARC has been shown to be involved in ECM remodelling in other tissues. Recently, our study demonstrated that SPARC is involved in wounding healing following cardial infarction and it does so by promoting migration of fibroblasts (Wu, Laser et al. 2006).

1.5. Aim of the study:

1, As outlined above, the hypertrophic process is not entirely beneficial. Several genetic and exogenous inhibitors of cardiac hypertrophy have been described in the past few years, some of which have been demonstrated to be beneficial to cardiac remodelling. The integrins are thought to play an important role in the development of hypertrophy. The integrin αv inhibitor ECM12197, a compound that was studied as an anti-angiogenic and an anti-cancer drug and that is also called cilengitide, may prevent cardiac hypertrophy. The present study was designed to clarify the contribution of the integrin αv subunit in the cardiac remodelling process upon pressure overload (aortic banding).

2, Resent research suggests that initiation and inhibition of cardiac hypertrophy involves multiple signalling pathways. We hypothesize that integrin αv inhibitors may modulate cardiac hypertrophic responses through typical hypertrophic signal transduction by pathways involving FAKFak, Src, and MAPK.

3. In addition to changes in the myocytes, hypertrophy also includes changes in the extracellular matrix and the microvasculature. Modulation of ECM gene expression is thought to be involved in the hypertrophic response. Integrins can signal through the membrane in either direction: The extracellular activity of integrins is regulated from the inside of the cell (inside-out signalling), while integrin binding to the ECM elicits signals that are transmitted into the cell (outside-in signalling). In order to identify the genomic targets of the αv-mediated outside-in-signalling, we performed microarray analyses probing a total of 96 cell adhesion and extracrellular matrix genes. In this study, we want to reveal changes in the components and organization of cytoskeletal proteins, integrins and ECM during development of hypertrophy in vivo,

2. Methods and materials

2.1. Animal

Wild type adult male and female C57/black mice 135 (Charles River, Sulzfeld, Germany), weighing 22-25 g at the beginning of the study were used. All the animals were closely monitored for clinical signs of heart failure by echocardiographic, hemodynamic, and molecular analyses, which were performed at 2 days, 7 days, and one month after aortic banding. All procedures were approved by the institutional animal research committee and kept under conventional conditions in the animal facilities in 'Medizinische Universitätsklinik' University of Würzburg. Experimental were performed in according to the animal care guidelines of the American Physiological Society.

2.2. Animal experiments

2.2.1 Experimental design

The study used wild-type mice.The experimental protocol for aortic banding has been previously described (Rockman, Hamilton et al. 1996) and is routinely performed in our laboratory. Mice are treated in vivo with a specific integrin α_v inhibitor or vehicle via osmotic minipumps inhibitor 1 day prior to the operation. Animals are sacrificed at day 2 and day 7 following aortic banding or sham operation following echocardiography and hemodynamic analysis. Mice with different pressure between right and left carotid artery less than 30mmHg will be excluded from the studies, as they generally do not show typical left ventricular hypertrophic response. Hearts are excised. Left ventricles are isolated and snap-frozen in liquid nitrogen for ECM gene-expression analyses and WB. Part of the heart is used for histopathological analysis.

2.2.2. Pressure overload hypertrophy in mice-

One of the most commonly used surgical intervention for pressure-overload induced hypertrophy is i.e., aortic banding. This system has been very well characterized and proven to be highly reproducible with a low mortality rate of 10-20% or less in experienced hands. Aortic banding is an excellent model system to

evaluate the process of development of left ventricular hypertrophy in response to hemodynamic stress. Furthermore, after several months, a subset of animals progresses into heart failure.

Pressure-overload LV hypertrophy was induced by aortic banding. Mice were anesthetized with tribromoethanol / amylene hydrate (Avertin; 2.5% wt/vol, 6 µL/g body weight IP). A topical depilatory agent was applied to the neck and chest, and the area was cleaned with betadine and alcohol. Mice were placed supine, A horizontal skin incision 0.5–1.0 cm in length was made at the level of the suprasternal notch. The thyroid was retracted, and a 2- to 3-mm longitudinal cut was made in the proximal portion of the sternum. This allowed visualization of the aortic arch under low-power magnification. A wire with a snare on the end was passed under the aorta between the origin of the right innominate and left common carotid arteries. A 6-0 silk suture was snared with the wire and pulled back around the aorta. A bent 27-gauge needle was then placed next to the aortic arch, and the suture was snugly tied around the needle and the aorta. After ligation, the needle was quickly removed. The skin was closed, and mice were allowed to recover on a warming pad until they were fully awake. The sham procedure was identical except that the aorta was not ligated.

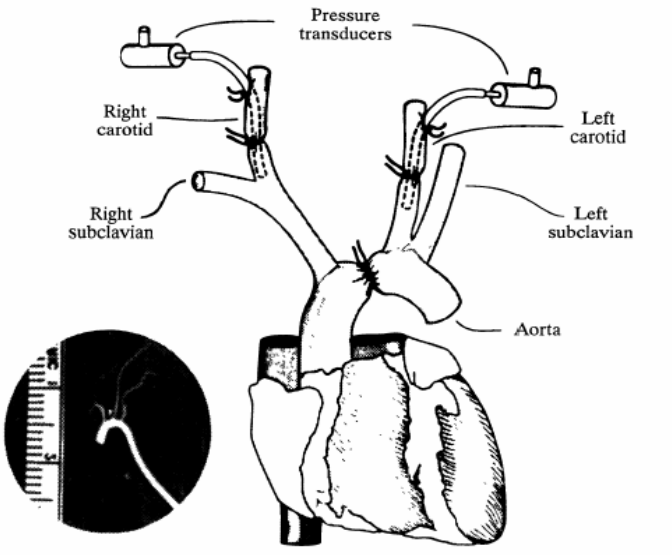

Fig. 2.1. Induction of hypertrophy Left ventricular pressure overload hypertrophy in these mice was induced by partial banding of the aorta. . A wire with a snare on the end was passed under the aorta between the origin of the right innominate and left common carotid arteries As a control, a thoracotomy was performed on the sham-operated mice without hemodynamic interventions. Under this condition, systemic arterial ressure remained unaltered for one week.

2.2.3 In vivo inhibitior of integrin αv

Mice were treated in vivo with a specific integrin α_v inhibitor 1 day prior to myocardial infarction. Alzet osmotic mini pumps were implanted subcutaneously. The release rate was 1µl/hour/animal of either a solution of 100 mg/ml of the integrin α_v inhibitor in 50% DMSO/PBS or DMSO/PBS alone (vehicle control).

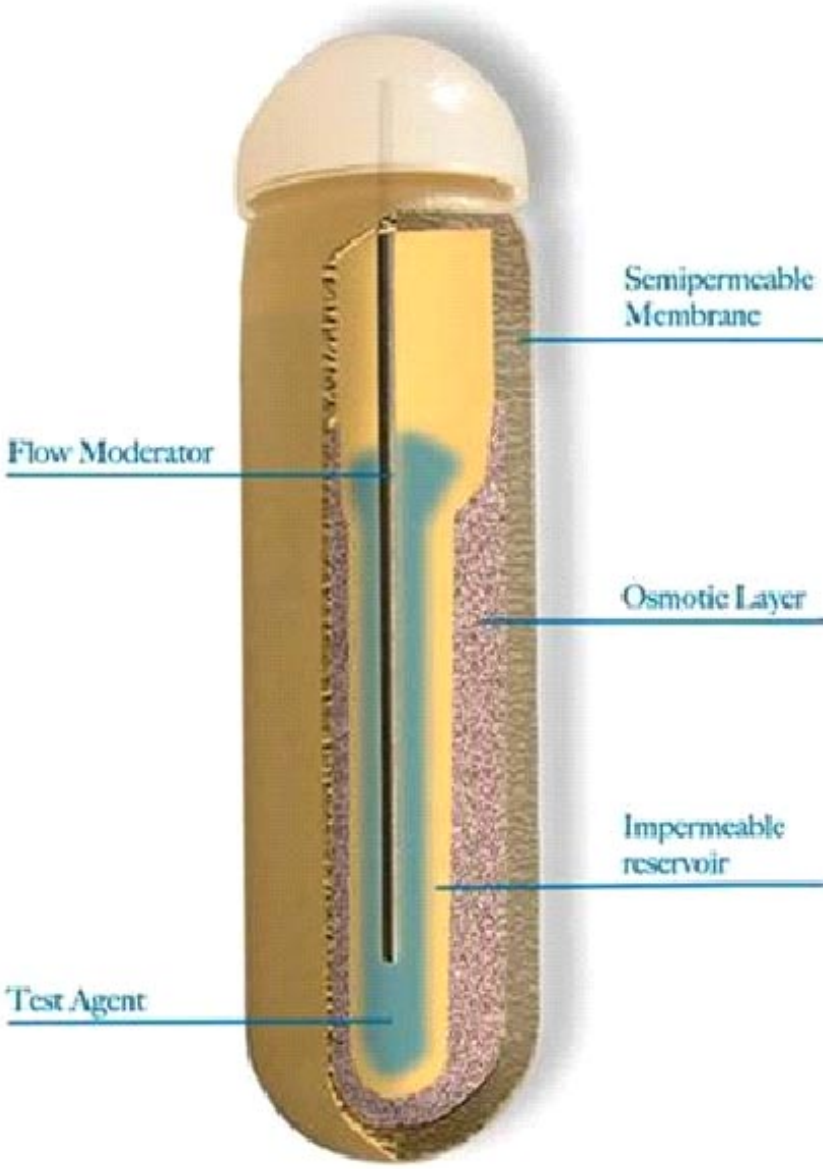

Fig 2.2. ALZET pump. The mini pump have 3 concentric layers: Rate-controlling, semi-permeable membrane; Osmotic layer and Impermeable drug reservoir as indicated. ALZET pumps work by osmotic displacement. Water enters the pump across the outer, semi-permeable membrane due to the presence of a high concentration of sodium chloride in the osmotic chamber. The entry of water causes the osmotic chamber to expand, thereby compressing the flexible reservoir and delivering the drug solution through the delivery portal.

2.2.4. Hemodynamic measurements

Hemodynamics is an important part of cardiovascular physiology dealing with the forces the pump (the heart) has to develop to circulate blood through the cardiovascular system. Adequate blood circulation (blood flow) is a necessary condition for adequate supply of oxygen to all tissues, which, in return, is synonymous with cardiovascular health, survival of surgical patients, longevity and quality of life.

Hemodynamic studies were performed either 2days, 7 days after aortic banding according to published protocols(Hu, Gaudron et al. 1998). under light isoflurane anesthesia and spontaneous respiration (isoflurane 1.5 vol% supplemented by 0.5 L of oxygen per minute).Millar carhteter into the carotid artery and gently advancing this into the left ventricular cavity. Left ventricular (LV) pressure curves were recorded after catheter placement in the LV cavity; systolic and diastolic blood pressure measurements were obtained on catheter withdrawal in the thoracic aorta. Pressure waves are recorded captured and analyzed with Chart software. Powerlab Systems (comprising an analog to digital hardwae unit an dChart software are used for monitoring cardiovascular sugnals. With recording and computation speeds of up to 200 KHz per channel (400KHz affregate). Different signals simulataneoulsy in real time can be recorded and calculated. All measurements were performed trained investigator blinded to treatment. Animals with heart rates below 400 bpm were excluded from morphometric, hemodynamic, echocardiographic, and molecular analyses. Simultaneous left and right carotid pressures were measured under anesthesia to assess the degree of pressure load.

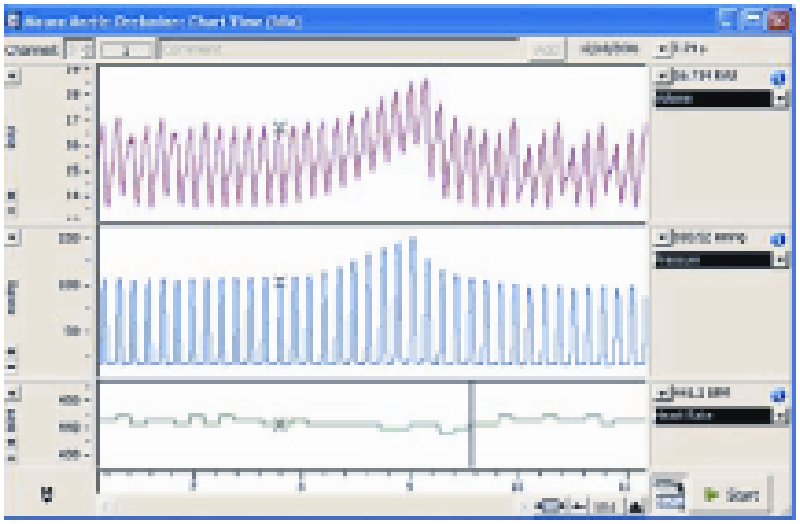

Fig. 2.3. Monitor of cardiovascular signal. Pressure signal were recoreded using a Poverlab and Chart software. Heart rates was calculated from the pressure signal in real time.

This technique provides the following indices of left ventricular function.

A, Left ventricular systolic pressure (LVSP) Unit:mmHg

B, Left ventricicular diastolic pressre (LVEDP) Units:mmHg

C, Heart Rate Units: bears perminuter.

D, Positive dp/dt (max): First derivative of LV pressure. This index is a measure of the rate of pressure development in the left ventricle .Units: mmHg/msec

E, Negative dp/dt (min): First derivative of LV pressure. This index is a measure of the rate of ventricular relaxation, or the rate of pressure decay on the LV Units:mmHg/msec.

2.2.5. Transthoracic Echocardiogram (M-mode)

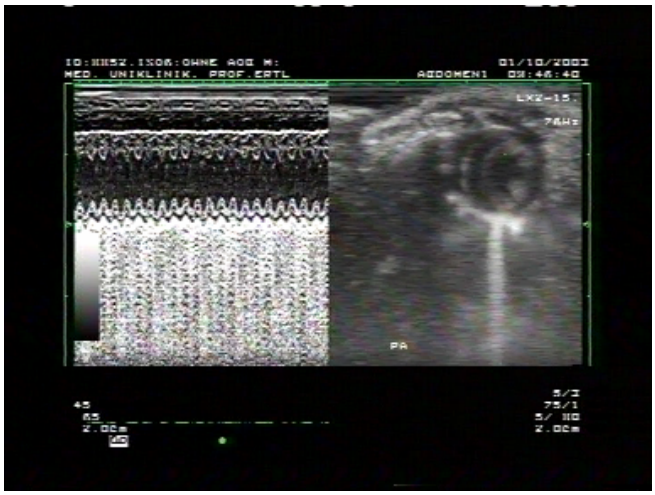

Fig 2.4. Transthoracic Echocardiogram (M-mode) The echocardiograph used a Toshiba Power Vision 6000 system with a 15-MHz frequency ultrasound probe under general anesthesia with tribromoethanol/amylene hydrate (Avertin; 2.5% wt/vol, 6 µL/g body weight IP) under spontaneous respiration at a minimum depth setting of 3 cm for 2D (on the right of the figure) and M-mode imaging.(In the left of the figure, from the same mouse in the parasternal short-axis view.) The 2D images were obtained in white-on –black format with M-mode imaging and the spectral Doppler tracings in black-on white format.

2.2.5.1. Echocardiography

Transthoracic echocardiography is the echocardiogram that is performed by positioning a probe, called a transducer, on the outside of the chest wall while the mouse is lying down. Sound waves lack the ability to travel through air, so a clear, gel-like substance is placed between the chest and the transducer to provide a

medium through which sound waves can travel. The gel also helps to improve the contact between the transducer and the skin. The transducer is placed in various positions on the chest to obtain different images of the heart.

2.2.5.2. Measurements.

Observatons were made in 47 intact, anesthetized mice. Mice were weighed and general anesthesia with tribromoethanol/amylene hydrate under spontaneous before the echocardiogram was performed. Heart rate are generally maintained at more thatn 400 per minute with these regimens The chest hair is removed with a topical depilatory agent. Limb leads were attached for electrocardiogram gating, and the animals are imaged in the left lateral decubitus with a 13_MHz linear probe In later studies, the transducer was placed on the left hemithorax. The correct transducer placement was judged by the round appearance of the left ventricular (LV) cavity after angulation and cranio-caudal transducer movements. Need to be careful that avoid excessive pressure, which can induce bradycardia. LV end-systolic and end-diastolic areas were calculated by manual tracings of the endocardial border followed by planimetry with the Nice software package (Toshiba Medical Systems) and transversal M-mode tracings were recorded with the cursor placed in the middle of the LV cavity.

Taken the guide of the 2D parasternal short-axis imaging plane, the LV M-mode tracing was obtained close to the papillary muscle level Measurements are taken for anterior and inferior wall-thickness, left ventricular and end-diastolic dimension.Two dimensional images are recorded in parasternal long-and short-axis projections with guided M-mode recordings at the midventricualr level in both views. Left ventricular (LV) cavity size and wall thickness are measured in at least three beats from each proection and and averaged. LV wall thickness [intervertricular septum (IVS) and posterior wall (PW) thickness] and internal dimensions at diastole and systole (LVIDd and LVIDs, respectively) are measured. LV fractional shortening [(LVIDd-LVIDs)/LVIDd], relative wall thickness [(IVS thickness +PWthickness)/LVIDd], and LV mass[1.05 (IVS thickness +LVIDd +PW thickness)^3-LVIDd^3] are calculated form the M-mode measurements.

2.2.6. Ventricular mass calculation

In wild type or integrin alpha v inhibition's mice with aortic banding or sham-operation, after echocardiography the experiment was terminated with an overdose of intraperitoneal sodium pentobarbital, the heart was excised, the atria and vessels were dissected free, and the ventricles are weighed. For normalizing the dates, body weight and the tibia are measured as well

2.3. RNA isolation

RNA was extracted from mouse heart with different treatments using TRIzol (Invitrogen) as described previously (Knepp, Geahr et al. 2003)Total RNA from 5-6 each groups of mice 2d or 1 wk after MI and sham-operated mice Total RNA from 4 groups of mice 2d or 1 wk after MI with or without integrin alphv with or without integrin alphv. 1ml of TRIZOL was added per tube containing 50-100 mg of heart tissue before homogenization. Then, homogenate was mixed well with 0.2 ml of chloroform per 1 ml of TRIZOL, and incubated at room temperature for 2-3 min. Following centrifugation (12000g for 15 min at 4 ºC), lower phase, interphase and upper phase of sample appeared in the tube. The upper phase was removed carefully and transferred to a new tube. RNA was precipitated by adding 0.5 ml of Isopropanol per 1ml of TRIZOL and incubated at room temperature for 10 min then centrifuged at 12000 *g* for 10 min at 4 ºC. After removing the supernatant, RNA pellets were mixed with 75% ethanol and then centrifuged at 7500g $\times$ 5 min at 4 ºC. After the supernatant was removed, the pellets were permitted to stand for air drying. RNA was dissolved in 20 μl of DEPC water and incubated at 55 ºC for 10 min.

To remove contaminating DNA 0.1 volume of 10X DNase 1 Buffer and 1ul of DNase 1 were added to the RNA (DNAfree, Ambion Inc.). The RNA solution was mixed gently and incubated at 37ºC for 20-30 min. The DNase Inactivation Reagent was resuspended by vortexing the tube and 0.1volume or 5ul was added to RNA solution. The tube was incubated for 2 min at room temperature and centrifuged at 10,000x g for 1 min to pellet the DNase Inactivation Reagent . The RNA was stored at -80˚C for later use.

RNA quality was analysed by electrophoresis in formaldehyde containing 1% agrose gels using a MOPS buffer. The integrity of the 18 s and 28 s rRNA bands are an incication of RNA quality. The concentration is md under UV light by using

spectrophotometer. Only the ratio at (280nm/ 260 nm) more than 1.8 are considered pure RNA and used for further gene array.

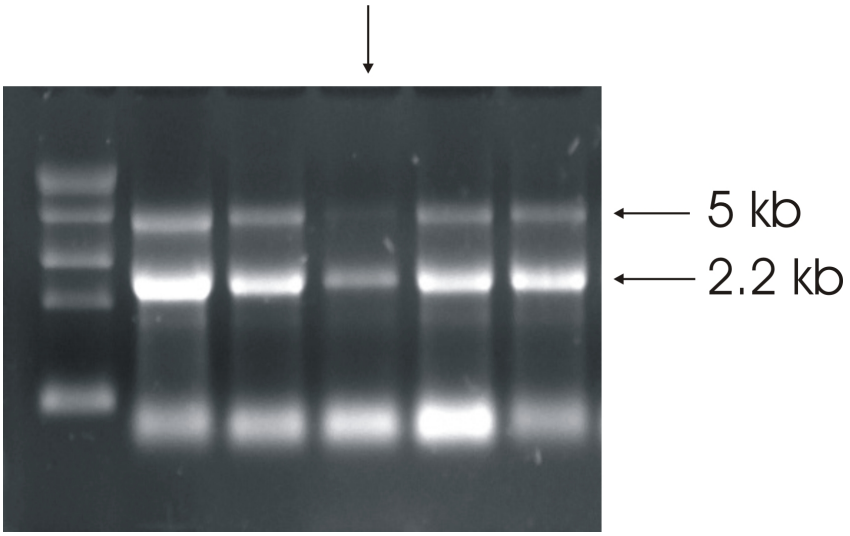

Figure 2.5. RNA electrophoresis Total RNA (5-10 ug) containing ethidium bromide are separated electrophoretically using a 1% formaldehyde agarose gel with a circulating running buffer of 1X MOPS for 2 hours .The two bands, upper 28S (5 kb), and the bottom 18S (2.2 kb), suggested a good quality of RNA. The RNA in the third lane indicated that the RNA was degraded.

2.4. Gene Array

2.4.1. RNA preparation and Gene Chip hybridization

RNA was extracted from mouse hearts with different treatments using TRIzol (Invitrogen) as described before. Total RNA from 4 groups of mice 2d or 7d after MI with or without integrin alphv was used as starting material and hybridized to the non-radioactive GEArray TM Q series cDNA array (SuperArray Inc.,USA). This array contain 96 sets of biotin deoxyuridine triphosphate (dUTP)-labeled cDNA probes (ECM-integrin related gene-specific cDNA fragments).These known genes encoding cell adhesion and extracellular matrix proteins were specifically generated in the presence a designed set of gene-specific primers Intensity differences > 2-fold in gene expression are considered significant and selected gene were further analyzed through Western blot analysis and immunofluorescence.

SouperArray Extracellular matrix Gene Array was using total RNA (5ug per membrane) as template for probe synthesis. The dUTP labeled cDNA were specifically generated by following protocols of Ampolabeling (LPR kit; Cat#:L-03N)

The RNA was reverse-transcribed by gene-specific primers with biotin-16-dUTP. Biotinylated cDNA probes were denatured and hybridized to the ECM-integrin related gene-specific cDNA fragments spotted on the membranes at 60C for 17 h. The GEArray membranes were then washed and blocked with GEA blocking solution, and incubated with alkaline phosphatase-conjugated streptavidin (washed twice with 2x saline sodium citrate buffer (SSC) 1% sodium dodecyl sulfate (SDS) and then twice with 0.1 x SSC/1% SDS at 60 C for 15 min each.) Chemiluminescent detection steps were performed by subsequent incubation of the membranes with alkaline phosphatase-conjugated streptavidin and CDP-Star substrate. The results were analyzed using ScanAlyze 2 software. All cDNA microrray experiments were performed twice with new filters. The positive and negative spots were identified and matched by at least two experiments are presented. Each array membrane comprised 96 marker gees, four positive controls including beta-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin A, and ribosomal protein L13a; and a negative control, bacterial plasmid pUC18. The relative expression levels of different genes were estimated by comparing its signal intensity with that of internal control GAPDH.

2.4.2 Post-Hybridization Data Analysis

The gene expression profile could easily distinguish during 4 different independence experiments. To quantify the results, we normalized the intensity data by first subtracting the average background (mean blank intensity) and then comparing intensity levels with the average intensity of the hybridization signal of the house keeping gene.

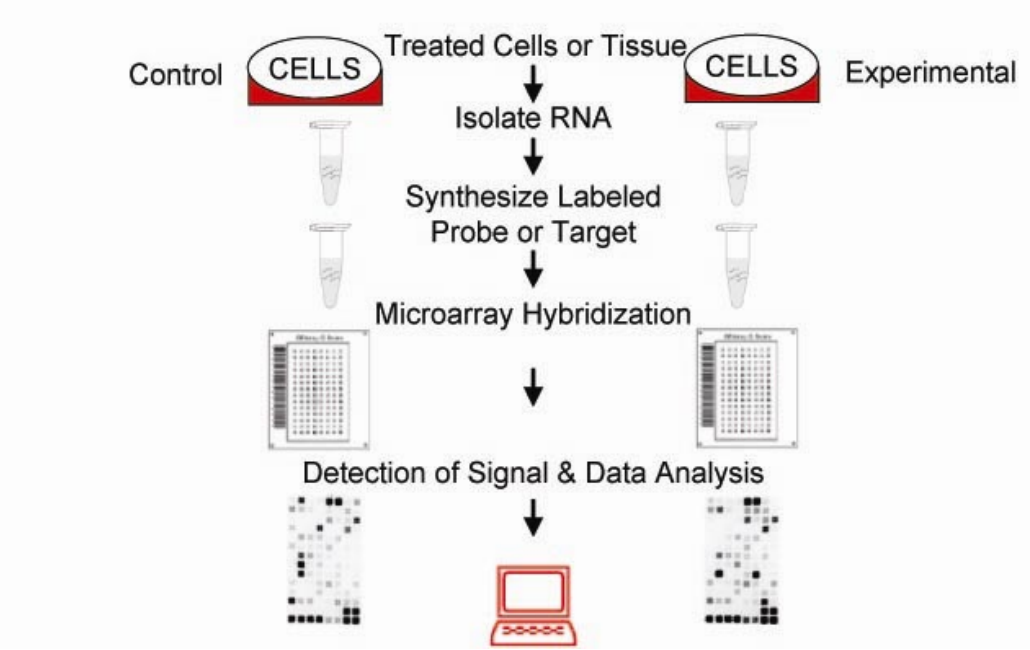

Figure 2.6. Process of cDNA gene array was shown as indicated.(from SuperArray Inc.,USA)

2.5. Protein methods

2.5.1. Protein extraction for Western blotting

Proteins were extracted from tissue in RIPA buffer or homogenisation of tissue in 2% Tris-Triton Lysis Buffer. Detergent soluble fractions were prepared from the supernatant, after 14000 *g* centrifugation for 10 min at 4°C to remove nuclei and the insoluble fraction then stored at- 80^{o} C. Protein concentration was determined by the Bradford protein assay using a BSA standard curve.

2.5.2. Western blotting

By adding an aliquot of Laemmli SDS sample buffer followed by 5 min boiling at 95°C, 15 µl of the samples from the heart tissue were separated on 7.5% -15% SDS polyacrylamide gels depending on the size or target protein and electrophoretically transferred to nitrocellulose membranes Plolyvinylidene Fluoride.membranes (Gelman Laboratory). Then, the proteins were transferred to Plolyvinylidene Fluoride membranes (Gelman Laboratory). After transfer, the membranes were blocked for 1 hour using 10% non-fat milk or 3% ABS) in TBST

Buffer. Blots were incubated with the primary antibody in TBST buffer overnight at 4˚C with light agitation. After incubation with primary antibody, the membranes are washed 5 times with large volumes of TBST and incubated with a horseradish peroxidase labelled secondary antibody in TBST buffer for 1 hour at room temperature, the blots were detected by enhanced chemiluminescence (ECL) accordining to the manufacturer's reonmmendations. The signal was detected by a digital imaging system (ALPHA Innotech ChemiImager 5500).

2.6. Material

2.6.1. Antibodies

Primary antibldy	Application	Size of detected protein(kDa)	Dilution	Ig	Distributor
Integrin αV	WB	25 and 27	1:500	rabbit polyclonal	Chemicon International, Inc.; Temecula, CA AB1930
Timp2	WB	24	1:1000	mouse anti-human	Chemicon International, Inc.; Temecula, CA MAB3310
fibronectin	Immu	220	1:500	mouse mouse rabbit	Chemicon International, Inc.; Temecula, CA MAB 88904 MAB1926
vitronectin	WB	65/57	1:500	rabbit	Santa Cruz Biotechnology, Inc.; Santa Cruz, CA
Collagen-3	WB	70	1:2500	Rabbit	Abcom, Cambridge, UK
TIMP1	WB	28	1:200	Mouse	Chemicon International, Inc.; Temecula, CA
TIPM2	WB	24	1:500	mouse	Chemicon International, Inc.; Temecula, CA
Fak	WB	125	1:1000	mouse	BD transduction laboratories; San Jose, CA F15020
Fak-p925	WB	125	1:1000	rabbit	Cell Signalling Technology ,Inc.; Danvers, MA 3284S

Src	WB	60	1:1000	mouse	Upstate Charlottesville, Virginia
Src-p-418	WB	60	1:5000	mouse	Cell Signalling Technology ,Inc.; Danvers, MA 2101S
ERK1/2	WB	44/42	1:1000	rabbit	Biosource Camarillo California USA
ERK1/2-p	WB	44/42	1:1000	rabbit	Biosource Camarillo California USA
p-38	WB	38	1:500	mouse	Santa Cruz Biotechnology, Inc.; Santa Cruz, CA
p-38-p	WB	38	1:500	rabbit	Promega Delta House UK
JUK1/2	WB	54/46	!:1000	rabbit	Biosource Camarillo California USA
JUK1/2-p	WB	54/46	1:1000	rabbit	Biosource Camarillo California USA
Integrin alpha-V	WB	25/27	1:1000	Rabbit polyclonal	Chemicon Inernational, Inc., Temecula, U.S.A. AB1930
Integin beta 1	WB	130/140	1:1000	Armenian hamster monoclonal	Santa-Cruz-Biotechnology,Inc., Santa Cruz, U:S.A
Integrin alpha 5	WB	150	1:500	Rabbit polyclonal	Chemicon Inernational, Inc., Temecula, U.S.A.

Pro-ANP	WB	15	1:500	Rabbit polyclonal	Chemicon Inernational, Inc., Temecula, U.S.A. AB5490
Vinculin	WB	113	1:1000	Rabbit polyclonal	Santa-Cruz-Biotechnology,Inc., Santa Cruz, U:S.A
GAPDH	WB	37	1:4000	Anti-mouse	Chemicon Inernational, Inc., Temecula, U.S.A. MAB 374

2.6.2.. Solutions and buffers

For DAN electrophoresis

Agarose gel

Agarose Ultrapure 2%
1x TAE
Ethidum bromide (0.5 µg/ml)

6x loading Dye (Agarose gel)

0.25 % bromphenol blue
30 % glycerol in water
0.25 % xylen cyanol

TAE (50x)

242 g Tris base
57.1 ml acetic acid
100ml 0.5M EDTA
Add H2O to 1 liter ,adjust pH to 8.5.

For RNA electrophoresis

MOPS running buffer (10x)

41.86 g MOPS
6.8 g $NaOAc.3H_2O$
3.8 g Na_4EDTA

	H_2O to 1L
RNA electrophoresis	1 g Agarose 88 µml H_2O DEPC 10 ml 10x MOPS buffer 2.3 µl EtBr 1.87 ml Formaldehyde
RNA loading dye	720 µl Formamide 160 µl 10xMOPS buffer 260 µl Formaldehyde 193 µl H_2O 267 µl 6x Bromophenol blue DAN loading dye

cDNA Array

10 x Dnase 1 Buffer	400 mM Tris-HCl (pH 7.5) 100 mM NaCl 60 mM $MgCl_2$
20 X SSC	175.3 g NaCl 88.2 G $Na_3Citrate.2H_2O$

Western blot

2% Tris-Triton Lysis Buffer	2 ml Triton-x 100 10 mM EGTA 200 µl NaVO3 (1000 x) Tris-HCL (100mM) H2O up to 100 ml Adjust pH to 7,4
PBS (10x)	2 g KCl

	2 g KH_2PO_4 80 g NaCl 11.5 g Na_2HPO_4
Western Blot Buffer 10x TBST	100 ml 0.5 M EDTA 12.1g Tris-Base
Running Buffer	24 g Tris-Base 116 g Glycine 20 ml 20% SDS 4L sterile H2O
RIPA Buffer:	1x PBS 1% Igepal CA-630 0.5% Sodium deoxycholate 4 L sterile H_2O
Transfer Buffer	12g Tris-Base 28 g Glycine 800 ml Methanol Sterile H_2O up to 1 liter pH 7,5 57.1 ml Glacial acetic acid
20% SDS	200 g SDS
PBST	1xPBS with 0.05% Tween 20
Stripping Solution	67g Guanidine-HCl (7M) 374mg Glycine (50 mM) 1.86mgEDTA (0,05) 740mg KCL (0,1 M) 156mg 2 (beta)-Mercaptoethanol (20 mM)

Sterile H2O up to 100 ml
adjust pH to 10,8 with 10 M NaOH

SDS Gels:

Buffer

1,5 M Tris-Base:
0,5 M Tris-Base
Ammoniu Persulfate (APS) 10 %
SDS 20%
Acrylamide 40% (premade solution)
Bisacrylamide 2% (premade solution)

Seperating SDS Gels

Buffers /%	7.5% (ml)	10% (ml)	12.5% (ml)
H20	16	13.8	10.9
1.5 M Tris-Base	11.3	11.3	11.3
20 % SDS	280µl	280 µl	280 µl
40 % Acryl	10.5	14	17.5
2 % Bis	4.1	2.7	2.2
TEMED	14µl	14 µl	14 µl
APS 10%	315 µl	315 µl	315 µl

4 % Stacking gels:

H_2O	9 ml
0.5 M Tris	1.75 ml
20% SDS	90 µl
40% Acryl	1.8 ml
2% Bis	1.05 ml
TEMED	14 µl
10% APS	210 µl

2.6.3. Chemicals and Kits

Chem Glow	Alpha Innotech corporation CA, USA
Formaldehyde	Merck
Isopropyl alcohol,	Sigma (St. Louis, MO)
trypsin	Sigma (St. Louis, MO)
2 (beta)-Mercaptoethanol	Sigma (St. Louis, MO)
ABS (albumin bovine serum)	Sigma (St. Louis, MO)
Acrylamide	Sigma (St. Louis, MO)
Agarose	Sigma (St. Louis, MO)
Ammoniu Persulfate	Sigma (St. Louis, MO)
Ammoniu Persulfate	Sigma (St. Louis, MO)
Bisacrylamide	Sigma (St. Louis, MO)
BrdU	Sigma (St. Louis, MO)
BSA	Sigma (St. Louis, MO)
Charcoal -stripped FCS	C.C.Pro
chemiluminescence (ECL	Sigma (St. Louis, MO)
Chloroform, agarose were from Sigma	Sigma (St. Louis, MO)
CPSR1	Sigma (St. Louis, MO)
Dextrose	Sigma (St. Louis, MO)
DMEM	Sigma (St. Louis, MO)
DNAse	Sigma (St. Louis, MO)
DEPC	Sigma (St. Louis, MO)
EDTA	Sigma (St. Louis, MO)
EtBr	Sigma (St. Louis, MO)
Ethanol	Merck
FCS	Sigma (St. Louis, MO)
Formaldehyde,	Sigma (St. Louis, MO)
Formalin	Sigma (St. Louis, MO)
Formamide	Merck
Glacial acetic acid	Sigma (St. Louis, MO)
Glycine	Sigma (St. Louis, MO)
Guanidine-HCl	Sigma (St. Louis, MO)
H2O DEPC	Sigma (St. Louis, MO)

HEPEA	Sigma (St. Louis, MO)
KCl	Sigma (St. Louis, MO)
KH2PO4	Sigma (St. Louis, MO)
Lab-Tek chamber slides	Nunc Inc., Naperville, IL, USA
Methanol	Sigma (St. Louis, MO)
MgSO4.7H2O	Sigma (St. Louis, MO)
MOPS	Sigma (St. Louis, MO)
NuPAGE LDS Sample Buffer	Invitrogen, (Carlsbad, CA)
NuPAGE 4-10% Bis-Tris Gel	Invitrogen, (Carlsbad, CA)
NuPAGE MOPS SDS Running Buffer	Invitrogen, (Carlsbad, CA)
NuPAGE Transfer Buffer	Invitrogen, (Carlsbad, CA)
Na2HPO4 anhydrogenous	Sigma (St. Louis, MO)
Na2HPO47H2O	Sigma (St. Louis, MO)
Na4EDTA	Sigma (St. Louis, MO)
NaCl	Merck
NaH2PO4	Sigma (St. Louis, MO)
NaN3	Sigma (St. Louis, MO)
NaOAc.3H2O	Sigma (St. Louis, MO)
NaOAc.3H2O	Sigma (St. Louis, MO)
Nitrocellulose ECL Membranes	Sigma (St. Louis, MO)
PVDF Membran filter paper	invitrogen
Penicillin	Sigma (St. Louis, MO)
RnaseZAP	Ambion Inc.
PAP pen	Daido Sangyo Co., Ltd., Tokyo, Japan
Plolyvinylidene Fluoride.membranes	Gelman Laboratory
Sodirmazide	Sugma
Tissue.tec	Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands
Tris base	Sigma (St. Louis, MO)
Tween 20	Sigma (St. Louis, MO)
Vitamin B12	Sigma (St. Louis, MO)
LPR Kit	Supper Array Bioscience Corporation
Taq PCR Master Mix kit	QIAGEN (Hilden ,Germany
DANfree Kit	Ambion Inc.)

Bio-Rad protein Assay	Bio-Rad (munic Germany)
Protease inhibitor cocktail set III	Calbiochem (Darmstadt, Germany.)
Mouse/Rat PDGF-BB ELISA kit	R&D systems (Inc. MN ,U.S.A)
Mouse/Rat TGF-β1 ELISA kit	R&D systems (Inc. MN ,U.S.A.)
Osteonectin ELISA kit	US Biological (Swampscott, USA)
PCR SuperMix	Invitrogen, (Carlsbad, CA)

2.7. Softwares and websites

- Microsoft Office ,
- EndNote program
- Microsoft Access
- Adobe photoshop
- Adobe reader
- ScanAlyze 2
- Image Arena von Tom Tee
- Chart 5
- Sigma
- Startview
- CoreIDRAW

WebPrimer-http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer
The Web Primer provides tools for the purpose to design primers. Current choices are limited to sequencing and PCR alignment
NCBI Nucleotide- ttp://www.ncbi.nlm.nih.gov/entrez/query.fcgi
The Entrez Nucleotides database is a collection of sequences from several sources, including GenBank.
NCBI BLAST-http://www.ncbi.nlm.nih.gov/BLAST/
(Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used

to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

2.8. Lab devices

- ChemiImager^{TM} 5500 from ALPHA Innotech. (San Leandro, CA).
- Mastercycler PCR System from eppendof (Hamburg, Germany)
- Ultrospec 3000 spectrophotometer from Pharmacia Biotech AB (Cambridge, England)
- Microscopes
- UV lamp from Bio RAD
- Incubators
- Vacuum dryer
- Autoclaves
- Centrifuges
- Heating blocks
- Water bath

3. Results

3.1. Integrin αv is required for early cardiac hypertrophy and survival following aortic banding in mice

3.1.1. Mortality

Mortality rate during observation period (day 2 and day 7 after AB) was higher in integrin αv inhibitor treated mice than that in un-treated mice following aortic banding (AB). Survival rates were reduced after AB. In the group of Integrin inhibitor-AB 63% versus un-treated-AB 87% at day 2 following AB; Integrin -inhibitor-AB 33% versus un-treated AB 77% at day 7 after AB; Deaths in sham-operated mice (n=1 in KO sham. n=0 in WT sham group) occurred in a single case during the surgery, which may have been due to a lower anaesthesia tolerance, but no post-surgical mortality was observed. (Fig. 3.1)

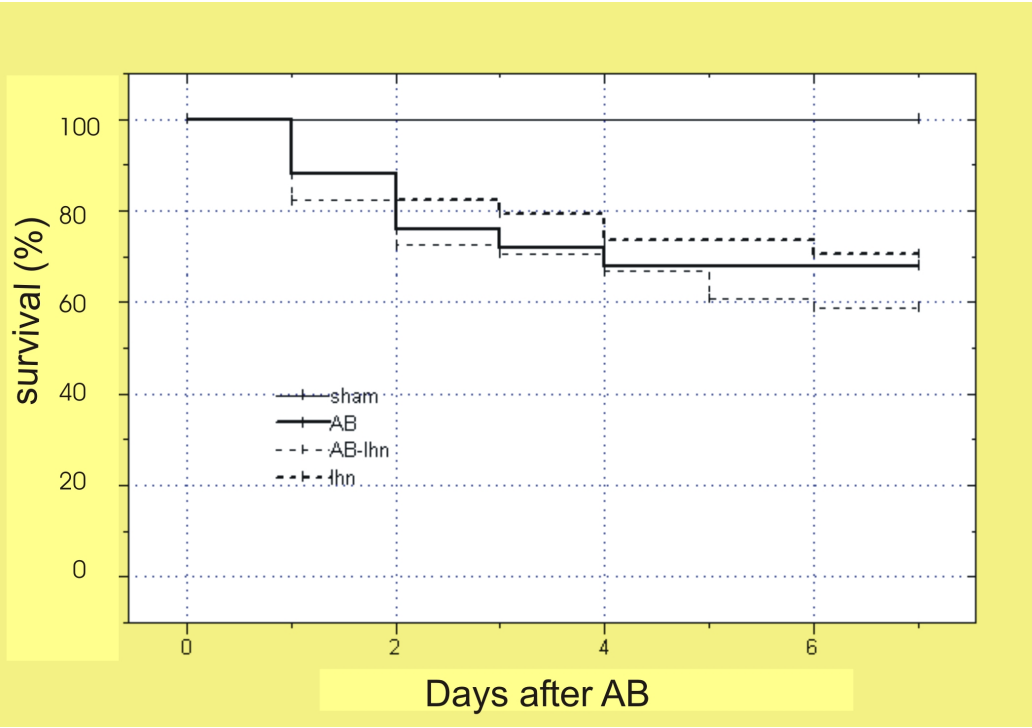

Fig 3.1. Survival rates in different groups of mice. Mortality was negligible in sham-operated mice and increased in both experimental groups after AB over 7 days observation period. Application of the alpha v inhibitor via mini pumps reduced survival rates in mice following aortic banding as indicated. (log-rank *P*=0.144).

3.1.2 Morphometry

Table 1

A) Morphometric data after aortic banding 2d

	sham	sham-inh	AB-2d	AB-inh-2d
	(n=12)	(n=12)	(n=12)	(n=12)
BW, g	25.1 ± 1.40	24.8 ± 1.6	25.6 ± 0.9	24..9 ± 0.5
LW, mg	115.1 ± 11.8	144.7 ± 4.6	231.5 ± 16.6 *	205.1 ± 22.5 #
HW,g	115.1 ± 5.80	109.1 ± 3.8	119.2 ± 2.8	107.4 ± 5.9
Tibia L, mm	18.3 ± 0.10	18.0 ± 0.2	18.1 ± 0.10	18.1 ± 0.2
HW /BW	4.2 ± 0.3	4.3 ± 0.2	4.3 ± 0.1	5.0 ± 1.2
HW/Tibia	6.1 ± 0.1	5.4 ± 0.2	6.6 ± 0.1	5.8 ± 0.3
LW/Tiba L	**6**.6 ± 0.8	8.1 ± 0.2	12.8 ± 0.9*	11.7 ± 1.2 #

B) Morphometric data after aortic banding 7d

	sham	sham-inh	AB-7d	AB-inh-7d
	(n=12)	(n=12)	(N=12)	(N=12)
BW,g	25.7 ± 1.4	25.8 ± 1.5	26.16 ± 0.9	24.3 ± 0.8
LW, mg	125.1 ± 11.8	184.7 ± 11.7	186.9 ± 19.1	200 ± 11.7
HW,g	110.1 ± 4.8	107.1 ± 5.6	143.7 ± 3.8	120.5 ± 4.0
Tibia L, mm	18.3 ± 0.10	18.0 ± 0.2	18.1 ± 0.10	18.1 ± 0.20
HW /BW	4.5 ± 0.4	4.3 ± 0.4	5.1 ± 0.9	5.4 ± 1.2
HW/Tibia	6.4 ± 0.1	5.8 ± 0.3	7.8 ± 0.3*	6.6 ± 0.3
LW/Tiba L	***6***.8 ± 0.3	7.4 ± 0.1	10.1 ± 1.1*	11.5 ± 0.7#

Morphometric data in animals at 2 days A) and day 7 B) following AB. Statistically significant differences between sham-operated and aortic banded mice of each group are indicated. Values are means ± SEM. BW, body weight; LW, lung weight; HW, heart weight; Tibia L, Tibia length; HB/BW, heart weight to body weight ratio; All measurements were performed 2 days (2d) and 7 days (7d) after aortic banding or sham operation. The data were included after AB only when pressure gradients between left and right carotid are higher than 30 mmHg. *p< 0.05 AB vs sham; #P<0.05, Inhibitor – AB-7d vs Inhibitor-sham-operated

Transverse aortic banding or constriction was utilized to provoke hemodynamic loading of the murine left ventricle as has been performed previously (Rockman, Hamilton et al. 1996). As shown in Table 1 this animal model was found to be adequate to mechanical stimulus for hypertrophy without the development of ischemic changes or cardiac failure (Rockman et al, 1991). At the time point of 7 days after AB, there was a significant increase of heart wall in the left ventricle. Seven days after AB, Lung weight and lung weight to body weight ratios were significantly increased than that in sham-operated mice. The absolute gain in heart weight and heart weight to tibia ratios, which were significantly higher in AB-WT mice than in sham operated mice at day 7 ($p<0.01$). However, this increase was not observed in integrin αv inhibited mice following AB as compared to control group at any time point studied (Figure 3.2).

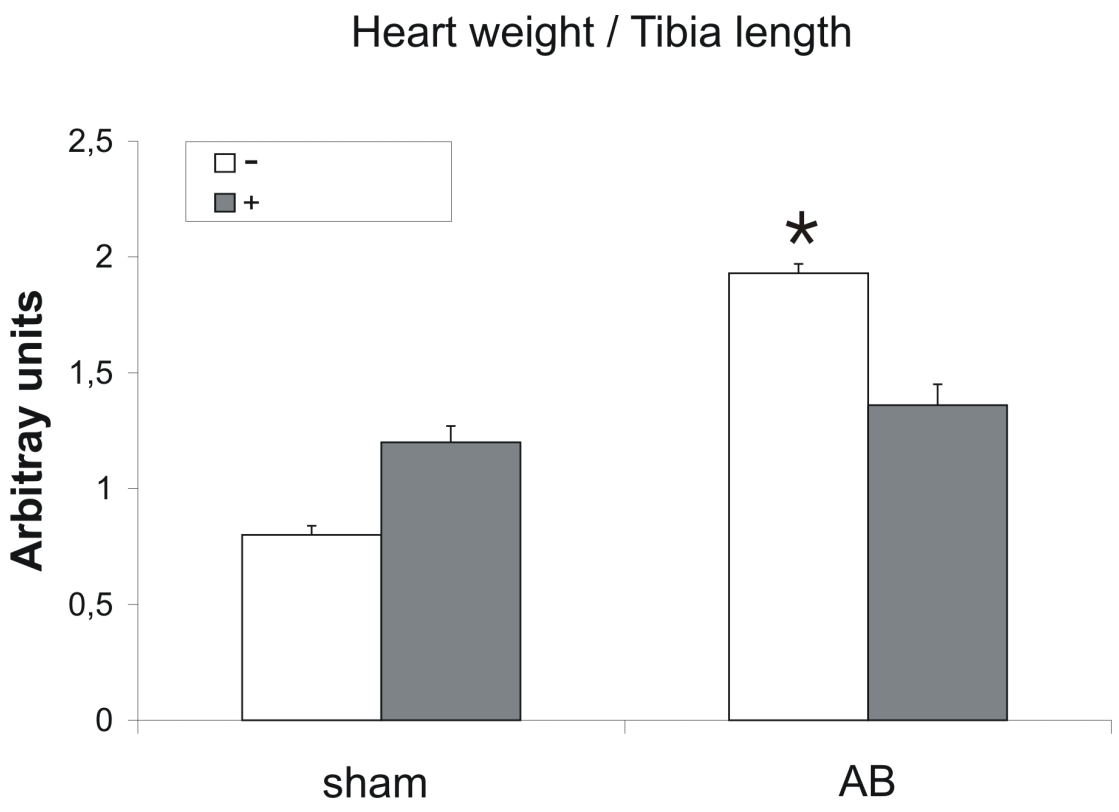

Fig 3.2. Value of heart weight/ Tibia length : Heart weight compare to tibia length ratio in integrin αv inhibited group and in wild type group at day 7 following aortic banding or sham operated. Bars represent the values of two independent experiments. Open bars and close bars indicate wild type without out (-) and with the integrin αv inhibitor (+) respectively (n=12 for each group, *P<0.01 versus corresponding sham operated group. There was no difference in the integrin alpha v inhibited group between sham operated and aortic banding group.

3.1.3 Transthoracic Echocardiography

To investigate the geometric remodelling that occurred in response to pressure overload, we examined the mice by serial echocardiographic analysis following aortic banding. The M-mode echocardiography provides quantitative data about contractile function. The analysis of echocardiograph is the procedure most often used in mouse studies. It is optimized for higher sample frequency and smaller heart size to accommodate the mouse's diminutive size and resultant rapid heart rate. 2 days, 7 days following AB, the consequences of the experimentally sustained pressure overload on LV morphology and function were non-invasively assessed using echocardiography. The data from these procedures provided a detailed and holistic view of cardiac function. Data comparisons from wild type mice with or without application of integrin αv inhibitor via mini-pumps following aortic banding/ sham operation were used to understand the effect of the integrin αv in the model of pressure overloaded heart in the context of the whole heart function. Data were shown in Table 2.

As compared to sham-operated mice, there were no significant changes of heart rates in any groups after AB. In contrast to corresponding sham-operated mice IVS (interventricular septum), FW (posterior wall thickness), LVD (left ventricular diameter) and FS (LV fractional shortening) did not differ significantly between sham-operated and aortic banding 2 days post-surgery. Increased inter-ventricular septum, posterior wall thickness and reduced end-systolic, left ventricular diameters, reduced end-systolic and end-diastolic left ventricular volume were observed following AB 7 days in comparison to sham-operated mice. This represents a typical pattern of concentric hypertrophic remodelling, which occurs in response to pressure overloaded conditions as an adaptive compensatory mechanism. In contrast, 7 days after aortic banding, mice receiving the integrin αv inhibitor only showed a mild thickening of ventricular walls without change in relative wall thickness. However, the left ventricular internal dimensions, diameter and left ventricular volume in mice treated with integrin alpha v inhibitor were significant increased as compared to that without treatment of integrin alopha v inhibitor, which indicated a substantial chamber enlargement associated with integrin αv (fig 4).

Application of the integrin alpha v inhibitor reduced LV fractional shortening (FS) at day 7 after AB as compared to that in untreated AB mice, indicating a major

deterioration of contractile function. This also implicates the dilation of left ventricle in the integrin αv inhibited mice. Findings here are in line with the reduced survival rates in integrin αv inhibitior treated mice after aortic banding as shown in fig. 3.1

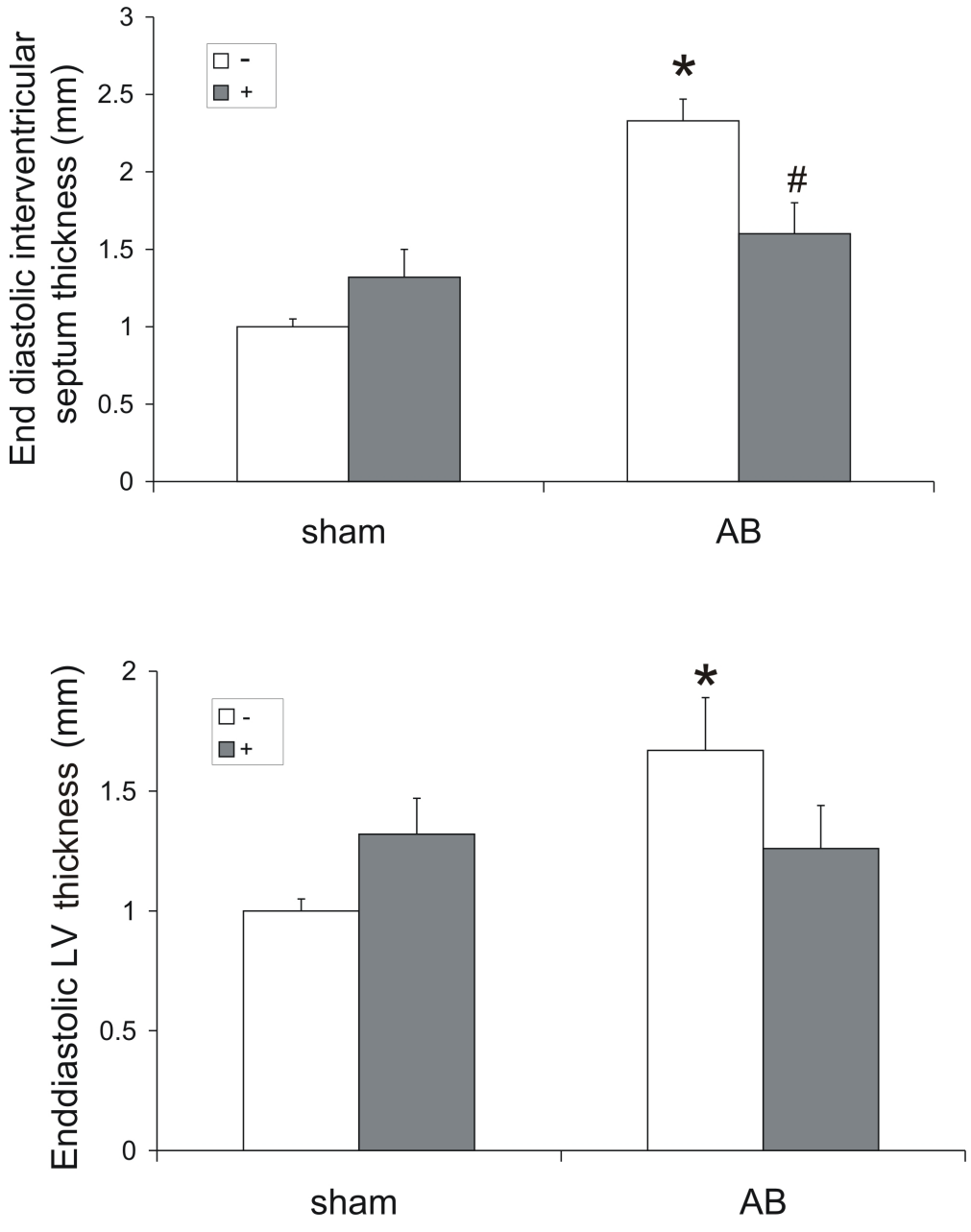

Figure 3.3. Effect of integrin αv inhibitor on heart wall thickness following AB. End-diastolic inter-ventricular septum thickness and end-diastolic left ventricular thickness was obtained by trans- thoracic echocardiography at day 7 after aortic band / sham operation. Bars represent the values of two independent experiments. Open bars and close bars indicate with out or with application of the integrin αv inhibitor via mini pumps respectively. (n=12 for each group) *p< 0.01 AB vs sham; # P<0.01 treatment of inhibitor vs without inhibitor.

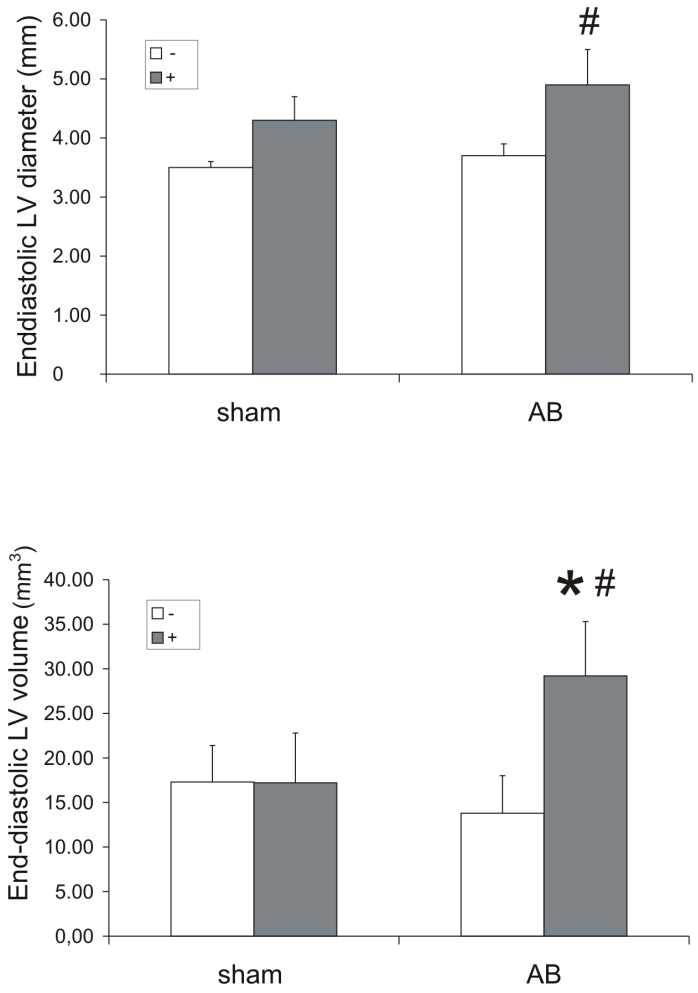

Figure 3.4, **Dilated left ventricle after AB associated with treatment of integrin αv inhibitor.** End-diastolic left ventricular diameter and LV volume were obtained by trans- thoracic echocardiography at day 7 after aortic banding / sham operation. Bars represent the values of two independent experiments. Open bars and close bars indicate without and with the treatment of integrin inhibitor respectively (n=12 for each group) *p< 0.01 AB vs sham; # P<0.01 integrin alphav treated vs untreated.

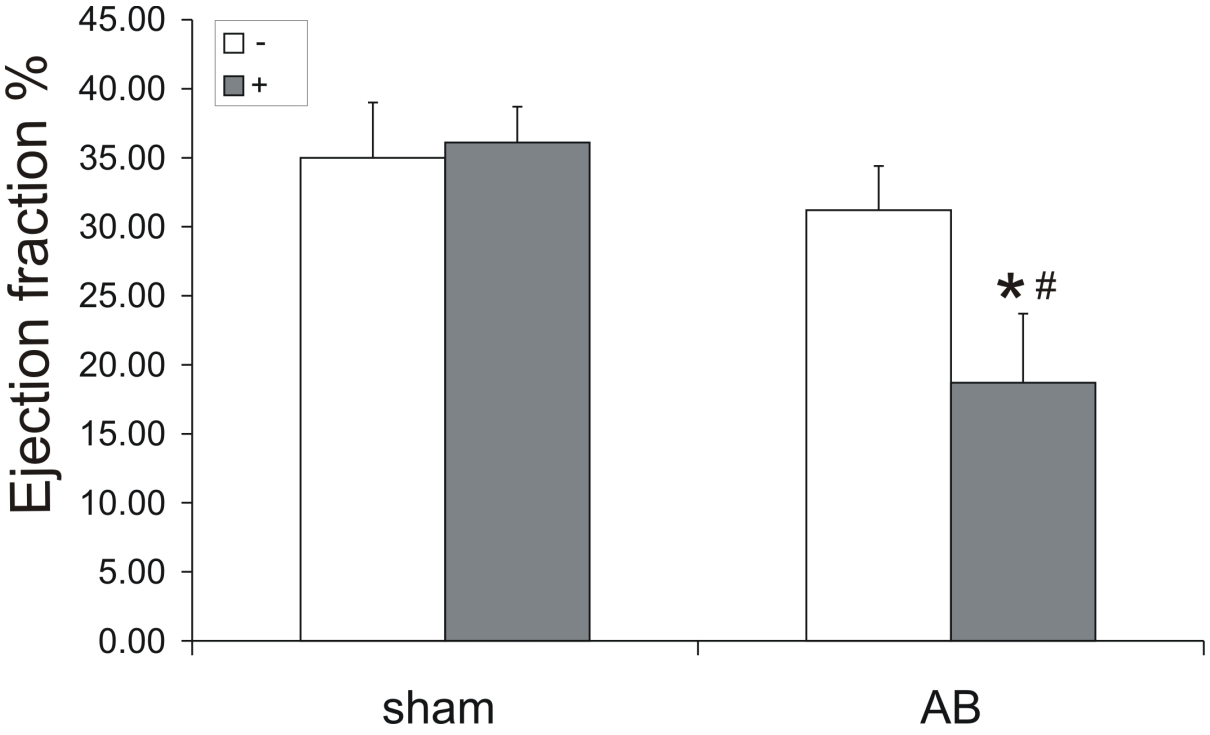

Fig 3.5, Left ventricular function 7 days after aortic banding. (AB) Cardiac function was evaluated non-invasively by trans- thoracic echocardiography at day 7 after aortic band / sham operation. Bars represent the values of percent fractional shortening. Open bars and close bars indicate with-out and with the treatment of integrin αv inhibitor respectively. (n=12 for each group) *p< 0.01 AB vs sham; # P<0.01 integrin inhibitor treated vs untreated of intregrin inhibitor.

Table 2,

A) Echocardiography measurements 2d after AB

	sham	sham-inh	AB-2d	AB-inh-2d
	(n=12)	(n=12)	(n=12)	(n=12)
HR,bmp	472 ± 12	456 ± 11	508 ± 18	464 ± 66
FWs,mm	1.00 ± 0.1	0.90 ± 0.10	1.50 ± 004 *	1.40 ± 0.60
IVSs,mm	0.90 ± 0.11	1.00 ± 0.21	1.70 ± 0.31*	1.60 ± 0.31
LVIDs,mm	2.93 ± 0.07	2.94 ± 0.18	2.73 ± 0.10	2.58 ± 0.40
S-Vol,mm^3	6.70 ± 0.30	7.20 ± 0.28	7.00 ± 0.15	11.2 ± 3.20
FWd,mm	1.03 ± 0.04	1.00 ± 0.14	1.21 ± 0.10	1.01 ± 0.14
IVSd,mm	0.80 ± 0.01	0.81 ± 0.07	1.20 ± 0.26*	0.90 ± 0.51
LVIDd,mm	3.50 ± 0.20	3.81 ± 0.20	4.00 ± 0.20	4.11 ± 0.50
D-Volmm^3	18.30± 3.10	19.20 ± 2.28	15.70± 2.30	18.80 ± 5.10
FS%	43.2 ± 3.10	39 ± 2.28	42.5 ± 5.30	24 ± 5.10

B) Echocardiography measurements 7d after AB

	sham	sham-inh	AB-7d	AB-inh-7d
	(n=12)	(n=12)	(n=12)	(n=12)
HR,bmp	452 ± 16	496 ± 16	508 ± 18	464 ± 66
FWs,mm	1.00 ± 0.01	1.33 ± 0.22	1.67 ± 0.15 *	1.26 ± 0.38
IVSs,mm	1.00 ± 0.01	1.32 ± 0.01	2.33 ± 0.03 *	1.60 ± 0.30 * #
LVIDs,mm	2.73 ± 0.10	2.73 ± 0.31	2.48 ± 0.26	4.02 ± 0.73 * #
Vols,mm^3	6.73 ± 2.60	6.07 ± 3.00	4.80 ± 3.40	21.40 ± 9.0 * #
FWd,mm	1.00 ± 0.06	0.97 ± 0.06	1.38 ± 0.19 *	0.86 ± 0.21#
IVSd,mm	0.80 ± 0.01	1.20 ± 0.38	1.93 ± 0.56 *	1.36 ± 0.50 *#
LVIDd,mm	3.50 ± 0.10	4.30 ± 0.40	3.70 ± 0.20	4.90 ± 0.60 #*
Vold,mm^3	17.30 ± 4.10	17.20 ± 9.62	13.80 ± 4.20	29.2 ± 15.10 *#
FS%	42.0 ± 4.10	38.1 ± 2.6	35.2 ± 3.20	18.7 ± 6.1 *#

Echocardiographic measurements obtained from mice with or without the application of integrin αv inhibitor. All measurements were performed 2days (2d), 7 days (7d) after aortic banding/ sham operation. HR, heart rate; FWs and FWd present posterior wall thickness at systole and diastole; IVSs and IVSd: interventricular septum at systole and diastole; LVIDs and LVIDd: left ventricular internal dimensions at systole and diastole; Vols and Vold: left ventricular volume at systole and diastole; FS% presents fractional shortening. Values are means ± SEM, Statistical comparison was done with two-way ANOVA. *p< 0.05 AB vs sham; # P<0.05 IAB-inh vs AB.

3.1.4. Hemodynamic effects of integrin αv inhibitor

Table 3

A) Hemodynamic data 2d after AB

	sham	sham-inh	AB-7d	AB-inh-7d
	(n=12)	(n=12)	(n=12)	(n=12)
HR	505 ± 16	533 ± 16	538 ± 18	559 ± 66
S-BP,mmHg	94 ± 13	105 ± 8	111 ± 14	132 ± 32
LVEDP,mmHg	3.7 ± 1.1	6.3 ± 3.1	5.3 ± 0.6	4.3 ± 0.9
dp/dt max	6164 ± 739	9877 ± 1058	7566 ± 566	8700 ± 884
dp/dt min	5737 ± 613	8800 ± 461	7042 ± 519	7700 ± 537

B) Hemodynamic data 7d after AB

	sham	sham-inh	AB-7d	AB-inh-7d
	(n=12)	(n=12)	(n=12)	(n=12)
HR	508 ± 26	543 ± 24	488 ± 33	533 ± 24
S-BP,mmHg	100 ± 8	105 ± 16	117 ± 15	123 ± 27
LVEDP,mmHg	3.6 ± 1.1	5.3 ± 1.1	8.3 ± 1.0*	5.2 ± 0.9
dp/dt max	6144 ± 456	9750 ± 345	6207 ± 410	6371 ± 750
dp/dt min	5737 ± 613	8000 ± 321	5983 ± 466	5800 ± 466

All measurements were performed A) 2days (2d), B) 7 days (7d) after aortic banding/ sham operation. The data were included after AB only when gradients of pressure between left and right carotid were higher than 30 mmHg. HR represents heart rate, S-BP indicate systolic blood pressure; LVEDP indicates Left ventricular end-diastolic pressure: dp/dt max: velocity of systolic left ventricular pressure development, dp/dt min: velocity of diastolic LV relaxation, Values are means ± SEM. Statistical comparison was done with two-way ANOVA. *p< 0.05 AB vs sham.

Hemodynamic measurements were performed to investigate whether integrin α_v inhibition has an influence on myocardial performance following aortic banding. (Table3). As shown in Fig3.6, LVEDP was increased after aortic banding 7 days (8.3±1.0 mmHg) as compared to sham operated hearts (3.6±1.1 mmHg; $p<0.05$, n=12). Suggesting the presence of diastolic dysfunction and diminished left ventricular compliance. Treatment with an integrin α_v inhibitor abolished the increase in LVEDP as compared with sham operated following the operation on day 7. No significant difference were observed for cardiac performance like LVDP, LVSP, dP/dt and dP/dt neither on day 2 nor on day 7 following aortic banding. Heart rate did not reveal significant changes when the integrin α_v inhibitor was used.

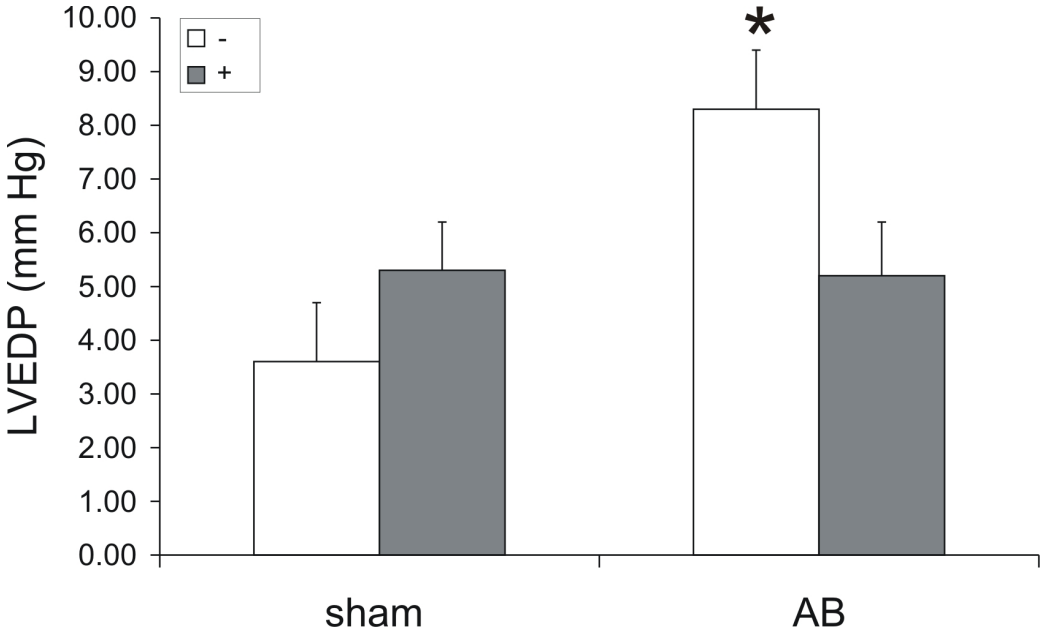

Fig 3.6. Reduced ventricular end diastolic pressure (LVEDP) by inhibition of integrin αv after AB. LVEDP was significantly increased after AB in wild type mice, the mice with a integrin αv inhibitor did not differ significantly following AB as indicated. Treatment with an integrin α_v inhibitor abolished the increase in LVEDP (5.2 ± 0.9 mmHg) as compared with sham operated following the operation on day 7. (AB) LVEDP were evaluated non-invasively by trans- thoracic echocardiography at day 7 after aortic band / sham operation. Bars represent the values of LVEDP in mmHg. Open bars and close bars indicate wild type without out and with the treatment of integrin αv inhibitor respectively. (n=12 for each group) *p< 0.01 AB vs sham; # P<0.01 vs WT-AB.

3.2. Integrin αv is required for pressure overload -activated protein kinase activity during heart remodelling

3.2.1 Activation of Fak and Src in the mice heart following aortic banding 7 day was abrogated by treatment of integrin αv inhibitor

The extracelluar domain of integrin binds to proteins of the ECM or to counter-receptors on the other cells, whereas the cytoplasmic domain forms links with cytoskeletal proteins such as α-actinin and focal adhesion kinase (FAK). FAK has been identified as the key cytoplasmic tyrosine kinase that transmits integrin-mediated signals in several cell types. Tyrosine phosphorylation of FAK at Tyr-397 (proximal to the kinase domain) creates a binding site for the SH2 domain of another non-receptor tyrosine kinase, Src, resulting in formation of a bipartite complex of two tyrosine kinases (Taylor, Rovin et al. 2000).The formation of the FAK·Src complex results in the activation of Src and the subsequent activation of downstream signals leading to the regulation of cellular processes such as growth, migration, and apoptosis (Laser, Willey et al. 2000). With the use of antibodies for Fak, Fak at Tyr-397, Src, and c-Src at Tyr-416, we evaluated the expression of these proteins in mice heart upon aortic banding or sham-operation at day 2 and day 7 that were treated with the specific alpha v integrin inhibitor or vehicle via osmotic minipumps.

Aortic banding at day 2 induced the phosphorylation of Fak at Tyr-397 site (3.9±1.4 fold, n=12, $P<0.05$) and phosphorylation of Src at Tyr-416 site 3 (3.3±0.9 fold, n=12, $p<0.05$) in wild type mice as compared to sham-operated mice. At day 7 following aortic banding, the activation of phosphorylation Fak and phosphorylation of Src are (3.5±1.4 fold, n=12, $P<0.05$) and (2.9±0.5 fold, n=12, $P<0.05$) fold. However, the activation of phosphorylation of FAK and phosphorylation Src were reversed at day 7 after AB by treatment of integrin αv inhibitor. This suggested that integrin αv subunit is required for activation of Fak/Src upon hemodynamic pressure overload

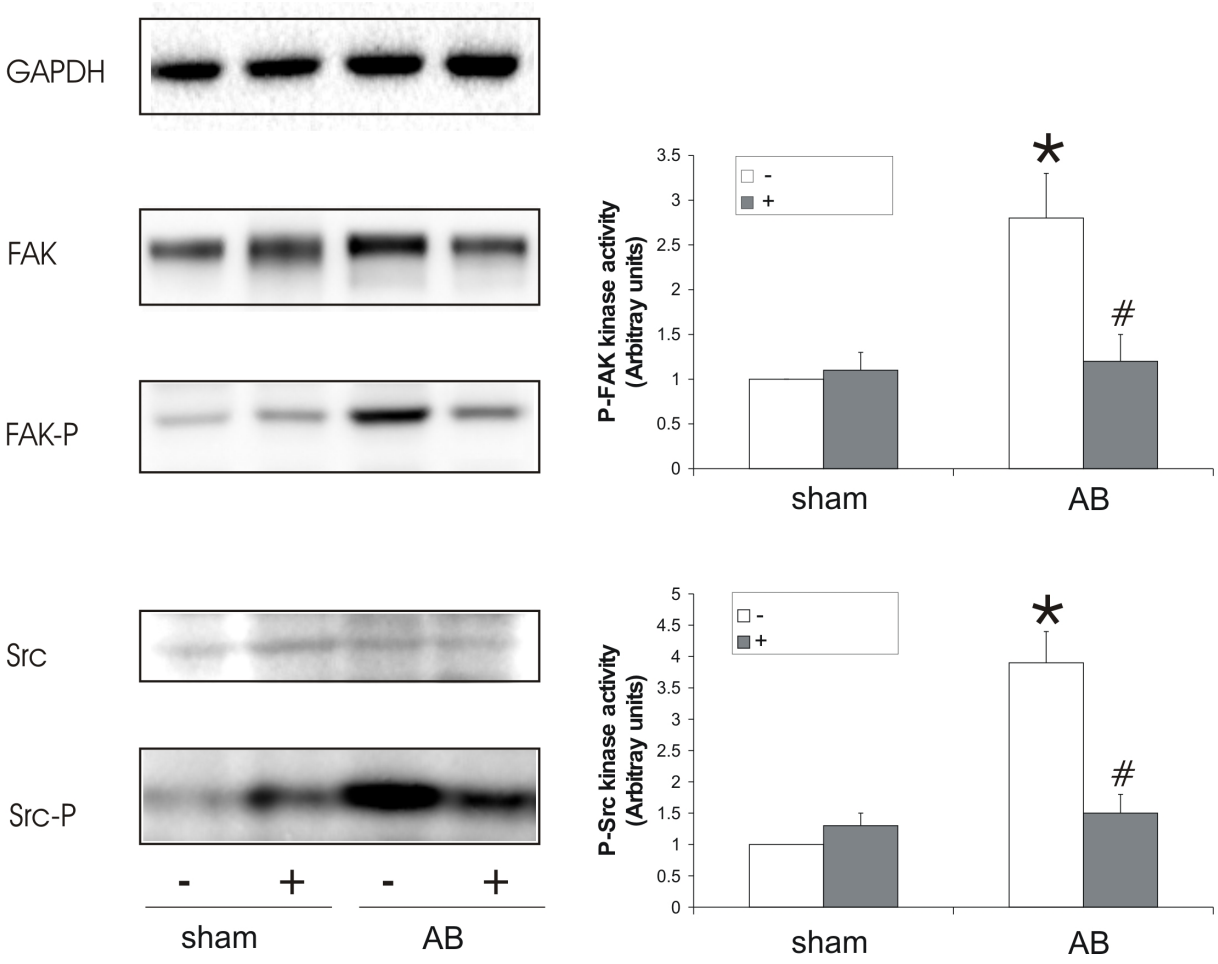

Fig. 3.7. Reduction of activation of FAK tyrosine phosphorylation-925 and Src phosphorylation in the heart under pressure overload by treatment of integrin αv inhibitor. Western blot analysis was performed on LV lysates in mice heart treated with a specific alpha v inhibitor (+) or vehicle (-) via osmoticminipumps after 7 days aortic banding or sham-operation, as described in Methods. Activities of FAK or Src were measured from 15 ug of extracted protein by Western blot (WB) with specific antibodies against Y925 autophosphorylation site, FAK proteins (amount of total FAK), Y216 Src autophosphorylation site or Src proteins (amount of total Src). Protein loading was verified using anti-GAPDH antibody. No differences in the amount of FAK proteins or Src proteins were observed in lysates from any of the heart samples by WB Analysis. Representative Western blots showing FAK and Src activity in the left and quantitative analysis of 12 Western blot experiments showing in the right side. Open bars and close bars indicate animals without or with integrin inhibitor respectively. Phosphorylation FAK and phosphorylation Src levels are shown as fold changes from sham-operated mice without treatment of integrin inhibitor. Values are mean ± SEM; n= 12; * p< 0.05 vs. sham-operated. #p<0.05 treated with inhibitor vs. untreated with integrin inhibitor.

3.2.2 Regulation of ERK1/2 and p-38 expression in heart after AB

Upon integrin-induced phosphorylation of some kinases like FAK and c-Src, they become activated, then may recruit Grb2 /Sos and finally initiate the Ras/ERK signal transduction pathway. (Parsons and Parsons 1997) To exam ERK1/2 and p-38 activity in compensated hypertrophy we performed Western blotting with anti-ERK, anti-phosphorylation ERK1/2, anti-p38, and anti-phosphorylation -p38 following aortic banding 2 days and 7 days. There were rapid activation of phosphorylation ERK1/2 after 2 days of aortic banding (3.3±0.4-fold increase, n=12, $p<0.05$), and day 7 of aortic banding (3.4±0.5-fold increase, n=12, $p<0.05$) as shown in figure 3.10. Phospho-p-38 was also activated rapidly at 2 day after aortic binding (4.2 ±0.4 fold-increase, n=12, $p<0.05$) and at day 7 of aortic banding (5.8 ±0.5, n=12, $p<0.05$). No difference neither in amount of ERK ½ nor in amount of p-38 proteins was observed in lysates from any of the heart samples by Western blot analysis. Interestingly, these up-regulation of phosphorylation -ERK and phosphorylation-p-38 were abolished at day 7 after aortic banding by using the integrin αv inhibitor (figure 3.10) however, the abolishment didn´t significantly differ at day 2 after aortic banding.

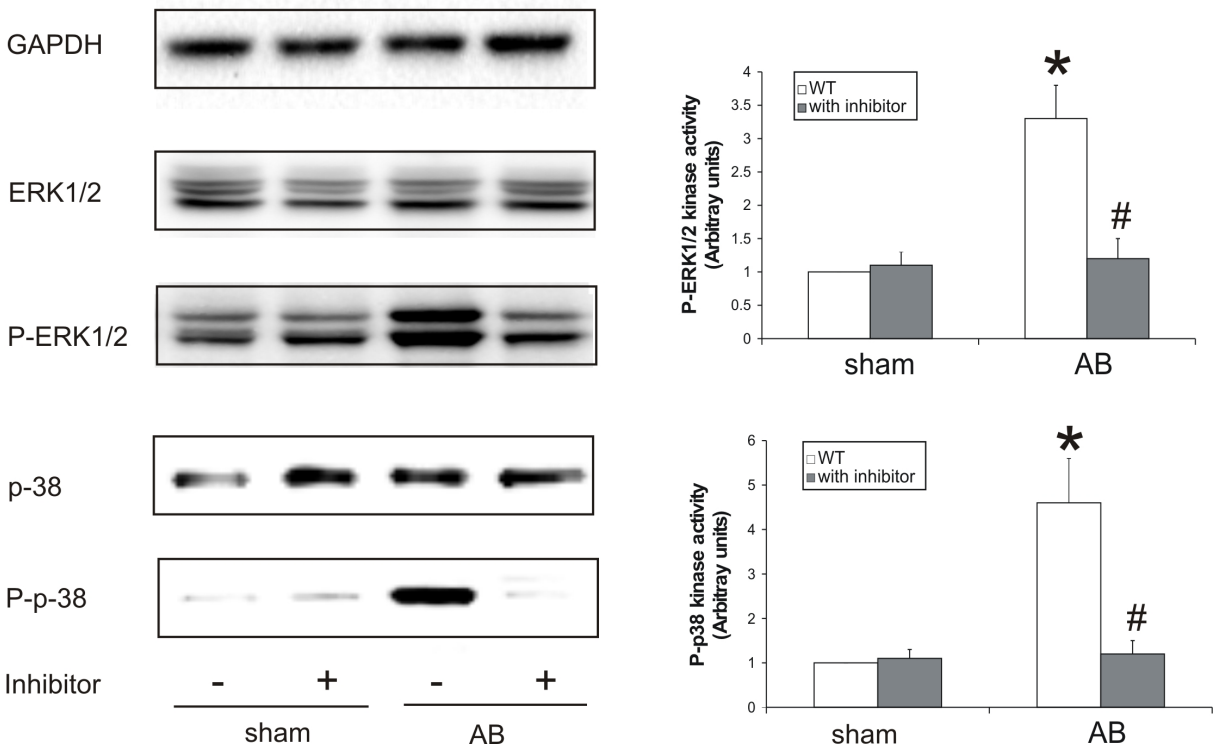

Figure 3.8. Reduction of activation of ERK and p-38 kinases at day 7 under pressure overload. .Western blot analysis was performed on LV lysates in mice heart upon aortic banding or sham-operation at day 7 that were treated with specific alpha v inhibitor (+) or vehicle via (-) osmotic minipumps as described in Methods. Activity of FAK or Src was measured from 15 ug of extracted protein by Western blot (WB) with specific antibodies against ERK phosphorylation , ERK, FAK, p-38 phosphorylation and p-38 proteins. Protein loading was verified using anti-GAPDH. No differences in the amount of ERK proteins or p38 proteins were observed in lysates from any of the heart samples by WB Analysis. Representative Western blots showing ERK and p-38 activity on the left and quantitative analysis of 12 Western blot experiments showing on the right side. Open bars and close bars indicate animals without or with integrin inhibitor respectively. Phosphorylation ERK and phosphorylation p-38 levels are shown as fold changes from sham-operated mice untreated of integrin inhibitor. Values are mean ± SEM; n= 12; * p< 0.05 vs. sham operated animals. # P<0.05: treatment of integrin inhibitor vs. untreated with integrin inhibitor.

3.2.3. Effect of integrin αv on ANF expression following aortic banding.

The amount of atrial natriuretic peptide (ANP), a marker for the severity of heart dysfunction and predictive for patients survival (Daugaard, Lassen et al. 2005; Pelzer, Loza et al. 2005) was investigated by Western blots. In untreated mice, a significant increase of pro-ANP was observed in either 2 days or 7 days after AB (2.7±0.17-fold, $p>0.05$ and 4.4±0.23-fold, $p<0.05$ respectivly) as compared to sham-operated mice. Interestingly, the increase of ANP expression at day 7 following AB was abolished by application of integrin αv inhibitor via mini pump. This is in line with the data from echocardiography that showed the absence of a hypertrophic response in the mice treated with integrin inhibitor.

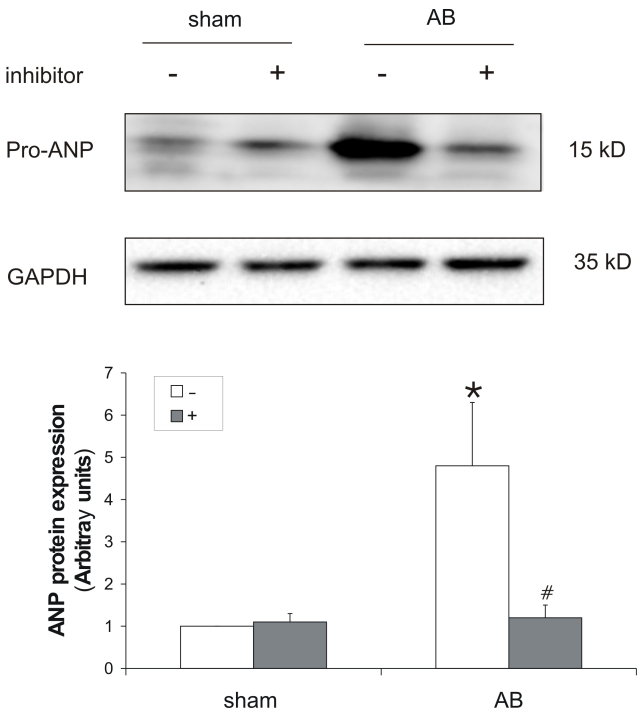

Fig. 3.9. Po-ANP expression after aortic banding. .Western blot analysis was performed on LV lysates in mice heart upon aortic banding or sham-operation at day 7 that were treated with specific alpha v inhibitor (+) or vehicle (-) via osmotic minipumps as described in Methods. LV pro-ANP–to-GAPDH ratios were comparable among sham-operated mice with inhibitor and without inhibitor. Aortic banding increased LV pro-ANP expression to significantly higher extent as compared to sham-operated in WT mice. The induction could not be observed in the mice treated with integrin αv inhibitor as indicated. Representative Western blot of LV pro-ANP expression was shown in upper lane (Values are mean ± SEM, n=13,. *P<0.05 vs. sham-operated, #p< 0.05 : treatment of integrin αv inhibitor vs. without integrin αv inhibitor.

3.3. ECM gene expression and its modulation by integrin alpha v inhibitor following aortic banding

Remodelling of the extracellular matrix is critical during the development of hypertrophy, Our data showed that investigation of downstream kinase signalling revealed activation of the p38 Mitogen-Activated Protein Kinase (MAPK), the Extracellular signal-Regulated Kinases 1 and 2 (Erk 1 and 2), Focal Adhesion Kinase (FAK), and tyrosine-phosphorylation of c-Src in wild-type hearts upon aortic banding, which was blunted in the mice receiving the integrin αv inhibitor via mini pump. After interaction with the extracellular matrix, signals are transmitted by integrins to the cell cytoplasm through a process termed “outside-in signalling”. In order to investigate the targets of the Integrin αv-mediated outside-in-signalling, to reveal the interplay of integrin-mediated signal transduction and hypertrophy with Extracellular Matrix (ECM) deposition we performed microarray analyses probing a total of 96 cell adhesion and extracrellular matrix genes. Gene expression profiling was performed on the aortic banding –induced pressure over load and sham-operated mouse heart. The distribution of the gene fragments, positive, negative controls and the complete gene list are available at www.superarray.com.The use of Ampolabeling-LPR derived probes provides more accurate and sensitive gene expression profiling results in our cDNA microarrays. The expression levels were analysed and shown as -fold intensity in comparison with sham operated samples displayed in Table 5. The used arrays encompass 96 cDNAs encoding cell adhesion molecules, extracellular matrix proteins, and additional proteins that play central roles in cell-extracellular matrix interactions as well as tissue remodelling. Upon total RNA isolation from individual heart tissues 7 days after the intervention, mRNA was reverse transcribed using gene specific primers in the presence of biotin-labeled UTP before hybridization to the array and signal detection. Following normalization of individual hybridization experiments, the expression levels in infarcted hearts were compared to sham-operated samples. Whereas none of the analysed 96 genes showed significant down-regulation during the course of the study, a total of 18 genes were found to be induced more than 2-fold after aortic banding 7 days. eg.,fibronectin (2.32 ± 0.94), collagen (8.11 ± 2.2), Adamts -1 (3.51 ± 0.81), SPARC (3.78 ± 0.12), and Tissue inhibitor of metalloproteinase 2 (TIMP2, 2.23 ± 0.98) were strongly up-regulated, whereas this up-regulation was abolished in mice with treatment of integrin αv

inhibitor. An increase of integrin αv gene expression upon aortic banding was observed (Fig 3.9) and this up-regulation was further conformed in protein level (Fig 3.10).

3.3.1. Integrin αv expression in myocardium

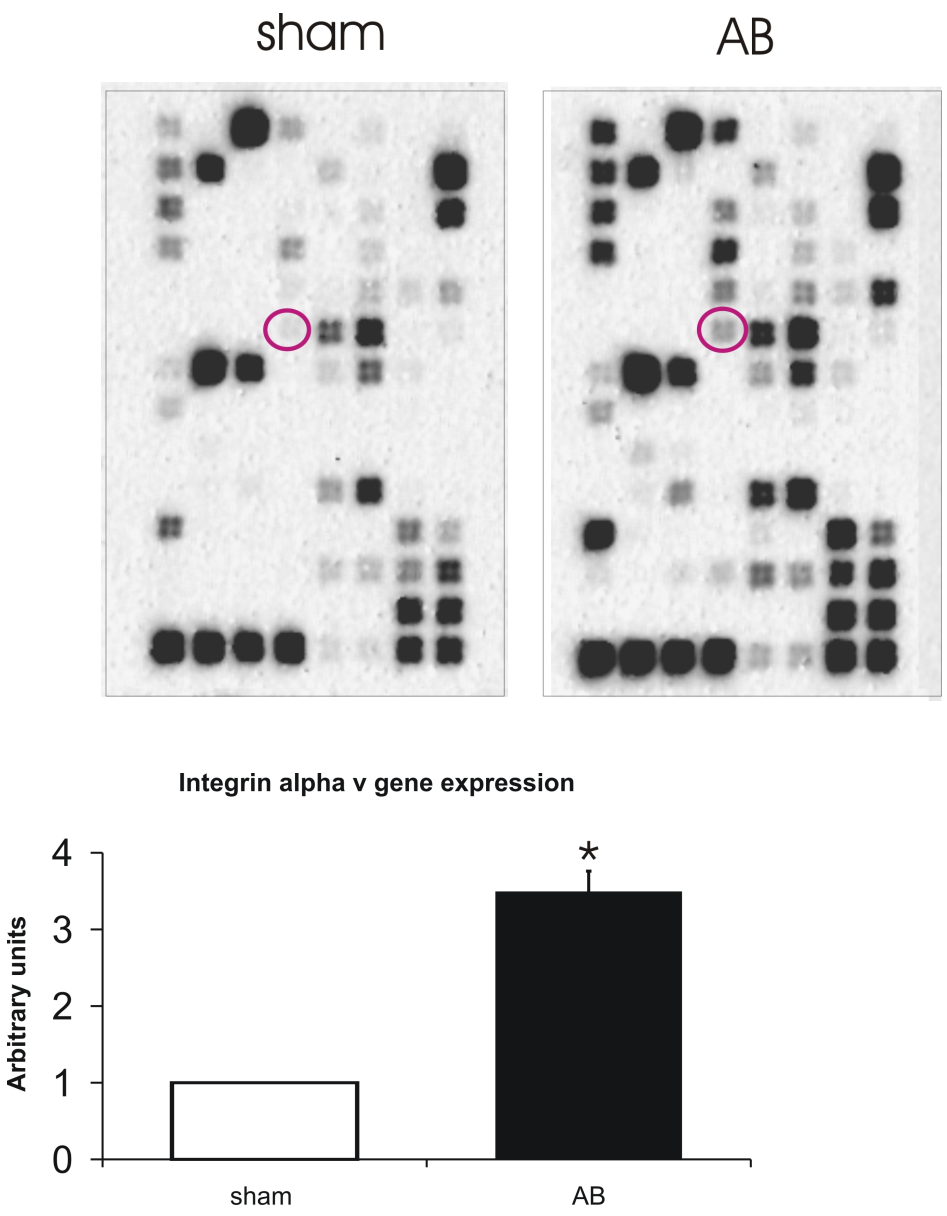

Fig 3.10. Induction fo integrin αv gene expression in myocardium upon aortic banding RNA was extracted from mice heart (n=6) after aortic banding 7 days with or without integrin αv inhibitor. Total RNA (5μg/membrane) was reverse-transcribed and labeled with biotin, and gene expression levels were detected using Nonrad-GEArray Q series kit and LPR kit. Expression of Integrin αv in a representative sham-operated and aortic banding were indicated by circles. Analysis of 6 individual animals showing below the image, Data are expressed as fold change from corresponding sham-operated mice.open bar and close bar indicate sham and aortic banding animals

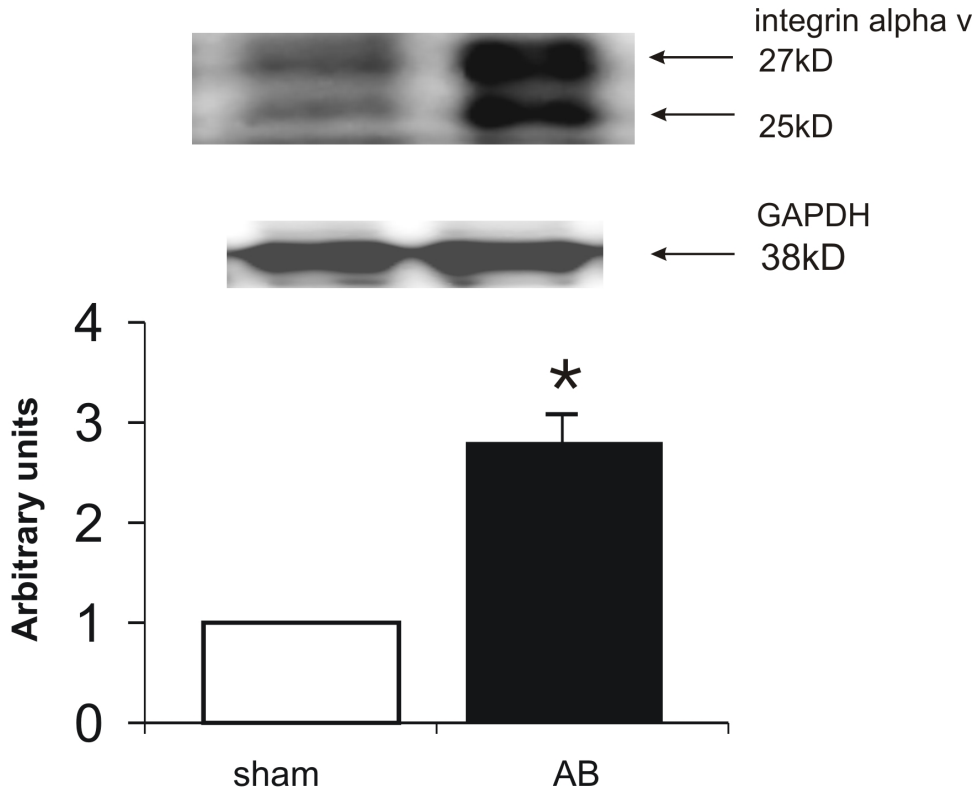

Figure 3.11. Enhanced Integrin αv protein level after aortic banding in heart ventricle. Western blot analysis was performed on LV lysates prepared as described in Methods. Integrin αv expression in day 7 after AB is shown upon panels as indicated. Protein loading was verified using anti-GAPDH. Analysis of Data are expressed as fold changes from corresponding sham-operated mice. Open bar and close bar represent sham-operated and aortic banding mice respectively. Values are mean ± SEM; n= 12; * = p< 0.01 AB vs. sham operated animals;

3.3.2. Regulation of ECM gene expression of upon pressure overload

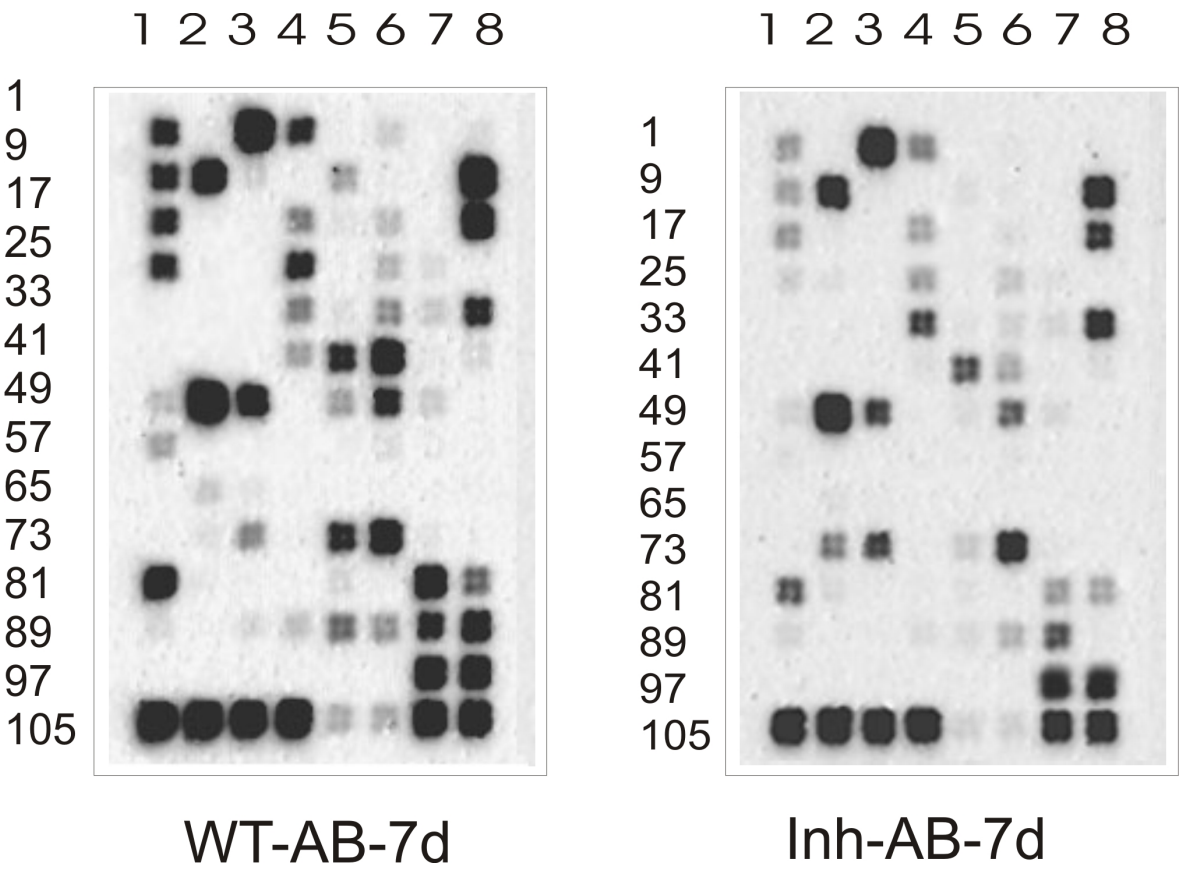

Figure 3.12. Representative expression profile of integrin–related genes after AB in mice heart detected by cDNA expression array system. RNA was extracted from mice heart (n=6) after aortic banding 7 days with or without integrin αv inhibitor. Total RNA (5μg/membrane) was reverse-transcribed and labelled with biotin, and gene expression levels were detected using Nonrad-GEArray Q series kit and LPR kit. Blanks: (100-102), negative control genes; PUC18DNA (97-99), positive control genes: glyceraldehydes-3-phosphate dehydrogenase (103,104), cyclophilin A (PPIA) (105-108), ribosomal protein L13a (109,110), and β-actin (111,112). All positive controls showed a clear hybridization signal, whereas blanks and negative controls consistently showed the absence of any hybridization signal. Membranes were hybridized with labelled RNA previously isolated from individual animals.

Table 4, Changes of gene expression associated with integrin αv following AB

Spot location	Gene name	fold induction (n=5) AB/sham	AB-Inh / sham
Cell adhesion molecules			
17	CEACAM 1	1.88 ± 0.77	1.34 ± 0.50
9	Catnb	2.08 ± 0.47	0.98 ± 0.78
38	Integrin alpha 6	2.41 ± 0.30	0.77 ± 0.12
39	Integrin alpha 7	2.10 ± 0.63	1.01 ± 0.17
44	Integrin alpha v	3.49 ± 0.23	3.61 ± 0.17
46	Integrin beta 1	2.16 ± 0.29	1.20 ± 0.14
50	Integrin beta 5	3.40 ± 0.63	2.93 ± 0.14
39	Integrin alpha 7	2.20 ± 0.63	1.01 ± 0.17
77	PECAM 1	2.41 ± 0.51	1.31 ± 0.32
78	tPA	2.02 ± 0.37	1.11 ± 0.23
81	ELAM-1	2.26 ± 0.30	0.71 ± 0.16
95	VCAM-1	1.83 ± 0.35	1.82 ± 0.16
Extracellular matrix proteins			
20	Collagen 1a1	8.11 ± 2.27	2.21 ± 2.27
31	fibronectin	2.32 ± 1.04	1.47 ± 0.42
54	Laminin B1	1.80 ± 0.15	1.12 ± 0.09
87	SPARC	3.77 ± 0.12	0.86 ± 0.29
96	Vitronectin	2.07 ± 0.14	0.76 ± 0.20
Proteases			
1	Adamts -1	3.51 ± 0.81	0.95 ± 0.25
Protease inhibitor			
93	Timp2	2.23 ± 0.98	1.51 ± 0.91

The listed genes displayed on average a more than 2-fold difference between the sham-operated and the aortic banding animals 2 days after the intervention. The expression levels are shown as -fold increase compared to sham operated samples after normalization using the internal control (GAPDH). Values represent mean ± SEM of independent hybridizations of RNA isolated from 6 animals for each treatment for each time point. CEACAM 1: carcinoembryonic antigen-related cell adhesion molecule 1; Catnb: beta-catenin, PECAM1: platelet/endothelial cell adhesion molecule (CD31 antigen); (tPA): tissue plasminogen activator; ELAM-1: Endothelial Leucocyte Adhesio Moleule-1; VCAM-1: vascular cell adhesion molecule 1.anti-angiogenic protein; Adamts -1: A disintegrin-like and metalloprotease (reprolysin type) with trombospondin type 1; Timp2: Tissue inhibitor of metalloproteinase 2. Values are means ± SEM, (n=5)

Adamts1 1	Adamts8 2	Bsg 3	Casp8 4	Casp9 5	Catna1 6	Catna2 7	Catnal1 8
Catnb 9	Cav 10	Cd44 11	Cdh1 12	Cdh2 13	Cdh3 14	Cdh4 15	Cdh5 16
Ceacam1 17	Cntn1 18	Col18a1 19	Col1a1 20	Col4a2 21	Cst3 22	Catnd2 23	Ctsb 24
Ctsd 25	Ctse 26	Ctsg 27	Ctsh 28	Dcc 29	Ecm1 30	Fn1 31	Icam1 32
Itga2 33	Itga2b 34	Itga3 35	Itga4 36	Itga5 37	Itga6 38	Itga7 39	Itga8 40
Itgae 41	Itgal 42	Itgam 43	Itgav 44	Itgax 45	Itgb1 46	Itgb2 47	Itgb3 48
Itgb4 49	Itgb5 50	Itgb6 51	Itgb7 52	F11r 53	Lamb1-1 54	Lamc1 55	Mmp1a 56
Mgea5 57	Mmp10 58	Mmp11 59	Mmp12 60	Mmp13 61	Mmp14 62	Mmp15 63	Mmp16 64
Mmp17 65	Mmp19 66	Mmp2 67	Mmp20 68	Mmp23 69	Mmp24 70	Mmp3 71	Mmp7 72
Mmp8 73	Mmp9 74	Ncam1 75	Ncam2 76	Pecam 77	Plat 78	Plau 79	Plaur 80
Sele 81	Sell 82	Selp 83	Serpinb5 84	Serpine1 85	Serpinb2 86	Sparc 87	Thbs1 88
Thbs2 89	Thbs3 90	Thbs4 91	Timp1 92	Timp2 93	Tnc 94	Vcam1 95	Vtn 96
PUC18 97	PUC18 98	PUC18 99	Blank 100	Blank 101	Blank 102	Gapd 103	Gapd 104
pia 105	Ppia 106	Ppia 107	Ppia 108	Rpl13a 109	Rpl13a 110	Actb 111	Actb 112

figure 3.13. Distribution of genes in mouse heart chip. The chip encompasses 96 genes that encode cell adhesion and extracellular matrix proteins. These proteins play key roles in mediating cell-cell, cell-tissue and cell-extracellular matrix interactions. The PCR fragment of each gene was printed on a Nytran+ membrane with rectangular area of 23x35 mm.There are 96 spots including blanks: (100-102), negative control: PUC18DNA (97-99), positive control genes: glyceraldehyde-3-phosphate dehydrogenase (103,104); cyclophilin A (PPIA) (105-108), ribosomal protein L13a (109,110) and beta-actin (111, 112).

4. Discussion

In the present study, we demonstrated that integrin- mediated adhesion and signalling play significant roles in the cardiac hypertrophic response. The major findings are as follows; A) integrin αv subunit is required to sustain compensatory cardiac hypertrophy in response to hemodynamic pressure overload, as AB in integrin αv inhibited mice led to a dilated cardiomyopathy but not a compenensatory hypertrophy observed in controls; B) significant activation of p-38 Mitogen-Activated Protein Kinase (MAPK), the Extracellular signal-Regulated Kinases 1 and 2 (Erk ½), Focal Adhesion Kinase (FAK), and tyrosin-phosphorylation of c-Src in wild type AB heart were blunted in integrin αv inhibition's mice. C) 7 days after AB 18 ECM genes were up-regulated more than 2-fold (n=6), e.g. collagen (8.11 ± 2.2), Fibronectin (2.32 ± 0.94), secreted protein, acidic and rich in cysteine (SPARC, 3.78 ± 0.12), : A disintegrin-like and metalloprotease (reprolysin type) with trrombospondin type 1(Adamts -1, 3.51 ± 0.81) and Tissue inhibitor of metalloproteinase 2 (TIMP2, 2.23 ± 0.98), whereas this up-regulation was abolished in mice that were treatEd by integrin alpha v inhibitor via mini pump.

4.1. Integrin αv is required for cardiac hypertrophy

Our main attention was focused on the α_v integrin, because it appears to play a major role in several processes relevant to remodelling, such as binding and activation of matrix metalloproteinases (Meerovitch, Bergeron et al. 2003) and growth factors (Trusolino, Serini et al. 1998), as well as cell proliferation (Cruet-Hennequart, Maubant et al. 2003), migration, and differentiation. The effects of the integrin αv subunit on the development of hypertrophy are unknown. To target the role of integrin αv in response to pressure over load, we used a specific integrin αv inhibitor: EMD 121974, a substance that is being used as an antiangiogenesis and anticancer drug, also called cilengitide. (Taga, Suzuki et al. 2002) Cilengitide inhibits two receptor proteins: integrins αvβ3 and αvβ5, which are useful for inhibiting bone resorption, treating and preventing osteoporosis, and inhibiting vascular restenosis, diabetic retinopathy, macular degeneration, angiogenesis, atherosclerosis, inflammatory arthritis, cancer, and metastatic tumor growth.

Integrin αv gene expression was hardly detected in mice myocardium as well as in protein level in our study. Previously study showed that distribution of αv integrin during mouse embryonic development is dynamically regulated in the glia of the central nervous system and in skeletal muscle (Hirsch, Gullberg et al. 1994) . In adult striated muscle, the amount of alpha v subunit dramatically declined and immunostaining was no longer detectable. (Hirsch, Gullberg et al. 1994) During development, alpha v was weakly evident in heart and endothelia of blood vessels, mesonephric tubula, smooth muscle of the digestive tract, and bronchia. Comparative analysis of the localization of alpha v, alpha 3, and alpha 5 integrin subunits indicated that alpha v has a unique and highly regulated distribution pattern. (Tarone, Hirsch et al. 2000). However, after aortic banding, integrin αlpha v expression was significant increased in the hypertrophic heart tissue either in mRNA level or in protein level, which indicated that alpha-v integrin expression is associated with the hypertrophic response.

In the present study, we used five parameters (LV weight, heart wall thickness; diameter of LV; volume of LV and ANP expression) to document left ventricular hypertrophy. In mice only treated with integrin alpha v inhibitor, there were few abnormal cardiac function, but were not tolerate hemodynamic loading. After two days following aortic banding ratio of heart weight to tibia as compared to wild type did not differ to significant difference, however, there was a significant increase of the posterior wall thickness; interventricular septum, and ANP expression at either day 2 or day 7 after aortic banding, whereas, no increases of these parameters were shown in the mice that were treated with integrin inhibitor, suggesting integrin αv is required in the development of cardiac hypertrophy.

Cardiac hypertrophy has been considered as an adaptive response of the heart to a variety of intrinsic and extrinsic stimuli. The hypertrophic response, during which cardiomyocytes increase in size without undergoing cell division, initially serves to compensate for decreased cardiac output, however, some researches revealed that hypertrophy process is not entirely beneficial, The view is further supported by using some inhibitor of hypertrophy in clinic trials, such as a calcium antagonist, nicardipine SR, and an angiotensin-converting enzyme (ACE) inhibitor, they all reduced left ventricular hypertrophy and improved left ventricular systolic and diastolic function (Sumimoto, Ochi et al. 1997), this reduced the risk for several endpoints like death and progression to heart failure. We questioned wether the

reduction of the hypertrophic response by treatment of integrin alpha v inhibitor is also beneficial? If so, the antiangiogenesis and anticancer drug EMD (integrin alpha v inhibitor) may contribute to prevent hypertrophy that leads to dilated cardiomyopathy and heart failure.

Prevention of cardiac hypertrophy through Integin αv inhibitor may be due to deactivated multiple hypertrophy signaling pathways triggered by pressure overload like Fak, Src and ERK1/2 signaling pathways. The effect of integrin αv inhibitor on development of hypertrophy was characterized by left ventricular dilatation, depressed systolic function and increased postoperative mortality, which is not beneficial. A cardiac hypertrophic response can be adaptive or maladaptive, not depend on change of heart wall thickness but rather a normal systolic function. Different inhibitors of cardiac hypertrophy play a different role, without hypertrophic response at the initial stage is beneficial or maladaptive, this may depend on different modulations of signal pathway and ECM expression, which means we can not intervene at any level and expect a similar effect on resulting cardiac phenotype.

The present study demonstrates that the remodelling of pressure overload is not simply involved in the initiation of biochemical signals but also that it coordinates cellular signalling machinery that controls ECM gene expression associated with load-induced cardiac myocyte hypertrophy. Our study suggested that integrin αv is required for the cardiac compensation upon pressure overload and it did so probably by activation of signalling transduction involved Fak, Src and MAPK pathway, and modulation of ECM gene expression profiling.

The mortality following operation was increased in the mice treated with integrin αv inhibition as compared with untreated mice. Furthermore, after aortic banding the FS% in mice treated with integrin αv inhibitor was significantly reduced as compared with that in AB mice, together with that enlarged left ventricular volume observed in mice treated with αv inhibitor, suggesting reduced systolic function in left ventricle associated with integrin alpha v inhibitor. In contrast, there was no significant change of fractional shortening in sham-operated as compared with that in AB mice in neither 2days nor 7days after aortic banding. From a functional point of view we found that following aortic banding 7 days, LVEDP is increased indicating a reduced compliance, which is consistence with results from other group that belong to a typical hypertrophic response following aortic banding. In contrast, we observed a significant decreased LVEDP after aortic banding in mice that were treated with the

α_v integrin inhibitor. This is in line with the 'soft heart' observed in integrin alpha v inhibition's mice. There was a significant increase in lung weight following aortic banding at day 2 in mice either with or without treatment of inhibitor as compared with that in sham operated suggesting pulmonary congestion. The difference could not differ significantly after day 7 following aortic banding as compared with sham-operated, which may due to a compensatory response. These findings revealed that integrin αv plays an important role in the compensatory hypertrophic response in the early remodelling of heart upon pressure overload.

4.2. Integrin alpha v mediated signal transduction is involved in development of hypertrophy upon pressure over load.

Myocardium remodelling after aortic banding has evolved to include not only changes in ventricular size and shape, but also cellular and molecular remodelling. Integrins are the most well-recognized class of ECM receptor, which are heterodimers of α and β subunits, change with development, hypertrophy, and aging. Integrin receptors are involved in signaling in both outside-in and inside-out manner on myocytes and fibroblasts (Giancotti and Ruoslahti 1999; Bouvard, Brakebusch et al. 2001). In this study we employed a well-characterized animal model in wild type mice following aortic banding that displayed compensated pressure overload hypertrophy (LV hypertrophy, normal LV function, and no pulmonary congestion) to examine the role of integrin on Src, FAK, several MAK kinases in cardiac hypertrophy. We determined that there were activations of Phosphorylation of FAK at Tyr-397; phosphorylation of Src at Tyr-416, the Extracellular signal-Regulated Kinases 1 and 2 (Erk ½), and p-38 in hypertrophic heart tissue. No changes were found in JNK activity after aortic banding in neither wild type nor integrin alpha v inhibition's mice. These findings are consistent with the study previously showed in guinea pigs that were performed by subtotal descending thoracic aortic banding (Takeishi, Huang et al. 2001). The activations showed in mice following aortic banding was blunted in integrin αv inhibition's mice that displayed dilated cardiopathy and congestive heart failure (dilated LV, depressed LV function, and pulmonary congestion), suggesting a critical role of integrin alpha v in the development of compansatary hypertrophy.

Cardiac hypertrophy requires coupling of intracellular signal transduction systems with transcription factors that activate and maintain the hypertrophic program. Over the past year, signaling pathways involving FAK kinases have emerged as critical regulators of cardiac hypertrophy. The cytoplasmic focal adhesion protein-tyrosine kinase (FAK) localizes with surface integrin receptors at sites where cells attach to the extracellular matrix (Calalb, Polte et al. 1995). Increased FAK tyrosine phosphorylation occurs upon integrin engagement with fibronectin.(Schlaepfer, Hanks et al. 1994)The FAK tyrosine -397 has been shown to be phosphorylated via an autophosphorylation process, being critical for FAK activation.(Calalb, Polte et al. 1995) Phosphoraylated Try-397 then recruits Src family kinases. The reduced phosphoraylated FAK-Try-397 in our study may lead to reduction of Src-Try-216 that was showed in our study in mice treated by integrin alpha v inhibitior after aortic banding. The activation of FAK is not well understood, but it is coupled to the assembly of focal adhesions. FAK may be recruited to nascent focal adhesions because it interacts, either directly or through the cytoskeletal proteins talin and paxillin, with the cytoplasmic tail of integrin β subunits (Giancotti and Ruoslahti 1999).

In addition, activation of Erk1/2 and P-38 were also observed in compensated hypertrophic heart but not in the enlarged cardiomyopathy suggesting these signals transduction may contribute a compensated response upon pressure overload. The indication was supported by another study that ERK1/2 MAPK activation leads to a concentric form of hypertrophy with enhanced cardiac function. (Bueno, De Windt et al. 2000). In contrast, JNK activation leads to maladaptive hypertrophy (Zhang, Gaussin et al. 2000; Ng, Long et al. 2001, Hayashida, Kihara et al. 2001)) it was reported that marked increases in JNK1 activities were observed 30 minutes after aortic banding, (Fischer, Ludwig et al. 2001)however, the activities in JNK were not observed in our study either 2 days or 7 days following aortic banding, indicating a very early and transient response of JNK to hemodynamic stress.

Taken together, the modulation of signal transduction in our study indicated that specific integrin alpha v- mediated signalling activation programmes may lead to specific cardiac phenotypes. The view was supported in human studies showing that activation of JNK and p-38-MAPKs are augmented in failing human hearts, while ERK activation remains on a physiologic level. Understanding the mechanisms,

through which differential activation of signal pathway may lead to distinctive cardiac phenotypes following pressure over load, is essential for the future clinical therapy.

4.3. Cross talk of ECM and integrin αv

The extracellular matrix has been recognized as a dynamic scenario that directly influences proliferation, survival, migration, and biosynthetic activities of cells. Extracellular matrix (ECM) proteins are the key component during the myocardium remodelling. In recent years, much attention has been focused on the role of cell-extracellular matrix connections in this process. Cardiac hypertrophy is characterized by both remodeling of the (ECM) and hypertrophic growth of the cardiomyocytes. Impaired integrin-mediated adhesion and signaling in myocardium might alter extracellular matrix expression. Here we showed increased expression of cytoskeletal association of the ECM proteins through cell surface accepter integrin αv in pressure-overloaded mice myocardium. These changes are accompanied by cytoskeletal binding and phosphorylation of focal adhesion kinase (FAK) at Tyr-397, c-Src at Tyr-416, activation of the extracellular-regulated kinases ERK1/2, and p-38. The linkage of ECM and cytoskeleton through integrin alpha v is important to preservation of myocardium function. Abrogation of integrin alpha v function, this linkage is disrupted. Delineation of how cell-membrane linked molecules of three classes: ECM, integrin and its signalling pathway is important in the process of heart remodelling.

Genes were found to be up-regulated resulting from pressure overload in our study. This is consistent with the concept that increased ECM is a main event in the remodelling of hypertrophy.(Bendall, Heymes et al. 2002). We have shown here an up-regulation of several integrins subunit in response to aortic banding such as integrin αv, integrin α6, integrin α7, integrin β1and integrin β3. Previous studies have shown that β1 integrins are linked to the hypertrophic response of cultured ventricular myocytes and the intact ventricle can cause increased expression of integrins such as α7 and β1 (Shai, Harpf et al. 2002). Our previous studies have demonstrated that vitronectin, the ligand of integrin alpha v induced induction of growth transfer factor beta 1, an important cytokine in the fibrotic response of the heart (Wu, Laser et al. 2006). Activation of transforming growth factor beta mediated by integrin was reported (Ma, Yang et al. 2003). It is known that TGF beta 1 plays a major role in

regulating the ECM synthesis and the cell-ECM interactions. TGF is thought to be the main mediator for cardiac fibrosis (Akiyama-Uchida, Ashizawa et al. 2002) that is involved in development of hypertrophy. These findings led us to the hypotheses that in our study loss of integrin alphav mediated TGF beta may take an important role in response to inhibited hypertrophy under a condition of pressure overload.

Increase of gene expression encoding ECM proteins that contain the Arg-Gly-Asp (RGD motif) such as collagen, fibronectin, and vitronectin were observed as a result from pressure overload in our study. Interstitial collagen accumulation has been extensively demonstrated to be increased at both mRNA and protein levels in pressure-overloaded cardiac hypertrophy previously (Brilla, Janicki et al. 1991). Increased LVEDP after aortic banding indicates abnormal myocardial diastolic stiffness. Previously study demonstrated that interstitial fibrosis, which associated with accumulation of collagen is responsible for heart diastolic stiffness (Brilla, Janicki et al. 1991). Thus the enlarged heart with depressed LVEDP in integrin alpha v inhibion's mice under the condition of hemodynamic overload may due to the reduced collagen amounts as compared to untreated AB mice, this would explain our observed phenotype with decreased LVEDP ("soft heart"). Collagen amount is regulated probably by integrin-mediated signal transduction through the RGD motif (inside-out signalling).

Increased cardiac ECM is due to a combination of increased biosynthesis and decreased degradation of ECM proteins, likely related to reduced MMP activity, mediated in part through the action of endogenous protease inhibitors (TIMPs) (Brew, Dinakarpandian et al. 2000; Volterrani, Giustina et al. 2000). The MMP can degrade several ECM components. The spectrum of ECM proteins that can be degraded is broad and includes fibronectin, tenascin, laminin native type-I and type-III collagens. Non-ECM proteins like pro-αv integrin cleaved by MT-MMPs (membrane type MMPs) (d'Ortho, Will et al. 1997; English, Puente et al. 2000; Sounni and Noel 2005). Altered expression and activity of various MMPs have been detected during cardiac remodelling induced by a variety of stimuli. In the present study TIMP2 was found to be significantly up- regulated in wild type mice after aortic banding, the increase was abolished and accompanied with a reduction of collagen expression in the mice treated with integrin alpha v inhibitor. Together with changed signalling transduction observed in integrin alpha v inhibitor treated mice, suggesting that activity of TIMP2 might be modulated by the signallings that were transmitted

from inside of cells through integrin alpha v (inside-out signal). These findings led us to hypothesize that reduced TIMP2 expression in integrin alpha v inhibition's mice may be, in part, responsible for an increased degradation of ECM, thus led to increases in left ventricular diastolic diameter.

In addition to MMP family members, an increase of ADAMTS-1 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif) gene expression after aortic banding was observed in the present study. Moreover, the upregulation was abrogated in the mice treated with integrin alpha-v inhibitor. The ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of proteases includes a group of 19 secreted enzymes with a more restricted spectrum of catalytic activities than the well known matrix metalloproteinases (MMP) (Nakamura, Hirohata et al. 2004). ADAMTS plays an essential role in cardiac development (Zhou, Weskamp et al. 2004) as ADAM19 null mice display a ventricular septal defect, abnormal aortic and pulmonic valve structures as well as abnormal vasculature. ADAMTS-1 was first described (Kuno, Kanada et al. 1997), there has been a rapidly expanding body of literature describing this gene family and the proteins they encode. ADAMTS1 is an extracellular metalloproteinase known to participate in a variety of biological processes that includes inflammation, angiogenesis, and development of the urogenital system (Torres-Collado, Kisiel et al. 2006).Our previously study have shown a significant increase of ADAMTs-1 gene expression in myocardial infarction (Wu, Laser et al. 2006), which is consistent with other results. (Manso, Elsherif et al. 2006).

We are interested in ADAMTS1 as a possible contributor to cardiac hypertrophy. ADAMTs are thought to be involved in cardiac hypertrophy as illustrated through studies of their shedding and subsequent activation of HB-EGF (heparin-binding epidermal growth factor). In turn, this activates MAPK signalling and cardiac hypertrophy (Asakura, Kitakaze et al. 2002). Integrin alpha v subunit is reported to bind to ADAM disintegrin domain. The disintegrin-like domains of many ADAMs are capable of acting as integrin ligands (Bridges, Sheppard et al. 2005). Thus, in our study the activity of MAPK and FAK/Src signalling upon pressure overload might lead to altered ADAMTs-1 expression through integrin alpha v. Agents that block the action of ADAM12, such as KB-R7785, not only attenuated cardiac hypertrophy resulting from pressure overload, but also improved cardiac function, which may therefore be useful in hypertrophy. However, in the present study, following aortic

banding there is an increased mortality and attenuated cardiac function in integrin alpha v inhibition's mice accompany with reduced expression of ADAMTS-1.

The function of ADAMTS-1 in the development of hypertrophy is unclear. ADAMTS-1 has been demonstrated to be involved in the inhibition of angiogenesis and shown to bind VEGF and efect the VEGF signalling pathway(Vazquez, Hastings et al. 1999; Luque, Carpizo et al. 2003), furthermore, it was found that ADAMTS-1 is a substrate for a number a MMPs. Proteolysis of ECM proteins alters integrin-mediated anchorage, focal adhesions, cytoskeletal architecture, and signaling molecules like FAK, MAPK signalling, which is involved in hypertrophic response. Degradation of cell adhesion molecules by ECM proteinases like ADAMT1 may release their linkage with the cytoskeleton and alter signalling.

Regulated adhesion molecules were detected in our study in the mice treated with integrin alpha v inhibitor under a condition of pressure overload, such as PECAM 1, ELAM-1, and VCAM-1. The precise mechanisms regulating these events remain largely undefined. Tyrosine phosphorylation and activation of FAK accompanies integrin-mediated adhesion, and dephosphorylation promptly occurs when cells are detached (Schaller 1996; Gao, Zoller et al. 1997; Aplin, Howe et al. 1998) It seems evident that there are extensive interactions between adhesion receptors particularly integrin and signalling components. A main role for the adhesion molecules may to organize membrane –proximal cytoskeletal structures that then serve a scaffolds for signalling cascades (Juliano 2002).cell–matrix and cell–cell adhesion is an important component of the dynamic remodeling process. Integrins, MMPs and ADAMST, as membrane associated molecules, are likely to be important independent or collaborative members of this molecular response pathway. Understanding the mechanisms by which pericellular proteinases are regulated and activated, the nature of their molecular targets, and how adhesion and proteolysis are integrated will provide exciting avenues for investigation of cardiac remodelling over the next few years. The alternation of extracellular matrix expression in integrin alpha v inhibition's mice may have a role to prevent cardiac hypertrophy in response to acute pressure-overload. Lack of integrin alphav, the signal elicited from ECM transfer to the inside of cells resulting reduced activation of those kinases, in turn, ECM thesis were modulated by reduced signal transduction. In conclusion, the cellular response is dictated by which ECM protein activates which specific integrin.

In addition, activation of integrins affects the inflammatory response may through ADAMs activity, which itself can control integrin-mediated gene expression.

We conclude that signaling downstream of integrin alpha v is mediated by the MAPK, FAK and c-Src pathways leading to an up-regulation of extracelluar matrix components necessary for the compensatory response of the heart.

It is a future challenge to dissect the molecular interconnetions between important signailing pathways such as FAK/MAPK cascade and aberrant ECM modulation during heart remodelling..

5. ABSTRACT

Integrins are transmembrane receptors transmitting mechanical signals from the extracellular matrix (ECM) to the cytoskeleton (outside-in-signaling). Many molecular defects in the link between cytoskeleton and ECM are known to induce cardiomyopathies. α_v integrin appears to play a major role in several processes relevant to remodeling, such as binding and activation of matrix metalloproteinases as well as regulation of cell proliferation, migration, and differentiation. We hypothesized that α_v integrin-mediated signaling is required for the compensatory hypertrophy after aortic banding (AB) and associated with the modulation of ECM protein expression. Mice were treated in vivo with a specific integrin α_v inhibitor or vehicle via osmotic minipumps starting 1 day prior to aortic banding (AB). At day 2 and day 7 following AB or sham-operation, the mice were examined by echocardiography and hemodynamic analyses were performed. Treatment of alpha v Integrin inhibitor led to a dilated cardiomyopathy and congestive heart failure in AB mice (dilated left ventricle, depressed LV function, and pulmonary congestion), but not to hypertrophy as observed in mice without inhibitor treatment. Investigation of downstream signaling revealed significant activation of the p38 Mitogen-Activated Protein Kinase (MAPK), the Extracellular signal-Regulated Kinases 1 and 2 (Erk 1/2), Focal Adhesion Kinase (FAK) and tyrosine-phosphorylation of c-Src in mice 7 days after AB. This response was blunted in mice treated with integrin alpha v inhibitor. Microarrays probing for a total of 96 cell adhesion and ECM genes identified various genomic targets of integrin alpha v mediated signalling. 7 days after AB 18 ECM genes were up-regulated more than 2-fold (n=6), e.g. collagen (8.11 ± 2.2), fibronectin (2.32 ± 0.94), secreted protein, acidic and rich in cysteine (SPARC, 3.78 ± 0.12), A disintegrin-like and metalloprotease (reprolysin type) with trombospondin type 1 (Adamts-1, 3.51 ± 0.81) and Tissue inhibitor of metalloproteinase 2 (TIMP2, 2.23 ± 0.98), whereas this up-regulation was abolished in mice that were treatd by integrin alpha v inhibitor via mini pumps. We conclude that signaling downstream of integrin alpha v is mediated by the MAPK, FAK and c-Src pathways leading to an up-regulation of extracelluar matrix components necessary for the compensatory response of the heart under a condition of pressure overload.

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Appendices

Gene description of gene array

Position	UniGene	Genebank	Symbol	Description
1	Mm.1421	NM_009621	Adamts1	Mus musculus a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif
2	Mm.100582	NM_013906	Adamts8	Mus musculus a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif
3	Mm.726	Y16256	Bsg	Mus musculus mRNA for basigin
4	Mm.10736	AF067834	Casp8	Caspase 8
5	Mm.88829	AB019600	Casp9	Mus musculus mRNA for caspase9
6	Mm.18962	NM_009818	Catna1	Mus musculus catenin alpha 1 (Catna1)
7	Mm.34637	NM_009819	Catna2	Mus musculus catenin alpha 2 (Catna2)
8	Mm.12878	NM_018761	Catnal1	Mus musculus catenin alpha-like 1 (Catnal1)
9	Mm.3476	NM_007614	Catnb	Mus musculus catenin beta (Catnb)
10	Mm.28278	NM_007616	Cav	Mus musculus caveolin, caveolae protein, 22 kDa (Cav)caveolin 1, caveolae protein, 22kD
11	Mm.24138	M27130	Cd44	Mouse CD44 antigen
12	Mm.35605	NM_009864	Cdh1	Mus musculus cadherin 1 (Cdh1)
13	Mm.14897	NM_007664	Cdh2	cadherin 2
14	Mm.4658	X06340	Cdh3	cadherin 3
15	Mm.2419	NM_009867	Cdh4	cadherin 4
16	Mm.21767	NM_009868	Cdh5	Mus musculus cadherin 5 (Cdh5),
17	Mm.14114	NM_011926	Ceacam1	CEA-related cell adhesion molecule 1,or carcinoembryonic antigen (CEA)-like

				antigen
18	Mm.4911	NM_007727	Cntn1	Mus musculus contactin 1 (Cntn1),
19	Mm.196000	NM_009929	Col18a1	Mus musculus procollagen, type XVIII, alpha 1 (Col18a1)
20	Mm.22621	U08020	Col1a1	Mus musculus FVB/N collagen pro-alpha-1 type I chain
21	Mm.181021	J04695	Col4a2	Mus musculus alpha-2 type IV collagen (GC is over 70%)
22	Mm.4263	NM_009976	Cst3	Mus musculus cystatin C (Cst3)
23	Mm.6680	NM_008729	Ctnnd2	catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)
24	Mm.22753	NM_007798	Ctsb	Mus musculus cathepsin B (Ctsb)
25	Mm.2147	NM_009983	Ctsd	Mus musculus cathepsin D (Ctsd)
26	Mm.33671	X97399	Ctse	cathepsin E
27	Mm.4858	X78544	Ctsg	cathepsin G
28	Mm.2277	U06119	Ctsh	cathepsin H
29	Mm.57226	NM_007831	Dcc	Mus musculus deleted in colorectal carcinoma (Dcc)
30	Mm.3433	NM_007899	Ecm1	Mus musculus extracellular matrix protein 1 (Ecm1)
31	Mm.193099	M18194	Fn1	Mouse fibronectin (FN) mRNA
32	Mm.28973	M31585	Icam1	Mouse (clone lambda-c5e) intercellular adhesion molecule 1 (ICAM-1)
33	Mm.5007	NM_008396	Itga2	Mus musculus integrin alpha 2 (Cd49b) (Itga2) (no Itga1 Homologue in Mouse)
34	Mm.26646	NM_010575	Itga2b	Mus musculus integrin alpha 2b (Cd41b) (Itga2b)
35	Mm.57035	NM_013565	Itga3	Mus musculus integrin alpha 3 (Cd49c) (Itga3)
36	Mm.1346	X53176	Itga4	Mouse integrin alpha-4 mRNA

37	Mm.16234	NM_010577	Itga5	Mus musculus integrin alpha 5 (fibronectin receptor alpha) (Itga5)
38	Mm.25232	NM_008397	Itga6	Mus musculus integrin alpha 6 (Itga6)
39	Mm.179747	NM_008398	Itga7	Mus musculus integrin alpha 7 (Itga7)
40	Mm.86983	AF041409	Itga8	Mus musculus integrin alpha8 mRNA
41	Mm.96	NM_008399	Itgae	integrin, alpha E, epithelial-associated
42	Mm.1618	AF065902	Itgal	Mus musculus strain DBA/2J integrin alpha L (Itgal) mRNA
43	Mm.4967	NM_008401	Itgam	Mus musculus integrin alpha M (Cd11b) (Itgam)
44	Mm.4427	NM_008402	Itgav	Mus musculus integrin alpha V (Cd51) (Itgav)
45	Mm.22378	NM_021334	Itgax	Mus musculus integrin alpha X (Cd11c) (Itgax)
46	Mm.4712	X15202	Itgb1	Mouse mRNA for fibronectin receptor beta-chain (VLA5-homolog.)
47	Mm.1137	NM_008404	Itgb2	Mus musculus integrin beta 2 (Cd18) (Itgb2)
48	Mm.8013	NM_016780	Itgb3	Mus musculus integrin beta 3 (Cd61) (Itgb3)
49	Mm.21117	L04678	Itgb4	Mouse integrin beta 4 subunit
50	Mm.6424	NM_010580	Itgb5	Mus musculus integrin beta 5 (Itgb5)
51	Mm.116903	NM_021359	Itgb6	Mus musculus integrin beta 6 (Itgb6)
52	Mm.58	NM_013566	Itgb7	Mus musculus integrin beta 7 (Itgb7)
53	Mm.20903	U89915	Jcam1	junction cell adhesion molecule1
54	Mm.148395	M15525	Lamb1	Mouse laminin B1
55	Mm.1249	J03484	Lamc1	Mouse laminin B2 chain
56	Mm.156952	NM_032006	Mcola-pending	Mus musculus collagenase-like A with Identity of 58.5% to human matrix metalloproteinase 1
57	Mm.202734	AF132214	MGEA5/Hy5	Mus musculus hyaluronidase 5 (Hy5)
58	Mm.14126	NM_019471	Mmp10	Mus musculus matrix metalloproteinase 10 (stromelysin 2)
59	Mm.4561	NM_008606	Mmp11	Mus musculus matrix metalloproteinase 11 (Mmp11)
60	Mm.2055	NM_008605	Mmp12	Matrix metalloproteinase 12

61	Mm.5022	NM_008607	Mmp13	Mus musculus matrix metalloproteinase 13 (Mmp13)
62	Mm.19945	NM_008608	Mmp14	Mus musculus matrix metalloproteinase 14 (membrane-inserted) (Mmp14)
63	Mm.7283	NM_008609	Mmp15	Mus musculus matrix metalloproteinase 15 (Mmp15)
64	Mm.42042	NM_019724	Mmp16	Mus musculus matrix metalloproteinase 16 (Mmp16)
65	Mm.42047	NM_011846	Mmp17	Mus musculus matrix metalloproteinase 17 (Mmp17)
66	Mm.131266	NM_021412	Mmp19	Mus musculus matrix metalloproteinase 19 (Mmp19)
67	Mm.29564	NM_008610	Mmp2	Mus musculus matrix metalloproteinase 2 (Mmp2)
68	Mm.103671	NM_013903	Mmp20	Mus musculus matrix metalloproteinase 20 (enamelysin) (Mmp20)
69	Mm.29373	NM_011985	Mmp23	Matrix metalloproteinase 23
70	Mm.21306	NM_010808	Mmp24	Mus musculus matrix metalloproteinase 24 (Mmp24)
71	Mm.4993	NM_010809	Mmp3	Mus musculus matrix metalloproteinase 3 (Mmp3)
72	Mm.4825	NM_010810	Mmp7	Mus musculus matrix metalloproteinase 7 (Mmp7)
73	Mm.16415	NM_008611	Mmp8	Mus musculus matrix metalloproteinase 8 (Mmp8)
74	Mm.4406	NM_013599	Mmp9	Mus musculus matrix metalloproteinase 9 (Mmp9)
75	Mm.4974	Y00051	NCAM	Mouse mRNA for neural cell adhesion molecule (NCAM)
76	Mm.30313	NM_010954	Ncam2	neural cell adhesion molecule 2
77	Mm.2822	NM_008816	Pecam	Mus musculus platelet/endothelial cell adhesion molecule (Pecam)
78	Mm.154660	NM_008872	Plat	Mus musculus plasminogen activator, tissue (Plat),
79	Mm.4183	X02389	Plau	Mouse mRNA for urokinase
80	Mm.1359	X62701	Plaur	M.musculus muPAR2 mRNA

81	Mm.5245	M87862	Sele	Mus musculus E-selectin protein mRNA
82	Mm.1461	NM_011346	Sell	selectin, lymphocyte
83	Mm.3337	M87861	Selp	Mus musculus P-selectin protein mRNA
84	Mm.4622	NM_009257	Serpinb5	Mus musculus serine protease inhibitor 7 (Spi7)
85	Mm.1263	M33960	Serpine1	Mouse plasminogen activator inhibitor (PAI-1)
86	Mm.5019	X16490	Serpine2	Mouse RNA for plasminogen activator inhibitor 2
87	Mm.35439	NM_009242	Sparc	Mus musculus secreted acidic cysteine rich glycoprotein (Sparc)
88	Mm.4159	M87276	Thbs1	Mouse thrombospondin 1 mRNA
89	Mm.26688	L07803	Thbs2	Mouse thrombospondin 2 (THBS2) mRNA
90	Mm.2114	L24434	Thbs3	Mus musculus thrombospondin 3 (Thbs-3)
91	Mm.20865	AF102887	Thbs4	Mus musculus thrombospondin-4 mRNA
92	MM.8245	NM_011593	Timp1	Mus musculus tissue inhibitor of metalloproteinase (Timp)
93	Mm.181969	NM_011594	Timp2	Mus musculus tissue inhibitor of metalloproteinase 2 (Timp2)
94	Mm.980	NM_011607	Tnc	Mus musculus tenascin C (Tnc),
95	Mm.1021	M84487	Vcam1	Vascular cell adhesion molecule 1
96	Mm.3667	NM_011707	Vtn	Mus musculus vitronectin (Vtn)
97	N/A	L08752	PUC18	PUC18 Plasmid DNA
100	Blank	Blank	Blank	Blank
103	Mm.5289	M32599	Gapd	Glyceraldehyde-3-phosphate dehydrogenase
105	Mm.5246	NM_008907	Ppia	Mus musculus peptidylprolyl isomerase A
109	Mm.13020	NM_009438	Rpl13a	Mus musculus ribosomal protein L13a (Rpl13a) 23 Kda highly basic protein)
111	Mm.103618	M12481	Actb	Cytoplasmic beta-actin

Abbreviations

A_{260}	absorbance at 260 nm
aa	amino acids
Ag	antigen
ANP	atrial natriuretic peptide
ATG	translational start site
BNP	brain natriuretic peptide
BSA	bovine serum albumin
BW	body weignt
cardiac failure	CF
cardiac fibroblasts	CFBs
cDNA	complementary DNA
CF	cardiac failure
Cre	Cre (causes recombination) recombinase
c-Src	c-Src-kinase
dilated cardiomyopathy	DCM
DMEM	Dulbecco's minimum essential medium
DMSO	dimethylsulfoxid
DNA	desoxyribonucleic acid
dp/dt max	velocity of systolic LV pressure development
dp/dt min	velocity of diastolic LV relaxation
ECM	extracellular matrix
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
Erk	Extracellular signal-regulated kinase
FAK	focal adhesion kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
FN	fibronectin
focal adhesion complexes	FACs
FS	fractional shortening

HW	heart weight
IGF	Insulin-like growth fachor
IVS	inventricular septum
Jnk	c-jun N-terminal kinase
Kb	kilobases
kDa	kilodalton
loxP	recognition site for Cre recombinase
LV	left ventricle
LVEDP	LV end diastolic pressure
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MI	myocardial infarction
mRNA	messenger RNA
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PFA	paraformaldehyd
RGD	arginine glycine aspartate
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	rotations per minute
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SPARC	secreted protein, acidic and rich in cystein
TGF	transforming growth factor
VN	vitronectin
WT	wildtype

Honours :

- **Awarded the first place in "Rudolf Thauer poster prize**" at the 71. Annual Conference of the German Society for Cardiology - heart research and circulatory research, Mannheim, Germany, 2005
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Posters and oral presentations

R. Wu, K. Schuh, N Burkard, M hahullber, H Han, K Hu, C Hauck, M. Laser, G. Ertl, O. Ritter. Ventricular myocyte-restricted targeting of Integrin beta1 abolishes compensatory cardiac hypertrophy due to altered expression of extracellular matirx components." *American heart Association, scientific sessions* **"November 12-15, 2006 (Chicargo, USA)**

R. Wu, M. Laser,C. Hauck, H. Han, K. Hu, K. Schuh, G. Ertl, O. Ritter. SPARC upon myocardial infarction orchestrates fibroblast migration and tissue remodelling in the heart."*Leopoldina Symposium, III Symposium on Carediovascular Healing Focus on Inflammation"***June23-24, 2006. (Wuerzburg, Germany)**

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R..Wu, M.Laser, H.Han, K.Hu, J.Varadarajulu, O.Ritter, C. Hauck Transient expression of SPARC upon myocardial infarction orchestrates fibroblast migration and tissue remodelling in the heart. "*Congress of the European Society of Cardiology"* September, 03-09, 2005 (**Stockholm, Sweden)**

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R..Wu, H.Han, K.Hu, M.Laser, O.Ritter Transient expression of SPARC upon myocardial infarction orchestrates fibroblast migration and tissue remodelling in the heart. Meeting of Mediziische Klinik Universität Würzburg "*Bad Brückenauer Wintertagung*". 16-17. Februar 2005 (**Bad Brückenau)**

R..Wu, H.Han, K.Hu, G. Ertl, M.Laser Characterization of cardiac specific excision of the β1 integrin gene in mice. "*8th annual symposium of Chinese Medical Association in Germany."* October 30-31,2004, **(Hamburg, Germany)**

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M.Laser, H.Han,, **R..Wu,** M.Joner, A.Grimm, C.Joha, T.Kupfer, C.Dienesch, K.Hu, J.Strotmann Integrin signaling in the hypertrophy in myocardium is required for survival and hypertrophic growth."*Congress of the European Society of Cardiology*." August 28 – September 01, 2004 **(Munich Germany)**

M.Laser, **R.Wu,** H..Han, , K.Hu, G.Ertl. Integrin signalling is activated and required after myocardial infarction and aortic banding in mice for early compensation and survival. "*70. Jahrestagung der Deutschen Gesellschaft für Kardiologie - Herz- und Kreislaufforschung"* April 15-17, **2004 (Mannheim, Germany)**

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1. Hallhuber[1] M, Burkard[1] N, **Wu RX**[1] Buch[2] MH, Engelhardt[3] S, Hein[4] L, Neyses[2] L, Schuh[5] K, Ritter[1] O (2006) Inhibition of nuclear import of calcineurin prevents myocardial hypertrophy. *Circulation research*. Sep 99 (6)

2. **Wu RX**., Laser M., Han H., Varadarajulu J., Schuh K., Hallhuber M., Hu K., Ertl G., Hauck C., Ritter O.(2006) Fibroblast migration after myocardial infarction is regulated by SPARC expression. *Journal of Molecular Medicine*. Jan 17;:1-12

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4. **Wu RX.,** Laser M., Han H., Hu K.,., Ertl G., Hauck C., Ritter O Schuh K Characterization of cardiac specific excision of the β1 integrin gene in mice- influence on the transcriptional response after transversal aortic constriction (manuscript in preparation)

5. **Wu R.X,** Laser M., Han H., Hu K., Schuh K., Ertl G., Hauck C., Ritter O. Targeting of alpha(v) integrins interferes with gene expression and signal transduction after aortic banding in mice (manuscript in preparation)

6. You J, Zhuang L, Cheng HY, Yan SM, Qiao YW, Huang JH, Tang BZ, Ma YL, Qu JY, **Wu RX**. (2005) andomized, controlled, clinical study of thymosin alpha-1 versus interferon-alpha in [corrected] patients with chronic hepatitis B lacking HBeAg in China. J Chin Med Assoc. 2005 Mar;68(3):154.

7. You J, Zhuang L, Tang BZ, Yang W, Ding S, Li W, **Wu RX**, Zhang H, Zhang Y, Yang S.(2001) A randomized controlled clinical trial on the treatment of Thymosin a1 versus interferon-alpha in patients with hepatitis B. World J Gastroenterol. Jun; 7(3).

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10. **Wu RX**., Gao L., Tian X.,.(1998) Treatment of HBV in patients with traditional Chinese medicine. *Combination of TCM and western medicine* 348, 23-30 (Chinese version)

11. **Wu RX.,** Gao L., Fan J et al.(1997) Pathological observation of the experimental cirrhosis of mice liver after administration with "anti-fibrosis No1",*Theory and Practice of Chinese Contemporary Medicine*, 823-29, Beijing Science and Technique Publishing House. (Chinese version)