Integrin α_v and Focal adhesion kinase -

promising targets to limit smooth muscle cell migration

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(Jeeva Varadarajulu)

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1. Introduction

1.1 Restenosis

Atherosclerosis is a pathological process in which deposits of extracellular lipids, mostly cholesterol, cellular waste products, calcium and other substances build up a plaque in the inner lining of an artery (Lusis, 2000). Plaques can grow large enough to induce a narrowing of the blood vessel, a process so-called stenosis. Arterial stenosis of the heart blood vessels triggers systemic hypertension and constitutes the major risk factor for congestive heart failure. Currently, percutaneous transluminal coronary angioplasty (PTCA, also known as coronary artery balloon dilation or balloon angioplasty) is the therapy of choice for coronary artery stenosis (Bult, 2000). During this non-surgical procedure, a catheter with a deflated balloon at its end is guided through to the narrowed end of the obstructed artery. Then the balloon is inflated, the pressure compresses the plaque and enlarges blood vessel inner diameter, ensuring easy blood flow again. Finally, the balloon is deflated and the catheter is removed. About 70-90% of PTCAs also involve the positioning of a stent.

Stents are wire mesh tubes used to keep arteries open after PTCA. This procedure is less traumatic and expensive than bypass surgery for patients with coronary artery disease. However, though approximately 1.5 million PTCAs are performed worldwide every year, nearly 30% of the treated patients are affected by restenosis, the re-narrowing of the blood vessel within 6 month after stent implantation (Bult, 2000). Since last decade, when stents were introduced, approximately 500000 PTCA/year have been performed in the U.S.A. However, nearly 150000 cases of restenosis have been reported every year. A slight reduction in the rate of restenosis from 30% to 25% could save as much as $750 million annually (Hillegass et al., 1994). This means that, although vascular interventions to correct stenosis show excellent short-term results, the long-term benefits are seriously compromised by the development of restenosis (Blindt et al., 2002). Over the past two decades, more than 650 studies have been reported on the design of new and effective strategies to prevent restenosis, unfortunately with limited success (in editorial comment of Faxon et al., 2002). Also clinical trials have been performed using stents with pharmacological agents to interfere with plaque formation or the intracellular pathways implicated in this process and they reduce restenosis significantly compared with angioplasty, but they have not eliminated restenosis. For these reasons, prevention of restenosis after PTCA has become the

“holy grail” for interventional cardiology and, the comprehension of the molecular mechanism of restenosis will not only improve the scientific knowledge of this process, but also allow designing of novel and successful strategies to prevent it.

1.1.1 Neointima formation

Within the mechanisms leading to the development of restenosis, vascular smooth muscle cells (VSMCs), the most numerous cells in vascular walls, are known to play a central role in the formation of the restenotic plaque. The intimal thickening of the vessel wall involved in restenosis after PTCA, or neointima formation, is thought to be initiated by the damage of the vessel wall, an event that triggers at the injured sites the conversion of VSMCs from a stationary, quiescent phenotype to a motile, replicative and secretory cell. The release of chemotactic stimuli, as well as haptotactic stimuli by inflammatory and endothelial cells, platelets and VSMCs stimulate the migration of the VSMCs from the middle layer of the vessels (also referred as media) to the injured sites (Axel et al., 1997; Grotendorst et al., 1981; Jones et al., 1996). According to their phenotypic changes, VSMCs start producing extracellular matrix (ECM) proteins and increase the expression of ECM-binding receptors, events that stimulate haptotaxis, the vectorial movement of VSMCs towards a positive gradient of adhesive ECM proteins. Recent studies have determined that the expression of ECM binding receptors on VSMC is a decisive factor in the formation of the restenotic plaque. Among these receptors, integrins play a fundamental role on VSMC migration and proliferation.

1.2 Integrins: a bridge between ECM proteins and intracellular molecular complex

Coordination of cell-cell and cell-matrix adhesion is essential for various cellular functions in multicellular organisms. Adhesion processes are coordinated by cell adhesion molecules, which connect cells with their environment. Among others, integrins, cadherins, selectins, syndecans and the immunoglobulin superfamily-related cell adhesion molecules (IgCAMs) have been implicated in these events (Juliano, 2002). Integrins constitute a major family of adhesion molecules to which the main receptors for ECM proteins such as fibronectin (FN), vitronectin (VN), collagen (CN), or laminin (LN) belong (Hynes, 1992). These molecules integrate the intracellular cytoskeleton with the ECM-rich extracellular environment by binding to intracellular protein complexes (Critchley, 2000). Signalling molecules, which are activated in an

adhesion dependent fashion, are also recruited into these complexes. As a result, ECM proteins, integrins, cytoskeletal proteins and signalling molecules assemble into aggregates on each side of the cellular membrane. On the other hand, integrins interact with cell surface receptors such as the intracellular adhesion molecule-1 (ICAM-1), the vascular cell adhesion molecule-1 (VCAM-1), other growth factor receptors (Meredith et al., 1996) and also bind to co-receptors present on other cells, bacterial polysaccharides, or viral coat proteins (Danen and Sonnenberg, 2003). As a consequence, integrins exert diverse cellular functions including cell adhesion, migration (Hynes, 1992), spreading (Ruoslahti and Reed, 1994), shape, polarity (Schwartz and Ginsberg, 2002), proliferation (Lauffenburger and Horwitz, 1996), apoptosis (Assoian and Marcantonio, 1997), survival (Schaller, 2001), angiogenesis (Luscinskas and Lawler, 1994), cell differentiation (Hynes, 1992), remodelling in normal development, homeostasis of multicellular organisms (Schwartz et al., 1995), organogenesis, regulation of gene expression (Ross and Borg, 2001) and maintenance of the immunity (Schwartz et al., 1995). Integrins are not only involved in normal cellular processes but also pathological conditions such as atherosclerosis, restenosis, invasive processes like tumorigenesis, metastasis and a variety of inflammatory conditions (Schwartz et al., 1995; Simon et al., 1997). Evidence about the decisive role of integrins binding specificity on cell development has also been obtained from integrin-deficient mice. The loss of any integrin α or β leads to severe abnormalities, varying from imperfections to lethality at embryonic stage or after birth. In particular, the β1 subunit can bind to one of several different α subunits, deficiency in the β1 integrin subunit results in embryonic lethality, and this phenotype is much more severe than the lack of one of the α subunits with which it can combine.

At the structural level, integrins are glycosylated, heterodimeric, transmembrane receptors consisting of non-covalently associated α (120 kDa to 180 kDa) and β subunits (90 to 110 kDa; Giancotti and Ruoslahti, 1999; Hynes et al., 1987). Nearly 24 different integrins have been reported to be expressed as combinations of the 18 α and 8 β subunits already described and splice variant isoforms of individual subunits produce additional complexity (Burkin and Kaufman, 1999; de Melker and Sonnenberg, 1999; Song et al., 1993). Interestingly, a recent survey of the human genome has identified 6 novel α and 1 novel β subunit, however, their cellular expression has not been defined yet (Venter et al., 2001).

Each subunit consist of a large extracellular domain (700 to 1100 aa), a single transmembrane segment and a short intracellular cytoplasmic tail (ranging from 20 to 60 aa) that interacts with various proteins essential for the regulation of integrin affinity and cytoskeletal interaction (Aplin et al., 1998; Hynes, 1999). They were initially described as cell matrix adhesion molecules (Tamkun et al., 1986), but later it was found that they are also important signal transducers (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999; Shattil and Ginsberg, 1997). Characteristically, when specific ligands like ECM components bind to a particular integrin to form a cluster, the integrin induces a cascade of intracellular events termed outside-in-signalling; reciprocally, signals from inside of the cell modulate the integrin-binding affinity to ligands, which convert the integrin from a low-affinity or resting state to a high-affinity or primed (also known as activated) state (inside-out-signalling; Giancotti and Ruoslahti, 1999).

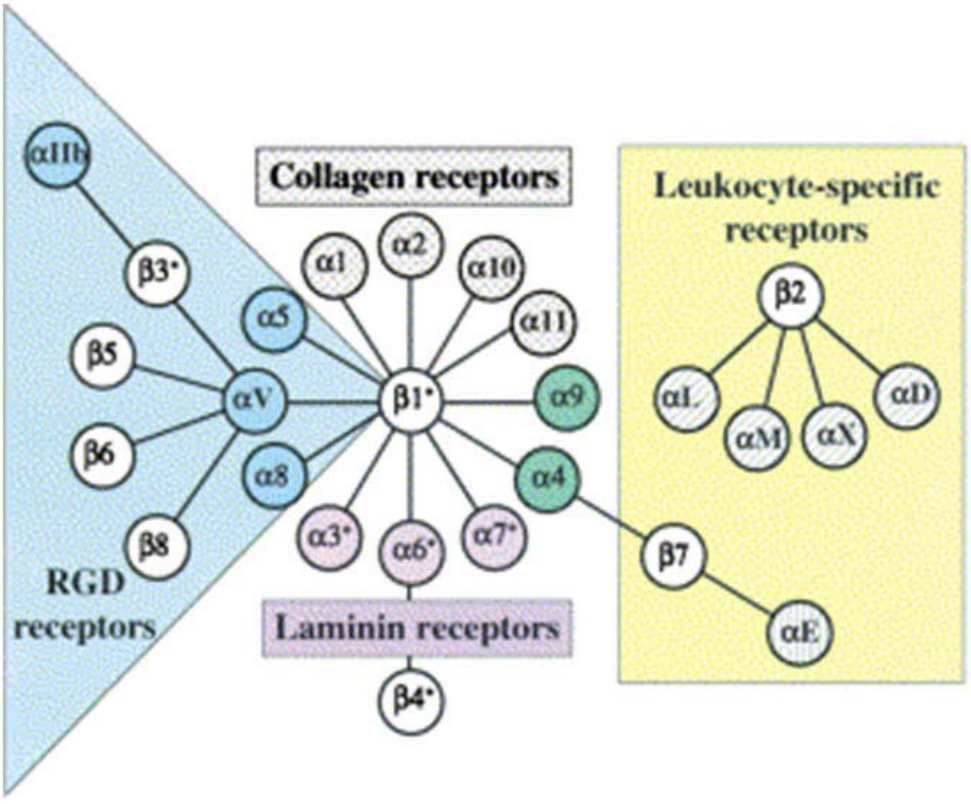

Fig. 1.1. The integrin receptor family. Integrins are made of α and β subunits. 8 α subunits and 18 β subunits associate together in different combinations resulting in at least 24 integrins. These can be subdivided into several subfamilies based on evolutionary relationship, ligand specificity and restricted expression pattern such as β_2 and β_7, which are specifically expressed in white blood cells. Vertebrates have a set of collagen receptors with I/A domains (α_1, α_2, α_{10} and α_{11}) and a pair of related integrins ($\alpha_4\beta_1$, $\alpha_9\beta_1$), which are restricted to chordates. Laminin receptors (purple) and RGD binding receptors (blue) are present throughout the metazoa. Splice variants in cytoplasmic domains were denoted by asterisks (This graphic is reproduced from Hynes, 2002)

Additionally, divalent cations such as Ca^{2+}, Mg^{2+} and Mn^{2+} modulate integrin-ligand binding; Ca^{2+} inhibits these interactions and stabilises a low-affinity conformation while Mn^{2+} stabilises a high-affinity conformation (Mould et al., 1995). Another important feature is that a single integrin binds to one or several ligands and that a single ligand interacts with several heterodimers of integrin subunits. For example, a particular ECM component may be recognized by more than one integrin in a competitive or cooperative manner and at the same time, one particular integrin could recognize several ECM components (Stupack and Cheresh, 2002). Several integrins bind strongly to the tripeptide Arg-Gly-Asp (RGD) sequence within specific ECM proteins such as VN, FN and other proteins. In addition to this RGD binding receptor subfamilies, laminin receptors, collagen receptors and also a set of leukocyte specific integrins are also present in vertebrates (Hynes, 2002; Fig. 1.1).

Integrins are mostly inactive when they are expressed in cells. Their expression can be abundant or low, and they can be expressed constitutively or may be induced by pathophysiological changes or by wounding. Normally in primary VSMC, the integrin expression of subunits α_1, α_3, α_5, α_v, β_1, β_3 and β_5 has been identified (Table: 1; Jones, 1996; Skinner et al., 1994). $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins are found to be upregulated after injury (Clyman et al., 1992).

Several studies have also shown that the $\alpha_v\beta_3$ expression is linked with invasion of various types of malignant tumours (Seftor et al., 1992; Zheng et al., 1999). In addition to VSMC (Albelda and Buck, 1990; Liaw et al., 1995b; Shattil, 1995; Yue et al., 1994). $\alpha_v\beta_3$ integrin is widely expressed on various cell types such as endothelial cells (Cheresh, 1987), keratinocytes (Kim et al., 1994), growth factor-stimulated monocytes and T lymphocytes (Huang et al., 1995; Murphy et al., 1994), leukocytes (Hendey et al., 1996; Lawson and Maxfield, 1995), monocyte-derived macrophages (Stern et al., 1996), fibroblasts (Hall et al., 1994) and platelets (Coller et al., 1991).

Integrin $\alpha_v\beta_3$ interacts with various ECM ligands including VN, osteopontin (ON), thrombospondin (TSP) and denatured CN, which initiates the downstream signalling pathways. Of the various factors that contribute to the development of restenosis, ECM molecules induced signalling events have drawn much attention in recent years. Thus, interference with such signalling pathways will be an effective target to prevent restenosis.

Table 1.1. Integrin expression in vascular smooth muscle (VSM). The table was modified from (Moiseeva, 2001). In some reports conflicting with regard to integrin expression conflicting reports have been reported such as α_1, α_5 and $\alpha_v\beta_3$ integrins; this is due to differences in detection levels, variations between species or variable levels of integrin expression in SMCs from sources of different origin (Smooth muscle; SM).

α	β	Expression in SM in vivo and in non-cultured isolated VSMCs	Expression in cultured vascular SMCs
α1	β1	Upregulated in developing aorta; high expression in human media in aorta, coronary arteries and other VSM; reduced expression in intimal thickening in human aorta. But high expression after injury.	Present in SMCs, Expression decreased in cultured SMCs to undetectable levels, modulated expression.
α2	β1	Expressed in ductus arteriosus. Not expressed in other SM.	Expressed in SMCs
α3	β1	Expressed in human media in arota, coronary arteries and other VSM.	Expressed in SMCs at high level
α4	β1	Expressed in newly formed blood vessels, in atherosclerotic intima, human embryonic arota, not in adult arota	Not expressed. Expressed in newborn ductus arteriosus SMCs.
α5	β1	Found in the media of human normal and atherosclerotic coronary artery, arota and other VSM. Ist expression is induced after a vascular injury in SMCs at the lumenal surface of the neointima.	Abundant in SMCs. Modulated expression
α6	β1/β4	Found in ductus arteriosus SM and in small Vessels. Not expressed in aortic SM.	Not expressed. Expressed in foetal ductus arteriosus SMCs.
α7	β1	Expressed at high levels in VSM	Expressed in foetal ductus arteriosus SMCs. Downregulated in cultured aortic SMCs.
α8	β1	Predominantly expressed in SM	Expressed in aortic SMCs.
α9	β1	Expressed in VSM	Not expressed in newborn ductus arteriosus SMCs.
αv	β1		Expressed in foetal ductus arteriosus and other vascular SMCs.
αv	β3	Expressed in Media of human normal and atherosclerotic arteries and ductus arteriosus. Very early upregulated after inury. Detected only in neointimal SMCs of injured arteries.	Expressed in vascular SMCs. Varied expression levels in SMCs from different sources
αv	β5	Expressed in ductus arteriosus and atherosclerotic intima. Very early upregulated after inury.	Expressed in SMCs.

1.3 The vascular extracellular matrix components supporting cellular processes

The ECM is a structural network of high molecular weight proteins including fibrillar collagens, glycoproteins, elastin, and polysaccharides (Timpl and Brown, 1996). TSP1 and 2, tenascins and SPARC (secreted protein, acidic and rich in cysteine) constitute additional ECM matricellular proteins (Bornstein, 1995; Sage and Bornstein, 1991). These proteins are self secreted by the cells and promote the disassembly of stress fibers and focal adhesions (FAs) while stimulating cell migration (Adams and Schwartz, 2000; Wenk et al., 2000). Additionally, VSMCs are surrounded by a basement membrane that contains LN, FN, CN type-IV and heparan sulphate proteoglycan (HSPG; Dingemans et al., 2000; Thyberg et al., 1990). This basement membrane is a structural support providing strength, elasticity and maintenance of the tissue, which also mediates intracellular signalling through cell surface receptors. ECM basement functions also as a physical barrier to filter selective molecules (e.g., glomerular basement membrane) and to sequester growth factors (Reviewed in van der Flier and Sonnenberg, 2001). As already mentioned, ECM proteins are involved in cytoskeletal reorganization, modulating many cellular processes through integrins. Degradation of ECM proteins is normally implied in morphogenesis, angiogenesis, growth, development and wound healing under normal conditions. However, this process is strictly regulated via several enzymes classified into four classes: matrix metalloproteinases (MMPs) and serine proteinases, cysteine and aspartic proteinases.

MMPs are the major class of enzymes that degrade most of ECM molecules. These enzymes are further classified as collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs), etc (Kuzuya and Iguchi, 2003). Deregulation of these enzyme functions is usually implicated in many pathological processes including arteriosclerosis (George et al., 1998), myocardial infarction and heart failure (Creemers et al., 2001), development and rupture of aneurysms (Pyo et al., 2000), restenosis following balloon angioplasty (Bendeck et al., 1994; Zempo et al., 1994), failure of vein grafts (George et al., 1998), metastasis (Stetler-Stevenson et al., 1993) and cancer (Coussen et al., 2002; Egeblad and Werb, 2002). Various MMPs were initially shown to be increased in cardiovascular pathological processes such as atherosclerotic plaques and post-angioplasty restenotic plaques since their function allows SMCs to migrate from media to intima. Many MMPs are involved in those pathological processes including MMP-1, -2, -3, -7, -9, -12, -13, MT-MMPs (Galis et al., 1994; Halpert et al., 1996; Li et al., 1996;

Okamoto et al., 2001; Rajavashisth et al., 1999; Sukhova et al., 1999; Uzui et al., 2002). Particularly, the increased expression of MMP-2 and MMP-9 correlate with increased VSMC invasion (Kurschat et al., 1999; Pyke et al., 1992). Studies to evaluate the role of MMP inhibitors to prevent cardiovascular complaints, particularly restenosis have revealed that although these inhibitors decrease intima formation, but they fail to inhibit restenosis (Bendeck et al., 1996).

1.4 Focal adhesions as signalling complexes

Cell-matrix adhesions trigger numerous responses that have important role in various cell functions including cell motility, cell proliferation, cell differentiation, regulation of gene expression and cell survival. Adhesion to substrates is mediated by specialized structures, which, are formed by all adherent cells, but their distribution can be varied. The well-characterised adhesions are focal adhesions (FA) and focal complexes. FAs are flat, elongated structures and often located near to the periphery of cells (Abercrombie and Dunn, 1975; Izzard and Lochner, 1976; Sastry and Burridge, 2000) whereas focal complexes are small adhesions at the periphery of cell.

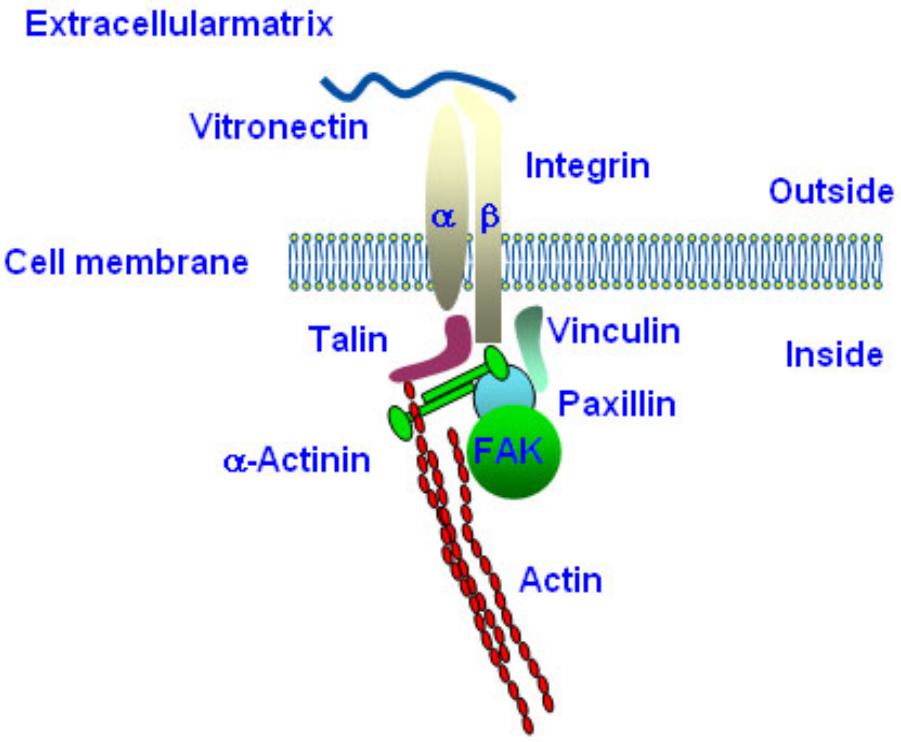

Fig. 1.2. Schematic representation of focal adhesion. Integrin engagement with VN leads to recruitment of several intracellular proteins like talin, vinculin, paxillin etc to form focal adhesion (FA) complex. This protein complex further interacts with actin cytoskeleton. The proteins indicated in the fig. are marker proteins of FA. FAK; focal adhesion kinase, α and β represent the integrin subunits respectively.

FAs mediate strong adhesion to the substrate, and they anchor to actin cytoskeleton by many other proteins. Until now, more than 50 proteins have been associated with FAs. Those proteins are scaffold-signalling molecules, GTPases and other types of enzymes including protein kinases, phosphatases, proteases, lipases and their substrates as well as various adaptor proteins (Fig. 1.2). Interactions between these proteins through different domains lead to signalling cascades (Chrzanowska-Wodnicka and Burridge, 1996; Hotchin and Hall, 1995; Nobes and Hall, 1995; Ridley et al., 1992; Yamada et al., 2003).

However, an ordered recruitment of those components in FAs requires tyrosine phosphorylation and the two major tyrosine kinases involved in FAs are focal adhesion kinase (FAK) and Src family of tyrosine kinases.

1.5 Focal adhesion kinase

FAK is a non-receptor tyrosine kinase (Hanks et al., 1992; Schaller et al., 1992) involved in cell migration (Cary et al., 1996), cell survival (Burridge and Chrzanowska-Wodnicka, 1996) and cell adhesion (Frisch et al., 1996). It belongs to the FAK family (Clements and Koretzky, 1999) that additionally comprises proline-rich tyrosine kinase (PyK2) also called cell adhesion kinase (CAK-β), related adhesion focal tyrosine kinase (RAFTK) or calcium-dependent protein tyrosine kinase (CADPTK; Avraham et al., 1995; Lev et al., 1995; Sasaki et al., 1995; Yu et al., 1996).

1.5.1 Expression pattern of FAK and Pyk2

FAK is evolutionarily conserved in multicellular organisms including humans (Weiner et al., 1993), rodents (Hanks et al., 1992), chicken (Schaller et al., 1992), xenopus (Hens and DeSimone, 1995; Zhang et al., 1995), drosophila (Palmer et al., 1999). Expression of FAK is ubiquitous in almost every cell type and increased expression of FAK is observed in many human cancers, including breast (Owens et al., 1995), prostate (Tremblay et al., 1996), colon (Owens et al., 1995), thyroid (Owens et al., 1996) and ovarian cancer tissues (Judson et al., 1999). Expression of Pyk2 is more restricted than that of FAK; Pyk2 is expressed very high in brain and lower in the liver, kidney, spleen, lung and cells of haematopoietic origin (Avraham et al., 1995; Lev et al., 1995; Sasaki et al., 1995).

1.5.2 Structural domains and the interaction partners of FAK

The N-terminal end of FAK consists of a region of about 300 aa called FERM domain, which shares sequence homology with erythrocyte band 4.1 protein/ERM (ezrin/radixin/moesin) proteins (Chishti et al., 1998; Girault et al., 1999) and allows proteins to interact either intermolecularly or intramolecularly. The N-terminal domain of FAK interacts with the kinase domain of FAK and deletion of the N-terminal domain increases its own catalytic activity suggesting that it acts as a negative regulator (Cooper et al., 2003; Schlaepfer and Hunter, 1996; Toutant et al., 2002). Point mutation studies reveal that the first subdomain of the FERM-like domain in FAK influences the phosphorylation state and the regulation of FAK within the cell (Cohen and Guan, 2005). Interaction of FAK N-terminal domain with activated platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) has also been identified (Fig. 1.3; Golubovskaya et al., 2002; Schaller et al., 1995; Sieg et al., 2000). Such interactions have also been shown with several other receptor tyrosine kinases including ErB2, ErB3, the IGF1 receptor, and Eph2A (Manes et al., 1999; Miao et al., 2000; Vartanian et al., 2000). It has been shown that FAK function downstream of several growth factors and also is an important molecule integrating biochemical signals and biological responses from both growth factors and integrins. N-terminal domain binds directly *in vitro* to peptides corresponding to cytoplasmic tail of β integrin (Schaller and Parsons, 1995). However, evidence for such interaction *in vivo* is still lacking.

The central domain of FAK is the kinase domain (Schaller and Parsons, 1994). It shares the similarity with other receptor and non-receptor tyrosine kinase domains. The crystal structure of this domain has been solved and a characteristic disulphide bond in the N-terminal lobe of the kinase has been identified. This disulphide bond influences the kinase activity of FAK (Nowakowski et al., 2002). FAK exhibits increased kinase activity (Guan and Shalloway, 1992; Lipfert et al., 1992) and tyrosine phosphorylation (Burridge et al., 1992; Guan et al., 1991; Hanks et al., 1992; Kornberg et al., 1991; Lipfert et al., 1992) upon integrin activation, with the major phosphorylation site identified as Tyr397 (Fig. 1.3). FAK autophosphorylates itself at Tyr397 which is essential for phosphorylation of other FA proteins (Chan et al., 1994; Eide et al., 1995; Schaller et al., 1999; Schaller et al., 1994) and subsequent phosphorylation of Tyr576 and Tyr577 within FAK. Kinase-dead mutant studies reveal that this mutated FAK retains most of the FAK functions suggesting that it acts mainly as a scaffold rather than like kinase (Tachibana et al., 1997).

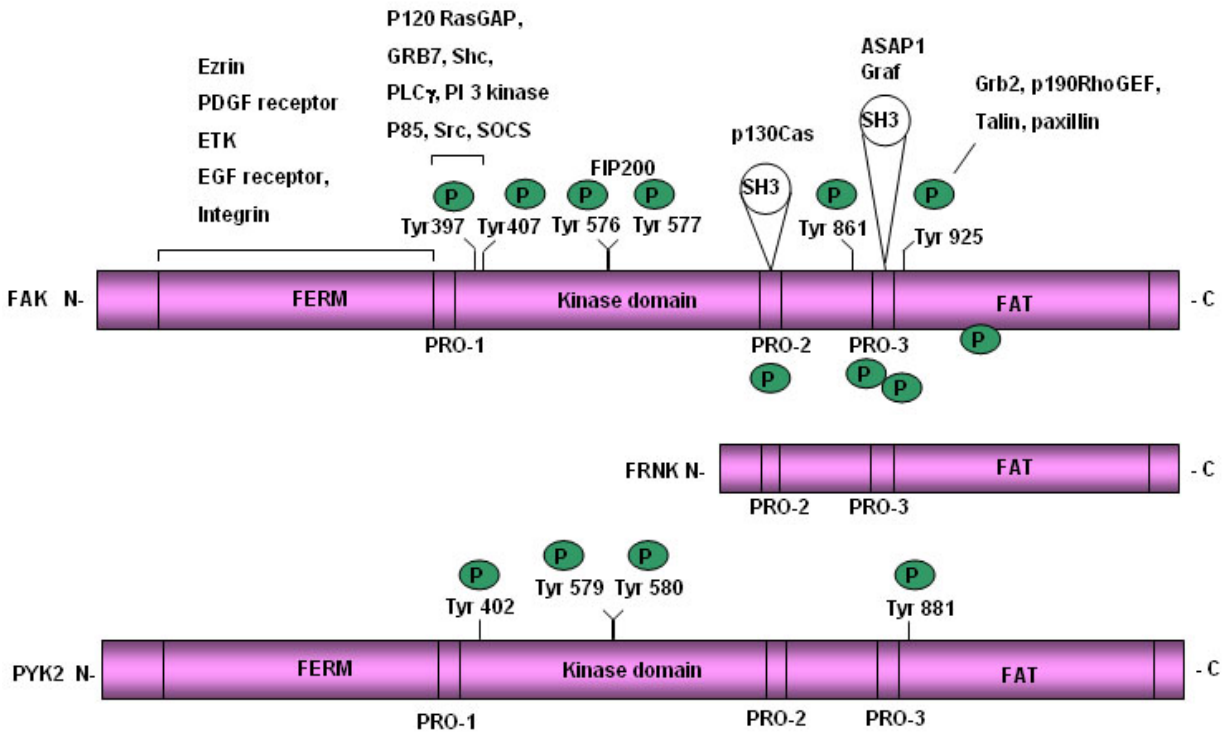

Fig. 1.3. Schematic representations of FAK and Pyk2 structural domains. FAK contains a central kinase domain flanked by an N-terminal domain and C-terminal domain. It has six tyrosine phosphorylation sites including Y397 autophosphorylation site and four serine phosphorylation sites. The interacting proteins and their binding sites are indicated in the fig. Pyk2 another member of FAK family shares a similar domain arrangement with FAK. It has four tyrosine phosphorylation sites. The proline rich sequences (PRO) and tyrosine phosphorylation (Tyr) with amino acid position of both molecules are depicted in the fig. The fig is modified from (Mitra et al., 2005).

The C-terminal domain of FAK can be subdivided into a focal adhesion-targeting domain (FAT) that is involved in localization of FAK to the FA sites (Schlaepfer et al., 1999) and the region between the catalytic domain and the FAT domain. The sequence present in the FAT domain is essential and also sufficient for FAK recruitment to FAs (Hildebrand et al., 1993; Klingbeil et al., 2001). FAT domain contains four helix bundles that resemble structure of other FA proteins, including vinculin, Cas and α-catenin (Arold et al., 2002; Hayashi et al., 2002; Liu et al., 2002a). FAK binds indirectly to integrin via its carboxyl domain, which bind to FA proteins such as paxillin and talin. Paxillin binds to the integrin α_4 subunit, whereas talin binds various integrin β subunit cytoplasmic tails (Calderwood et al., 1999; Liu et al., 1999; Patil et al., 1999). Moreover, paxillin serves as a docking partner by binding with other FA proteins such as vinculin.

The biological activities of FAK require recruitment of FAK via its FAT domain to FA sites. Therefore, it is interesting that the C-terminal non-catalytic domain of FAK comprising the FAT (904-1012) domain can be expressed as a separate transcript. The product of this transcript has been termed FRNK (FAK-related non-kinase) (Hildebrand

et al., 1993; Schaller et al., 1993; Taylor et al., 2001). FRNK is expressed in restricted set of cells, predominantly in lung, intestine and functions as a negative regulator of FAK activity as it displaces FAK from FA sites (Nolan et al., 1999; Richardson and Parsons, 1996; Sieg et al., 1999; Xiong et al., 1998). It blocks the tyrosine phosphorylation of FAK and substrates of the FAK/pp60Src complex (Richardson and Parsons, 1995). Point mutation of FRNK (Leu 1034 to Ser) disrupts the ability of FRNK to inhibit FAK. Inhibition by FRNK can be overcome by expressing Src. Moreover, FAK mutations in the FAK C-terminal domain, which inhibit its recruitment to the FAs, inhibit phosphorylation (Cooley et al., 2000; Shen and Schaller, 1999). Both FAK family kinases lack Src homology 2 or 3 domains (SH2 or SH3), present in many cytoplasmic tyrosine kinases. But the C-terminal domain of FAK contains proline rich binding sites for SH2 or SH3 domain containing other FA proteins including $p130^{CAS}$, Grb2, Graf and phosphatidylinositol 3 kinase (PI3 kinase). Y925 accommodates Grb2 upon phosphorylation by providing a binding site for the Grb2 SH2 domain (Schlaepfer and Hunter, 1996) and initiate a Ras/MAP kinase pathway. $p130^{CAS}$ is an adapter protein, which mediates interaction with the FAK proline rich region. The SH3 domain of $p130^{CAS}$ interacts with the sequences around proline (Pro712-Pro715) of FAK (Polte and Hanks, 1995) and also at the second site Pro878-881. Other SH3 domain containing protein such as GRAF, a regulator of small GTPase binds to FAK around the sequence Pro878. FAK and GRAF interaction suggests that FAK is involved in G protein signalling. In addition, ADP ribosylation factor (ARF), a member of small GTPase family protein is also shown to interact with proline rich domain of FAK (Liu et al., 2002b; Randazzo et al., 2000; Taylor et al., 1998; Taylor et al., 1999). FAK also has four sites for serine phosphorylation within this domain (Ser722, Ser843, Ser846 and Ser 910). However, their role in FAK function is not studied so far.

1.5.3 Downstream signalling of FAK

FAK activation is initiated by integrin engagement with its ligand (Schaller et al., 1999). Tyrosine phosphorylation of many proteins is an important mechanism involved in signalling events occurring at FA sites. Tyrosine phosphorylation at FA creates docking sites for SH2 containing proteins. The phosphorylation on Y397 of FAK, for example, creates a high affinity-binding site recognized by SH2 domain-containing proteins such as Src family kinases (Fig. 1.4). FAK association with Src kinase leads to formation of multimolecular signalling complexes in which FAK serves as a scaffold. Src kinases then phosphorylate additional residues on FAK or on FAK associated proteins

such as p130^{CAS} and paxillin (Cary et al., 1998; Schaller and Parsons, 1995; Vuori et al., 1996).

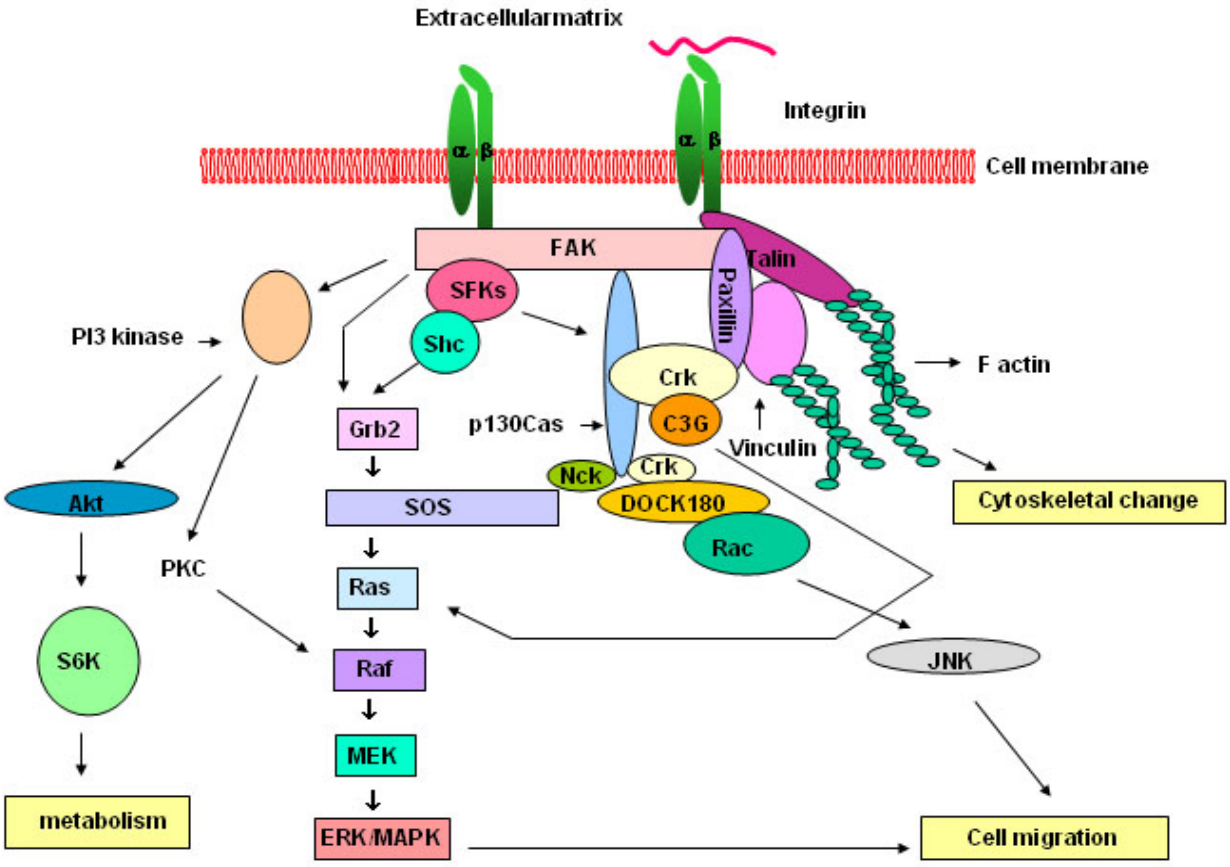

Fig. 1.4 Integrin mediated downstream events of FAK activation and it's signalling: Integrin engagement with specific ECM ligands leads to FAK phosphorylation at Tyr397, subsequently Src is recruited into this site by binding via its SH2 domain. Recruitment of Src family kinase (SFK) stimulates phosphorylation of other phosphorylation sites within FAK and also in other FA proteins including p130^{CAS} and paxillin. As a consequence, paxillin phosphorylation involves in regulation of actin cytoskeleton by binding with other FA protein such as vinculin. Vinculin interacts with cytoskeleton by its F actin binding site and regulates the cytoskeletal remodelling. Phosphorylated paxillin also binds with Crk, an adaptor protein. Crk can associate SOS and also another guanine nucleotide exchange factor C3G leading to activation of MAPK pathway. FAK interacts with SH3 domain of p130^{CAS} a multi functional adapter protein by the proline rich site and may require Crk downstream of p130^{CAS}. Crk and Nck adapter protein binding to p130^{CAS} might lead to enhanced cell migration through the activation of pathways involving by either binding to SOS complex or by JNK MAP kinase cascade. Tyrosine phosphorylation of p130^{CAS} can recruit Crk/Dock 180 complexes that may signal through Rac to activate JNK signalling. Src family PTKs can also promote Shc tyrosine phosphorylation and Grb2 binding to Shc at Tyr317 promotes the SOS complex. Therefore, Ras/MAP kinase pathway is activated. The second pathway is mediated by FAK association with PI 3 kinase directly or through Src and involved in activating MAP kinase pathway. Two protein serine/threonine kinases Akt and S6K have been identified as downstream effectors of PI 3 kinase which function in modulating cell metabolism.

It is also essential for recruitment of SH2 domain containing proteins including PI3 kinase, phospholipase C and Grb7 (Akagi et al., 2002; Chen et al., 1996; Chen and Guan, 1994a; Han and Guan, 1999). Five additional sites within FAK are phosphorylated when Src kinases bind to FAK. These phosphorylation sites are important for cell adhesion-induced activation of FAK and downstream signalling

(Calalb et al., 1995; Owen et al., 1999). Although tyrosine phosphorylation and activation of FAK accompanies integrin-mediated adhesion, dephosphorylation promptly occurs when cells are detached (Aplin et al., 1998; Parsons et al., 2000; Schaller, 1996). The phosphorylation at Tyr397 and Tyr925 accommodates Grb2 upon phosphorylation by providing a binding site for the Grb2 SH_2 domain (Hildebrand et al., 1996; Schlaepfer et al., 1994). The Grb2–initiated recruitment of its binding partner SOS, a guanine nucleotide exchange factor for Ras sets the stage for activation of Ras followed by activation of the downstream MAP kinase cascade comprising Raf-1, Mek, and Erk (Fig. 1.4; Hildebrand et al., 1996; Schlaepfer et al., 1994). This signalling events lead to activation of Erk1/2.

There are two additional signalling pathways regulated by FAK leading to activation of Erk1/2. One is via binding of $p130^{CAS}$ to the FAK proline-rich domain and second is via association of FAK with Shc, an adaptor protein (Gu et al., 1999; Schlaepfer and Hunter, 1996). Another pathway mediated by FAK is associating with PI 3 kinase directly or through Src and involved in activating MAP kinase pathway. Previous studies have demonstrated that integrin-induced Erk activation occurs independently of FAK activation by growth factors such as PDGF, EGF and FGF (Fibroblast growth factor; Huang et al., 2004; Lin et al., 1997; Wary et al., 1996). Integrins as well as growth factor induced Erk activation participate in cell migration. All these reports suggested that it is essential to identify the molecular mechanisms by which growth factor and ECM mediated signalling pathways in VSMC converge to regulate proliferation and migration of VSMC in order to prevent restenosis.

1.5.4 Crosstalk between integrin and growth factor signalling

Besides interacting with ECM components, integrins co-operate with several growth factor receptors including EGFR, PDGFR and fibroblast growth factor receptor (FGFR) and stimulate various cellular signalling events (Giancotti and Ruoslahti, 1999; Huynh-Do et al., 1999). For example, $\alpha_v\beta_3$ integrin clusters with PDGF receptor or insulin receptor and form complexes (Bartfeld et al., 1993; Schneller et al., 1997; Vuori and Ruoslahti, 1994). These complexes, established upon the integrin clustering, lead to crosstalk between two different receptors (Miyamoto et al., 1996; Plopper et al., 1995). Because many signalling mechanisms between integrin engagement and growth factor stimulation are similar, there is an opportunity for cross talk between these two pathways.

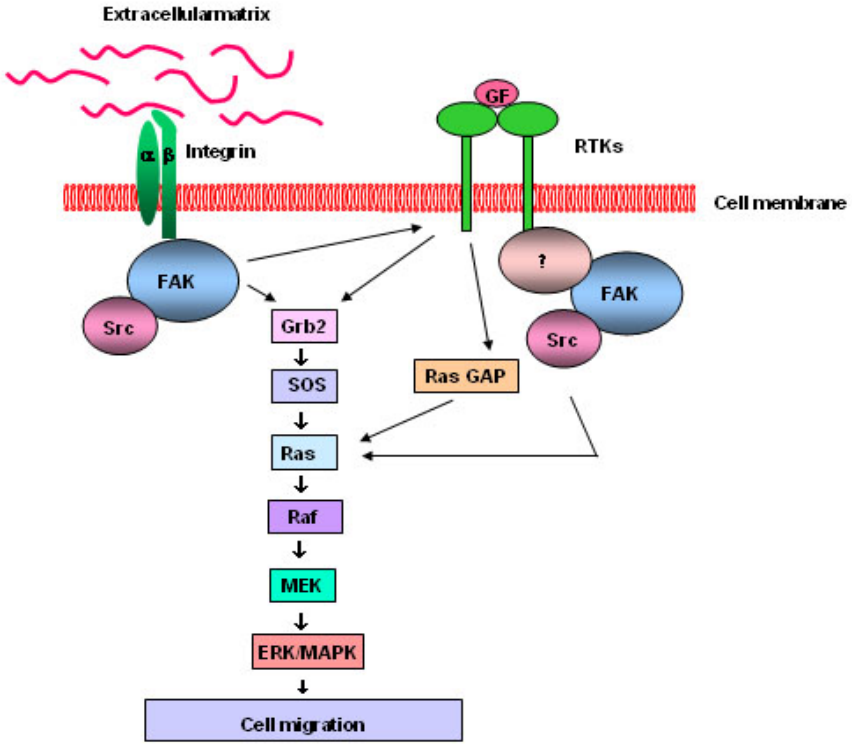

Fig. 1.5 Integrin and growth factor (GF) signalling pathways converge in multiple points to modulate the cell function. In this cooperative signalling pathway, engagement of either integrin or growth factor receptor with their respective ligands leads to activation of Ras through various signalling molecules. It results in subsequent stimulation of Raf, MEK and finally the MAP kinases/Erk. RTK; receptor tyrosine kinase, α and β-integrin subunits.

One of the main converging point in these two pathways is FAK. While FAK phosphorylation stimulated by growth factors contribute to increase in cell migration, FAK knockout cells does not migrate towards chemotatic response against PDGF which indicates FAK function is required and essential for PDGF stimulated migration (Sieg et al., 2000). This impaired motility can be rescued by re expressing of FAK in those FAK knockout cells. Integrins co-operate with growth factors to promote signalling pathways such as MAP kinase pathway (Fig. 1.5). However, even in the absence of growth factor, the integrin binding to ECM proteins trigger the MAP kinase pathway (Moro et al., 1998; Schlaepfer et al., 1994; Wary et al., 1998). Interestingly, growth factors do not stimulate those pathways in suspended cells underlying the importance of cell adherence to ECM proteins via integrins (Aplin and Juliano, 1999; Aplin et al., 2001; Assoian and Schwartz, 2001; Howe et al., 2002; Schwartz and Assoian, 2001). But constitutively activated FAK is able to rescue this effect (Renshaw et al., 1999; Renshaw et al., 1997). It is well known that growth factors such as PDGF modulate the expression of number of proteinases including MMPs. Interruption of such signalling pathways by anti-PDGF inhibit VSMC migration by regulating MMPs. These

observations suggested that targeting of both integrin initiated FAK signalling and growth factor cooperative signalling might have significant impact on cell migration.

1.5.5 The functional relevance of FAK in cell migration

FAK is implicated in many biological functions. One of them is promoting VSMC migration. By activating FAK, growth factors as well as cytokines trigger chemotaxis; ECM proteins trigger haptotaxis (Schlaepfer et al., 1999). Earlier studies demonstrate that the activity of FAK correlates with endothelial cell migration (Romer et al., 1994). FAK knockout mice are dying at the age of embryonic 8.5 days with defects that suggest abnormalities in mesoderm migration. It indicates the importance of FAK in embryonic development (Ilic et al., 1995). Microinjection of a FRNK related construct impairs the endothelial cell motility by inhibition of FAK (Gilmore and Romer, 1996). Overexpression of FRNK leads to reduction in protease secretion and inhibition of migration, invasion and proliferation (Hauck et al., 2002; Hauck et al., 2001a; Slack et al., 2001). Further, inhibition of FAK or deletion of the FAK gene affects cell migration (Ilic et al., 1995), whereas overexpression of FAK enhances cell motility (Cary et al., 1996). Taken together, FAK signalling cascade triggered by both integrin as well as growth factors contribute to the migratory response of SMCs, therefore FAK appears to be an interesting target to interfere with aberrant VSMC migration.

1.5.6 Interference of FAK signalling

Since FAK is essential during cell adhesion process, many cellular mechanisms exist to modulate its function. For example, a 200kDa FAK family interacting protein (FIF200), originally identified as a binding partner for C-terminal domain Pyk2 in yeast two hybrid system, was found to bind FAK and inhibits its catalytic activity *in vitro* (Schaller, 2001). In addition, dephosphorylation of tyrosine residues from activated FAK through phosphatases plays major role in regulating FAK mediated signalling. FAK-regulating tyrosine phosphatases are protein-tyrosine phosphatases PTP-PEST (Shen et al., 1998) and protein tyrosine phosphatase 1B (PTP1B; Liu et al., 1998). Moreover, Shp-2, a SH2 domain-containing protein has been suggested to inhibit the FAK tyrosine phosphorylation which leads to impaired FA formation and cell migration (Tsuda et al., 1998).

The phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which was identified as a tumour suppressor gene, has some influence in FAK signalling in cancer development. It negatively regulates FAK by dephosphorylation (Li et al., 1997;

Steck et al., 1997) and leads to impair in cell motility (Tamura et al., 1998). Another mechanism, which is equally effective, is proteolytic cleavage of FAK by the enzymes caspases 3 and 7 (Levkau et al., 1998; Wen et al., 1997). Caspase-mediated cleavage of FAK during apoptosis generates a FRNK-like peptide (Gervais et al., 1998). Further, FAK proteolysis is related to an increase FA turnover in transformed and apoptotic cells through the actions of calpains and caspases (Fincham et al., 1995; Wen et al., 1997). The FA turnovers in this event are extensive and lead to complete loss of cell adhesion. Inhibition of FAK with antisense oligonucleotides in tumour cell lines (Xu et al., 1996) or microinjection of chick embryo fibroblast cells with a monoclonal antibody against FAK induced apoptosis (Hungerford et al., 1996) suggesting the influence of various factor's involved in FAK function. These observations indicate that FAK signalling is essential for various cell functions, which are involved, in both physiological and pathological conditions.

1.6 The aim of study

The aim of this study is to investigate if targeting the integrin α_V directly or integrin α_V-initiated signals such as FAK activation is efficient to abrogate migration of hCASMCs and to identify novel interaction partners for N-terminal domain of FAK.

1. Direct interference with integrin α_V function was done by using specific pharmacological inhibitor against integrin α_V and the effect of such inhibitor on the integrin initiated intracellular signals was also monitored.

2. In another strategy, AAV mediated overexpression of FRNK was employed to interfere in integrin-initiated FAK signalling events in primary hCASMCs. Effect of AAV mediated FRNK overexpression was assessed *in vivo* porcine restenosis model.

3. Finally, to explore the putative interacting partners of FAK, rat smooth muscle cell library was screened with N-terminal domain of FAK by bacterial two-hybrid system.

The studies presented here establish some of the potential targets whose suppression either by pharmacological agents or by dominant negative expression of specific genes via AAV mediated gene delivery can prevent hCASMC migration.

2. Targeting of α_v integrins interferes with FAK activation, smooth muscle cell migration and invasion

2.1 Introduction

Neointimal formation is thought to involve VSMCs that have dedifferentiated from a contractile to a secretary form, which is characterized by proliferation, migration and synthesis of ECM. Mainly, modulation of SMC phenotype and altered expression of receptors on their surface is crucial in the development of restenosis (Assoian and Marcantonio, 1997; Thyberg et al., 1990). As mentioned earlier, integrin-ECM interaction plays a main role in multiple cellular activities that are relevant to these pathological events including cell migration, invasion and MMP secretion. The mechanism behind those processes is mainly implicated by intracellular signalling events, which, is predominantly initiated by inappropriate integrin activation.

There are different integrins present in the vessel wall. But mainly, the expression of integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ is upregulated after the injury. Recent evidence has shown that integrin α_v is upregulated in atherosclerotic plaques. Various animal studies in baboons, rats, rabbits, and pig models also revealed that β_3 integrin expression is stimulated by PTCA (Corjay et al., 1999; Slepian et al., 1998; Srivatsa et al., 1997). Although, these integrins are expressed in normal vessel, increased expression was observed in diseased vessels. In particular, some ligands of $\alpha_v\beta_3$ integrin (TSP, VN, ON, fibrin and fibrinogen) are enriched only in injured or diseased vessels. Normally, these integrins are known to interact with RGD containing motifs, a common binding motif found in integrin ligands. Therefore, these integrins and their ligands are highly involved in intracellular signalling events, which modulate various cellular functions after injury. Further, early studies have shown that $\alpha_v\beta_3$ integrin is involved in the migration of SMCs and endothelial cells (Liaw et al., 1994; Liaw et al., 1995a; Yue et al., 1994). Thus, $\alpha_v\beta_3$ upregulation may have influence also in restenosis by regulating cell migration. It is already well established that antibodies against $\alpha_v\beta_3$ not only interfere with ligand occupancy, but also inhibit endothelial cell movement during angiogenesis (Brooks et al., 1994). Moreover, various animal studies also demonstrated that blockade of $\alpha_v\beta_3$ integrin reduces the intimal thickening, which correlates with abundant apoptosis in the injured vessel wall (Coleman et al., 1999; van der Zee et al., 1998). Studies have shown that interruption of such integrin-ligand interactions could suppress cellular growth or induce apoptotic cell death (Brooks et al., 1994; Meredith et al., 1993;

Montgomery et al., 1994; Varner et al., 1995). As a consequence, those interferences limit the application of integrin blocking in clinical trials.

Apart from integrin family receptors, receptors from growth factor signalling have also major impact on those pathological processes. A wide variety of growth factors such as PDGF, FGF, transforming growth factor-β (TGF-β), EGF, ILGF-1 (insulin like growth factor-1), vascular endothelial growth factor (VEGF), have been also implicated in the development of atherosclerosis and restenosis (Couffinhal et al., 1997; Flaumenhaft et al., 1992; Hamon et al., 1995; Klagsbrun and Edelman, 1989; Lindner et al., 1991; Ruoslahti et al., 1992). Importantly, interference with integrin $\alpha_v\beta_3$ also regulates SMCs responses against various growth factors such as PDGF (Bilato et al., 1997; Choi et al., 1994), EGF (Jones et al., 1997), ILGF-I (Jones et al., 1996), TGF-β (Sajid et al., 2000) and α-thrombin (Stouffer et al., 1998) which indicates that cooperative signalling also can be altered. Together, the above presented experimental studies and others have revealed that factors affecting VSMC migration, proliferation *in vitro* as well as *in vivo* intimal inhibition. However, none of the drugs is promising for clinical applications because such agents are normally cytostatic or cytotoxic which, although effective, result in the disruption of normal vascular repair mechanisms (Losordo et al., 2003). Therefore, the heterogeneity of receptor subtypes and the intracellular signalling systems connected to these receptors may be important factors, which should be further considered.

The primary objective of the present study was to find, whether a specific pharmacological inhibitor against integrin α_v could inhibit integrin-initiated downstream pathways and to study the signalling molecules such as FAK, the main player in many integrin-dependent cellular functions. And also it provides us new insights into the essential mechanism behind the physiological and pathophysiological functions influenced by ECM-ligand interactions. Interrupting such ECM-ligand induced signalling by pharmacological inhibitors subsequently may lead to reduced neointima formation after vascular injury. Together, pharmacological integrin α_v specific inhibitors are feasible for the treatment of restenosis.

2.2 Results

2.2.1 Characterization of human coronary artery smooth muscle cells

2.2.1.1 Analysis of cytoskeletal proteins in hCASMCs by immunofluorescence

Primary human coronary artery smooth muscle cells (hCASMCs) were obtained from clonetics (San Diego, CA) and maintained in smooth muscle cell (SMC) medium. The purity of hCASMCs was assessed by immunofluorescence staining with anti SMC specific actin antibody. More than 95% of the cells stained positive for the SMC specific actin isoform (Fig. 2.1). To further confirm their identity of the hCASMCs, the cells were analyzed for the expression of SMC specific actinin by double staining for both actinin and actin. The cells showed positive staining for SMC-specific actinin confirming the identity of SMCs (Fig. 2.1).

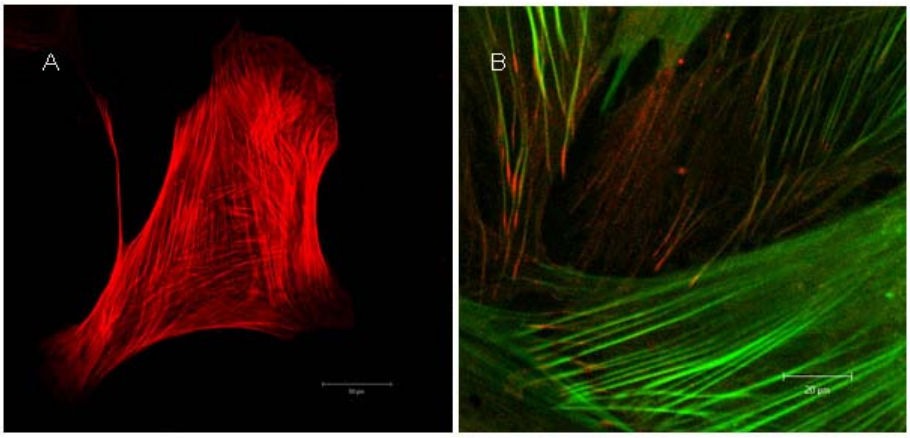

Fig. 2.1. Immunofluorescence staining of hCASMCs against SMC-specific actin and actinin. Cells grown on gelatine-coated cover slips were fixed with PFA and stained for SMC-specific actin or actinin. The stained samples were analyzed by confocal microscopy. A) Actin staining (decorated in red) of hCASMCs. B). Double staining of actin (decorated in red) and actinin (decorated in green) in hCASMCs. In Fig. A the bar represents 50 µm and in Fig. B the bar represents 20 µm.

2.2.1.2 Expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in hCASMCs

Expression of α_v integrins in hCASMCs was analyzed by both flow cytometry and confocal immunostaining. Flow cytometry analysis with integrin α_v specific antibodies revealed the expression of both integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ subtypes in hCASMCs when compared to isotype matched control antibodies (Fig. 2.2). To confirm further the presence of these integrins in hCASMCs, confocal immunostaining was performed using anti-$\alpha_v\beta_3$ and anti-$\alpha_v\beta_5$ antibodies conjugated with FITC (in green) and actin (in red) was costained using phalloidin-TRITC (Fig. 2.2).

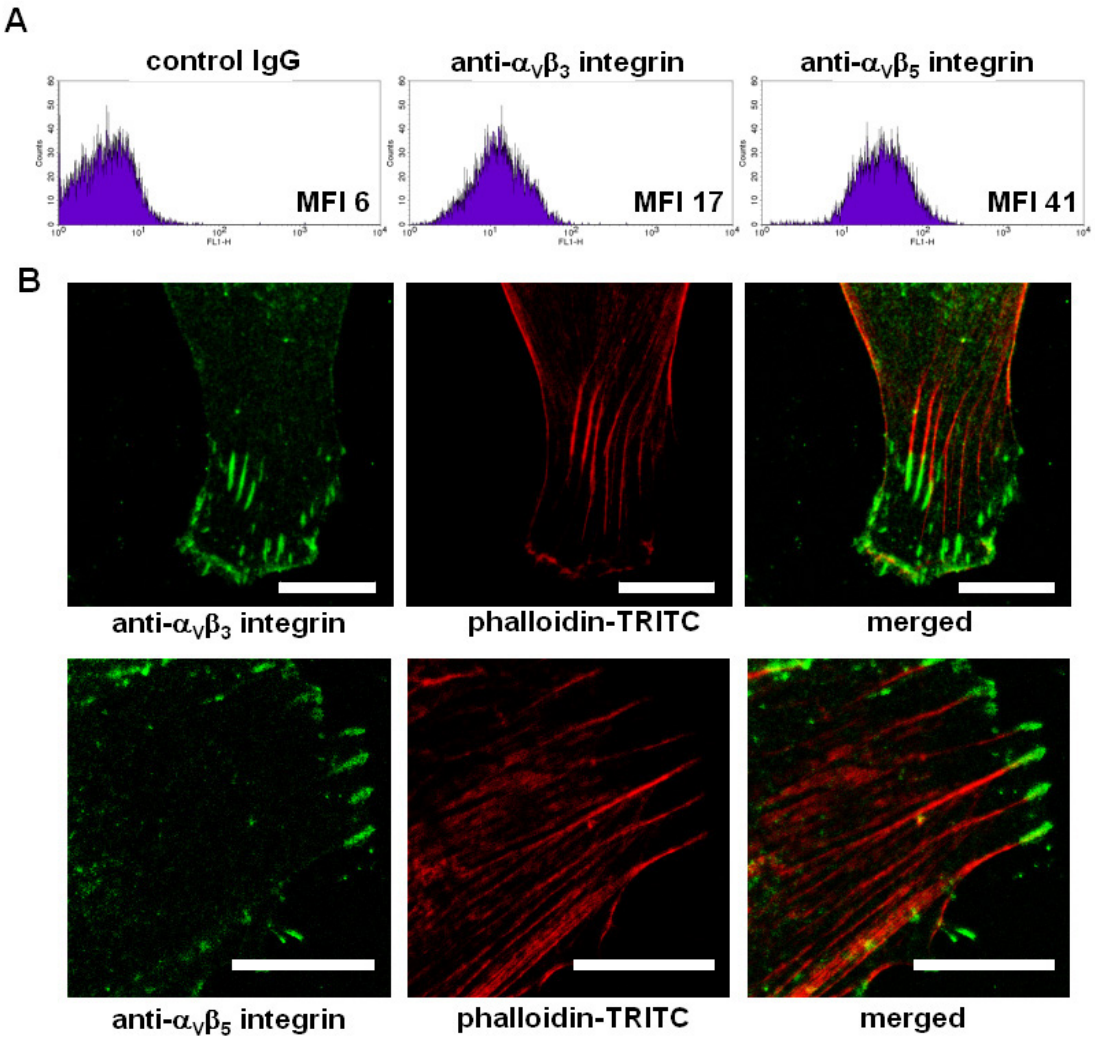

Fig. 2.2. Integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expression and localization in primary hCASMCs. (A). Analysis of integrin expression by FACS analysis**.** hCASMCs were stained with monoclonal antibodies directed against integrin $\alpha_v\beta_3$, integrin $\alpha_v\beta_5$, or an isotype matched control antibody. The cells were analyzed by flow cytometry using a FACS Calibur and mean fluorescence intensity (MFI) of each sample is indicated. (B) Immunofluorescence co-staining of integrin $\alpha_v\beta_3$ or integrin $\alpha_v\beta_5$ and actin in hCASMCs**.** The cells grown on gelatine-coated glass cover slips were fixed with ice-cold acetone, co-stained for integrin and actin using the indicated FITC-labelled monoclonal antibodies and TRITC-phalloidin and images were analyzed by confocal microscopy. Bars represent 20 µm.

The results from these studies confirmed the expression of these two integrin subtypes in hCASMCs that were predominantly localized to both FAs and focal contact points at the cell–substrate interface. Furthermore, analysis of their subcellular localizations indicate the presence of these two integrin subtypes at the organizing centres of the actin filaments, as evidenced by their colocalization with actin.

2.2.1.3 Expression of FA proteins in hCASMCs

SMCs are known to express a wide variety of adhesion molecules and FA proteins (Sajid et al., 2003). To confirm the expression and localization of FA marker proteins such as vinculin and FAK, immunofluorescence staining was performed against vinculin

and FAK (Fig. 2.3). The results indicate that FA proteins were predominantly located in FAs where integrins interact with the ECM.

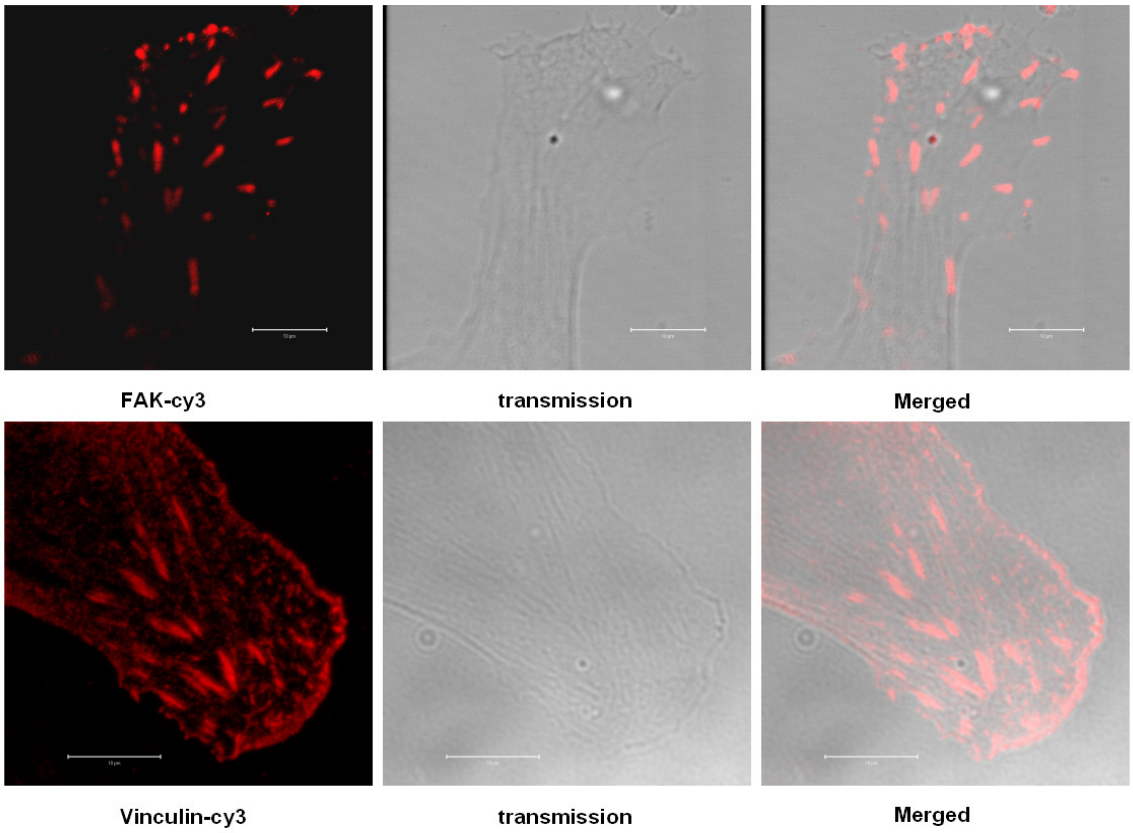

Fig. 2.3. Expression of FA proteins in hCASMCs. hCASMCs grown on gelatine-coated glass cover slips were fixed with ice-cold acetone, stained with monoclonal antibodies against-FAK (upper panel) or vinculin (lower panel), and images were analyzed by confocal microscopy. Bars represent 20 µm.

2.2.2 Effect of various ECM proteins on tyrosine phosphorylation in hCASMCs

ECM proteins are potent stimulants of signal transduction events that modulate many cellular processes such as cell proliferation, survival, adhesion, migration and other cellular functions. To study the effect of various ECM proteins on the tyrosine phosphorylation status of cellular proteins, hCASMCs were trypsinized and replated on various integrin ligands such as VN, FN or CN (Fig. 2.4). Serum-starved hCASMCs were detached by limited trypsin treatment, which disrupts the integrin engagement to ECM. The cells were taken in suspension (SUS) for 45 min and then replated on cover slips coated with various ECM proteins as indicated in the fig legends. After 45 min incubation, the cells were fixed with acetone and processed for immunofluorescence microscopy with monoclonal antibodies against phospho-tyrosine. Of the various ECM proteins tested, CN, FN and VN, but not a non-specific ligand such as poly-L-lysine (PL) exhibited a strong signal for tyrosine phosphorylation at the FAs and focal contacts (Fig. 2.4. A, B and C).

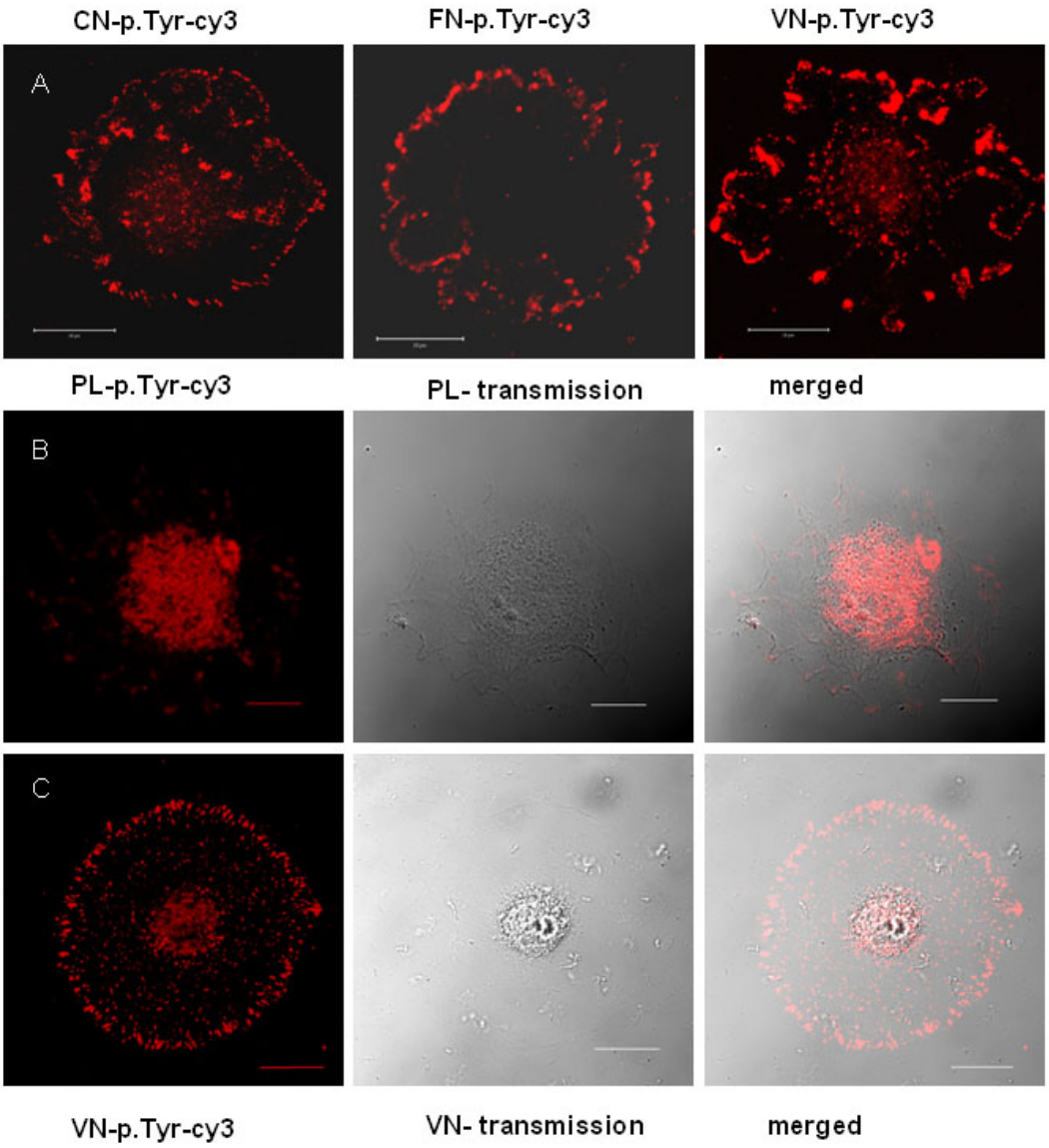

Fig. 2.4. Analysis of phosphorylated proteins in ECM-replated hCASMCs by immunofluorescence staining. (A) Serum-starved hCASMCs were replated on ECM proteins such as CN (5 µg/ml), VN (5 µg/ml) and FN (5 µg/ml), fixed with ice-cold acetone and processed for immunofluorescence microscopy with monoclonal antibodies against phospho-tyrosine. The samples were analyzed by confocal microscopy. Bars represent 20 µm. (B) Serum-starved hCASMCs were replated on VN (5 µg/ml) and PL (5 µg/ml; C) and fixed with ice cold acetone and stained for phosphotyrosine. The samples were analyzed by confocal microscopy. Bars represent 20 µm.

2.2.3 Effect of integrin stimulation by VN on tyrosine phosphorylation of cellular proteins

The increased tyrosine phosphorylation is one of the initial events of integrin binding to many ECM proteins such as VN and FN. ECM proteins-induced downstream signalling events were further studied in the hCASMCs by performing the replating assay combined with biochemical analysis. The suspended cells were either lysed directly or replated onto VN-coated cell culture dishes for 1 hr and then lysed. Western blotting was performed in the whole cell lysates (WCL) using anti-phospho tyrosine

antibody. Interestingly, cells that were replated on VN showed tyrosine phosphorylation of several cellular proteins, including a major ~116 kDa tyrosine phosphorylated protein band (Fig. 2.5). Other protein bands that showed a moderate increase in tyrosine phosphorylation include ~40 and ~66 kDa proteins (Fig. 2.5).

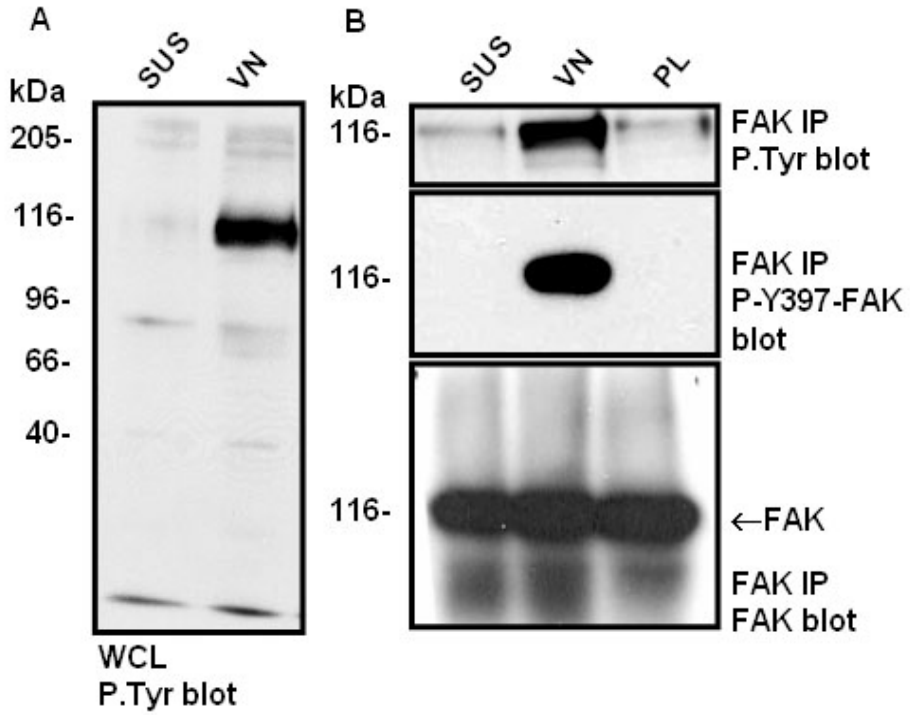

Fig. 2.5. Effect of VN-stimulated integrin activation on protein tyrosine phosphorylation in hCASMCs. A) Serum-starved hCASMCs were lifted with trypsinization and then either kept in SUS or replated on VN-coated plates (VN; 10 µg/ml in PBS). The WCLs were analyzed by Western blotting with anti-phosphotyrosine antibody. B) Serum-starved hCASMCs were kept in SUS or replated onto VN (10 µg/ml in PBS) or PL (10 µg/ml in PBS) coated plates. After lysing the cells, FAK was immunoprecipitated (IP) and the samples were analyzed by Western blotting with either anti-phosphotyrosine antibody (P.Tyr; top panel) or phospho-specific antibody to the Tyr-397 autophosphorylation site of FAK (middle panel). To demonstrate similar level of proteins in the samples, the same blot was stripped and probed with a monoclonal anti-FAK antibody (lower panel).

These changes showing VN-mediated increase in tyrosine phosphorylation, including the major ~116 kDa protein band, were not observed in the case of either suspended cells or treated cells by plating onto PL. The data obtained from these experiments are consistent with the results obtained from immunofluorescence staining for tyrosine phosphorylation and suggests the possibility that the ~116 kDa protein might represent the one localized predominantly at FAs.

2.2.3.1 Identification of FAK as the major VN-stimulated phospho-protein

Previous studies demonstrate that during integrin activation, several integrin-associated proteins with an apparent molecular weight of 110–130 kDa undergo tyrosine phosphorylation and some of them were identified as FAK and the adapter protein $p130^{CAS}$ (Polte and Hanks, 1995; Schaller et al., 1992). Therefore, to test the

possibility that the major tyrosine phosphorylated protein during VN stimulation is one of these proteins, FAK was immunoprecipitated from WCLs prepared either from cells that were kept in SUS or from replated cells onto VN or PL-coated culture dishes and the samples were analyzed by Western blotting with either anti-phosphotyrosine antibody or phospho-specific antibody to the Tyr-397 autophosphorylation site of FAK. Compared to SUS cells, replating cells on VN increased dramatically FAK tyrosine phosphorylation (Fig. 2.5; top panel). However, cells replated on PL, a cell attachment substrate that does not stimulate/engage integrins, did not result in enhanced FAK tyrosine phosphorylation, suggesting that it was the VN-mediated integrin stimulation that led to FAK tyrosine phosphorylation. To demonstrate further that phosphorylation in FAK occurs at the Tyr-397 autophosphorylation site, the same membrane was re-probed with a phospho-specific antibody directed against P-Y397 site of FAK (Fig. 2.5; middle panel). The result from such studies demonstrated that VN stimulation can result in phosphorylation of FAK at this site and that such phosphorylation was absent both in the case of suspended cells and the PL replated cells (Fig. 2.5; middle panel). The results cannot be attributed to different amounts of immunoprecipitated FAK, since probing the same membrane with anti-FAK antibody confirmed the equivalent amount of immunoprecipitated FAK in all samples. Taken together, these data demonstrate that FAK in hCASMCs is strongly activated in response to VN-binding to integrins.

2.2.4 Dose-dependent stimulation of FAK phosphorylation by VN

The concentration dependence of FAK activation by VN was verified by using various concentrations of VN coated cell culture dishes. Serum-starved hCASMCs were replated onto the VN-coated dishes or kept in SUS.

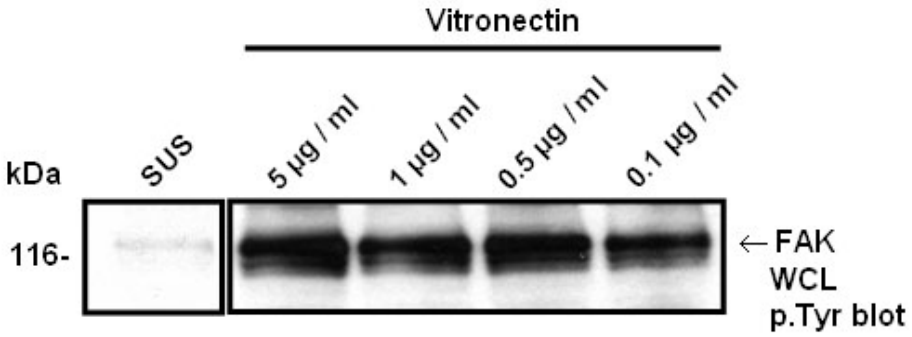

Fig. 2.6. Dose-dependent stimulation of FAK phosphorylation by VN. Serum-starved hCASMCs were replated onto dishes coated with various concentrations of VN or kept in SUS. The replating assay, SDS-PAGE and Western blot were carried out as described in the methods section. The blot was probed with anti-phosphotyrosine antibody.

The result shows that FAK tyrosine phosphorylation was increased at concentrations of VN coating as low as 0.1 µg/ml (Fig. 2.6). However, at higher concentrations of VN (5 µg/ml), a stronger tyrosine phosphorylation of FAK (about 1.5–2 fold higher than the level seen with the 0.1 µg/ml concentration) was observed (Fig. 2.6).

2.2.5 Time-dependent stimulation of FAK phosphorylation by VN

To analyse the kinetic of FAK phosphorylation, cells were replated onto VN for varying times. For this, serum-starved hCASMCs were replated on VN coated culture dishes as it was described earlier. The attachment of cells was observed under the microscope at regular intervals. Cells were found to begin spreading with leading lamillipodia in 45 min and well spreaded in about 90 min with no noticeable changes in morphology when culturing was extended for 6 hrs. Cells were lysed at the indicated time points and immunoprecipitation and Western blotting were performed as before. These studies exhibited that the tyrosine phosphorylation of FAK occurred as early as 45 min of VN stimulation, and it remained nearly the same for at least 6 hrs (Fig. 2.7).

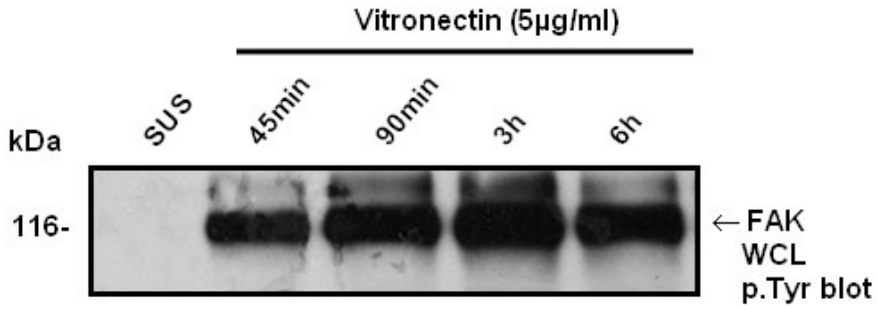

Fig. 2.7. Time-dependent stimulation of FAK phosphorylation by VN. Serum starved hCASMCs were replated onto VN (5 µg/ml)-coated dishes or kept in SUS. After the indicated time points, cells were lysed and SDS-PAGE and Western blot analyses were carried out as described in methods section. The blot was probed with anti-phosphotyrosine antibody.

Furthermore, consistent with the confocal immunostaining data, the tyrosine phosphorylation of FAK in the Western blot showed similar kinetics. As expected, there was no detectable level of the tyrosine phosphorylated FAK in SUS cells, similar to the results obtained in previous experiments. These results clearly indicate that VN is a rapid and potent stimulant of FAK tyrosine phosphorylation, which could persist for at least 6 hrs.

2.2.6 Inhibition of tyrosine phosphorylation in VN replated hCASMCs by an integrin α_v specific inhibitor

To demonstrate that VN-induced tyrosine phosphorylation is mediated specifically via α_v type integrins, a small peptidomimetic inhibitor of integrin α_v was used during

replating of hCASMCs on VN protein-coated culture dishes. For this, a dose dependent effect of α_v inhibition on FAK tyrosine phosphorylation in VN replated cells was studied. The WCLs were prepared in the presence or absence of integrin α_v inhibitor from VN replated hCASMCs. The Western blot analysis illustrated that the integrin α_v inhibitor was able to block dramatically the tyrosine phosphorylation in a concentration dependent manner.

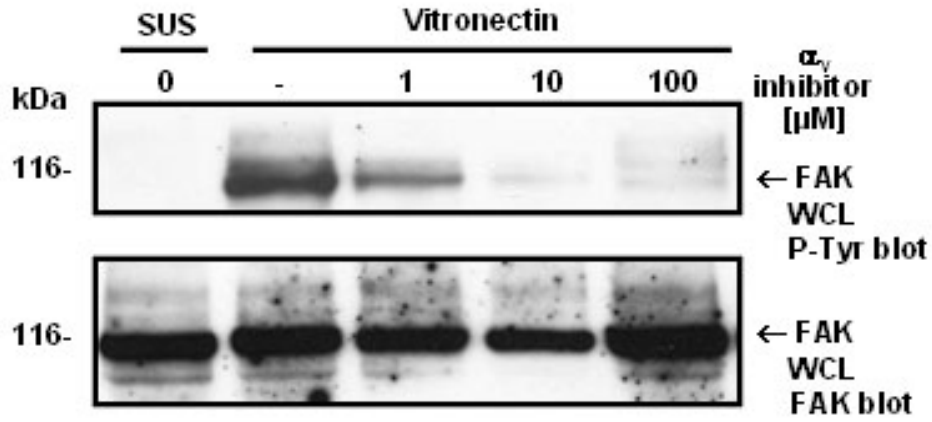

Fig. 2.8. Blockage of VN-induced FAK phosphorylation by integrin α_v inhibitor. The hCASMCs were either kept in SUS or replated onto VN-coated (2.5 μg/ml in PBS) plates. α_v inhibitor at varying concentrations as indicated in the fig was added to the suspended cells prior to their replating. The Western transfer was carried out and the blot was probed with anti-phosphotyrosine antibody (upper panel). To show a similar level of proteins in all the samples, the membrane was stripped and reprobed with antibody against FAK (lower panel).

At concentrations as high as 10 μM, the integrin α_v inhibitor completely abrogated the tyrosine phosphorylation of FAK (Fig. 2.8). The lower panel shows the presence of equal amount of FAK protein in all samples.

2.2.7 Interference of tyrosine phosphorylation by integrin α_v inhibitor in immunofluorescence assay

To further test the interference of the integrin α_v inhibitor with the ECM-induced tyrosine phosphorylation of proteins in hCASMCs, confocal immunofluorescence analysis was performed. Replating assay was done in the presence or absence of the integrin α_v inhibitor using VN coated cover slips. In the absence of the integrin α_v inhibitor, the phosphorylated proteins were seen in the periphery of the cells where the FAs were present (Fig. 2.9). However, in the presence of the integrin α_v inhibitor, peripheral accumulation of tyrosine phosphorylation was completely blocked. That is, instead of punctate staining of tyrosine phosphorylation, diffused signal was observed. This observation supports the previous results with biochemical assays where the

tyrosine phosphorylation of FA proteins in Western blot was dramatically abrogated by the integrin α_v inhibitor (Fig. 2.9).

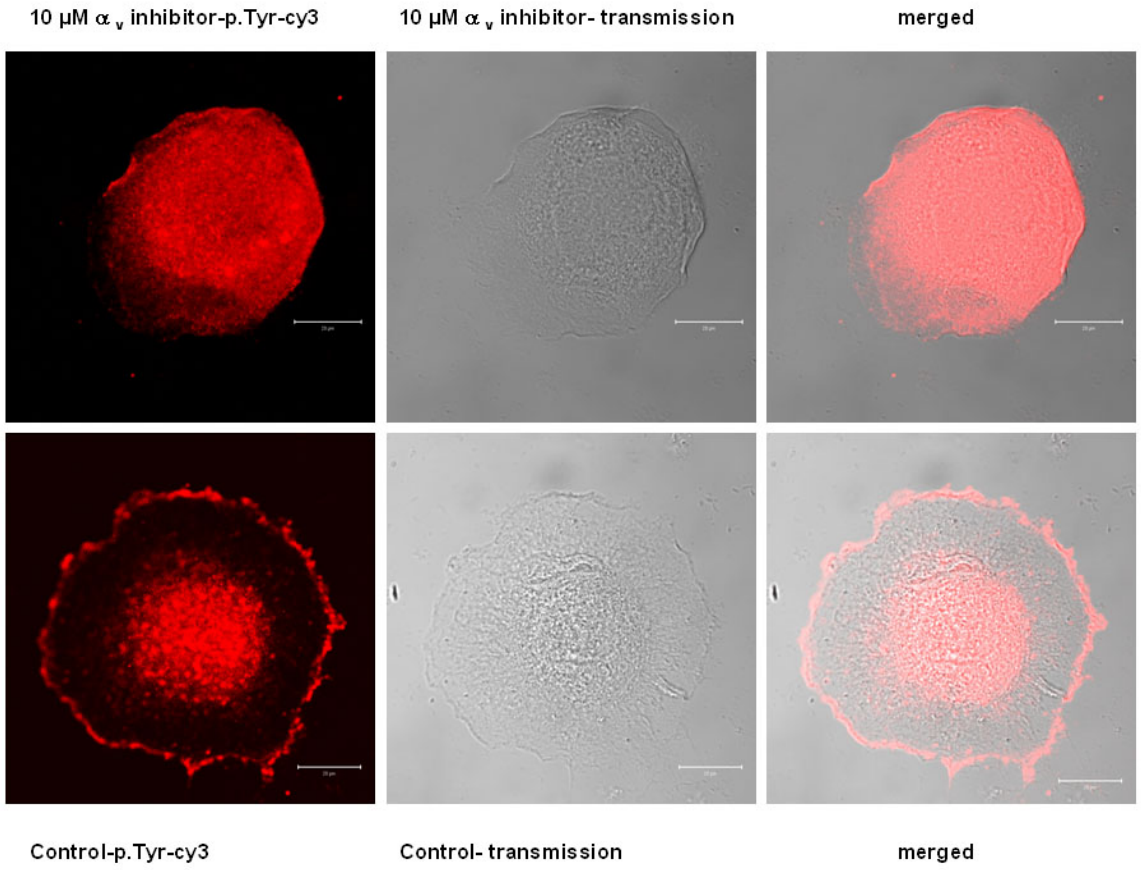

Fig. 2.9. Loss of VN-triggered tyrosine phosphorylation at focal contacts by integrin α_v inhibitor treatment. Serum-starved hCASMCs were replated onto VN-coated (5 µg/ml) glass cover slips in the absence or presence of 10 µM integrin α_v inhibitor. Attached cells were fixed with acetone and stained with monoclonal antibodies against phospho-tyrosine and Cy3-coupled secondary antibodies. The samples were analyzed by confocal microscopy. Bars represent 10 µm.

2.2.8 Demonstration of VN-induced cell migration

In addition to cell adhesion, ECM proteins are known to play an important role in cell migration. To demonstrate the VN-induced cell migration of hCASMCs, haptotaxis assays were performed using modified Boyden chambers (Fig. 2.10). Serum-starved hCASMCs were detached and the cells were added on top of the VN-coated chambers. The migration chambers were placed in 24-well plates containing suspension medium. After 3 hrs of incubation, the chambers were removed and the top part was wiped out with a cotton tip in order to remove non-migrating cells. The cells were then washed and fixed with methanol/acid fixative and stained with crystal violet staining solution to determine their migration to the other side of the membrane. The stained cells were counted under the microscope from 3 randomly chosen fields. The results (Fig. 2.10)

show that VN, even at a concentration of 0.5 μg/ml, caused hCASMCs migration. In addition, VN dose-dependent increase in migration was observed when compared to control cells that exhibited no appreciable migration when BSA was used for coating.

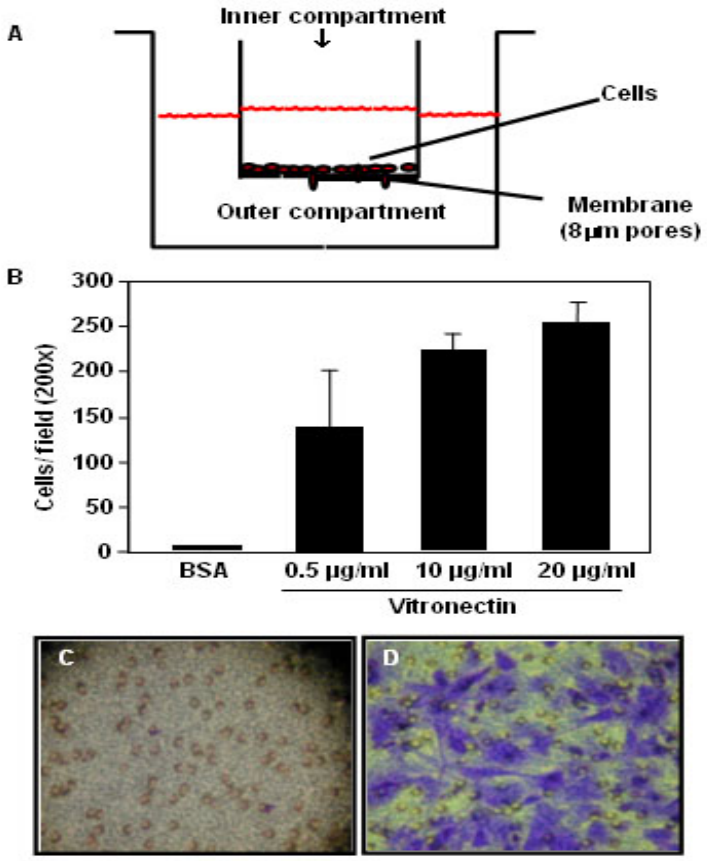

Fig. 2.10. Demonstration of VN-induced cell migration. The modified Boyden chambers were coated with various concentrations (0.5 μg/ml, 10 μg/ml, and 20 μg/ml diluted in DMEM) of VN or BSA (0.25% in DMEM as control). Migration assay was performed as described in the methods section. The cells were counted in three random fields/chamber at 200X. Results represent the average ± -standard deviation of two repetitions. A. A model showing a modified Boyden chamber. B. Cell migration in response to different VN concentrations is shown. C. Micrograph of the lower membrane of the BSA-coated control chamber. D. Micrograph of the lower membrane of the VN-coated chamber exhibiting migrating cells stained with crystal violet solution.

2.2.9 Demonstration of FN-induced cell migration

FN, which interacts primarily with $\alpha_v\beta_5$ integrin, is also known to stimulate the migration and growth of various cell types including SMCs (Glukhova et al., 1991). Therefore, to test whether FN, similar to VN, can mediate the migration of hCASMC, haptotaxis assay was performed with various concentrations of FN (Fig. 2.11). The results demonstrate that hCASMCs migration towards FN was also dose dependently increased when compared to BSA coated control (Fig. 2.11). Migration was observed at FN concentration as low as 1 μg/ml, and this was found to increase in a dose-dependent manner in 5 and 10 μg/ml concentrations. The results shown in this

experiment demonstrate that FN, similar to VN, is also a very efficient stimulant of hCASMCs migration.

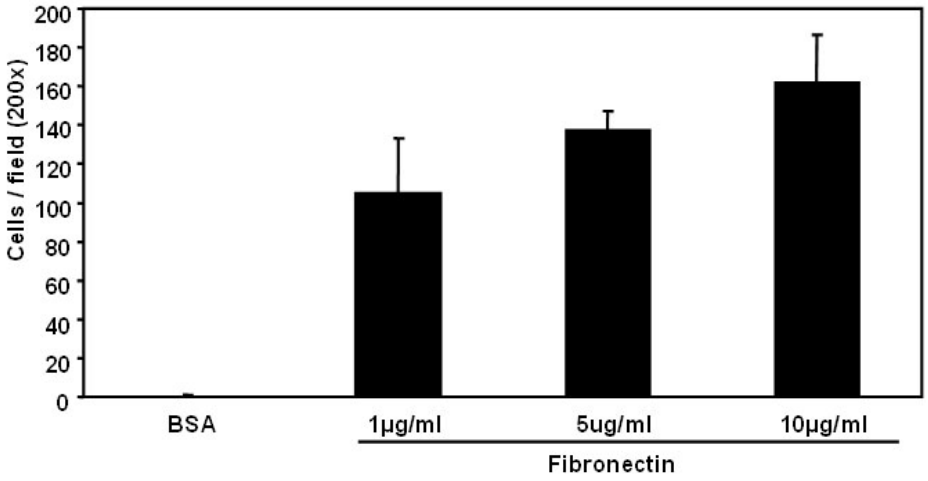

Fig. 2.11. Dose dependent stimulation of FN induced migration. The modified Boyden chambers were coated with various concentrations (1, 5, 10 µg/ml) of FN. The control chambers were coated with BSA. Over night serum starved hCASMCs were used and migration assay was performed as described in methods.

2.2.10 Effect of integrin α_v inhibitor on VN induced cell migration

To demonstrate that VN-mediated hCASMCs migration is mediated via α_v-integrins, migration assay was performed in the presence of various concentration of the integrin α_v inhibitor. The integrin α_v inhibitor was added to suspended hCASMCs and also included in the migration chambers containing 24 well plates that were coated with VN. The results show that the integrin α_v inhibitor can effectively inhibit VN-induced migration of hCASMCs in a dose-dependent manner. VN-mediated migration of hCASMCs was almost completely blocked at higher concentrations of the integrin α_v inhibitor (100 µM; Fig. 2.12).

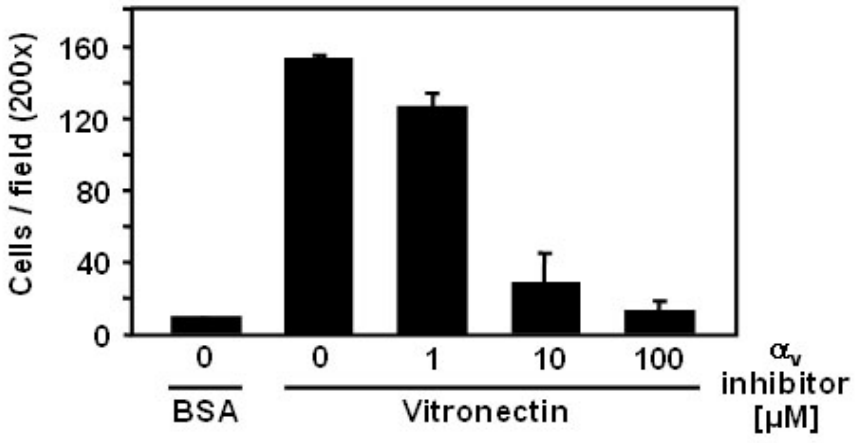

Fig. 2.12. Effect of integrin α_v inhibitor on VN-induced cell migration. VN (5 µg/ml in DMEM) was used for coating the modified Boyden chambers. The control chambers were coated with BSA in SUS medium. The serum-starved hCASMCs were seeded in presence or absence various concentrations of integrin α_v inhibitor. The haptotaxis was carried out as described in methods section.

2.2.11 PDGF-BB stimulated chemotaxis of hCASMC migration

Growth factor such as PDGF is known as a critical regulator for both normal and altered cell migration and proliferation (Heldin and Westermark, 1999). In order to determine the effect of the PDGF-BB on the migration of hCASMCs, chemotaxis assay was performed. For this, both sides of the modified Boyden chambers were coated with CN, which is diluted in PBS. Overnight serum starved hCASMCs were taken in SUS and seeded on the migration chambers. Various concentration of PDGF-BB was added to the lower compartment of the migration chambers. The chemotaxis assay was carried out as described in the methods section. At higher PDGF-BB concentrations, the migration of hCASMCs was increased markedly, and a dose dependent migratory response of hCASMCs to PDGF-BB was observed (Fig. 2.13).

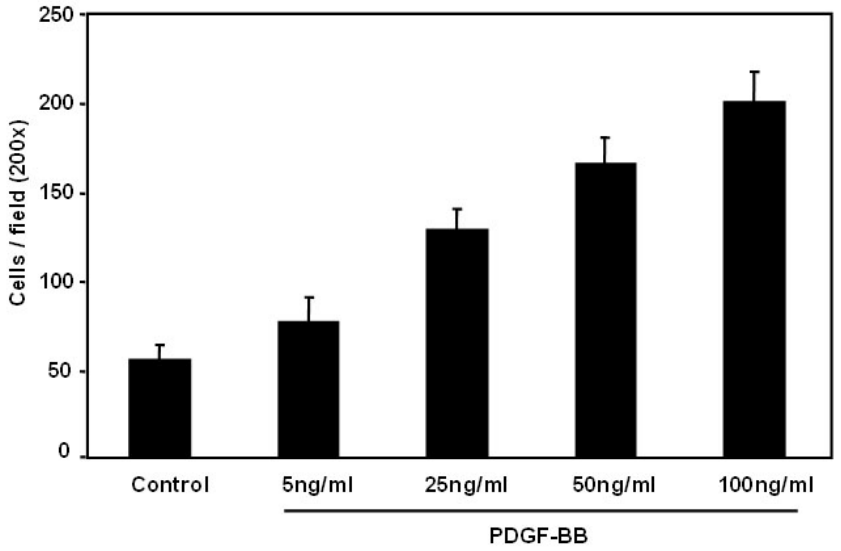

Fig. 2.13. Dose response of PDGF-BB induced cell migration. The modified Boyden chambers were coated with CN (10 µg/ml) containing tube for overnight at 4°C. Serum-starved hCASMCs were seeded on the modified Boyden chamber and PDGF-BB at various concentrations was used to stimulate the chemotaxis. The control chambers were kept in SUS medium without PDGF-BB. After 3 hrs incubation, the cell migration was stopped and processed for migration analysis as described in the methods section.

2.2.12 Integrin α_v inhibitor inhibit PDGF-induced hCASMC migration

The results from replating and migration assays suggested that ECM proteins are potent stimulators of FAK mediated signalling pathways. PDGF-BB also stimulated the migration of hCASMCs in a similar manner. Therefore, to test the possibility that PDGF-BB-induced chemotaxis of hCASMCs requires the involvement of α_v integrin; the α_v inhibitor employed in the previous experiments was used again for these studies. The experiments were performed in serum starved hCASMCs that were seeded on the VN coated chambers and stimulated with 50 ng/ml of PDGF-BB in the presence or absence of various concentrations of integrin α_v inhibitor. The results from this migration assay

showed that the integrin α_v inhibitor was able to block the PDGF-BB-induced chemotaxis, indicating that the migratory effect induced by this growth factor requires the involvement of α_v integrin (Fig. 2.14).

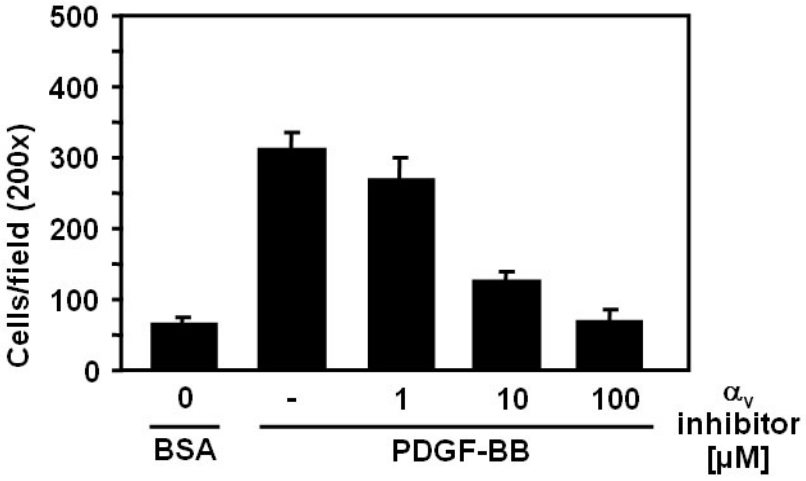

Fig. 2.14. Integrin α_v inhibitor inhibits PDGF-BB induced hCASMCs migration. The modified Boyden chambers were coated with VN (500 ng/ml) containing tube for overnight at 4°C. Serum-starved hCASMCs were seeded on the chamber and 50 ng/ml of PDGF-BB was prepared in SUS medium and added to the 24-well plate containing 400 µl of SUS medium. The control chambers were kept in wells containing SUS medium without PDGF-BB. The migration assay was performed in the presence or absence of integrin α_v inhibitor as indicated in the fig. After the incubation, the cell migration was stopped and processed as described in methods section.

2.2.13 Evaluation of matrigel invasion by hCASMCs

To evaluate the invasive potential of hCASMCs, the modified Boyden chambers were overlaid with various concentrations of commercially available reconstituted

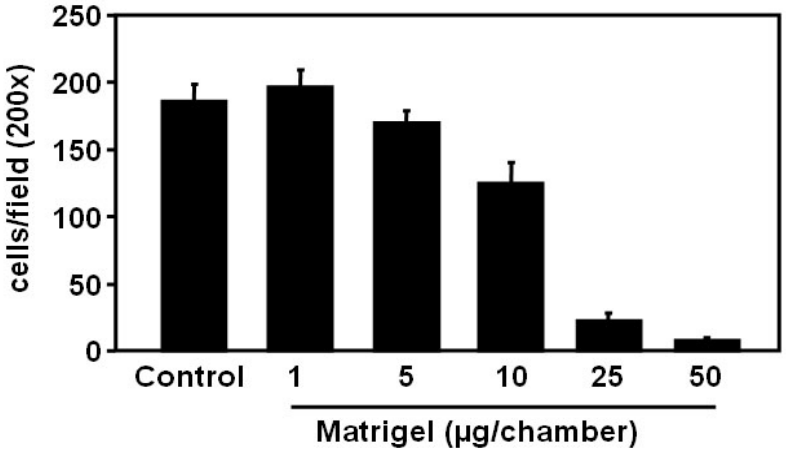

Fig. 2.15. Evaluation of matrigel invasion by hCASMCs**.** Serum-starved hCASMCs were seeded on the different concentrations of matrigel coated chambers. The control chambers were coated with CN (10 µg/ml) diluted in PBS. Invasion of hCASMCs towards FCS (5%) was allowed for 24 hrs. Then, cells that had invaded to the lower compartment were fixed, stained and counted in three random fields/chamber at 200X. Bars represent mean ± standard deviation from 3 random fields taken from each of 2 separate chambers.

basement membrane (growth factor reduced matrigel) in order to mimic an *in vivo* condition. The lower compartment was filled with SMC medium containing 5% FBS as chemoattractant. Serum-starved hCASMCs were allowed to migrate for 24 hrs in order to establish the optimal matrix concentration for invasion. These studies indicated that the hCASMCs were able to migrate up to a concentration of 10 µg/chamber of matrigel (Fig. 2.15).

2.2.14 Inhibition of α_v integrin impairs invasive motility of hCASMCs

To determine the role of α_v integrin on the hCASMCs invasion through the matrigel coated chambers, the various concentrations of α_v inhibitor was used in the invasive assay. The reconstituted basement membrane was overlaid in the chambers with the optimized concentration from the previous experiment. The hCASMCs were serum starved and taken in SUS as in the previous experiments. The cells were seeded on the matrigel-coated chambers with or with out integrin α_v inhibitor and incubated for 24 hrs. The results from these studies indicate that the integrin α_v inhibitor at concentration as high as 10 µM was sufficient to block the invasive potential of the hCASMCs (Fig. 2.16).

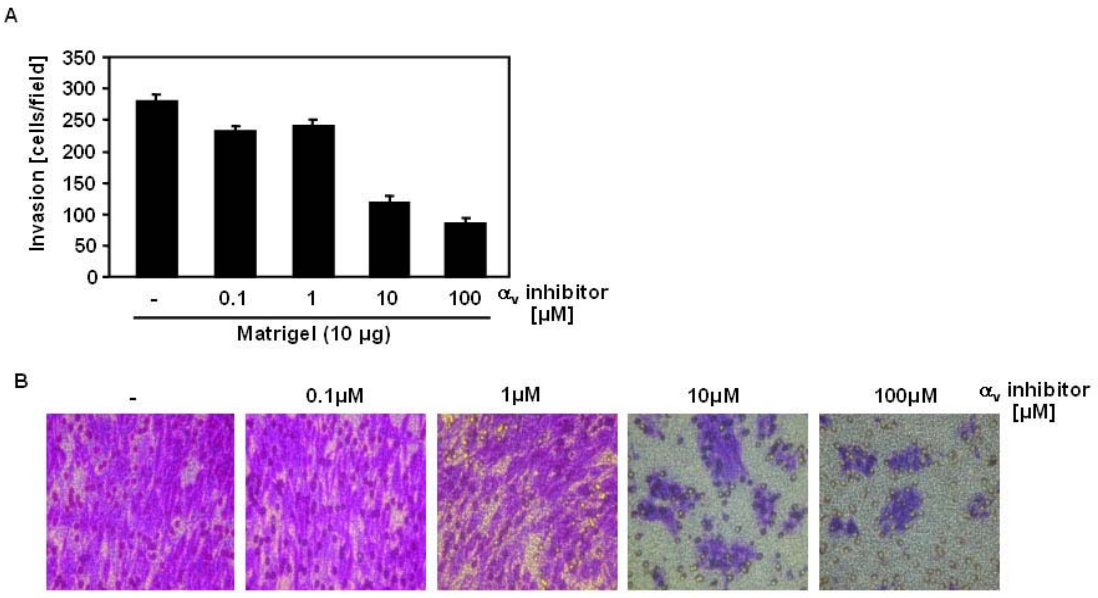

Fig. 2.16. Inhibition α_v integrin impairs invasive motility of hCASMCs. A. Serum starved hCASMCs were added with or without the integrin α_v inhibitor into the modified Boyden chambers overlaid with reconstituted matrigel. Results represent the average ± standard deviation from 3 random fields taken from each of the two separate chambers. The graph shows the cell invasion through matrigel. B. Microphotograph shows crystal violet stained invading cells through matrigel present on the lower side. Note the loss of invasion when increasing concentration of integrin α_v inhibitor was added to the hCASMCs.

2.2.15 Reduction of MMP secretion in hCASMCs by integrin α_v inhibitor

Since α_v integrin inhibition resulted in a substantial loss of invasiveness of hCASMCs, it is possible that this effect is mediated via the loss of MMP secretion. To test this possibility, the effect of adding integrin α_v inhibitor on MMP secretion was analyzed. For this, the hCASMCs were plated on VN-coated plates for 16 hrs in the presence or absence of the indicated concentrations of integrin α_v inhibitor. Conditioned medium collected from these cell experiments was further analyzed by gelatine zymography. Integrin α_v inhibitor reduced substantially MMP-2 secretion by hCASMCs in a dose dependent manner. The zymogram results also suggest that the mechanism for the severely impaired migration in the presence of higher concentrations of the integrin α_v inhibitor was due to reduced secretion of MMP level. Furthermore, the integrin α_v inhibitor did not cause any non-specific effect on cell adhesion, and they were lower in spreading (Fig. 2.17).

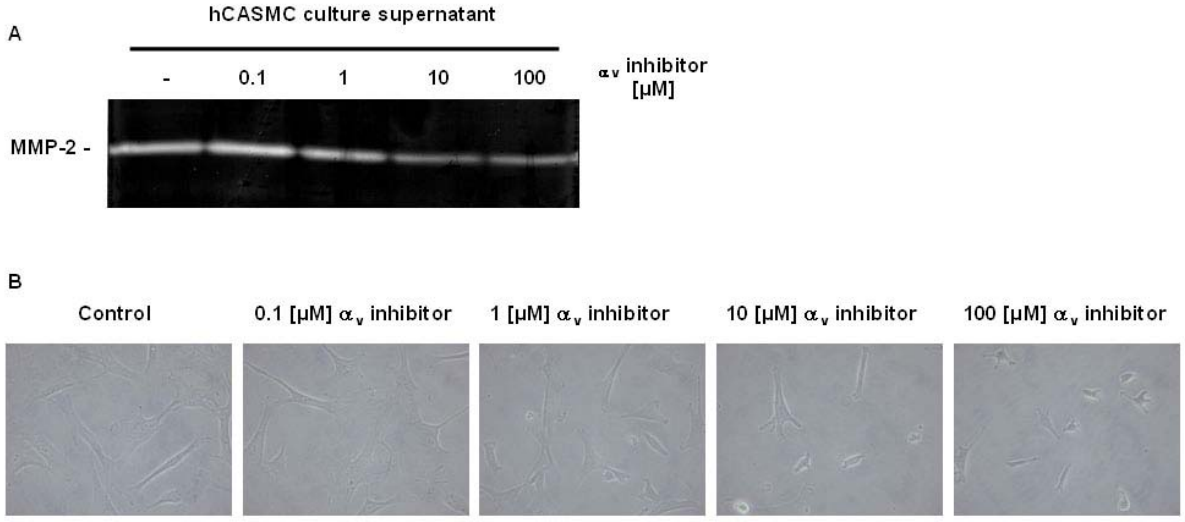

Fig. 2.17. A. Loss of MMP secretion of hCASMCs by integrin α_v inhibitor. hCASMCs were plated onto VN coated dishes for 16 hrs in the presence or absence of the indicated concentrations of integrin α_v inhibitor. Conditioned medium from each culture was analyzed by SDS-PAGE gelatin zymography. White bands against a dark background are corresponding to gelatinolytic area of MMP-2. B. Micrograph shown in phase contrast microscopy illustrates the hCASMCs cells plated on VN in the presence or absence of the integrin α_v inhibitor.

2.2.16 Integrin α_v inhibitor does not induce apoptosis

The dramatic effect of integrin α_v inhibitor on both tyrosine phosphorylation cellular proteins and migratory response against haptotatic and chemotatic factors suggested the importance of α_v integrin during ECM and growth factor induced signalling. Previous

studies by other research group (Albelda and Buck, 1990; Brooks et al., 1994) show that some of the integrins, such as $\alpha_v\beta_3$ integrins, can regulate apoptosis in certain cell types. Therefore, to determine whether the treatment with integrin α_v inhibitor would result in apoptosis, hCASMCs were treated with 10 and 100 μM of the integrin α_v inhibitor.

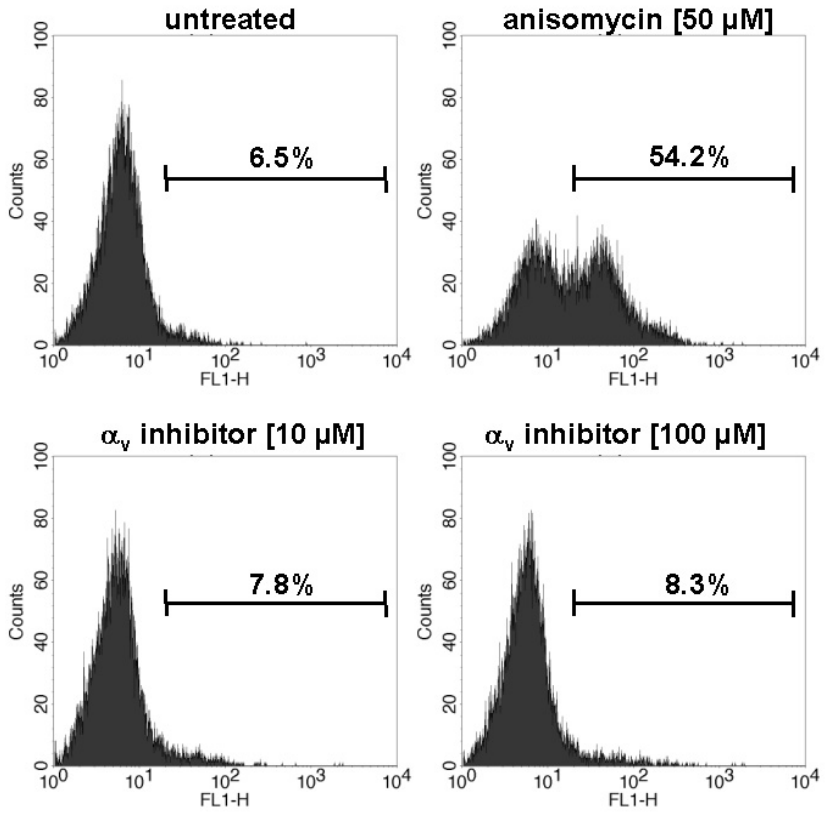

Fig. 2.18. Effect of α_v integrin inhibition on hCASMCs apoptosis. hCASMCs were grown for 18 hrs in the presence or absence of the indicated concentrations of either integrin α_v inhibitor or anisomycin, Cells were stained with FITC-coupled annexin-V and analyzed by flow cytometry to detect the apoptotic cells. Numbers indicate the percentage of hCASMCs staining positive for annexin-V.

The cells grown in the absence of any inhibitor served as untreated control whereas those treated with 50 μM anisomycin, a well-known inducer of apoptosis, served as positive control. While anisomycin treated cells readily exhibit a substantial level of apoptosis by staining positive for annexin-V, both integrin α_v inhibitor treated and untreated cells did not exhibit any appreciable level of apoptosis (Fig. 2.18). These studies demonstrate that the loss of α_v integrin function alone is not sufficient to cause apoptotic cell death of hCASMCs.

2.3 Discussion

Restenosis is one of the major drawbacks of PTCA, a common procedure for the treatment of stenotic blood vessels. Of the various factors that contribute to the development of arterial restenosis, alteration in cell adhesion receptors such as integrins has drawn much attention in recent years. In view of the possible role that these cellular receptors may play in pathological processes, recent efforts have been directed towards investigating *in vitro* expression, distribution of these integrins and disruption of integrin-ligand interaction mediated signalling events.

ECM-integrin interaction induces SMC migration and proliferation, thus influences the development of restenosis. Therefore, exploring the molecular mechanisms that connect integrins with hCASMC migration has great potential for therapeutic interventions. Specifically to dissect the mechanisms underlying the ECM-integrin interaction initiated signals, both specific integrin subtypes involved and the importance of FAK activation was characterised. Functionally, blocking integrin α_v-dependent signalling events lead to reduced haptotaxis and chemotaxis migration. Moreover, it impaired cellular invasion by reducing the secretion of MMP-2. In contrast, the integrin α_v inhibitor did not induce apoptosis. Therefore, an integrin α_v inhibitor could be suitable for prevention of restenosis and could potentially be combined with the application of stents.

The initial study was to determine the involvement of ECM-integrin interaction in signalling pathways by replating hCASMCs on VN coated plates. Since tyrosine phosphorylation is one of the initial events, which occur upon the interaction of integrins with ECM ligands, it can contribute to FA formation and subsequent signalling events. In particular, upon integrin engagement with ECM ligands, FAK has been shown to undergo autophosphorylation at the Tyr-397 residue that serves as a docking site for the recruitment of several other signalling proteins (Chen et al., 1996; Han and Guan, 1999; Schaller et al., 1994; Xing et al., 1994). Many studies revealed that FAK shows increased kinase activity (Guan and Shalloway, 1992; Lipfert et al., 1992) and tyrosine phosphorylation upon integrin activation in a variety of cell types, due to the autophosphorylation at Y397 (Burridge et al., 1992; Guan et al., 1991; Hanks et al., 1992; Kornberg et al., 1991; Lipfert et al., 1992). Western blot analyses with anti-phospho tyrosine antibody of WCL from those VN replated cells clearly indicate that FAK is the major tyrosine phosphorylated protein. In addition, the tyrosine phosphorylation in response to VN occurred in a dose-dependent manner.

Furthermore, immunoprecipitation combined with Western blot analysis from the same lysates demonstrated that Tyr-397 phosphorylation of FAK only on VN replated cells not on the negative controls. It suggested that the increased phosphorylation at the autophosphorylation site of FAK at Tyr 397 reflects an enhanced enzymatic activity of FAK. VN is major ligand for integrin $\alpha_v\beta_3$ and it indicates that $\alpha_v\beta_3$ integrin engagement with VN mediates FAK Tyr-397 phosphorylation. These results also correlate with observation from immunofluorescence assays, which support the idea that these ECM proteins are potent stimulators of tyrosine phosphorylation.

Further, these data from replating assays and immunofluorescence studies on FAK tyrosine phosphorylation and activation are supportive of several earlier studies that include: (i) FAK tyrosine phosphorylation is highly increased in response to SMC plating on FN (Polte et al., 1994), (ii) FAK can enhance the rate of cell spreading on FN (Owen et al., 1999; Richardson et al., 1997), (iii) specifically, binding of $\alpha_v\beta_3$ to VN results in an increase in FAK tyrosine phosphorylation (Zheng et al., 1999), (iv) FAK gets activated by tyrosine phosphorylation following integrin-mediated cell adhesion (Hanks and Polte, 1997), (v) During the cell adhesion on ECM, a number of FA proteins become tyrosine phosphorylated which leads to FA formation (Guan et al., 1991), (vi). A number of signalling events are associated with FA formation (Burridge et al., 1992). Although many studies have used pharmacological inhibitors to interrupt the signalling events mediated by integrin-ligand interaction, they failed to limit restenosis in humans.

To explore the possibility of interference with ECM-Integrin interaction, the replating assay was performed with specific integrin α_v inhibitor. Integrin α_v inhibitor dramatically blocked the tyrosine phosphorylation of FAK suggesting that the integrin α_v initiated signalling events lead to the activation of FAK. Together, the results from immunofluorescence assays and the biochemical assays support the idea that tyrosine phosphorylation of FA proteins at the focal complexes or FAs were abrogated by the integrin α_v inhibitor. Particularly, FAK tyrosine phosphorylation was blocked due to the interference of integrin-ligand interaction.

Since, integrin interaction with ECM proteins and the associated recruitment of signalling proteins are important for cellular functions, the presented study further focused on the mechanism of ECM-integrin interaction during hCASMC migration by using various stimulants. In this context, FA formation and their constant turnover are critical events for cell migration. $\alpha_v\beta_3$, which interacts preferentially with VN, have been shown to contribute significantly to the formation and turnover of FAs. This integrin is also able to interact with various other ECM proteins such as FN, ON, fibrinogen,

denatured or proteolysed CN and other matrix proteins. In contrast, some integrins interact only with specific ligand e.g. $\alpha_5\beta_1$ binds only with FN (Hynes, 1992; Ruoslahti and Pierschbacher, 1986).

To explore the integrin subtype that contributes significantly to hCASMCs migration, FN and VN were used in cell migration studies. Analysis of migratory behaviours using various concentrations of VN and FN exhibited a dose-dependent increase in migration of hCASMCs. These data suggest a possible role for the integrin binding to these ligands in hCASMCs migration. Interestingly, treatment of cells with integrin α_v inhibitor blocked the VN-induced migration and the loss of cell migration was found to occur in a dose-dependent manner. This reduced migration of hCASMCs towards VN also correlated with the reduction of FAK tyrosine phosphorylation. Collectively, these data demonstrate that α_v integrin is essential for migration of hCASMCs. It also points to a possible role of FAK autophosphorylation events in the cell migration. These data are in consistent with previous reports that FAK is involved in many cellular functions particularly migratory response of various cell types (Gilmore and Burridge, 1996; Hauck et al., 2001b; Sieg et al., 1999)

Many studies have also shown that besides ECM proteins, there are several other molecules such as chemokines, PDGF and IGF-I also involved during SMC migration (Axel et al., 1997; Grotendorst et al., 1981; Jones et al., 1996). To understand the intracellular signalling pathways of SMCs movement from media to the intima, *in vitro* models of chemoattractant directed SMCs migration across a modified Boyden chamber have frequently been used (Hauck et al., 2000). Chemotactic assays were performed with PDGF-BB as a chemoattractant using modified Boyden chambers. Results from such assays explained that PDGF-BB is one of the effective stimulators of hCASMC migration. Surprisingly, integrin α_v inhibitor is able to inhibit hCASMCs migratory response against chemotatic stimulants as in the case of haptotaxis. It indicates that the cooperative signals from integrins and growth factor signalling can be abrogated by integrin α_v inhibitor.

In vivo, the SMCs are normally surrounded by ECM proteins, which include collagens, elastin and proteoglycans. In case of restenosis, remodelling the ECM after the injury facilitates migration and proliferation of SMC in this complex three dimensional network and several proteinases play an essential role in this. In order to mimic *in vivo* situation, matrigel, which provides a three dimensional environment, was overlaid on top of the modified Boyden chamber during invasion assays. The integrin α_v inhibitor did not only block the migratory response induced by ECM proteins but also

the ability of invasiveness through matrigel. One possible reason for this impaired invasion is due to the reduction of MMP-2 secretion in hCASMCs by the integrin α_v inhibitor. Together, the current results provide evidence for MMP meditated hCASMC migration *in vitro*. Previous studies have shown MMP-2 and MMP-9 activities were increased which correlated with SMC migration after the injury (Kurschat et al., 1999; Pyke et al., 1992). Importantly, the integrin α_v inhibitor does not induce apoptosis in treated hCASMCs. This data demonstrate that treatment of α_v inhibitor is not alone sufficient to induce apoptosis.

Currently available therapies to prevent restenosis normally target a single stimulator of SMC migration. Majority of such clinical trials often failed to prevent the restenosis in humans. Failure of those trails could be attributed to the complex pathophysiology of restenosis wherein several stimulators promote SMC migration. Often, inhibition of a single stimulant induced SMC migration was overcame by some other redundant cytokines, chemoattractants or mitogens. In addition, redundancy of multiple receptor subtypes induced signalling events also contributes the SMC migration during the restenosis. Therefore, interference of any one of the stimulating factors is not sufficient to inhibit the restenosis cascade. The integrin α_v inhibitor, used in this study, showed its ability to inhibit multiple points in signalling pathways, thus could be useful for prevention of the restenosis. In the case of integrin α_v blockades, the route of drug delivery, the minimum dose and duration of the drug, and systemic side effects associated with the treatment are not well established, and they warrant further studies *in vivo*. The above results confirm the hypothesis that interference with α_v mediated cell matrix interactions leads to reduce hCASMCs migration *in vitro*.

3. Adeno-associated virus mediated overexpression of FRNK interferes with SMC migration *in vitro* and neointima formation *in vivo*

3.1 Introduction

Numerous studies have shown that SMCs proliferation and migration have an essential role in vasculargenesis and wound healing processes. They not only play an important role in development but also in many pathological processes such as atherosclerosis and restenosis (Owens, 1995; Schwartz et al., 1990b). For example, excessive growth and migration of SMC can lead to neointima formation in the injured vessels. Recently it has been shown that signalling mechanisms involved in regulating cell migration was triggered by both integrin and growth factors (Giancotti and Ruoslahti, 1999). Both ECM induced signalling and growth factor signalling converges to modulate SMC proliferation and migration in such pathological processes. FAK is one of the main key mediator facilitating migration by coordinating both chemotatic and haptotatic cues. Overexpression of FAK influences in increased migration and elevated FAK expression was observed in tumours (Akasaka et al., 1995; Cary et al., 1996; Owens et al., 1995). Further, several indications has shown that cells from FAK deficient mice showed reduced rate of migration (Ilic et al., 1995, Ilic et al., 1996).

Microinjection or overexpression of FRNK, a C-terminal domain of FAK, leads to reduced cell motility and also delay in formation of FAs and spreading (Gilmore and Romer, 1996; Richardson and Parsons, 1996). PDGF and EGF induced cell migration can be reduced by overexpression of FRNK (Hauck et al., 2000; Taylor et al., 2001). FRNK also impaired cell invasion and experimental metastasis (Hauck et al., 2002). Adenoviral mediated overexpression of FRNK also reported to interfere in PDGF-BB stimulated primary VSMC proliferation (Sundberg et al., 2003). Therefore, interference of FAK signalling by FRNK would be a powerful target for abrogating SMC migration and subsequent reduction in case of neointima formation.

3.1.1 Adeno-associated viral (AAV) vectors

In late 1960s, AAV was identified as a contaminant defective satellite virus in an adenoviral stock. It is a single stranded DNA virus belongs to the family parvoviridae and genus dependovirus (Srivastava et al., 1983). Currently, there are ten members in this genus. The first five members (AAV1, AAV2, AAV3, AAV4 and AAV5) belongs to

primates and other five members belongs to bovine, canine, avian, equine and ovine (Snyder and Flotte, 2002). Wild type AAV is a nonenveloped virus, which is incapable of autonomous replication and spread. The 4.7kb genome contains genes responsible for replication, site specific integration (rep gene) and structural capsid proteins (cap gene; Hermonat et al., 1984; Tratschin et al., 1984). AAV virion consists of three proteins VP1, VP2 and VP3 (Berns and Giraud, 1996). 90% of the protein component of virion is made up of VP3, the remaining 10% is made up of VP1 and VP2 in an equal ratio (Smith-Arica and Bartlett, 2001).

AAV vectors are based on non pathogenic virus with broad host range from animals to humans and a wide variety of cell types. In AAV vector system, 96% of the parental genome encoding the viral proteins has been deleted and the interested genes were flanked by inverted repeats to generate an AAV vector plasmid (Samulski et al., 1989). They are generally defective without helper free virus. It requires co-infection with helper virus such as adenovirus (Atchison et al., 1965), herpes virus and vaccinia virus (Schlehofer et al., 1986), which provides proteins necessary for replication in the host cells. In the absence of helper virus, AAV remains latent within the cell. Their replication can be induced by various stress inducing factors like ultraviolet irradiation, hydroxyurea, heat shock or several carcinogens (Schlehofer et al., 1986 ; Yakobson et al., 1987; Yalkinoglu et al., 1988). Heparan sulphate proteoglycan (HSPG) was identified as a receptor for AAV2 and is important for binding stage of AAV infection (Summerford and Samulski, 1998). $\alpha_v\beta_5$ integrin and FGF receptor has also shown to be essential for mediating infection (Qing et al., 1999; Summerford et al., 1999). AAV viral vectors were studied in many models for cystic fibrosis (Flotte et al., 1993), parkinson's diseases (Kaplitt et al., 1994), anaemia (Kessler et al., 1996) and brain tumours (Okada et al., 1996). Further, they have also been used in wide range of studies using liver, brain, muscle, heart and other tissues (Acland et al., 2001; During and Ashenden, 1998; Flotte, 2001; Maeda et al., 1998; Ye et al., 1999).

3.1.2 AAV helper free virus system

AAV helper free system provides infectious recombinant AAV-2 virions without the use of adenovirus. The interesting gene can be cloned into the pAAV-MCS vector or pAAV-IRES-hrGFP. The adenoviral genes products such as E2A, E4 and VA RNA gene, which are essential for production of infectious AAV particles, are from the plasmid pHelper. The genes responsible for replication and packaging are provided by

pRc vector. Co-transfecting all three plasmids in the 293T cells by using calcium chloride method produced the viral particles (Fig.3.1).

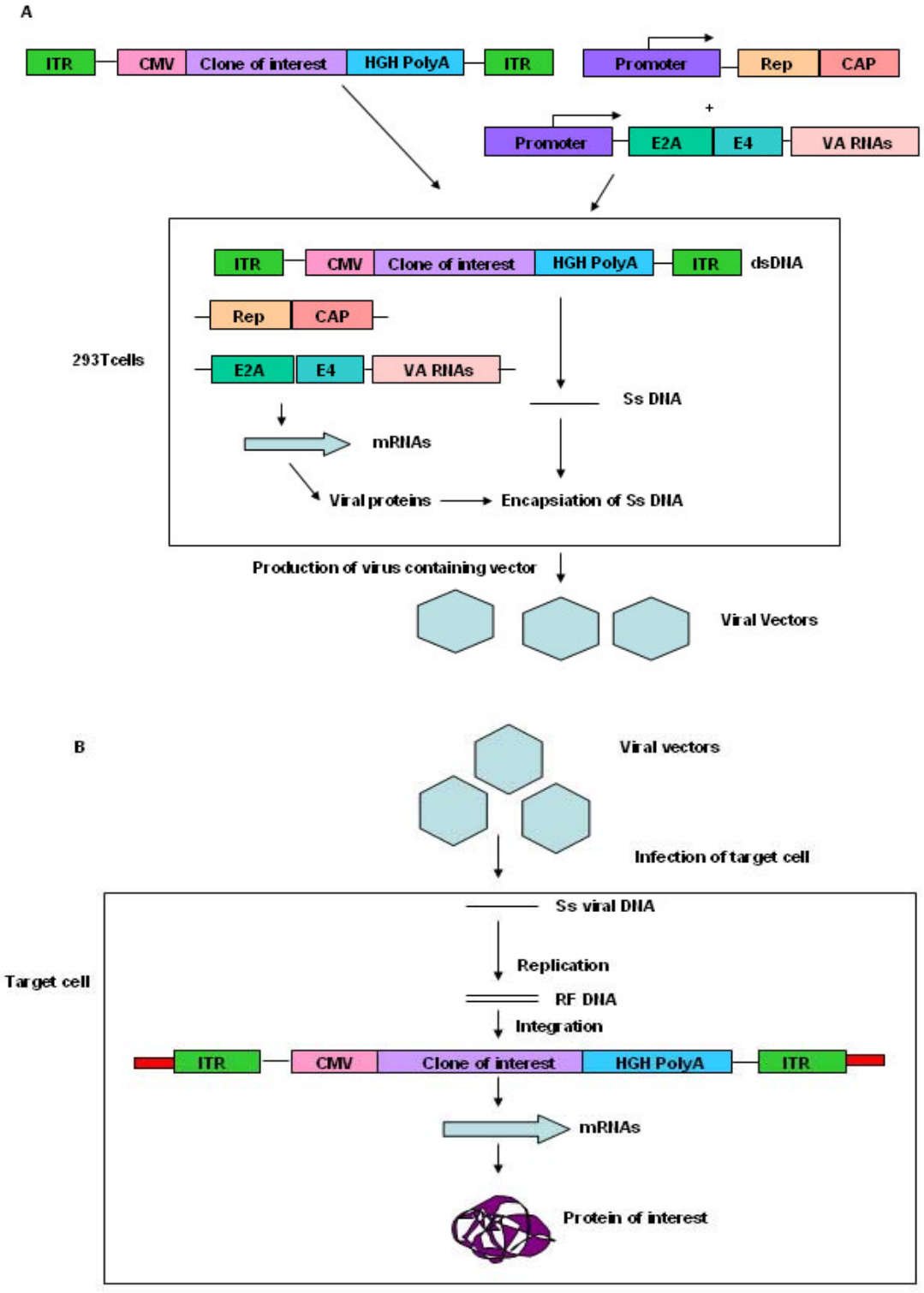

Fig. 3.1. Strategies for generation of AAV-2 vectors. A) The 293Tcells were cotransfected with three plasmids including pAAV-MCS or pAAV-IRES-hrGFP vectors containing gene of interest flanked by inverted terminal repeats (ITRs), the pRC vector which has rep and cap gene and pHelper plasmid that contains viral genes (E2A, VA, and E4). pAAV-IRES-hrGFP vector containing GFP is used to monitor the transfection efficiency. B) The viral particles containing supernatants were collected from the transfected cells and used to infect the target cells. After the infection, the target cell will express the gene of interest (The fig was modified from stratagene user manual).

3.1.2.1 Advantages and disadvantages of AAV helper free vectors

AAV vectors have several advantages for application in gene therapy. Preclinical studies revealed that AAV is not associated with any known human diseases, and none of its serotypes close to their zootropic relatives, that cause any known diseases in animals. It neither has any cytopathic effects nor elicits immunological responses since AAV coding sequence, which is responsible for antigenic viral proteins are eliminated from the vector. It has potential site-specific integration in human chromosome 19 named as AAVS1, which allows the therapeutic genes to be transmitted to further cell progeny. Furthermore, persistent long-term gene expression and lack of toxicity continue to appear very attractive for clinical applications. However, AAV has certain limitations to use as a vehicle in gene therapy. They have limited packaging size (less than 5 kb) and also it is difficult to achieve high titer viral production. In addition, it would be difficult to use in the tissue specific application since it infects wide range of cell types. Also, theoretical risk of insertional mutagenesis (may be deregulation or disruption) is another limitation during the usage of AAV vectors.

In view of the above advantages, AAV helper free vector system mediated overexpression of FRNK was employed to interfere FAK, a central molecule involved in SMC migration, in both *in vitro* and *in vivo* model system.

3.2 Results

3.2.1 Production of pAAV-MCS-FRNK and pAAV-LacZ viral particles

To interfere with FAK-mediated signalling, an endogenous inhibitor of FAK, FRNK has been used in several studies (Hauck et al., 2002; Richardson and Parsons, 1996; Taylor et al., 2001; Xu et al., 2000; Zhao and Guan, 2000). In contrast to FAK, which is ubiquitously expressed, FRNK is not present in most cells. To overexpress FRNK, a recombinant viral vector would be suitable to deliver the FRNK cDNA into the hCASMCs.

To produce the recombinant FRNK encoding AAV particles, the pAAV-MCS vector was digested with EcoRI and XbaI. The FRNK fragment comprising sequence coding for a triple-HA-tag at 3`end were amplified by PCR from plasmid pcDNA 3.1-FAK-HA (Fig. 3.2). After digestion with EcoRI and XbaI, FRNK-HA tag was ligated into digested pAAV-MCS and transformed into *E. coli* DH5α strain. Positive clones were identified; isolated and recombinant plasmids were purified.

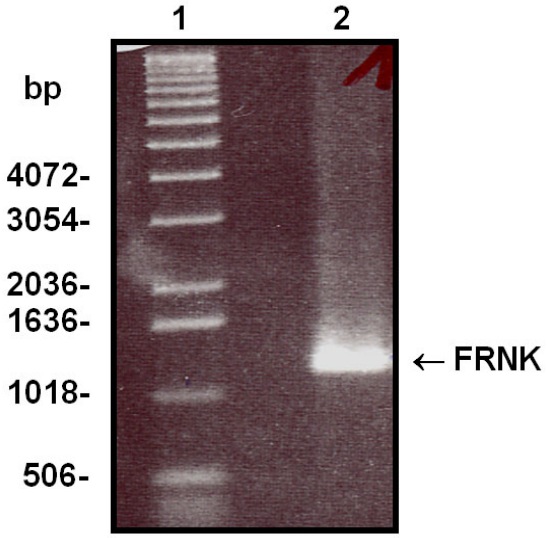

Fig. 3.2. Construction of pAAV-MCS-FRNK. FRNK cDNA was amplified from plasmid pcDNA3.1-FAK-HA using the primers FRNK–HA sense and FAK-anti-short. The PCR product was loaded onto an agarose gel. Lane1-1Kb DNA ladder; lane2-FRNK-HA fragment.

Subsequently, the pAAV-MCS-FRNK or pAAV-LacZ together with pHelper and pAAV-Rc plasmids were used for cotransfecting the 293T cells using the calcium chloride precipitation method (Fig. 3.3). The viral particles were harvested by repeated freeze thawing of the cells after the indicated time. The viral suspensions of pAAV-MCS-FRNK and pAAV-LacZ were used then to infect hCASMCs.

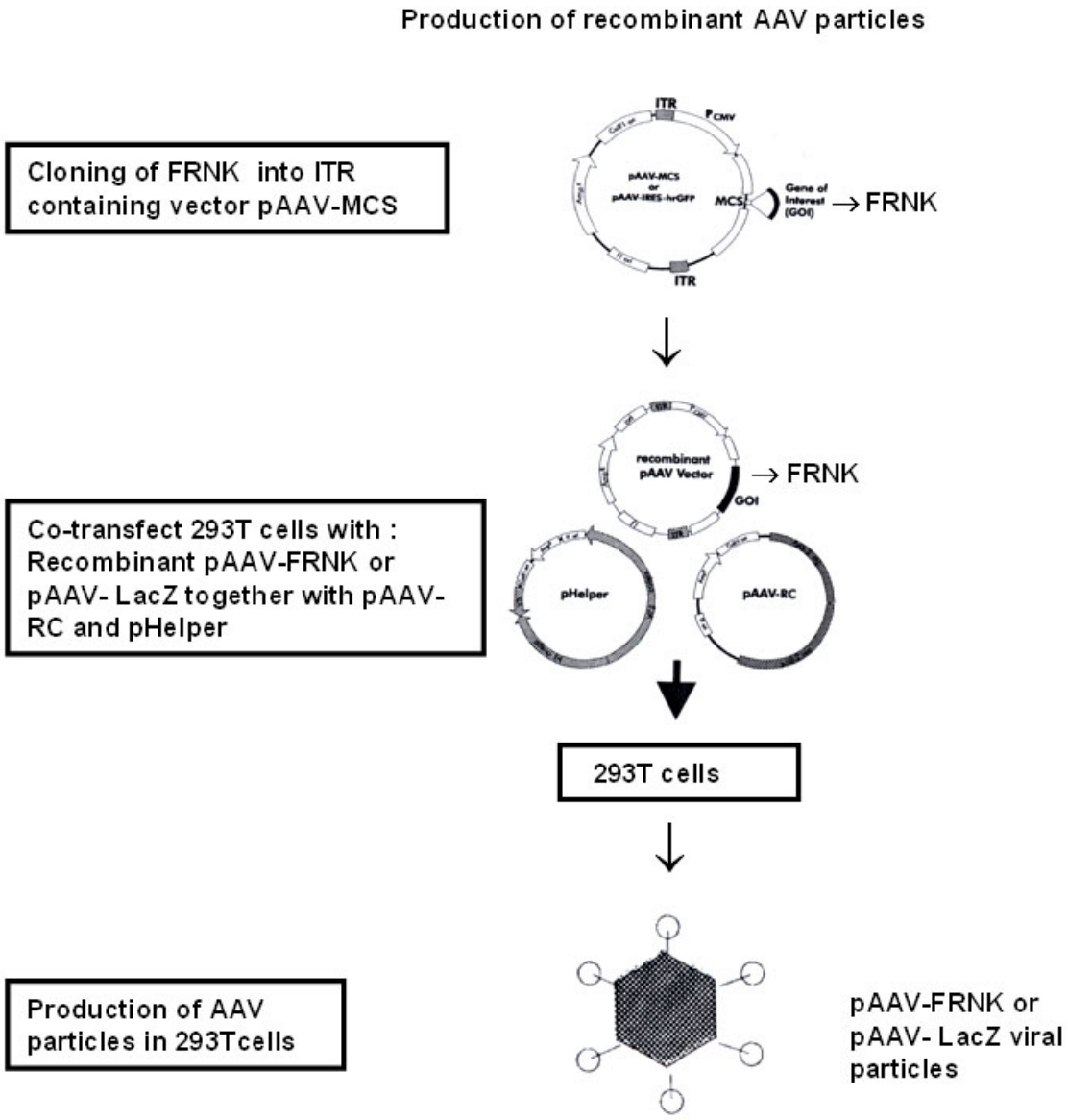

Fig. 3.3. Production of pAAV-MCS-FRNK and pAAV-LacZ viral particles. pAAV-vector has an ITR-flanked multiple cloning site (MCS) which can be used to insert the gene of interest for packaging into AAV particles. The MCS of this vector was used to insert the PCR-amplified FRNK-HA fragment. The resulting recombinant pAAV-MCS-FRNK or pAAV-LacZ, pAAV-RC and pHelper plasmids were used for cotransfection of 293T cells by the calcium chloride method. After 54 hrs, the cells were harvested, suspended in 0.1 M Tris buffer with 0.15 M NaCl pH 8 and the viral particles were released by repeated freeze thawing. The suspensions were centrifuged at 16000 rpm for 10 min and the viral supernatants were collected for further use.

To determine the effectiveness of viral gene transduction, hCASMCs infected with recombinant pAAV-LacZ-encoding AAV viral particles were used. Five days after infection, β-galactosidase staining revealed efficient infection of hCASMCs with pAAV-LacZ viral particles (Fig. 3.4). Also the pAAV-MCS-FRNK and mock were used to infect the hCASMCs. These cells were lysed to determine the overexpression of FRNK.

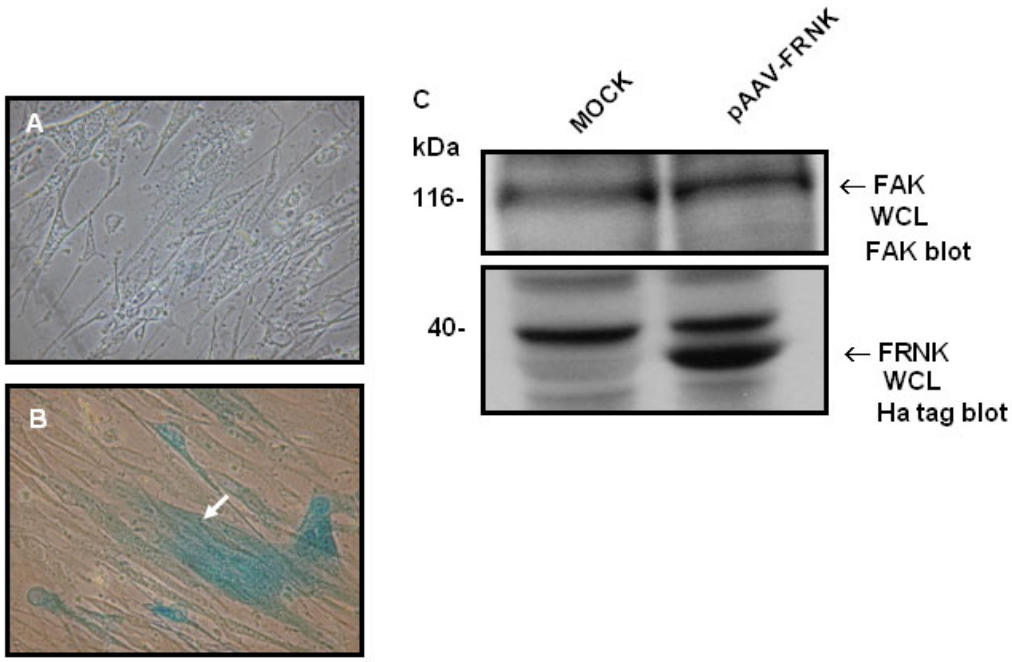

Fig: 3.4. The expression of pAAV-MCS-FRNK and pAAV-LacZ viral particles in hCASMCs. The pAAV-MCS-FRNK (8 µg) or pAAV-LacZ (8 µg), pHelper (8 µg), and pAAV-Rc (8 µg) plasmids were used for transfecting the 293T cells using the calcium chloride precipitation method. The virus-containing 293T cells were harvested after 54 hrs of transfection. pAAV-MCS-FRNK and pAAV-LacZ viral particles containing supernatants were used to infect primary hCASMCs as described in the methods. A) and B) After 5 days, the β-galactosidase staining was performed on uninfected control cells (A) and on pAAV-LacZ-infected hCASMCs (B). (C) hCASMCs lysates from mock and pAAV-MCS-FRNK transfected cells were analysed by SDS-PAGE and Western blotting with anti-FAK (top panel) and anti-HA-tag antibodies (lower panel). Arrow indicates pAAV-LacZ-postive cells.

The lysates were separated by SDS-PAGE and analysed by Western blotting. The blots were probed with α-HA-tag antibody that recognizes the HA-tagged, virus-encoded FRNK. In addition, the same blots were probed with anti-FAK antibody to detect the FAK level in the virus-infected hCASMCs. Here, the results show that the level of FAK in pAAV-MCS-FRNK infected cells does not change in comparison to uninfected cells (mock; Fig. 3.4; upper panel). Clearly, the infection with the pAAV-MCS-FRNK shows the expression of HA-tagged FRNK in hCASMCs (Fig. 3.4; lower panel).

3.2.2 Effect of pAAV-MCS-FRNK virus on VN-induced hCASMC migration

To determine whether FRNK expression in hCASMCs is also able to block the FAK dependent functions such as cell migration, cells infected with pAAV-MCS-FRNK and mock were serum starved for overnight and then used for migration assay. As observed in Fig. 3.5, VN-induced cell migration was strongly decreased in pAAV-MCS-FRNK-infected cells. These data suggest that FRNK delivered by a viral vector might be useful as a negative regulator of hCASMC migration.

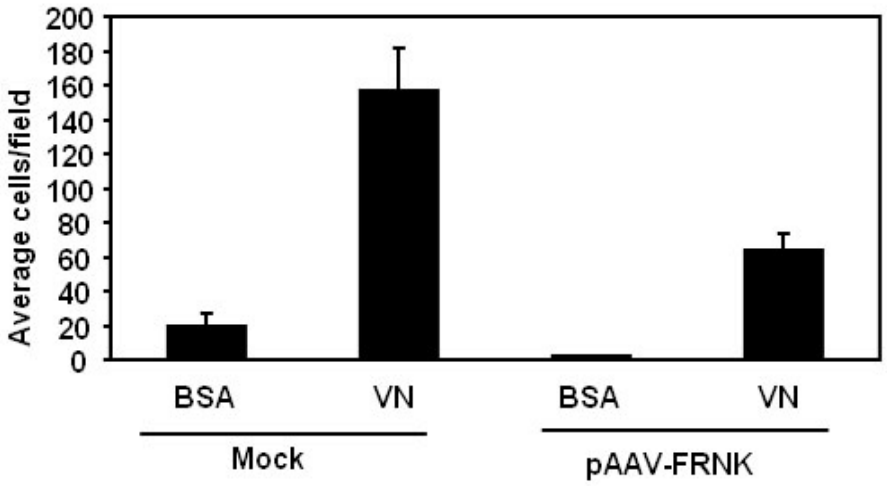

Fig. 3.5. Effect of pAAV-MCS-FRNK virus on VN induced hCASMC migration. Serum starved pAAV-MCS-FRNK and mock-transfected cells were analysed in a modified Boyden chamber haptotaxis assay. The graph shows mean values ± standard deviation of the cell migration of infected (pAAV-MCS-FRNK) and uninfected (mock) cells on BSA or VN (10 µg/ml) coated modified Boyden chambers.

3.2.3 Construction of pAAV-IRES-GFP-FRNK

In order to monitor effective viral production, FRNK was also cloned into mutiple cloning site of pAAV-IRES-hrGFP vector. The pAAV-IRES-hrGFP vector was digested with EcoR I and Sal I. FRNK was amplified and PCR purification was done by using Qiagen purification kit. The PCR fragments were digested with EcoR I and Sal I (Fig. 3.6).

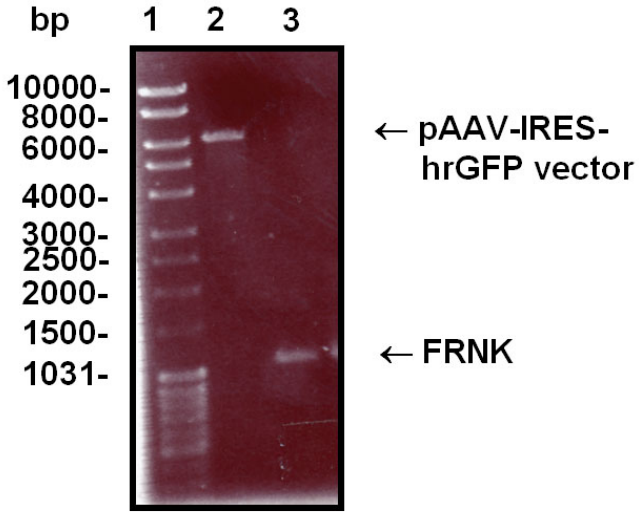

Fig. 3.6. Construction of pAAV-IRES-GFP-FRNK. FRNK was cloned into MCS of pAAV-IRES-hrGFP. The pAAV-IRES-hrGFP vector was digested with EcoR I and Sal I. FRNK was amplified by using the primer-FRNK-KOZAK-sense and FRNK without stop codon ANTI from the template PBS SK (+) – FRNK-HA tag. The PCR fragments and pAAV-IRES-hrGFP vector were digested with EcoR I and Sal I. Lane 1.1 kb. Lane 2. Digested pAAV-IRES-hrGFP vector. Lane 3. Digested FRNK PCR fragments.

The digested PCR fragments were ligated with digested pAAV-IRES-hrGFP vector and transformed into *E.coli* DH5α. The positive clones were identified; isolated and recombinant plasmids were purified. The pAAV-IRES-hrGFP containing FRNK was subsequently used for virus production.

3.2.4 Production of AAV-GFP-FRNK viral particles

pAAV-IRES-GFP-FRNK, pHelper and pAAV-Rc plasmids were used for cotransfecting the 293T cells using the calcium chloride precipitation method. The virus is named as AAV-GFP-FRNK. The viral particles were harvested by repeated freeze thawing of the cells after the indicated time. The viral suspensions were used to infect the cells. The green fluorescent cells (GFP) were counted in the fluorescent microscope. Then, cells were lysed in RIPA buffer. Lysates prepared from AAV-GFP-FRNK and mock infected hCASMCs were analysed by Western blot using an anti flag antibody to detect FRNK. These experiments revealed that FRNK was expressed in AAV-GFP-FRNK infected cells. In the mock-infected cells, there was no expression of FRNK. It indicates that the viral vectors were efficiently transduced and leads to overexpression of FRNK in hCASMCs (Fig. 3.7).

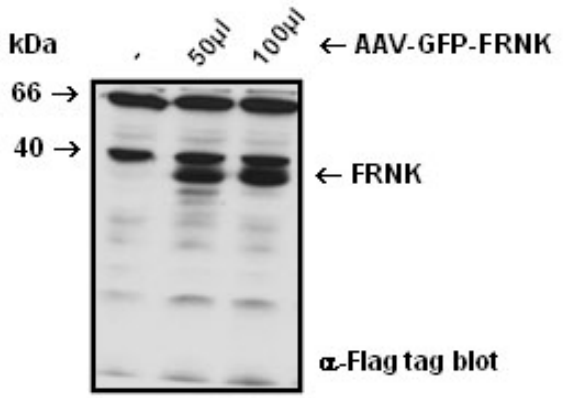

Fig. 3.7. Expression of AAV-GFP-FRNK. The pAAV-IRES-GFP-FRNK (10 µg), pHelper (10 µg), and pAAV-Rc (10 µg) plasmids were used for cotransfecting the 293T cells using the calcium chloride precipitation method. After 54 hrs, the virus-containing 293T cells were harvested. The AAV-GFP-FRNK viral particles were used for infecting 293T cells as described in methods. After 3 days, the cells were lysed and Western blotting was performed. The blot was probed with anti-Flag tag antibody. Lane 1. AAV-GFP infected cells. Lane 2 and 3. AAV-GFP-FRNK transfected cells.

3.2.5 Standardization of high titer AAV-GFP-FRNK viral particles

To improve the titration of the AAV-GFP-FRNK viral particles were produced as described earlier. There are various parameters which influence the production of high titer viral stocks such as concentration of the plasmids used for the transfection, different time of harvesting and different type of lysis. To optimize these parameters, various amounts of plasmids such as 5 µg, 8 µg and 10 µg of each plasmid were used

to transfect the 293T cells. The result shows that the viral production is dependent on the amount of the three plasmids. 293T cells that were infected from those viral suspensions show that increasing amounts of plasmid will lead to an increase in the titer of the virus. Transfection with 10 µg of each plasmid result in the maximum amount of GFP-positive cells indicating the increase in the viral titer when compared to other transfections with lower amount of plasmids (Fig. 3.8).

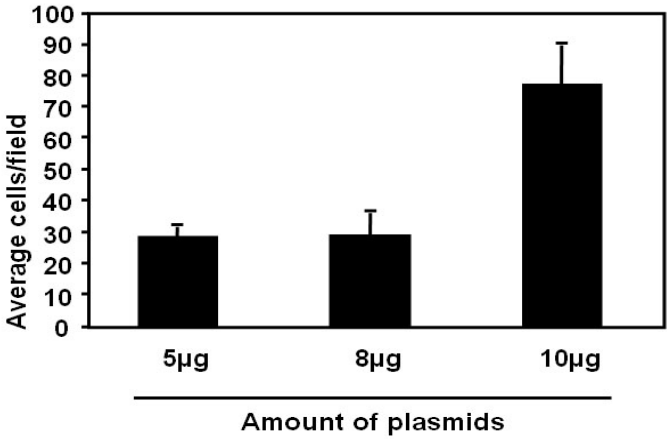

Fig. 3.8. Production of AAV-GFP-FRNK viral particles by using different amount of plasmids. pAAV-IRES-GFP-FRNK, pAAV-RC and pHelper plasmids were used for transfection of 293T cells by the calcium chloride method. Different amount of plasmids such as 5 µg, 8 µg and 10 µg of each plasmid were used to transfect the 293T cells. The cells were harvested and suspending into DMEM produced the viral particles. The viral particles were used to infect 293T cells. After 6 days, GFP cells were counted in the microscope. The graph shows mean values ± standard deviation of infected cells.

To determine the appropriate harvesting time for the viral production after the transfection in 293T cells, the cells were harvested at 62 hrs, 68 hrs, 74 hrs and 84 hrs.

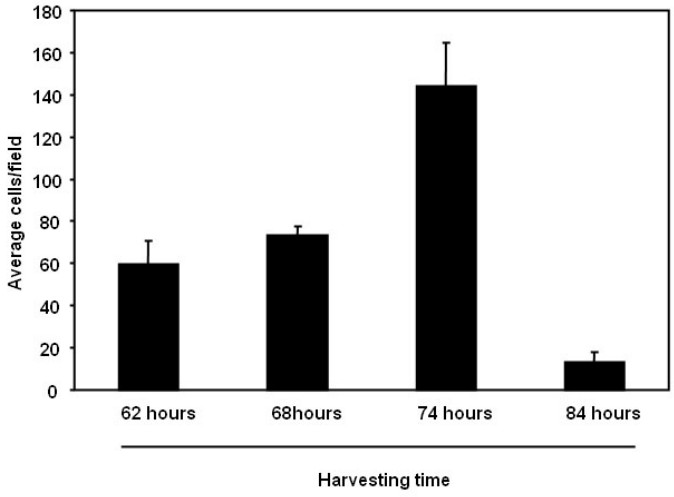

Fig. 3.9. Harvesting of AAV-GFP-FRNK viral particles after different following transfection. pAAV-IRES-GFP-FRNK, pAAV-RC and pHelper plasmids were used for transfection of 293T cells by the calcium chloride method. After 62, 68, 74 and 84 hrs the cells were harvested and finally the viral particles were produced by suspending in DMEM. The 500 µl of viral particles were used to transfect the 6 well plates containing 293T cells. After 3 days, the GFP cells were counted in the microscope. The graph shows mean values ± standard deviation of infected cells.

Then the viral suspensions were used for infection. The expression of GFP cells suggested that appropriate harvesting time would be 74 hrs after the transfection (Fig. 3.9) because virus harvested after 74 hrs shows increased level of GFP expression compared to the other harvesting time points. In order to find the suitable lysis condition for viral production, AAV particles containing 293T cells were lysed by different methods using freeze thawing in DMEM, DMEM with 50 mM Tris, syringe, 0.1 M Tris and TBS. The viral suspensions were further used to infect the 293T cells. More number of GFP expressing 293T cells were found in samples infected with viral suspensions from cells lysed in DMEM than in other lysis conditions (Fig. 3.10).

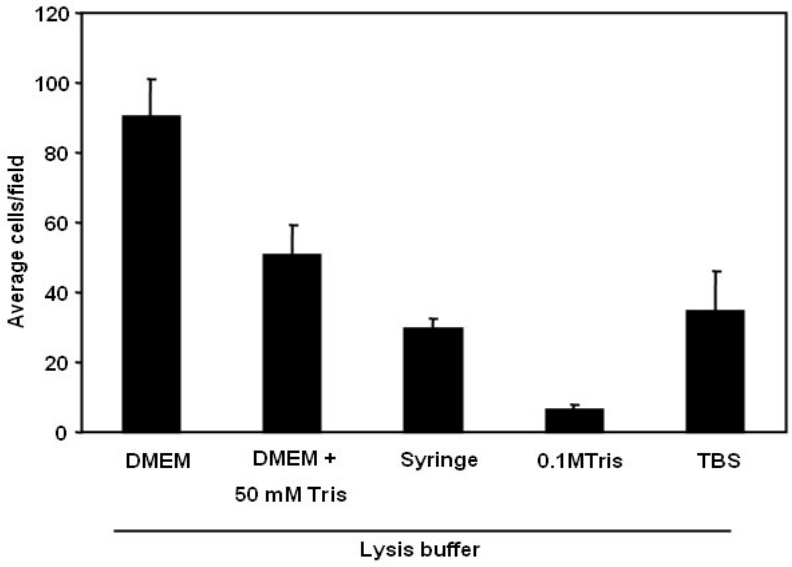

Fig. 3.10. Production of AAV-GFP-FRNK viral particles by using different type of lysis. pAAV-IRES-GFP-FRNK, pAAV-RC and pHelper plasmids were used for transfection of 293T cells by the calcium chloride method. 10 µg of plasmid was used to transfect the 293T cells. The cells were harvested 74 hrs. The cells were lysed using different kinds of lysis buffers or syringe. The viral particles were used to transfect the 293T cells. After 3 days, the GFP cells were counted in the microscope. The graph shows mean ± standard deviation of infected cells.

3.2.6 AAV mediated overexpression of FRNK suppresses neointimal formation after coronary stent implantation in porcine coronary artery model

To interfere with neointima formation in a porcine coronary artery model *in vivo*, the AAV-GFP-FRNK and also the AAV-GFP control viral particles were produced by cotransfecting 10 µg of all three plasmids. The virus was harvested at 74 hrs and lysed in DMEM. The viral supernatants were concentrated using AAV2 concentrating kit. To test this concentrated viral preparation, 293T cells were infected with viral supernatants and cells were lysed. The lysates prepared from AAV-GFP-FRNK and AAV-GFP control virus transfected 293T cells were analysed by Western blot using a flag

antibody. The results revealed that FRNK was expressed in AAV-GFP-FRNK lysates (Fig. 3.11).

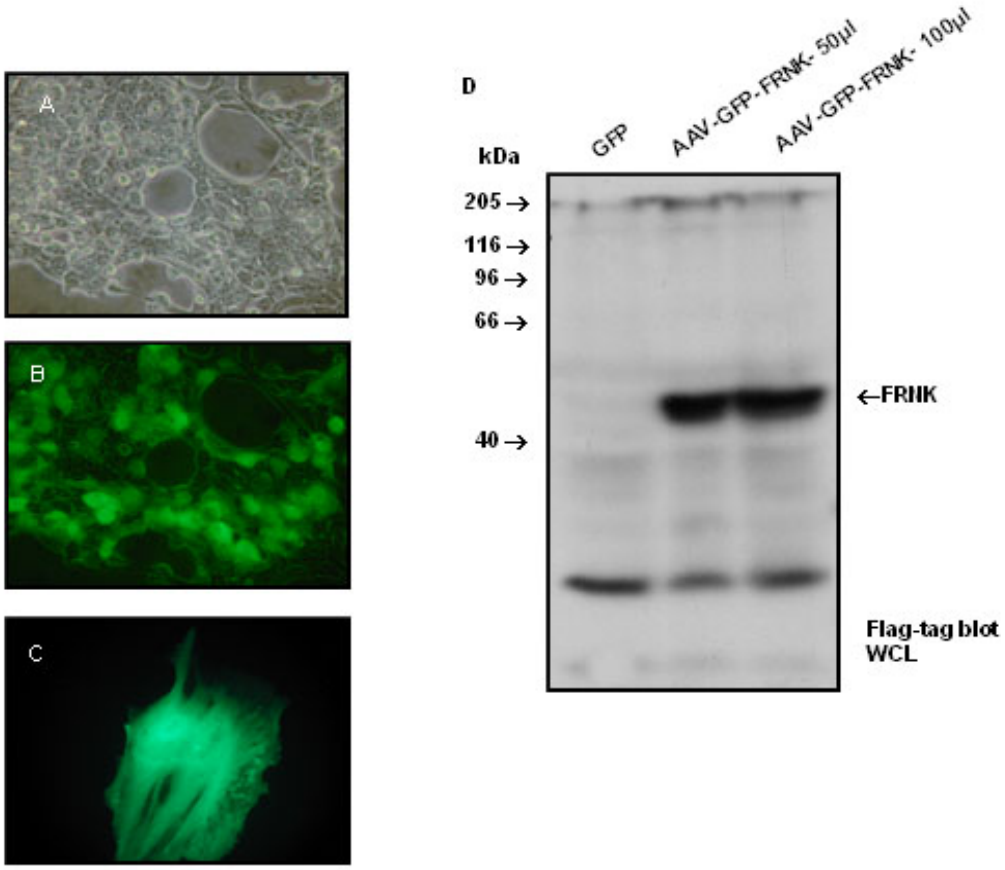

Fig. 3.11. Production of AAV-GFP-FRNK and AAV-GFP control viral particles. The pAAV-IRES-GFP-FRNK (10 µg), pHelper (10 µg), and pAAV-Rc (10 µg) plasmids were used for cotransfecting the 293T cells using the calcium chloride precipitation method. After 74 hrs, the virus-containing 293T cells were harvested in DMEM. The AAV-GFP-FRNK virus containing supernatant was used for infecting 293T cells as described in methods. A) Transfected 293T cells in transmission (200X magnification). B) GFP expressing 293T transfected cells (200X magnification). C) hCASMCs infected with AAV-GFP-FRNK (400X magnification). After 4 days, the infected cells were lysed and Western blotting was performed. The blot was probed with anti–flag tag antibody. D) AAV-GFP-FRNK expression in 293T cells. Lane 1. AAV-GFP control infected cells. Lane 2. 50 µl of AAV-GFP-FRNK infected cells. Lane 3. 100 µl of AAV-GFP-FRNK infected cells.

To evaluate the ability of FRNK to prevent intimal hyperplasia after stent implantation, two different groups (3 pigs per group, 2 stents/pig) of animals were subjected to stent implantation either with AAV-GFP-FRNK or AAV-GFP control virus (Table 3.1). One of the AAV-GFP-FRNK treated pig received double amount of virus (see the table 3.1). Those stents were successfully implanted in the left anterior descending coronary artery (LAD).

Table 3.1. Table showing treatment groups and number of implants performed in the porcine restenosis model.

Treatment Groups		Number of Implants
AAV-GFP-FRNK treated	1x	4
	2x	1
AAV-GFP control treated	1x	6

All pigs were until 28 days after the PTCA in the care facilities. Repeated angiograms were performed and X-rays of the vessel showed that the stents confirm well to the contours of the vessel lumen. All stents appear widely and evenly expanded.

Table 3.2. The morphometric data from AAV-GFP-FRNK and AAV-GFP control stents implanted pigs. Lumen area, neointimal area, % area of stenosis, intimal thickness, injury score and medial area are calculated from AAV-GFP-FRNK and AAV-GFP control stent implanted pigs 28 days after the implantation. Values are expressed as mean ± SD.

Treatment Group	Lumen Area (mm^2)	Neointimal Area (mm^2)	% Stenosis	Intimal Thickness (mm)	Injury Score	Medial Area (mm^2)
AAV-GFP-FRNK treated n=5	4.14 ± 0.55	2.33 ± 0.85	36.30 ± 13.55	0.27 ± 0.14	0.75 ± 0.07	1.45 ± 0.25
AAV-GFP control treated n=6	3.35 ± 0.51	3.09 ± 1.58	47.59 ± 23.31	0.41 ± 0.29	0.84 ± 0.28	1.69 ± 0.40
p-value	0.3209	0.3635	0.3662	0.3537	0.5167	0.2781

The injury score was measured according to the Schwartz method (Schwartz et al., 1992). The cross section of each stent was measured with digital morphometry to determine the areas within the external elastic lamina (EEL), internal elastic lamina (IEL) and lumen. Percentage area of stenosis was calculated using the formula [(Neointimal area/internal elastic lamina area) x 100]. The medial area was then calculated by subtracting the IEL area from the EEL area. Neointimal thickness was measured as the distance from the inner surface of the each stent strut to the luminal border. The results revealed that the injury score was similar in AAV-GFP-FRNK and AAV-GFP control treated groups. However, there is a slight reduction in the neointimal thickness for the AAV-GFP-FRNK-treated animals (2.33 ± 0.85 mm^2) were observed when compared to the AAV-GFP control treated animals (3.09 ± 1.58 mm^2; Table 3.2).

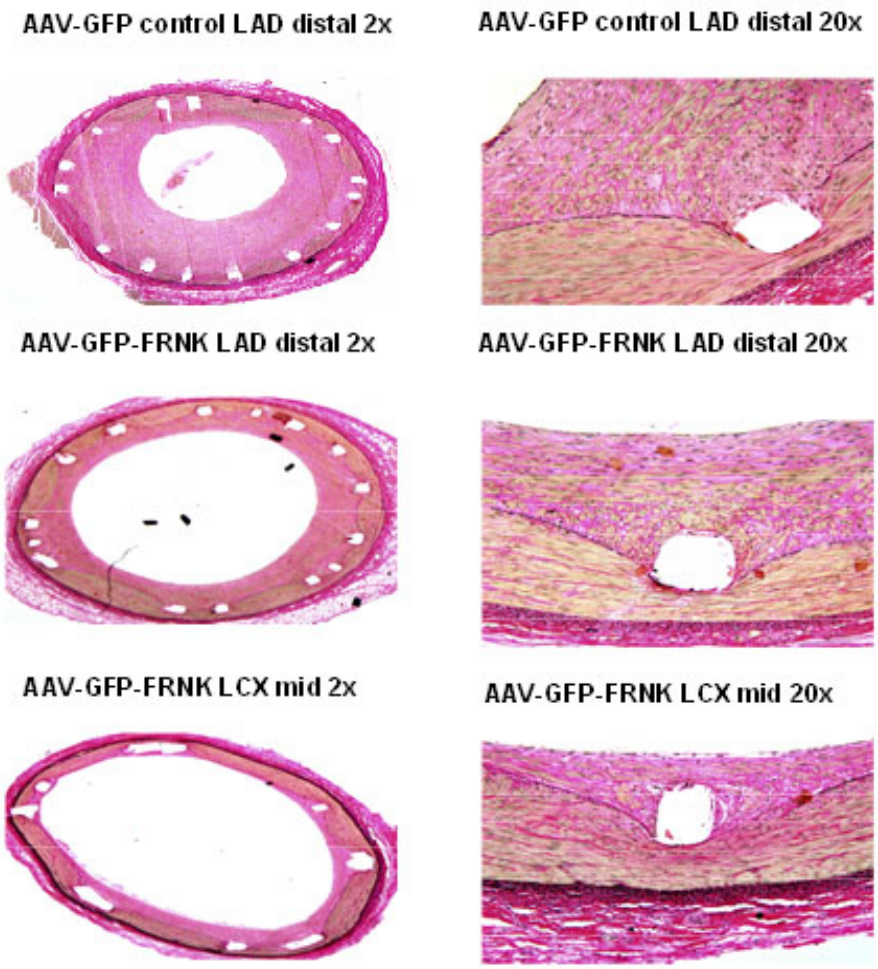

Fig. 3.12. Representative histological specimens from AAV-GFP control and AAV-GFP-FRNK coated stents in pig coronary arteries at 28 days after implantation. The vessels were dehydrated in ethanol and embedded in methylmethacrylate plastic. After polymerization, 2-3 mm sections were sawed from distal or middle area of each stent. 4-5 μm sections were cut in a microtome and stained with haematoxylin and eosin and elastic movat pentachrome stains. All sections were examined under the light microscopy.

The histological staining was performed for each cross section taken from either distal area or middle area. There is no significant reduction in the % stenosis however there was a trend towards a reduction in neointimal area and thickness in AAV-GFP-FRNK treated animals in small animal scale. An inflammatory reaction consisting predominantly of macrophages and giant cells was also less in the AAV-GFP-FRNK-treated group when compared to AAV-GFP control-treated group. Macrophages observed in these areas were often foamy in character. However, no eosinophils and no inflammation were observed extending into the adventitia (Fig. 3.12).

3.3 Discussion

The present study demonstrates that AAV gene delivery of FRNK can result in an impairment of hCASMCs migration *in vitro* as well as a reduction in neointimal formation after oversized stent implantation in the porcine coronary artery. AAV is non pathogenic, incapable of autonomous replication and spread. For this purpose, AAV normally needs a helper virus for its replication. A common strategy to produce AAV is co-infection with adenovirus, which is eliminated from the final AAV-containing cell supernatant. However, this approach still retains some functional adenoviral genes that can be cytopathic to the recipient (Johnson et al., 1992; Roizman and Jenkins, 1985). In addition to its cytopathic effect, adenoviral genes can also induce immunological responses to transduced cells (Schulick et al., 1995). The AAV-helper free system, used in this study, is completely free of helper virus like adenovirus.

Previously, replication defective adenoviral vectors also have been used as gene transfer vehicles for cardiac myocytes *in vitro* and *in vivo*. They were shown to transduce cardiac myocytes with 100% efficiency, but gene expression was transient due to its episomal feature (Kirshenbaum et al., 1993). Second generation adenoviral vectors were developed with low antigenicity, but the duration of gene expression also decreased (Robbins and Ghivizzani, 1998). Studies also revealed that retroviral vectors and adenoviral vectors were successful in gene transfer into other vascular cells, their low transduction efficiency and transient expression, respectively, are major limitation for their applications. Unlike the retroviral system, AAV is able to transduce non-dividing cells. Further, it has improved long term gene expression makes it attractive as vehicle for gene transfer. Since all viral proteins in this vector are eliminated, it is useful in gene therapy of many diseases. Also, AAV helper free system does not have any lethal effect and is safe to use as a delivery system. Moreover, it does not elicit neutralizing antibodies against AAV capsid protein so that it can be administered repeatedly. Among all receptors which mediate AAV infection, HSPG receptor has been shown to be essential for binding stage of AAV infection (Summerford and Samulski, 1998). In some cell types, where HSPG receptor expression is mainly on the basal side of the cell, the nonexposed area of those cells, AAV has shown to have low transduction efficiency (Bals et al., 1999). However, therapeutic value of AAV mediated gene transfer is also studied in several cardiac diseases (Kimura et al., 2001; Melo et al., 2002).

The pAAV-LacZ virus efficiently transduced hCASMCs *in vitro*. Infected cells were stained positive for X-Gal staining. In infected cells, transgene expression was found in a large proportion of the cell population. However, Yao *et al* (Yao and Wang, 1995) suggested that AAV can transduce the endothelial layer and advential layers but not the medial layer containing VSMC in *ex vivo* rat aorta. The reason might be intima act as a barrier between the various cell layers. And also, one should consider the cells in culture might have different transduction efficiency than in the medial layer SMC of the aorta. It might be due to several reasons such as difference in cell cycle status or changes in SMC phenotypic change. Other groups also showed that SMCs in cell culture were transduced at higher efficiency than cells in the medial layer (Maeda et al., 1997).

Next, using AAV viral system FRNK was overexpressed to suppress the hCASMC migration by abrogating FAK mediated cell signalling. The choice of FRNK as a therapeutic gene was based on *in vitro* and *in vivo* evidences that it inhibits cell migration by interrupting FAK signalling. Although, the suggestion that the FAK is important for cell motility is not novel, many other groups have reported this function of FAK in a variety of cell types and inhibition of FAK by FRNK constructs results in decreased endothelial cell motility (Romer et al., 1994). Further, several studies reported that there is an increase in the number and size of FAs in FAK knock out fibroblasts and fibroblasts transfected with FRNK. It leads to subsequent decrease in cell migration and spreading (Gilmore and Romer, 1996; Ilic et al., 1995). As a result of overexpression of FRNK, FAK phosphorylation is decreased and phosphorylation of some other FA proteins such as paxillin and tensin is also reduced (Richardson et al., 1997; Richardson and Parsons, 1996). But the effect of overexpression of FRNK can be overcome by expression of Src (Richardson et al., 1997). Furthermore, perlecan-stimulated FRNK expression was correlated with decreased FAK tyrosine phosphorylation and the inhibition of cell proliferation (Walker et al., 2003). Thus, accumulating evidence suggested that FAK plays an important role in cell migration and FAK may serve as a major target to block SMC migration in case of restenosis. Accordingly, AAV-helper free system was used to overexpress the endogenous inhibitor of FAK in order to block the SMC migration both *in vitro* and *in vivo*. The results show that AAV mediated FRNK has significant impact on both *in vitro* and *in vivo*.

Results from VN haptotaxis assays revealed that pAAV-MCS-FRNK overexpression is able to abrogate the migration of hCASMCs. This is in consistent with several reports that overexpression of FRNK is able to block angiotensin II induced tyrosine

phosphorylation, PDGF stimulated chemotaxis and haptotaxis (Richardson et al., 1997; Sieg et al., 1999; Taylor et al., 2001). These results suggest that targeting of FAK or downstream signalling events will have a significant impact on SMC in case of restenosis.

The current study shows that AAV mediated overexpression of FRNK prevents neointima formation in porcine coronary stent model. Porcine arteries are very similar to human vessels in size and histology. The pig has similar cardiovascular morphology and physiology like humans (Muller et al., 1992; Schwartz et al., 1990a). FRNK suppressed injury induced SMC migration and subsequently resulting in reduction of neointima formation. The morphometric analysis showed that injury score is equal in both the groups. But interestingly, the neointimal area in AAV-GFP-FRNK treated groups was less than that in the AAV-GFP control treated group. In contrast, the luminal area in the AAV-GFP-FRNK remained larger than that AAV-GFP control group. Particularly in AAV-GFP-FRNK system, the stent-implanted vessels did not show any inflammation or other kind of side effects indicating that it is safer to use in clinical trials. Similarly, other reports have shown the successful AAV based expression of LacZ gene in different *in vivo* settings with no toxicity or inflammation. AAV based expression of the paclitaxel suppressed the neointima formation at 28 days but not in 90 days. It should be taken into the account whether the AAV-GFP-FRNK is able to reduce the neointima formation also in longer periods. Eventhough, results from AAV-GFP-FRNK experiments and other groups have shown many advantages of this system, its low transduction efficiency or low titer virus might be the major limitation of using the AAV based helper virus free system. Using conventional transfection methods to generate 10^{13} particles would require at least 20 and up to 20000 10-cm cell culture plates of 293Tcells (Allen et al., 2000). There is always difficult to compare many strategies due to difference in vector transduction assays. However, by exploring about unique biological properties of AAV will be helpful in such therapeutic interventions.

4. Identification of FAK N-terminal domain interaction partners by bacterial two-hybrid system.

4.1 Introduction

FAK is a critical mediator of integrin signalling which is important in various cellular functions. Upon integrin activation, FAK interacts with SH2 domain containing proteins such as Src family kinases, PI3 kinase, phospholipase Cγ and Grb7 leading to mutimolecular signalling complexes (Chan et al., 1994; Chen and Guan, 1994b; Han and Guan, 1999; Zhang et al., 1999). Protein-protein interactions of FAK with other signalling molecules are important for assembly of such molecular complexes. The C-terminal domain of FAK interacts with many proteins such as $p130^{CAS}$, Graf, paxillin and talin (Chen et al., 1995; Harte et al., 1996; Hildebrand et al., 1995; Hildebrand et al., 1996). The interaction of the FAK C-terminal domain with these molecules links integrins with small GTPase and serine/threonine kinase signalling pathways.

Interaction of the FAK N-terminal domain with growth factor receptors coordinates signalling between integrins and growth factor receptors (Sieg et al., 2000). Further, the FAK N-terminal FERM domain was shown to engage in several protein-protein interactions or membrane-protein interactions. The FERM domain is also known to associate intramolecularly with the kinase domain. The crystal structure of the FAK FERM domain shows that the tri-lobed architecture found in ERM family proteins is present in FAK, however, the confirmation of the domain is modified due to the unique arrangement of the F3 subdomain (Ceccarelli et al., 2005).

Although, the N-terminal domain of FAK plays a significant role in many cellular functions, only few interaction partners are known currently. As a potent mediator of integrin signalling, it is essential to understand the various interactions of FAK. To uncover novel protein-protein interactions the two-hybrid system is a widely used technique to screen large cDNA libraries. Yeast, bacterial and mammalian two hybrid systems exist to screen for hard to find binding partners. Moreover, these techniques enable also the detailed characterization of known interactions.

In order to understand the function of N-terminal domain of FAK and as a starting point to dissect its interactions, here the bacterial two-hybrid system was employed to identify its putative interaction partners.

4.2 Results

4.2.1 Plasmids

To understand the role of FAK in signalling pathways, a bacterial two-hybrid screen was performed to identify putative proteins that interact with the N-terminal domain of FAK. The following constructs were made for the screening assay.

Bait construct

Mouse FAK-N-terminal domain was cloned into pBT vector (pBT-FAK-NT) (Stratagene), which has Cam resistance.

Prey plasmid

A cDNA library derived from rat aortic smooth muscle cells was amplified as described in methods and was used as prey plasmids to find out the interaction partners of FAK the N-terminal domain.

Initially, control experiments were performed to verify the reliability of the screening system. The experiments were aimed to check auto-transactivation of the bait plasmids as well as to determine the co-transformation efficiency of the plasmids

4.2.2 Determination of auto-transactivation

Auto-transactivation of the reporter genes by the bait alone would rule out the use of this two hybrid system from the beginning. Therefore, the pBT-FAK-NT construct and other control plasmids (see the table 4.1) were cotransformed to check the auto-transactivation.

Table 4.1. Plasmids used for transformation. Plasmids (50 ng each) A and B were cotransformed and plated on either CTCK or TCK plates. The antibiotic resistance of respective plasmids are indicated in parentheses. + Indicates growth of colonies. – Indicates no growth.

A / B	pBT- LGF2 (cam)		pBT- (cam)		pBT- FAK-NT(cam)	
	CTCK	TCK	CTCK	TCK	CTCK	TCK
pTRG-Gal11(tet)	+	+	-	+	-	+
pTRG (tet)	-	+	-	+	-	+

	CTCK	TCK	cam	tet
pBT- FAK-NT (cam)	-	-	+	-
pTRG (tet)	-	-	-	+

The cotransfected samples were plated on CTCK, cam or tet plates and incubated at 30°C. While the known interacting proteins pTRG-Gal1 and pBT-LGF2 showed positive interaction, bacteria transformed with control plasmids did not grow on CTCK plates (Table 4.1).

4.2.3 Determination of co-transformation efficiency

Efficient co-transformation of both target and bait plasmids is necessary for the proper expression of both proteins. To determine the cotransformation efficiency, different combinations of plasmids were co-transformed and colonies were counted (Table 4.2). The colony forming unit (cfu) yield was calculated by using the formula cfu yield/µg = (cfu x Concentration of the plasmid used)/ Dilution factor. For example 268 colonies on the plate, cfu yield/µg = (268 x 0.05) / 5 =26800 cfu/µg.

Table 4.2. Plasmids used to determine co-transformation efficiency. Mixture of 50 ng of each plasmid was transformed in the reporter strain and plated on either CTCK plates or TCK plates as described in methods.

Plasmids (cotransfected)	CTCK plates (No. of clones)	TCK plates (No. of clones)
Gal11/LGF2	250	268
pBT-FAK-NT/pTRG	-	59

4.2.4 Screening of pBT-FAK-NT with RASMC cDNA library

The number of cotransformations will be determined based on the total number of colonies required to represent an entire library and cfu yield.

Table 4.3. Screening result of pBT-FAK-NT interacting with RASMC library

Bacterial two hybrid system	**pBT-FAK-NT (Cam)**
Total number of clones screened	1.6 x 10^6 clones
cfu/yield of transformation	26800 cfu/µg.
Amount of plasmid used	60 µg each plasmid
Number of CTCK plates used (150 mm)	32 plates
Number of colonies selected	260 colonies
Number of colonies shown positive for Both CTCK and TCK-X-Gal plates	120 colonies

Number of cotransformation = Total number of colonies to be screened/cfu yield for the co-transformation. So to screen 1.6 x 10^6 clones, 1.6 x 10^6/26800 = 60 µg. 60 µg of both plasmids were cotransfected and plated onto 32 dishes (150 mm). 250 colonies were selected and streaked on CTCK, TCK as well as TCK-X-Gal plates. 120 positive clones were shown positive for both growth on CTCK plates and also blue colonies on TCK-X-Gal (Table 4.3).

4.2.4.1 Isolation and re-cotransformation of single plasmids

The positive clones isolated from CTCK plates contain both bait and prey plasmids. To isolate the single prey plasmids, putative clones were grown in the presence of the antibiotic Tet since only the prey construct has Tet resistance gene while bait construct has Cam resistance. From the growing cultures single colonies were isolated after plating the culture in the Tet plates.

Once again to confirm that the transactivation observed was specific to the interaction between the fusion proteins and not because of auto-activation of cDNA library, the isolated prey plasmids were co-transformed with the bait plasmid, pBT-FAK-NT. The cotransformed plasmids grew on CTCK plates indicating that the isolated clones were indeed specifically interact with each other. The higher number of colonies containing plates was considered as strong interacting partners and such clones were further analysed by sequencing.

4.2.4.2 Identification of putative clones

The isolated clones were sequenced by using the primers mentioned in the methods. Sequences were analysed for length, orientation and then compared with the NCBI database (http://www.ncbi.nlm.nih.gov/Blast/). Several putative interacting proteins were identified (Table 4.4).

Table 4.4 Examples of putative interacting proteins for pBT-FAK-NT

Clones	Homologous database match
Clone1	*Rattus norvegicus* similar to putative MAPK activating protein
Clone 2	*Mus Musculus* Ubiquitin-conjugating enzyme E2G 2
Clone 3	*Homo sapiens* putative 17.9 KDa protein (AF155657)
Clone 4	Rat dipeptidase

4.2.5 The putative 17.9 kDa protein

Comparison of the nucleotide sequence of the clone 3 revealed a partial identity with Homo sapiens putative 17.9 KDa protein (gi:7688670). This clone was named as FUN1 (FAK binding protein with unknown function 1).

The rFUN1 was amplified from the isolated prey plasmid from two-hybrid screen using the primers indicated in methods. The infusion reaction was performed to clone the rFUN1 in pDNR dual vector and subsequently the cre-lox recombination was performed to clone rFUN1 into pLPS-3'EGFP. Peptide antibody was produced in rabbits against FUN1 peptide CRVQGGGRMEEQRS (Biogenes).

4.2.6 Comparison of FUN1 amino acid sequence from rat, mouse and human

The human FUN1 amino acid sequence was identified as G18. Human G18 contains a triple goLoco motif. It contains 160 aa and 84% identity with rat protein sequence. The mouse FUN1 contains 159 aa and 95% identity with rat protein sequence. The rat FUN1 contains 158 aa and also has triple goLoco motifs (Fig: 4.1).

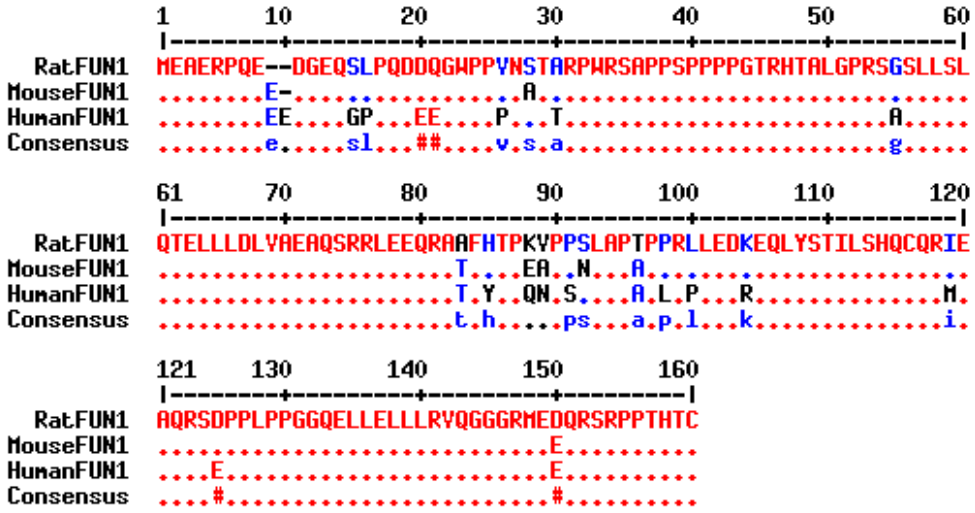

Fig: 4.1. Amino acid sequence analysis of rat (gi: 46237579, mouse (gi: 18314674) and human (gi: 17511735) FUN1. Amino acid alignment of FUN1. Identical, similar and different amino acid residues are indicated. Amino acid sequences were analysed by the website http://ribosome.toulouse.inra.fr/multalin/multalin.html.

4.2.7 Secondary structure prediction of rFUN1

The secondary structure prediction (McGuffin et al., 2000) of the rFUN1 revealed that the majority of the protein consists of random coil and three goLoco motif are predicted as α helices (Fig. 4.2).

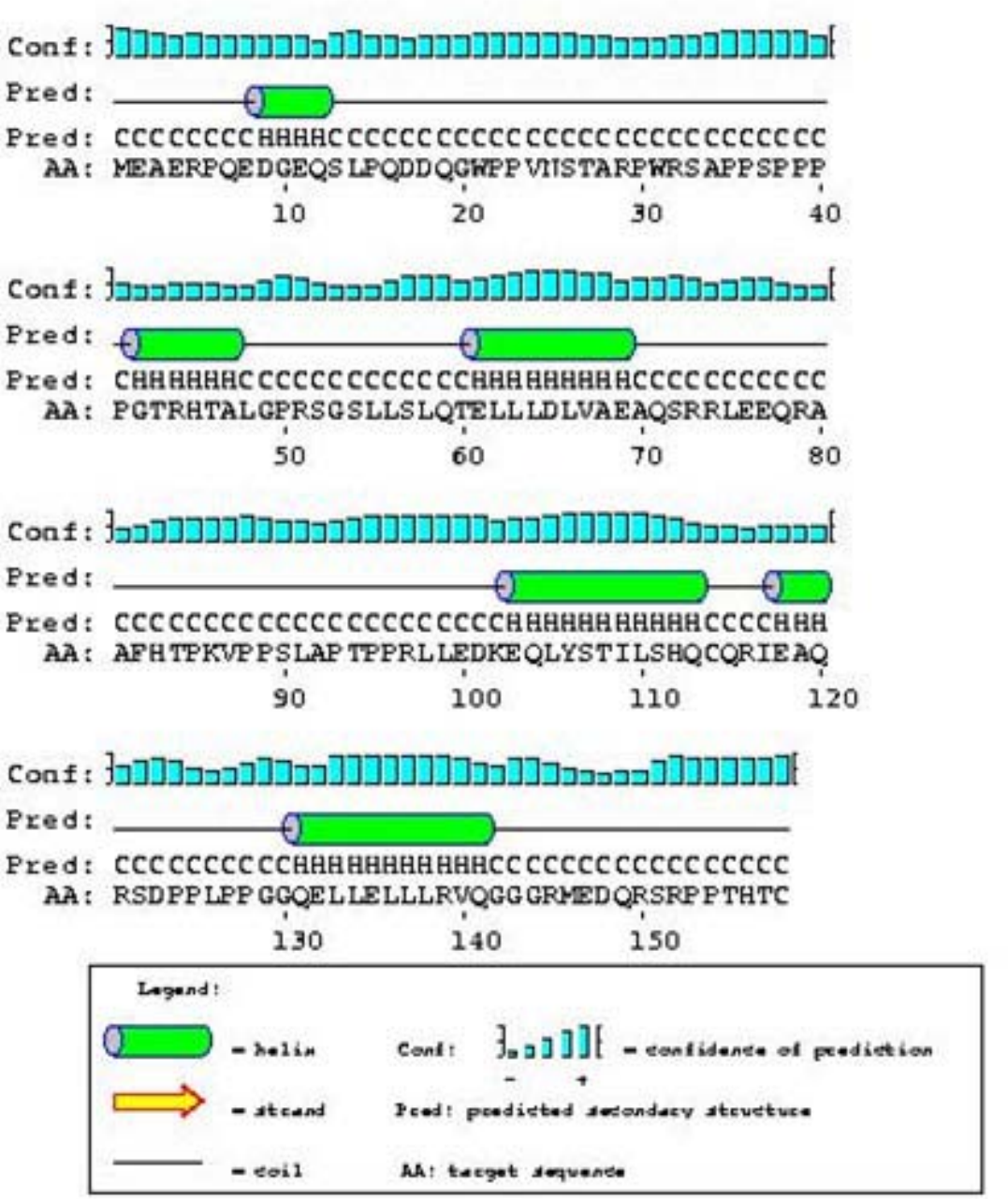

Fig: 4.2. Predicted secondary structure of the rFUN1 protein. Predicted secondary structure of the ORF of rFUN1 was performed using the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred).

4.2.8 Expression pattern of FUN1 in various cell types

Western blot analysis of WCL from various cell lines showed a major band around 17.9 kDa. The FUN1 expression was higher in neuronal cells and haematopoietic cells (Fig. 4.3).

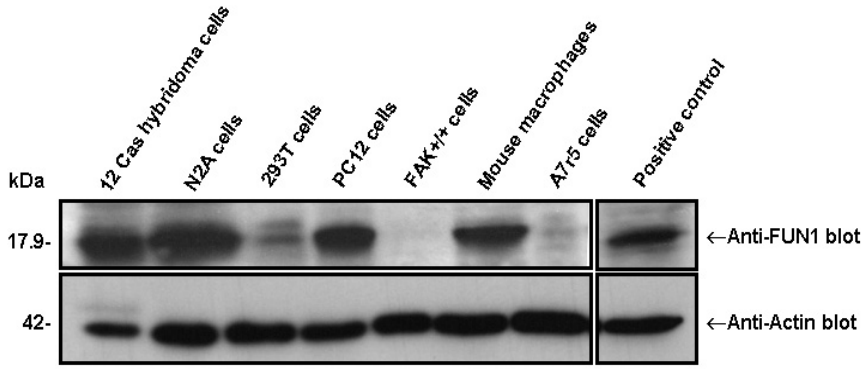

Fig: 4.3. Western blot analysis of WCL from various cell lines. WCL was prepared and Western blot was performed with FUN1 peptide antibody (upper part). Anti-actin Western blot was performed on the same samples as a loading control (lower panel).

4.2.9 Interaction of FAK N-terminal domain with rFUN1

To confirm the interaction of rFUN1 with FAK-N-terminal domain in eukaryotic cells, FAK-NT-myc was transfected either with control plasmid or with rFUN1-GFP in 293T cells. Samples were lysed in various buffers (see in the figure legends) and co-immunoprecipitated with GFP polyclonal antibody. Western blot analysis showed that the sample containing rFUN1 specifically interacted with FAK-NT-Myc in the samples lysed with RIPA buffer. Control vector transfected samples did not show any interaction under the same lysis condition (Fig 4.4).

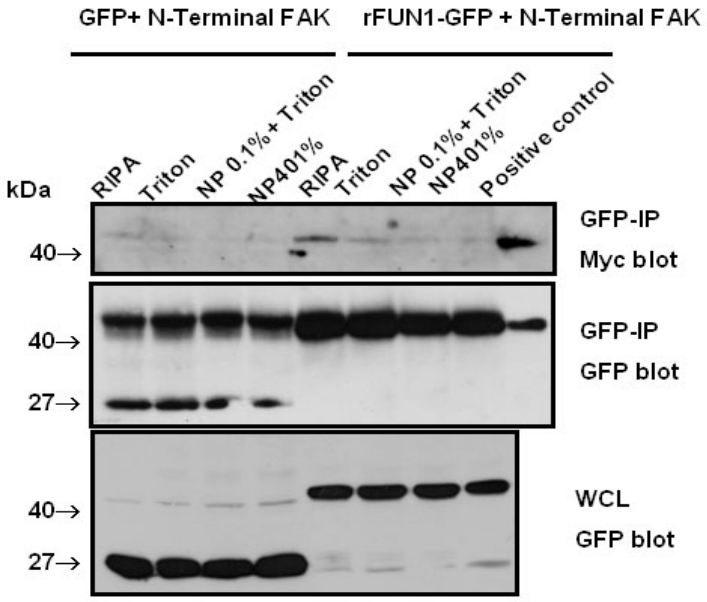

Fig 4.4. Interaction of FAK N-terminal domain and rFUN1 in eukaryotic cells. The 293T cells were transfected with N-terminal FAK and GFP or rFUN1-GFP vector. The cells were lysed with lysis buffer as indicated in the fig. The samples were immunoprecipitated with anti-GFP antibody. Western blot was performed with anti-myc antibody (upper panel) and anti-GFP antibody (middle panel). The lower panel shows expression of GFP and FUN1 in WCL (anti-GFP antibody).

4.3 Discussion

FAK promotes signalling events that modulate many cellular processes. It interacts with several signalling molecules and such protein-protein interactions play a central role in regulating FAK functions. As part of a broad strategy to identify putative interacting partners of the N-terminal domain of FAK, here the bacterial two-hybrid system was used to screen a rat smooth muscle cell cDNA library.

Bacterial two-hybrid screen identified few putative binding partners for the FAK N-terminal domain. Among these proteins, one encodes a putative protein that has approximate molecular weight of 17.9 kDa and was named as FUN1. FUN1 is identical to G-protein signalling modulator 3 and belongs to the family of proteins able to activate G protein signalling in the absence of a receptor. The human homologue also called as AGS4 or G18.1b. has been identified and characterized (Cao et al., 2004).

G18 family of proteins contain one or more goLoco motif (otherwise known as G protein regulators (GPR)) and represents diverse family of $G\alpha$ interacting proteins (Siderovski et al., 1999). They bind specifically to the $G_{i\alpha}$ subunit of the G proteins and stabilize the GTP bound confirmation thus acting as a guanine nucleotide dissociation inhibitor. GPR motifs can also actively displace β/γ subunits from G_α (Kimple et al., 2004).

GPR containing accessory proteins have broad cellular functions which dependent on their cellular expression and its unique GPR motifs. For example, mammalian protein LGN is important for assembly and organization of mitotic spindle and GPR-1 and GPR-2 in *C.elegans* is required for asymmetric cell devision in early embryo. RGS12 containing GoLoco motif or G protein regulatory motif proteins were also known to play significant role in G protein signalling events (Kimple et al., 2002). Activation of GPCR was shown to rapidly stimulate phosphorylation of FAK which is dependent on the extracelluar Ca^{2+} (Fan et al., 2005).

Given the diverse cellular functions of G protein and FAK, the interaction of FUN1 and N-terminal domain of FAK will have several functional implications. FUN1 also has three goLoco motifs and could potentially act as modulator of G protein signalling but it's role in FAK signalling and mode of interaction with FAK still remains to be investigated.

5. Conclusions

Restenosis is a major drawback of PTCA performed during the treatment of atherosclerosis. It is a multistep process which involves thrombosis, ECM elaboration, elastic recoiling, apoptosis, oxidative stress, intimal hyperplasia and unfavourable remodelling ultimately leading to neointima formation (Clowes et al., 1983; Isner et al., 1995; Lindner et al., 1993; Strauss et al., 1994; Tardif et al., 1997). During neointima formation SMCs migrate and invade through basement membrane and the chemotactic cytokines, growth factors and ECM proteins released during vessel injury act as a potent mediator of such migration. The critical step of migration of SMCs is its interaction with the cellular receptor such as integrins and subsequent signalling events.

Integrins, a major family of cell surface receptors, are glycoproteins, which integrate intracellular cytoskeleton with the ECM ligands such as VN, CN or LN. Integrin $\alpha_v\beta_3$ is one of the major integrins which was shown to be involved in angiogenesis, cell growth and cell migration including SMC migration during restenosis. Besides that it is also involved in tumour growth, local invasiveness, metastasis and wound healing (Brooks et al., 1995; Fujii et al., 1998; Mitjans et al., 1995; Sajid and Stouffer, 2002; Yeh et al., 1998). Its expression determines the invasiveness and metastatic potential of colon and breast cancer. In addition, other α_v integrins such as $\alpha_v\beta_5$ and $\alpha_V\beta_1$ also play an important role in SMC migration, angiogenesis and tumour growth (Kim et al., 2000; Moiseeva, 2001; Reinmuth et al., 2003; Stoeltzing et al., 2003). Therefore, among other integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ and their signalling events have received increased attention as potential therapeutic targets in the intervention of not only diseases like cancer, thrombosis and inflammation but also in restenosis.

Several integrins including $\alpha_v\beta_3$ recognize an Arg-Gly-Asp (RGD) sequence present in many of the ECM proteins. Blocking monoclonal antibodies of integrin α_v and synthetic RGD peptides have shown to be effective against cancer, tumour cell growth *in vivo* (Castel et al., 2001; Mitjans et al., 2000). RGD peptidomimetic SCH221153 antagonist interferes with integrin-ECM interactions and lead to the inhibition of endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (Urbinati et al., 2005). In an experimental breast cancer mice model, administration of cilengitide (the integrin α_v inhibitor) together with radio immunotherapy led to increased efficacy (Burke et al., 2002). Currently it is in phase II clinical studies as anticancer drug (cilengitide, EMD121974). In addition, RNA aptamer, that binds recombinant $\alpha_V\beta_3$, inhibits

proliferation of endothelial cell and survival by binding to its target (Mi et al., 2005). Further, Tat protein, a main transactivating protein of HIV accumulates and become immobilized in the ECM and is known to interact with $\alpha_v\beta_3$ integrin by its RGD peptide. Although antagonists of integrin α_v have tremendous therapeutic potential, it has several limitations. Since the integrin α_v is a major subfamily predominantly expressed in various cell types, blocking α_v would block all the signalling pathways associated with most integrin subtypes. In addition, those antagonists have less stability and high immunogenicity.

The present study aimed to explore the application of the integrin α_V inhibitor. Firstly, integrin α_v inhibitor was used to test its effect on hCASMC *in vitro*. It inhibits haptotaxis as well as chemotaxis of hCASMC induced by VN and PDGF-BB respectively. The blockade of hCASMC migration by integrin α_v inhibitor is associated with reduced activation of two major enzymes FAK and MMP-2. Although the integrin α_v inhibitor, used in this study, showed its ability to inhibit multiple signalling pathways, the route of drug delivery, the minimum dose and duration of the drug and systemic side effects associated with the treatment are not well established, and they warrant further studies *in vivo*.

ECM engagement with integrin triggers conformational change and clustering lead to remodelling of FA proteins in various cellular functions. One of the major proteins activated upon this event is FAK. Increased FA formation and subsequent reduction in the migration of the FAK null cells highlights the essential role of FAK in FA turnover. It suggests that FAK is a key component of integrin mediated signalling pathways regulating cell migration.

FRNK, the C-terminal domain of FAK, has binding site for proteins $p130^{CAS}$, Grb2, Paxillin, Graf and PI3 kinase (Harte et al., 1996; Hildebrand et al., 1993; Hildebrand et al., 1996; Schlaepfer et al., 1994). Many studies revealed that FRNK and FAT domain were able to displace native FAK from its binding proteins resulting in inhibition of FAK mediated effects on cell motility (Gilmore and Burridge, 1996; Hauck et al., 2002; Richardson and Parsons, 1996).

Given the important role of FAK in integrin-mediated cell migration, another aim of the project was to interfere with FAK-initiated signalling pathways by AAV mediated overexpression of FRNK. FRNK inhibited *in vitro* hCASMC migration and also reduced neointima formation in porcine restenosis model. However, it must be noted that the preliminary positive results should be extended in large-scale animal studies.

FAK structural domains facilitate the transmission of downstream signalling by recruiting several signalling and adapter proteins. In the current study, putative novel interaction partners of the N-terminal domain of FAK were identified by bacterial two-hybrid system. Several putative partners were identified and one among them was homologous to human AGS4 and named as FUN1. AGS family proteins are known to interact with different subunits or conformation of G proteins. They regulate the activation and deactivation cycle of G proteins. Although FUN1 identified as binding partner of FAK N-terminal domain by the bacterial two-hybrid screen, its functions in FAK signalling still remain to be investigated.

Together, the above results suggest that integrin-initiated FAK signalling events might be an interesting target to interfere with the pathological state of restenosis. It might also be employed in other disease states that involve integrin signalling.

6 Materials

6.1 Cells

hCASMC	Human coronary artery smooth muscle cells (adherent; Clonetics, San Diego, CA, USA).
293T cells	Transformed primary human embryonic kidney epithelial cell line (adherent).
RASMC	Rat artery smooth muscle cells (adherent).

6.2 Medium for cell culture

Medium for hCASMCs	Smooth muscle basal medium (Cambrex Biosciences, Walkersville, MD) supplemented with SMGM2 singlequots containing insulin, hFGF, gentamicin, fetal bovine serum (FBS) and hEGF (Clonetics, San Diego, CA, USA).
Medium for 293T cells	Dulbecco's Modified Eagles Medium (DMEM) supplemented with L-glutamine and 10% calf serum (CS; PAA laboratories).
Medium for RASMCs	DMEM supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% non essential amino acids (PAA laboratories).
Serum starvation medium	DMEM + 0.5% FCS
Suspension medium	DMEM + 0.25% bovine serum albumin (BSA)
Freezing medium	DMEM + 20% FCS + 10% dimethyl sulfoxide (DMSO)
Heat inactivated CS medium	DMEM + 2% heat inactivated CS (30 min incubation at 56°C)

6.3 Bacteria

***Escherichia coli* strains:**

Nova Blue	endA1, hsdR17 ($r_{K12}^{-}m_{k12}^{+}$), supE44, thi-1, recA1, gyrA96, relA1, Lac [F'proA^{+}B^{+}lacI^{q}Z ΔM15:Tn10 (Tet^{R})], (Novagene).

E. coli DH5α	F-, end1, hsdR17 (rk-, mk-), supE44, thi-1, recA1, gyrA96, relA1, λ-, Δ (argF-lac) U196, Φ80dlacZΔM15
XL1-Blue	MRF` Kan (strain harbouring the pTRG cDNA library) and XL1-Blue MRF´ Kan-Δ (mcrA) 183 Δ (mcrCB-hsdSMR-mrr) 173 end A1 supE44 thi-1 recA1, gyrA96 relA1 lac [F´ proAB $lacI^q$ ZΔM15 Tn5 (Kan`)].
BacterioMatch™ Two hybrid system reporter strain	Δ (mcrA) 183 Δ (mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA 1, gyrA96 relA 1 lac [F´ $lacI^q$ bla lacZ Kan^r]

6.4 Medium for bacteria

LB medium and agar plates

LB medium	10 g bacto-trypton, 5 g yeast extract and 5 g NaCl were dissolved in 800 ml of H_2O and pH was adjusted to 7, then filled up to 1 L and finally autoclaved.
LB-agar	LB medium supplemented with 1 M $MgCl_2$, 1.5% agar-agar and sterilized by autoclaving.
LB-sucrose agar	LB agar with 7% sucrose
Freezing medium	Equal mixture of 600 µl of LB-medium and 600 µl 50% glycerin

Antibiotics for medium and agar plates

After autoclaving LB–agar, it was cooled to 55°C. Then antibiotics were added to the medium and poured into bacterial Petri plates.

Antibiotics	Final concentration
Ampicillin (amp)	100 µg/ml
Chloramphenicol (Cam)	30 µg/ml
Kanamycin (Kan)	30 µg/ml
Tetracycline (Tet)	12.5 µg/ml

6.5 Antibodies, proteins and enzymes

6.5.1 Antibodies

Antibodies	Type	Name	Origin	Purchased From
Integrin $\alpha_v\beta_3$	Monoclonal	Clone LM609	Mouse	Chemicon
Integrin $\alpha_v\beta_5$	Monoclonal	Clone P1F6	Mouse	Chemicon
IgG1	Monoclonal	Clone DD7	Mouse	Chemicon
SMC specific actin	Monoclonal	Clone 1A4	Mouse	Sigma-Aldrich
Vinculin (Human)	Monoclonal	Clone HVIN-1	Mouse	Sigma-Aldrich
Phospho-tyrosine	Monoclonal	Clone 4G10	Mouse	Upsate Biotechnology
FAK	Monoclonal	Clone 77	Mouse	BD Biosciences
FAK	Polyclonal	A17	Rabbit	Santa Cruz
Phospho-FAK-P-397	Polyclonal	44-624	Rabbit	Biotechnology
Flag-tag	Monoclonal	Clone M2	Mouse	QCB BioSource
GFP	Monoclonal	Clone JL-8	Mouse	International
GFP	Polyclonal	Ab290	Rabbit	Sigma-Aldrich
HA-tag	Monoclonal	Clone 12CA5	Mouse	BD Biosciences
FUN1	Polyclonal		Rabbit	Abcam
				BioGenes

Antibodies	Company
Peroxidase-conjugated rabbit-anti-mouse	Jackson Immunoresearch
Peroxidase-conjugated goat-anti-mouse	Jackson Immunoresearch
Peroxidase-conjugated Protein A	ICN Biochemicals
Cy2-conjugated goat-anti-Rabbit	Jackson Immunoresearch
Cy3-conjugated goat-anti-Rabbit	Jackson Immunoresearch
Cy2-conjugated goat-anti-mouse	Jackson Immunoresearch
Cy3-conjugated goat-anti-mouse	Jackson Immunoresearch

Antibodies are conjugated with either FITC (5-(6)-carboxyflurescein-succinylester) or Rhodamin-(5-(6)-carboxytetraethylrhodamine-succinimidyl-ester). Annexin-V-FITC apoptosis detection kit (Sigma). Phalloidin Alexa-Fluor-546 (Molecular probes).

6.5.2 Proteins

Human purified vitronectin was obtained from Life Technologies. Fibronectin was purchased from ICN Biomedicals. Poly-L-lysine was from SIGMA. Gelatin was from

Merck. Growth factor reduced matrigel matrix was bought from BD Biosciences. PDGF-BB was from Upstate Biotechnology, Lake Placid, NY. Albumin Fraction V, bovine was from Roth. Protein A/G-plus sepharose was obtained from Santa Cruz Biotechnology.

6.5.3 Enzymes

Enzymes	Company
Taq-DNA-polymerase	Biolabs
Vent polymerase	Biolabs
Cre recombinase	Biolabs
In-Fusion enzyme	BD Biosciences
Restriction enzymes	Biolabs, Fermentas
T4-DNA-Ligase	Fermentas
Trypsin	PAA laboratories

6.6 Integrin α_v inhibitor and migration chambers

Integrin α_v inhibitor (EMD121974) was a generous gift form Merck KG, Darmstadt, Germany. Millicell modified Boyden chambers (Millipore, Bedford MA) were used in migration assays and invasion assays.

6.7 Plasmids

pDNR-Dual	ori_pUC*, loxP, MCS; SD, 6XHN-tag, (Cam^R), SacB (BD Biosciences)
pLPS-3'EGFP	ori_pUC*, loxP, SV40, (Kan^R), poly A signal (Clontech)
pBT	p15A origin of replication, MCS, lac-UV5 promoter, λcl ORF, (Cam^R), (Stratagene)
pTRG	ColE1 origin of replication, MCS, Lpp promoter, Lac UV5 promoter, RNAα ORF, (Tet^R) ORF.
pAAV-MCS	left AAV-2 ITR, CMV promoter, β-globin intron, MCS, human growth hormone (hGH) polyA signal, right AAV-2-ITR, f1 origin of ss-DNA replication, (amp^R) *(bl*a), ORF pUC origin of replication, Not I cleavage sites for sub cloning from pCMV-MCS.

pAAV-IRES-hrGFP	left AAV-2 ITR, CMV promoter, β-globin intron, MCS, 3× FLAG tag, internal ribosome entry site (IRES), hrGFP ORF, human growth hormone (hGH) polyA signal, right AAV-2-ITR, f1 origin of ss-DNA replication, (amp^R) *(bl*a) ORF, pUC origin of replication, Not I cleavage sites for subcloning from pCMV-MCS
pAAV-LacZ	left AAV-2-ITR, CMV promoter, human growth hormone (hGH) intron, β-galactosidase *(la*cZ) ORF, SV40 poly A signal, right AAV-2-ITR, f1 origin of ss-DNA replication, (amp^R) *(bl*a) ORF, pUC origin of replication, Not I cleavage sites for subcloning from pCMV-MCS.
pAAV-Rc	AAV-2 *rep g*ene, AAV-2 *cap g*ene, f1 origin of ss-DNA replication, (amp^R) *(bl*a) ORF, pUC origin of replication.
pHelper	adenovirus E2A gene, adenovirus E4 gene, adenovirus VA gene, pUC origin of replication, (amp^R) *(bl*a) ORF, f1 origin of ss-DNA replication.

6.8 Reagents and buffers

6.8.1 Reagents and buffers for eukaryotic cells

Gelatin solution	0.1% gelatine in 1x PBS, incubated in water bath for 45 min to 1 hr. Filter sterilized.
1X PBS	24 g NaCl, 0.6 g KCl, 3.42 g Na_2HPO_4 $7H_2O$, 0.6 g KH_2PO_4 dissolved in 1 L of H_2O. pH 7.4 and autoclaved.
PBS^{++}	1X PBS supplemented with 0.35 mM $CaCl_2$, 0.25 mM $MgCl_2$
0.025% Trypsin/EDTA	0.5% trypsin was diluted in 1x PBS.
DMEM Soybean trypsin inhibitor	0.25 mg/ml soybean trypsin Inhibitor (125 mg/500ml DMEM) and 0.25% BSA
Paraformaldehyde (PFA)	4 g PFA in 80 ml warm H_2O and 1 N NaOH was added to clarify, and then the pH was adjusted to 7.4 with 1 N HCl.10 ml of 10X PBS was added and filled up the H_2O to 100 ml.
Saponin solution	5% saponin in 1X PBS

Blocking solution	PBS^{++}, 10% CS, 0.2% saponin
Calcium chloride (2.5 M $CaCl_2$) solution	184 g $CaCl_2$.$2H_2O$ in 500 ml H_2O
2X HBS	16.4 g NaCl, 11.9 g HEPES, 0.21 g Na_2HP0_4 dissolved in 800 ml of H_2O. pH was adjusted to 7.1-7.5. Made up to 1 L and filter sterilized.
RIPA buffer	1% triton X-100, 50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 100 mM sodium fluoride (NaF), 0.1% sodium dodecyl sulphate (SDS) was added slowly. Made up to 240 ml. 1% of 1 mM dry deoxycholic acid was added slowly. It should not be in brown colour. 1 mM sodium orthovanadate was added dropwise. 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pefabloc, 10 μg/ml pepstatin (in MeOH), 10 μM benzamidin (in EtOH). H_2O was filled up to 250 ml.
Triton buffer	1% triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM EGTA, 1.5 mM $MgCl_2$, Filled up to 245 ml of H_2O. 1 mM sodium orthovanadate was added dropwise. 10 μg/ml leupeptin, 10 μg/ml aprotonin, 10 μg/ml pefabloc, 10 μg/ml pepstatin (in MeOH), 10 μM benzamidin (in EtOH). Made up to 250 ml of H_2O.
NP40 buffer	50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 0.5% NP40, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 10 μg/ml pefabloc, 10 μg/ml pepstatin (in MeOH), 10 μM benzamidin.
HNTG buffer	50 mM HEPES pH 7.4, 50 mM NaCl, 0.1% triton X-100, 10% glycerol, 0.5 mM Na_3VO_3, 50 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 10 μg/ml pefabloc, 10 μg/ml pepstatin (in MeOH), 10 μM benzamidin.

6.8.2 Reagents and buffers for molecular biology

Buffers for miniprep (Birnboin-Dooley method)

Buffer P1	50 mM Tris HCl, pH 8, 10 mM EDTA, 100 µg/ml RNAse A, pH 8.0, 4°C.
Buffer P2	200 mM NaOH, 1% (w/v) SDS.
Buffer P3	3 M potassium acetate, pH 5.5.
TE-buffer	10 mM of tris HCl, 1 mM of EDTA pH 8.

Reagents for agarose gel

DNA loading buffer	42 mg of bromophenol blue, 6.7 g of sucrose. Dissolved in 10 ml of H_2O.
GEBS	50 mM EDTA, 20% (w/v) glycerin 0.5% (w/v), sarcosyl 0.05% (w/v), bromophenol blue.
50x TAE buffer	242 g tris base, 100 ml EDTA pH 8 (0.5 M), 57.1 ml glacial acetic acid and H_2O. Made up to 1 L.
DNA marker	1 kb DNA ladder (Fermentas).

6.8.3 Buffers and reagents for proteins

Buffers and reagents for SDS-PAGE, coomassie staining and Western blot

Sample buffer 10 ml (2x)	2.5 ml stacking gel buffer (0.5 M Tris HCl pH 6.8), 2 ml 20% SDS, 2.5 ml H_2O, 2 ml glycerol, 1 ml β-mercaptoethanol, 1 mg bromophenol blue.
Sample buffer 100 ml (4x)	30 ml glycerin, 30 ml 20% SDS, 20 ml stacking gel buffer (0.5 M Tris HCl pH 6.8), 20 ml β-mercaptoethanol and 1 mg bromophenol blue.
Acrylamide solution	40% polyacrylamide.
APS	10% ammonium peroxodisulphate.
SDS	20% (w/v) sodium dodecyl sulphate.
TEMED	0.1% N, N, N', N'-tetramethyldiamine.
Stacking gel buffer	0.5 M tris pH 6.8.
Separating gel buffer	1.5 M tris pH 8.8.
Running buffer (10x)	25 mM tris HCl, 192 mM glycine, 0.1% SDS.
Western-transfer-buffer	6 g tris base, 28.8 g glycine, 430 ml methanol. It was degassed. 10 ml of 20% SDS was added and filled up to 2 L of H_2O.

Coomassie stain (membrane)	250 ml of isopropanol (2-propanol), 100 ml of glacial acetic acid, 650 ml of H_2O and 0.03% of coomassie.
Coomassie stain (gels)	1% coomassie brilliant blue R-250 in 5% acetic acid, 10% methanol.
Destaining solution (membrane)	500 ml methanol, 500 ml H_2O, 100 ml glacial acetic acid.
Destaining solution (gels)	800 ml H_2O, 100 ml isopropanol (10%) and100 ml glacial acetic acid (10%).
Blocking solution (Blotto)	2% BSA in TBST and 0.05% sodium azide.
10% Tween	50 ml of tween-20 (10%). Made up to 500 ml with H_2O.
10x TBS	121 g tris (25 mM), 175 g sodium chloride (125 mM) dissolved in 2 L H_2O and the pH was adjusted to 7.5.
Washing buffer-TBS/Tween	200 ml 10x TBS and 10 ml 10% tween, made up to 2 L H_2O
H_2O_2-solution	30% (V/V)-hydrogen peroxide
ECL Cocktail	0.5 ml p-coumaric acid (4-Hydroxycinnamic acid in DMSO, 90 mM), 1 ml luminol (5-amino-2, 3-dihydro-1,4-phtalazinedion in DMSO; 250 mM), 20 ml 1 M Tris (pH 8.5). Mixed and made up to 200 ml with H_2O and stored at 4°C.
Stripping Buffer	40 ml stacking gel buffer (0.5 M Tris HCl pH 6.8), 10 ml 20% SDS, 2 ml β-mercaptoethanol and made up to 250 ml with double distilled H_2O.
X-Gal (20 mg/ml)	X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galacto pyranose) was dissolved in N, N-dimethyl formamide. It was covered with aluminium foil and stored at -20°C.
Low molecular weight marker	5 mg each of lysozyme (14.4 KDa), soy bean trypsin Inhibitor (22 KDa), horseradish peroxidase (40 KDa), BSA (66 KDa) and lipoxidase (96 KDa) were dissolved in 5 ml of triton buffer and 50 µl aliquots were stored at -20°C. Prior to use, it was

	thawed and mixed with 200 µl of triton buffer and 250 µl 2x SDS sample buffer.
High molecular weight marker	5 mg each of horseradish peroxidase (40 KDa), BSA (66 KDa), lipoxidase (96 KDa), β-galactosidase (116 KDa) and myosin, rabbit muscle (205 KDa) were dissolved in 5 ml triton buffer and 50 µl aliquots were stored at -20°C. Prior to use, it was thawed and mixed with 200 µl triton buffer and 250 µl 2x SDS sample buffer.

Reagents for gelatin zymography

2.5% Triton X-100, 10 mM Tris pH 8, 50 mM Tris pH 8 with 0.5 mM $CaCl_2$

Staining solution	1% coomasie brilliant blue R-250 was dissolved in 5% acetic acid and 10% methanol.
Destaining solution	5% acetic acid and 10% methanol
4x non-reducing sample buffer	1.25 M tris HCl pH 6.8-10 ml, 20% SDS-20 ml, glycerol-20 ml, bromophenol blue–4.8 mg.

6.8.4 Reagents for migration assay

Fixative	75 ml 50% ethanol mixed with 25 M acetic acid
Disodium borate buffer	0.1 M disodium borate. pH 9.2,
Crystal violet stock solution	50 mg crystal violet in 1 ml of 96% ethanol
Crystal violet staining solution	Crystal violet (0.1%) stock solution was diluted (1: 100) in disodium borate buffer.
X-Gal staining solution	PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal (added just before use). It was stored at 4°C.

6.9 Chemicals and kits

AAV Helper-Free System (Stratagene), BacterioMatch™ Two–hybrid system, XR Plasmid cDNA library (Stratagene), BigDye terminator Cycle Sequencing (Applied Biosystems), Chemi Glow (Alpha Innotech corporation), Plasmid Midi Kit (Qiagen), QIAquick PCR Purification Kit (Qiagen), Triple Master PCR System (Eppendorf), Acetic acid (AppliChem), Acrylamide (Roth), Agar-agar (Roth), Agarose (Roth), Ammonium sulphate (AppliChem), Anisomycin (Calbiochem), Antibiotics (Roth (AppliChem), Ammonium per sulphate (Roth), Bromophenol blue (AppliChem), β-Mercaptoethanol (Roth), dNTPset (Biolabs), Calcium chloride (Roth), Chloroquine (Sigma), Coomaric

acid (Sigma), DMSO (AppliChem), Dimethyl formamide (AppliChem), Ethanol (AppliChem), Ethidium bromide (Roth), EGTA (AppliChem), EDTA (AppliChem), Glycine (AppliChem), Glycerin (AppliChem), Hydrogen peroxide (AppliChem), IPTG (Isopropyl-β-D-thiogalactopyranoside) (AppliChem), Isopropanol (AppliChem), Luminol (5-amino-2 (3-dihydro-1 (4- phtalazinedion) (Sigma), Methanol (AppliChem), Mounting medium (Sigma), Magnesium chloride (Roth), Paraformaldehyde (AppliChem), Sodium chloride (AppliChem), Saccharose (Roth), Saponin (Roth), Sodium azide (Roth), SDS (Roth), TEMED (Roth), Tris-base (AppliChem), Triton-X-100 (Roth), Trypton (Roth), Tween-20 (Roth), Virkit™ AAV 3 pack, X-gal (AppliChem), Yeast extract (Roth).

6.10 Lab devices

Gel electrophoresis chamber (Peqlab Biotechnologie GmbH), Autoclave (Varioklav 500), Laminar flow hood (Heraeus Hera Safe), Centrifuges (Heraeus Instruments Megafuge 1,0R, Heraeus Biofuge fresco, Heraeus Sepatech Biofuge 13, Heraeus Sepatech Megafuge 1,0R, Sorvall RC 5B Plus), Cell counting chamber (Marienfeld Neubauer improofed), Coverslip /glass slides (Knitte), Fridge and freezer (Privileg), Filmcasette (Rego x-ray casette), Film developer (Agfa Curix 60), Gel documentation (Biorad Gel Doc 2000), Glasswares (Schott, VWR Brand, Merck), Heating block (Grant Boekel BBA, Roth Block thermostat TCR 100), Incubator (Memmert), Ice machine (Scotsman AF-20), Microwave oven (Electronia MW 810), Microscopes (Nikon Eclipse TS 100, Zeiss Axiovert 100 m), Pipetman (Brand accu-jet), Pipette (Gilson Pipetman), pH Meter (Beckmann), PCR Machine (Eppendorf), Plasticwares (Eppendorf, Greiner, Costar), PVDF-Membrane (Millipore Immobilon), Photometer (Hach DR / 2010), Power supply (Consort Power), Rotor (Rotary Mixer), Shaker (Heidolph Duomax 1030, Heidolph Polymax 1040), Shaking incubator (Eb Edmund Bühler, Eppendorf Thermomixer compact), SDS-PAGE apparatus (Biorad), Semi-dry apparatus (Schleicher & Schüll), Sequenzer (ABI PRISM 310 Genetic Analyzer), Trolley (Scaltec SBA 32, Kern 470), UV-Sterilizer (Biorad), Vortexer (Heraeus Hera Cell), (KIKA MS2 Minishaker), Waterbath (Memmert, Julabo DC), X-ray film (Retina x-ray).

7. Methods

7.1 Cell culture methods

7.1.1 Cell culture

Cell culture dishes (10 cm) were coated with 5 ml of gelatine and incubated overnight at 4°C. hCASMCs were cultured on these gelatine-coated culture dishes up to 8 passages in smooth muscle basal medium. Cells were passaged by detaching with 0.025% Trypsin/EDTA and cultured at 37°C with 5% CO_2. Cells were serum-starved using serum starvation medium. 293T cells were maintained in DMEM with 10% CS and detached by using 0.5% trypsin. DMEM with 2% heat inactivated CS was used during the infection of AAV preparations. RASMC were cultured in DMEM with 10% FCS, non-essential aa, streptomycin and penicillin.

7.1.2 Freezing and thawing of cells

Semi confluent dishes were washed with PBS, trypsinized and centrifuged at 600rpm for 4 min. Approximately; 5 x 10^5 cells were suspended in 1 ml freezing medium and placed in cryovial. Vials were taken into styroporbox and kept at -80°C for 2-3 days. Finally, these vials were stored at liquid nitrogen containers.

To thaw the frozen cells, the cryovial containing the cells were taken from the liquid nitrogen containers and thawed quickly at 37°C. The cell suspension was mixed with fresh growth medium and centrifuged at 600 rpm for 3 min. The pellet was suspended in 10 ml medium and added into new cell culture plate, incubated in incubator at 37°c with 5% CO_2. After 24-48 hrs, the cells were sub-cultured further.

7.1.3 Cell counting

The cells were detached and counted for various experiments such as migration assays and immunofluorescence staining. After detaching the cells, 16-20 µl of suspended cells were added on to neubauer chamber. The cells were counted in 4 different fields containing 16 squares under phase contrast inverted microscope. The average number of the cells was multiplied by 10^4 to get the number of the cells/ml.

7.1.4 Transfection (Calcium phosphate DNA precipitation method)

293Tcells were splitted one day before transfection. Required amount of plasmids were added in 500 µl of sterile double distilled H_2O. 500 µl of 2X HBS was added. Then 50 µl of calcium chloride was added drop wise while vortexing. Those sub-confluent

plates were taken and 10 µl of chloroquine was added. Then calcium-DNA mixture was added to the cells drop wise. Cells were incubated at 37°C, 4.5% CO_2 and the medium was changed after 6-8 hrs. After 2 days, transfected cells were used either for whole cell lysates (WCL) or immunofluorescence assays.

7.1.5 Replating assay

Cell culture dishes were coated with ECM proteins such as VN and FN diluted in PBS at indicated concentrations and kept at 4°C for overnight. Overnight serum-starved cells were washed with 5 ml PBS and detached by using 2 ml of 0.025% trypsin/EDTA. 3 ml of soybean trypsin inhibitor was added to neutralize the trypsin. The cells were centrifuged at 600 rpm for 4 min and suspended in suspension medium. They were incubated at 37°C for 45 min to 1 hr. The cells were shaken periodically to avoid clumping of cells. Similar number of cells was replated on the VN, FN or PL coated plates. The cells were lysed directly from the suspension or replated cells were incubated for 45 min to 1 hr and after the attachment of the cells, the WCL were prepared.

To demonstrate the inhibition of VN induced tyrosine phosphorylation, replating assay was performed in the presence of integrin α_v inhibitor. Cell culture dishes were coated with indicated concentrations of VN. Integrin α_v inhibitor was added to the suspended hCASMCs at various concentrations. After 45 min in suspension, the cells were replated on VN-coated plates in presence or absence of the integrin α_v inhibitor. Cells were observed for attachment, and then WCLs were prepared.

7.1.6 Preparation of WCL

After attachment of replated cells from replating assays or in other experiments, cells were lysed using RIPA lysis buffer. Sephadex beads were added to the lysates and incubated on rotator for 5 min at 4°C. Then cell lysates were centrifuged at 13000 rpm for 2X 15 min and supernatants were collected. Similar amount of 2X sample buffer were added to the supernatants. The samples were heated at 95°C for 3-4 min.

7.2 Immunoprecipitation

The WCLs prepared from various experiments were used for performing immunoprecipitation. The antibodies against the interested proteins were added to the lysates and incubated in a rotator in the cold room for 1-4 hrs. After the incubation, protein-A beads were added and incubated further for 1 hr. Samples were washed with

different lysis buffers at indicated time. The 2X sample buffer was added to the samples and analysed by SDS-PAGE.

7.3 Protein techniques

7.3.1 SDS-PAGE and Western blotting

WCLs or IP samples were separated on SDS-polyacrylamide gel electrophoresis. The running gels were prepared by using different percentage of polyacrylamide from 8% to 15% depends on the protein size and degassed. After preparing the gel mixture, two plates were casted between the clamps. After assembling the apparatus, the gel was poured in between the glass plates. After polymerization, some water was poured above the gel. After 1 hr, water was removed above the gel. The stacking gel (see the table) was prepared and degassed, poured on the top of the separating gel and comb was set-up. Samples were loaded on the gel using the needle and electrophoresis was done using the running buffer at 100 V for 1.5-2 hrs.

Running gel	8%	9%	10%	12.5%	15%
Polyacrylamide	2 ml	2.25 ml	2.5 ml	3.1 ml	3.75 ml
Distilled H_2O	5.5 ml	5.25 ml	5 ml	4.3 ml	3.65 ml
0.5 M Tris pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
20% SDS	50 µl	50 µl	50 µl	50 µl	50 µl
10% APS	30 µl	30 µl	30 µl	30 µl	30 µl
TEMED	15 µl	15 µl	15 µl	15 µl	15 µl

Stacking gel	5%
Polyacrylamide	1.25 ml
Distilled H_2O	6.15 ml
0.5 M Tris pH 6.8	5 ml
20% SDS	50 µl
10% APS	30 µl
TEMED	15 µl

After the electrophoresis, the gel was transferred by Western transfer method. SDS-PAGE gels were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were made wet by methanol before the transfer. The gel membrane sandwich was prepared and transferred overnight at 30 V in a chamber containing

Western transfer buffer. After the transfer, membrane was taken and stained with coomasie staining solution and subsequently destained with destaining solution. Membranes were blocked with blotto for 2 hrs at room temperature and probed with respective primary antibodies as indicated in fig for overnight. They were washed with TBST buffer for 3X; 15 min. Respective secondary antibodies were added (3 µl for 15 ml TBST) and incubated further. After 2 hrs incubation with the secondary antibodies, blots were washed with TBST for 3X, 15 min to get rid of the unspecific antibodies bound to the blots. Blots were detected with ECL detection system (3 µl H_2O_2 in 10 ml ECL solution) and developed in the developing machine.

7.3.2 Stripping of membranes

Probed membranes were stripped to remove the antibodies by incubating the membrane with stripping buffer at 80°C for 5 min. The membrane was washed thoroughly with water and blocked again with the blotto for 2 hrs at room temperature. Then again for the second time, another primary antibody was added and processed like before. This procedure allows different proteins to be detected from the same membrane.

7.3.3 Gelatin zymography

Overnight serum starved hCASMCs were replated on VN coated (500 ng/ml) plates with or without integrin α_v inhibitor as described in other replating assays. Cells were incubated for another 24 hrs. Conditioned medium from each sample was collected after 1 day. Those samples were mixed with equal amount of non reducing sample buffer. Each sample was then loaded onto SDS-PAGE containing 1 mg/ml gelatine as a substrate. After electrophoresis, the gel was incubated with 2 changes of 2% Triton X-100. The gel was further incubated in 10 mM Tris pH 8.0 and replaced 2 times followed by 16 hrs incubation in substrate buffer (50 mM Tris, 5 mM $CaCl_2$) at 37°C. To detect the enzymatic activity, the gel was stained with staining solution and subsequently destained. The gelatinolytic activity was detected by transparent bands in blue stained background.

7.4 Cell migration assay

7.4.1 Haptotaxis

Cell migration assays were performed by using modified Boyden chambers of 12 mm diameter with 8 µm pores. These chambers were pre-coated with different

concentrations of VN or FN on the lower part of the membrane. Control chambers were coated with DMEM containing 0.25% BSA. The chambers were coated for 2 hrs at room temperature. After the incubation period, they were washed with PBS and dried.

Overnight serum-starved hCASMCs were detached by using 0.025% Trypsin/EDTA and the cells were suspended in suspension medium. Then hCASMCs (1.5 x 10^5/cells in 300 μl of suspension) were seeded and the chambers were placed in 24 well culture dishes containing 400 μl suspension medium. After 3 hrs incubation, the top of the chambers was wiped with cotton tips to remove the cells that had not migrated. Remaining cells on the lower side of the membrane were fixed with fixative for 15 min, stained with crystal violet staining solution, and washed with water to remove the excess crystal violet. After drying, stained cells were counted in three fields per chamber at 200X magnification. The result is expressed as mean ± SD of two independent chambers.

Haptotaxis cell migration assays were also performed in same manner with or without the addition of the integrin α_v inhibitor. The integrin α_v inhibitor was added to the suspended cells and also included during the migration in the 24 well plate.

7.4.2 Chemotaxis

Migration chambers were coated in CN or VN (1 μg/ml) diluted in PBS for overnight at 4°C. Serum starved, detached and suspended hCASMCs were seeded on the chambers. Then chambers were placed in 24 well plates containing suspension medium with or without various concentrations of PDGF-BB as described in fig. The chambers were incubated for 3 hrs and processed like in other migration assays.
Chemotaxis cell migration assays were also performed in same manner with or without the addition of various concentrations of the integrin α_v inhibitor. Integrin α_v inhibitor was added to the suspended cells and also included during the migration in a 24 well plate.

7.4.3 Invasion assays

Invasion assays were performed in modified Boyden chambers containing polycarbonate filters with an 8 μm pore size. The growth factor reduced matrigel was diluted in 100 μl water to obtain different concentrations. The diluted matrigel was added on top of the chambers and kept in the incubator for 1 hr. It was air dried for overnight in a sterile field. Next day, the matrigel was reconstituted by using DMEM and incubated at 37°C incubator for 2 hrs. Serum starved hCASMCs were seeded on top of the chambers and placed in 24 well plates containing normal smooth muscle cell

growth medium with 5% FCS as chemoattractant. The chambers were incubated for 1 day. After the incubation, the chambers were taken and wiped with cotton tip. The cells migrated to the lower surface were fixed, stained and counted. 3 random fields were counted at 200X magnification.

To demonstrate the effect of integrin α_v inhibitor on invasion assay was performed in the presence of integrin α_v inhibitor. Inhibitor was added to the suspended cells as well as in the 24 well plates.

7.5 Flow cytometry

7.5.1 Analysis of integrin expression

Cultured hCASMCs were detached with 0.025% Trypsin/EDTA and suspended in PBS, 0.25% BSA at 8 x 10^5 cells/ml. 200 µl of cell suspension was incubated on ice with 2 µg of FITC-labelled monoclonal antibody against integrin $\alpha_v\beta_3$ and integrin $\alpha_v\beta_5$ for 45 min. The cells were washed three times with PBS + 0.25% BSA and analysed using a FACS Calibur (Becton Dickinson). Isotype matched control antibody was used as negative control**.**

7.5.2 Apoptosis assay

Serum starved hCASMCs were incubated for 18 hrs with or without the indicated concentration of the integrin α_v inhibitor or 50 µM anisomycin. Cells were suspended in PBS + 0.1% BSA about 8 x 10^5 cells/ml. Apoptotic cells were stained using annexin-V-FITC apoptosis detection kit. Stained samples were analysed by FACS Calibur.

7.6 Immunofluorescence and confocal microscopy

The hCASMCs (2 x 10^4 cells) were seeded on the gelatin or ECM protein coated cover slips. The cells were fixed either with acetone for 15 min or with PFA. After fixation, cells were washed with PBS for 3 times and permeabilized with blocking solution for 5 min. The antibodies diluted in blocking solution were added to the cells and incubated for 45 min at room temperature in a humidified chamber. After three washing with PBS, samples were blocked with blocking solution for 5 min. Then, respective secondary antibodies were added and cells were incubated for another 45 min in a dark humidified chamber. Later, samples were washed 3 times, thoroughly,

again with PBS and mounted on glass slides using mounting medium. Images were acquired using confocal laser scanning microscope (Zeiss, Heidelberg, Germany).

Cell specimens from immunoflurescence assay were analysed by zeiss confocal laser scan fluorescence-inverted microscope equipped with two lasers, which uses a helium-neon laser (excitation wavelength at 543 nm) and an argon laser (excitation wavelength at 488 nm). The excitation spectra were separated by transmission filter. The specimens were observed through an oil immersion (40X) objective. The image sections were recorded as 1024 x 1024 pixels. These images were further processed by Adobe Photoshop 7.0.

7.7 Molecular biology techniques

7.7.1 Cloning

FRNK was also amplified and cloned into two different vectors including pAAV-MCS vector and pAAV-IRES-hrGFP vector. rFUN1 was amplified and cloned into pDNR dual vector. Then subsequently the cre recombination was performed and rFUN1 was cloned into pLPS-3'EGFP vector

7.7.2 Templates and oligonucleotides

FRNK was amplified from plasmid pcDNA3.1-HA FAK (WT), using the primers FRNK–HA sense (5'-ATAGAATTCATGGAATCCAGAAGACAGGCTACC-3') and FAK anti-short (5'-ATTATCTAGATCAAGCAGCGTAATCTGGAACGTCATATGG-3') and cloned into pAAV-MCS vector. FRNK was amplified by using the primer-FRNK-KOZAK-sense (5'-ATAGAATTCACCATGGAATCCAGAAGACAGGCTACC-3') and FRNK W/O STOP codon anti-(5'-ATAGTCGACGTGTCTGCCCTAGCATTTTCAGTC TTGC-3') from the template PBS SK (+)–FRNK-HA tag and then cloned into the vector pAAV-IRES-hrGFP. The putative interaction partners for N-terminal domain of FAK were sequenced using pBT forward primer-5'-TCCGTTGTGGGAAAGTTATC-3' and pBT reverse primer-5'-GGGTAGCCAGCAGCATCC-3'. rFun1 was amplified using primers rFUN1-IF-sense-(5'-GAAGTTATCCGACACCATGGAGGCTGAAAG ACCCC-3') and rFun1-IF-antisense w/o stop codon (5'-ATGGTCTAGAAAGCT TCCGCAGGTGTGTGTAGGGG-3'), then infusion reaction was performed to clone into the pDNR dual vector. After the cre recombination the clones were analysed by using check 1-sense GCTCACCGTCTTTCATTGCC and check2-antisense TCCGCTCATGAGACAATAACC.

7.7.3 PCR mixture

PCR was performed to amplify specific DNA fragments using plasmid DNA as template and specific oligonucleotide primers. Taq DNA polymerase and vent polymerase were added in the PCR mixture for amplification and proof reading respectively.

PCR mixture contains (50 µl)

1 µl	Sense primer (100 pmol)
1 µl	Anti-sense primer (100 pmol)
1 µl	dNTPs (20 mM)
4 µl	$MgCl_2$ (25 mM)
5 µl	10x PCR-buffer + $(NH4)_2SO_4$
0.5 µl	Taq-polymerase
0.5 µl	Vent-polymerase
1 µl	Template plasmid (10-100 ng)
36 µl	distilled H_2O

Amplification in thermocycler was done using the following reaction cycles:

Hot start	94°C	5 min
Denaturation	94°C	30 sec
Hybridization	55-60°C	30 sec (Temperature of annealing suitable for primers, calculated according to: T_m= (G/C) ∗4°C+ (A/T) ∗2°C-2°C)
Polymerization	72°C	50 sec (30 cycles of denaturation, hybridisation and polymerisation)
Final elongation	72°C	5 min

PCR products were analysed by DNA agarose gel electrophoresis. To analyze the clones by PCR, following reaction mixture was used.

Master Mix:

	For each sample
20 mM dNTPs	0.2 µl
10X PCR buffer + $(NH_4)_2SO_4$	1.5 µl
25 mM $MgCl_2$	1.6 µl
Primer 1 (1: 10)	0.4 µl
Primer 2 (1: 10)	0.4 µl
H_2O	11 µl

-15 µl master mix in a PCR-tube was incubated.
-0.2-0.5 µl template was added and PCR-Hot start was done.

Enzyme mix:

	For each sample
H_2O	4.3 µl
10X PCR Buffer + $(NH_4)_2SO_4$	0.5 µl
Taq-polymerase	0.2 µl

To check the clones by PCR in thermocycler following reaction conditions were used.

Hot start	94°C	5 min
Denaturation	94°C	30 sec
Hybridisation	55°C	30 sec
Polymerisation	72°C	2 min (20 cycles of denaturation, hybridisation and polymerisation)
Final elongation	72°C	5 min

7.7.4 Agarose gel electrophoresis

PCR products, digested plasmids and plasmids were analysed by agarose gel electrophoresis. Agarose was dissolved in 1X TAE buffer and heated in a microwave oven. Agarose gel was polymerized in gel electrophoresis apparatus. Probes were loaded on the slots with 6X sample buffer and electrophoresis was carried out in 1X TAE buffer. After 1-2 hrs, the gel was visualized by ethidium bromide staining.

7.7.5 Visualization of DNA

DNA fragments were resolved on 0.7% to 1.5% agarose gel depending on fragment size. After the electrophoresis, gels were stained in ethidium bromide bath for 15-20 min. Ethidium bromide stained gels were visualised in gel documentation machine.

7.7.6 PCR purification and gel extraction

For cloning, the digested fragments from vector and inserts which was visualised in the UV-illuminator. The specific fragments were extracted using clean scalpel. Gel extraction was done by using instructions from QIAquick® gel extraction Kit (Qiagen). PCR products were purified by QIAquick® PCR purification kit (Qiagen)

7.7.7 In-Fusion cloning reaction

In-fusion cloning method was used for cloning the FUN1 sequence. In-fusion PCR kit was used for this purpose. The oligonucleotides were designed with special in-fusion sequence of about 15 bp sequence in the 5`end. Using gradient PCR amplification with various annealing temperature, annealing temperature was detected and specific sequence was amplified. After the amplification, PCR products were loaded on the agarose gel and purified from the gel.

The amplified PCR-products and pDNR-Dual linearized vectors were used for in-fusion reaction. The concentration of PCR product was measured by comparing the standard. 50-100 ng of PCR products were used for the reaction. The following reaction mixture was used.

1 µl	10X BD In-Fusion reaction buffer
1 µl	10X BSA (500 µg/ml)
0.5 µl	Linearized pDNR-Dual vector (100 ng/µl)
50-100 ng	PCR-product
1 µl	1:10 diluted BD In-Fusion enzyme (20 U/µl)
X µl	Double distilled H_2O
10 µl	Final volume

The reaction mixture was incubated for 30 min at room temperature and further incubated on ice. The fusion product was transformed into competent *E.coli* and selected on LB/X-Gal/amp plates. Growing clones were further streaked out on the amp plates and incubated at 37°C for overnight. Next day, plasmids were isolated by mini prep and resolved on the agarose gel. Positive clones were confirmed by PCR or by sequencing.

7.7.8 Cre-lox site specific recombination

BD Creator DNA Cloning Kit was used for cre-lox recombination. pDNR-Dual vector containing specific PCR-fragment was used as donor vector and pLPS-3'EGFP vector was used as acceptor vector, which has loxP sequence of P1 bacteriophage. Cre recombinase specifically recognizes the loxP site and allows rapid transfer of gene of interest from pDNR-Dual in frame with the splice donor (SD) site. This transfer automatically allows the gene to be in frame with EGFP. The following reaction mixture was used in the reaction

1 µl	10X Cre-reaction-buffer
1 µl	10X BSA
0.5 µl	Cre-recombinase
100 ng	Donor vector
200 ng	Acceptor vector
X µl	Distilled H_2O
10 µl	Final volume

The reaction mixture was incubated at room temperature for 15 min and heat inactivated at 70°C for 5 min. The reaction mixture was transformed into competent *E. Coli.* The samples were plated on Cam with 7% sucrose containing LB agar plates. The selected clones were analysed further as like in other experiments.

7.7.9 DNA digestion with restriction enzymes

Restriction digestion of plasmids with specific restriction enzymes was used for cloning the interested PCR fragments or to confirm the clones for the presence of gene of interest in the construct. Plasmids and digestion enzymes mixture was incubated at 37°C for from few hrs to overnight. The restriction enzyme concentration and DNA concentration is variable in every experiment.

7.7.10 Dephosphorylation of DNA

3 µl of Shrimp Alkaline Phosphatase (SAP) was added to the digested vector samples to avoid self-ligation and incubated for 30 min. After incubation, samples were heat inactivated at 70°C for 15 min.

7.7.11 Ligation

In order to find the optimal ratio for ligation, the digested vector and inserts were loaded on the agarose gel before ligation. The 10 µl of ligation mixture was prepared with T4 ligase. Ligation mixtures were incubated at 16°C or at room temperature for 1 or 2 hrs.

7.7.12 Preparation of competent bacterial cells

The bacterial strain was streaked on the respective antibiotic containing plates. Next day, single colony was inoculated into 2X 10 ml LB medium containing same antibiotics and incubated for overnight at 37°C, 225 rpm in a shaking incubator.

Next day, 4X 200 ml LB medium containing same antibiotics was inoculated with 4 ml of overnight culture and the cultures were incubated in a shaking incubator at 37°C. The absorbance was periodically measured until it reaches 0.6 to 0.8 OD. After reaching this OD, cultures were transferred into sterile bottles. It was centrifuged in a pre-cooled centrifuge at 3000 rpm for 5 min at 4°C. The pellets were resuspended in 100 ml of 0.1 M $CaCl_2$ (cold) in the cold room. Cells were centrifuged again at 3000 rpm for 5 min at 4°C.The pellet was suspended again in 10 ml of 0.1 M $CaCl_2$ (cold). The suspension was incubated in a tube on ice for 30-60 min. After incubation, 4 ml of glycerin was added and 100 µl aliquots were frozen at -80°C. Prior to use, the competent bacteria was thawed on ice.

7.7.13 Transformation of bacteria

Competent *E.coli* cells stored at -80°C were thawed on ice. 1-2 µl of plasmid or 10 µl of ligated sample was added to tube containing 100 µl of competent *E.coli*. The samples were incubated on ice for 30 min and then heat shocked at 42°C for 90 sec in a preheated water bath. Samples were rapidly cooled on the ice for few min and 1 ml of LB medium was added and incubated at 37°C for 1 hr in a shaker. The agar plates were prepared in sterile conditions and then stored at 4°C until they were needed. Prior to use, they were warmed at room temperature. After incubation, culture was transferred into an eppendorf tube and centrifuged at 3000 rpm for 3 min. The pellet was suspended in small volume of supernatant and spreaded on agar plates. The plates were inverted and incubated in the incubator at 37°C. The clones were streaked on the same antibiotic plates and subsequently mini prep was done.

7.7.14 Plasmid purification

Miniprep: Birnboin-Dooley method

Bacterial colonies were streaked on respective antibiotic plates. The bacterial culture was suspended in 300 µl of ice-cold P1 buffer and vortexed to suspend the cells. Then 300 µl of buffer P2 was added and incubated for 5 min at room temperature. After incubation, 300 µl of ice cold buffer P3 was added, incubated further for 10 min at room temperature and mixed by shaking. The samples were spun down at 13000 rpm for 30 min, supernatants were transferred into new tube and 0.7 volume (560 µl) of isopropanol was added into the tubes and incubated for 2-3 min. Samples were again

centrifuged for 30 min at 13000 rpm, washed with 70% ethanol and centrifuged for 5 min. DNA was air dried and dissolved in 40 µl of TE buffer.

Mini-prep (Qiagen and eppendorf purification system)

To get high purity of plasmids, the Qiagen or Eppendorf purification systems were used for preparation of plasmids.

Midi-prep (Qiagen and Nucleobond®AX plasmid (macherey-Nagel) purification kit)

To obtain large quantity of plasmid DNA, the Qiagen plasmid or Nucleoband plasmid purification kits were used.

7.7.15 Spectrophotometric measurement of DNA amount

Aliquot of the DNA sample was diluted and absorbance was read in spectrophotometer at wavelength of 260nm and 280nm. Concentration of DNA was obtained by multiplying absorbance and dilution factor with standard factor.

7.7.16 DNA precipitation

To get pure quality of plasmid DNA, prepared plasmid samples were ethanol precipitated. Plasmids (in Tris/EDTA (TE) buffer or in distilled H_2O) were taken, 1/10 volume of 3 M sodium acetate; pH 5.2 and 2.5 volume of 100% ethanol (cooled at -20°C) was added. Samples were incubated for 30 min at -80°C or 1-2 hrs on ice. After the incubation, samples were centrifuged at 13000 rpm for 10 min. Pellets were washed with 70% ethanol, dried and eluted in TE buffer or distilled H_2O.

7.7.17 Sequencing

To check the appropriate insert, plasmids were sequenced using either forward or reverse primer.

Reaction mixture:

Premix	2 µl
Big dye terminator sequencing buffer	2 µl
Plasmid	0.1-0.5 µg
PCR-product (0.2-5 kb)	10-100 ng
Primer	50 pmol
H_2O	Filled up to10 µl.

The reaction mixture was prepared without DNA or PCR product. The samples were kept on the thermocycler. After hot start, the DNA mixture was added and amplified using the following PCR program:

96°C 2 min
96°C 30 sec
45-60°C 15 min
60°C 4 min
25 cycles.

After amplification, the reaction mixture was further processed for DNA precipitation. To the entire amplified PCR mix, 90 µl of H_2O, 10 µl of 3 M sodium acetate pH 5.2 and 250 µl of 100% ethanol was added and mixed. Samples were centrifuged at 13000 rpm for 15 min. The pellets were washed with 70% ethanol, centrifuged at 13000 rpm for 5 min and dried at room temperature. DNA was diluted in 20 µl of Hidi. Samples were transferred in sequencing tube and sequenced using an ABI sequencer.

7.7.18 Bacterial two-hybrid system

Amplification of rat smooth muscle cell library

22 ml of TGY medium was inoculated with 20 µl of main aliquot of bacteria. 400 µl of this culture was added into each plate of 54 plates (150 cm). The dishes were spread with this aliquot and incubated at 30°C for 24 hrs. After the incubation, colonies were scraped, suspended into TGY medium and centrifuged at 13000 rpm for 15 min. Half of the pellets were used for plasmid preparation using Qiagen plasmid preparation kit and other half of the pellets were frozen at -80°C for further use.

Control experiment 1

Co-transformation of plasmids

A / B	pBT-LGF2 (Cam)		pBT (Cam)		pBT-FAK-NT (Cam)	
	CTCK	TCK	CTCK	TCK	CTCK	TCK
pTRG-Gal11 (tet)						
pTRG (tet)						

Each plasmid was diluted to 0.1 µg/µl in a 40 µl total volume. Respective plasmids 1 µl of A and 1 µl of B plasmids were incubated for 30 min with the reporter strain on ice. The samples were heat shocked at 42°C for 75 sec, N2Y medium was added and

incubated for 1 hr at 37°C. 100 µl of bacterial suspension was added on CTCK, TCK plates and spread and incubated at 37°C for overnight.

Screening of the cDNA library with pBT-FAK-NT

The LB plates containing CTCK and TCK antibiotics were prepared in 150 mm bacterial plates. From the co-transformation efficiency, the DNA mixture was prepared. 21.3 µl of DNA mixture was added into 30 tubes and incubated with reporter strain for 45 min. Heat shocked at 75°C. 1.6 ml of T2Y medium was added and incubated for 90 min. The bacterial suspension was taken in the eppendorf and spun down for 2 min at 3000 rpm. Approximately, the pellet was suspended in 250 µl of volume supernatant and spreaded on the antibiotic plates. The plates were incubated on 30°C for two days; the colonies were streaked on TCK plates. 290 clones were selected and streaked further on X-Gal containing TCK plates as well as CTCK plates. The clones that are positive for the CTCK and X-Gal-TCK plates were considered as positive interacting partners. Furthermore, these clones were harbouring both plasmids of bait and target plasmids. To isolate the single target plasmids, positive clones were inoculated on tetracycline containing LB-medium for overnight. Next day, 20 µl of culture was taken and spreaded on tet plates; growing clones were selected again and streaked on tetracycline as well as Cam plates. Clones that are growing only on Tet plates were considered as single plasmids. Those clones were frozen again for further use and sequenced with the specific primers.

7.8 AAV virus production, infection and evaluation

7.8.1 pAAV-MCS-FRNK and pAAV-LacZ virus production

pAAV-MCS-FRNK plasmid was co-transfected with pHelper and pRc plasmids in 293T cells by calcium chloride method. After 6 hrs, the medium was changed by DMEM containing 10% CS. In the same way β-galactosidase (pAAV-MCS-LacZ) AAV was also produced by co-transfecting the three plasmids, pAAV-LacZ, pHelper and pRc plasmids. Then after 54 hrs, cells were collected in medium and centrifuged at 1000 rpm for 5 min. Cell pellets were suspended in 0.1 M Tris buffer with 0.15 M NaCl pH 8.0. Freeze–thaw cycle was carried out 3 times in ethanol ice bath. Finally, the tubes were centrifuged at 16000 rpm for 20 min. Virus containing supernatants were collected and kept for further infection.

7.8.2 AAV-FRNK-GFP and AAV-GFP control virus production

pAAV-IRES-hrGFP-FRNK plasmid was co-transfected with pHelper and pRc plasmids in 293T cells by calcium chloride method. After 6 hrs, the medium was changed by DMEM containing 10% CS. 18 hrs later cells were serum starved using DMEM with 0.25% BSA. In the same way pAAV-IRES-hrGFP AAV was also produced by co-transfecting the three plasmids, pAAV-IRES-hrGFP, pHelper and pRc plasmids. To improve the quality of the virus, various amount of plasmids, and different time of harvesting and different lysis condition were used during harvesting. The viruses were prepared with optimized condition. Finally, the tubes were centrifuged at 16000 rpm for 20 min. The virus containing supernatants were collected and kept for further infection.

7.8.3 Infection of human SMC with pAAV-MCS-FRNK and pAAV-LacZ

hCASMCs or 293T cells were infected with supernatant prepared as described above. The confluent 293T cells were splitted into semi confluent in 6 well plates. Before the infection, the medium was aspirated, infections were carried out in presence of DMEM containing 2% heat inactivated CS. After 2 hrs, DMEM containing 10% CS was replaced.

7.8.4 β-galactosidase staining

The pAAV-LacZ virus-infected hCASMCs were washed with PBS and fixed with 2% paraformaldehyde. The cells were stained with X-Gal containing staining solution at 37°C for 2 hrs. The blue cells were counted in three fields at 200X magnification.

7.8.5 Cell migration inhibition by pAAV-MCS-FRNK construct

After 5 days incubation, pAAV-MCS-FRNK or control virus infected hCASMCs were serum starved for overnight. These serum-starved cells were used in the migration assay. The haptotaxis assay was performed in the manner described above (see haptotaxis assay). The migration chambers were coated with 10 µg/ml of VN. Cells were seeded on the modified Boyden chambers and incubated for 3 hr. After the incubation, chambers were removed and the top part was wiped off with a cotton tip in order to remove non-migrating cells. Then cells were fixed and stained. Stained cells were counted in three fields at 200X magnification. Results are expressed as mean ± SD.

7.9 Application of AAV-GFP-FRNK in porcine restenosis model

7.9.1 Animal preparation

This investigation conforms to guide for the care and use of laboratory animals published by US National Institute of Health. 6 domestic pigs were used in this study. All the animals were fed Ticlopidin 250 mg per day (Tiklyd, Sanofi-Synthelabo, Germany) and aspirin at dose of 100 mg starting 4 days prior to the coronary intervention until 4 weeks after the stent implantation. The pigs were intubated and mechanically ventilated. Anesthesia was performed intravenously with piritramid 0.3 – 0.6 mg/kg BW, midazolam 0.2 mg/kg BW every 45 minutes and pancuronium 0.04 mg/kg BW every 1.5 hr.

7.9.2 Interventional protocol

The right carotid artery was carefully dissected and a 6 French sheath was inserted into that vessel. Medication was given via an i.v-line placed in an ear vein. After preparation the left coronary artery was intubated with a 6F JL 3.5 guiding catheter. Under fluoroscopy guidance a bare metal stent (Size 3 mm x 10 mm, Eucatech, Rheinfelden, Germany) was implanted in the proximal part of the LAD (left anterior descending coronary artery) and in the proximal part of the circumflex artery each with an inflation pressure of max 12 atmospheres for 20 s. A total of 11 stents were implanted into the coronary arteries of 6 domestic pigs. After that a double balloon perfusion catheter was placed in each of the arteries directly in the stent region. The balloons were mounted on the catheter with a distance of 15 mm. Thus inflation of the balloons created a complete occlusion of the stented segments lying within the balloons. Via an injection lumen in the catheter either AAV-GFP (placebo) solution or AAV-GFP-FRNK solution was injected in the coronary segment situated between the proximal and distal balloon. Injection time was 3 minutes with a flow of 1 ml/min. Afterwards all intracoronary devices were removed and a control angiography was done. The animals were kept for 28 days in a stable under daily observation. After 28 days the animals were sacrificed.

7.9.3 Experimental design

After euthanasia, hearts were collected from the sacrificed animals. They were evaluated for neointima formation. The vessels and hearts were taken and radiography was performed to locate and access the stent placement. The stented vessel segments

were dehydrated in a graded series of ethanol and embedded in methylmethacrylate plastic. After polymerization, two to three millimeter sections were sawed from the proximal, mid and distal portions of each stent. Sections from the stents were cut on a rotary microtome at four to five microns, mounted and stained with hematoxylin, eosin and elastic movat pentachrome stains. All sections were examined by light microscopy for the presence of inflammation, thrombus, neointimal formation, vessel wall injury and localized toxic effects associated with drug-coated stents. A vessel injury score was calculated according to the Schwartz method. A numeric value from 0 (no injury) to; 1 = break in internal elastic membrane; 2 = perforation of the media; and 3 = perforation of the external elastic membrane. Digital morphometry was used to obtain all morphometric measurements. Quantitative measurements of cross sectional areas including neointima area, lumen area, external elastic lamina (EEL), internal elastic lamina [IEL], intimal thickness assessed at both AAV-GFP control treated and AAV-GFP FRNK treated injured sites. Neointimal thickness was measured as the distance from the inner surface of each stent strut to the luminal border. Percent area stenosis was calculated with the formula [(Neointimal area/IEL area) x 100]. Ordinal data were collected on each stent section and included strut apposition to the vessel wall, fibrin deposition, granuloma reactions and haemorrhage around the stent struts and were expressed as a percentage of the total number of struts in each section. An overall inflammation and fibrin value was scored for each section (value 0-3). Endothelial coverage was semi-quantified and expressed as the percentage of the lumen circumference covered by endothelium.

7.9.4 Statistics

ANOVA (analysis of variance) tests were used to calculate the significance of differences between cumulative frequency distribution of the treatment groups. A value of $P \leq 0.05$ was considered statistically significant. All the data are presented as the mean value $\pm$ SD.

8. Summary

The prevention of restenosis after percutaneous coronary intervention is a major task for researchers and clinicians in cardiovascular pharmacology. Nearly 1.5 million PTCA are performed every year worldwide and, due to the implantation of stents, most of the cases can be treated successfully. 60% of those patients develop restenosis within 6 months. SMC migration and ECM deposition are known to be responsible for neointima formation. Among many processes, integrin initiated signalling events play a central role in SMC migration. Many integrins recognize a specific RGD sequence which is present in several ECM proteins and cell surface immunoglobulin super family molecules. Until now, there are various integrin antagonists such as antibodies, cyclic peptides, peptidomimetics, and non-peptides have been shown to interfere with such pathological situations indicating the importance of integrin initiated signalling pathways in SMC migration. Therefore, in this study SMC migration induced by ECM proteins was inhibited either using pharmacological inhibitor or by overexpressing the endogenous inhibitor of FAK by AAV vector system.

In the first part of the thesis, the effect of integrin-ligand stimulation on hCASMCs was studied. The tyrosine phosphorylation of many cellular proteins was observed from serum starved hCASMCs replated on VN but not on PL coated plates. The major tyrosine phosphorylated protein was identified as FAK by immunoprecipitation and also phosphorylation was found at Tyr 397, the autophosphorylation site of FAK. Further, VN induced the dose dependent migration of hCASMCs in haptotaxis assay.

The integrin α_v inhibitor was used to block those ECM stimulated integrin signalling pathways and cell migration. It inhibited the ECM stimulated tyrosine phosphorylation in a dose dependent manner. Interestingly, specific potent antagonism of integrin α_v abrogated both ECM induced haptotaxis and growth factor induced chemotaxis. The inhibition of migration is consistent with the replating assay results that show interference with integrin induced signalling pathways particularly the FAK tyrosine phosphorylation. The integrin α_v inhibitor also is able to interfere with hCASMC invasion through matrigel by reducing MMP-2 secretion. Importantly, integrin α_v inhibitor did not induce the apoptosis in hCASMCs.

FAK is a key player in many cellular events and its involvement in cell migration was extensively studied in various cell types. The present study explored the function of FAK in hCASMC migration by overexpression of FRNK, the C-terminal domain of FAK.

Overexpression of FRNK inhibited the *in vitro* SMC migration as well as the neointima formation in a porcine restenosis model *in vivo*.

The last part of this thesis focused on the identification of putative binding partners for the N-terminal domain of FAK by bacterial two-hybrid screen. One of the interesting binding partners was a putative protein of 17.9 kDa. Its human homolog is AGS4, which acts as a GTPase activator. The preliminary results revealed that it is able to interact with N-FAK domain and its expression is high in haematopoietic cells.

Taken together the above results suggest that integrin α_v and FAK are promising targets for inhibition of SMC migration. Disruption of FAK-mediated signalling pathways by a pharmacological inhibitor or by overexpression of FRNK, which acts as dominant-negative regulator, resulted in decreased migration of SMCs and thus can lead to reduction of neointima formation.

9. Zusammenfassung

Die Prävention einer Restenose nach PTCA ist eines der wichtigsten Ziele für Forscher und Kliniker. Etwa 1,5 Millionen Interventionen werden weltweit jährlich durchgeführt und mit Hilfe von Stentimplantationen können die meisten Patienten erfolgreich behandelt werden. Jedoch kommt es in bis zu 60 % der Fälle zu einer Restenosierung des behandelten Gefässes innerhalb von etwa 6 Monaten. Für die Entwicklung der Neointima, Hauptursache der Restenose, sind Wanderung glatter Gefässmuskelzellen (GMZ) und Ablagerungen von Proteinen der extrazellulären Matrix (EZM) verantwortlich. Signalkaskaden, die über Integrin Rezeptoren vermittelt werden, spielen in der Migration von GMZ eine zentrale Rolle. Viele Integrine binden über eine spezifische Aminosäuresequenz, die sogenannte RGD Sequenz, die in verschiedenen EZM Proteinen und in auf Zelloberflächen gebundenen Immunglobulinen vorkommt. Bisher konnte von verschiedenen Integrinantagonisten, wie Antikörpern, zyklischen Peptiden, Peptidomimetika und Nicht-Peptiden, gezeigt werden, dass die die pathologische Reaktion vermindern können, was auf die Bedeutung der Integrin vermittelten Signalkaskaden in der GMZ Migration hinweist. Wir konnten zeigen, dass die Wanderung von GMZ sowohl mit einem pharmakologischen Inhibitor, wie auch durch die endogene Überexpression eines FAK Inhibitors, der über ein AAV Vektorsystem übertragen wurde, gehemmt werden konnte. So stellt die Blockade der Integrin vermittelten Signalkaskaden ein vielversprechendes Ziel für die Inhibition der Restenose nach PTCA dar.

Im ersten Teil der Arbeit konnten wir nach Stimulation von humanen GMZ mit Vitronektin (VN) eine verstärkte Tyrosinphosphorylierung (PTyr) verschiedener zellulärer Proteine nachweisen. Dabei zeigte sich eine besonders signifikante Phosphorylierung eines Proteins, das mittels Immunpräzipitation als *“focal adhesion kinase”* (FAK) identifiziert wurde. Die erhöhte PTyr zeigte sich auch am Tyrosinrest FAK Tyr-397, der Autophosphorylierungsstelle der Kinase. Die erhöhte PTyr von FAK war abhängig von der Stimulation durch VN und nicht zu beobachten, wenn die GMZ auf Poly-L-Lysin ohne spezifische Rezeptor Ligand Interaktion adhärierten.

Mit Hilfe eines Integrin α_V Inhibitors konnte diese rezeptorvermittelte Aktivierung in einer dosisabhängigen Weise verhindert werden. Die Inhibition der durch VN stimulierten Migration (Haptotaxis) mit Hilfe des α_V Inhibitors korrelierte mit der Reduktion der Aktivierung Integrin vermittelter Signalwege, im Besonderen der PTyr von FAK. Interessanterweise konnte die Blockade von Integrin α_V nicht nur die durch

VN stimulierte Haptotaxis, sondern auch die durch Wachstumsfaktoren induzierte Chemotaxis hemmen. Die Migrationsrate wurde mit Hilfe eines modifizierten Boyden-Migrationskammer Experiments ermittelt, das ein *in vitro* Modell zur Untersuchung von Zellwanderung darstellt. Der α_V Inhibitor hemmte auch die Invasion der GMZ in eine Matrigel Matrix und die Sekretion der Matrixmetalloproteinase 2. Eine Apoptose wurde bei den verwendeten Konzentrationen nicht induziert.

FAK stellt ein wichtiges Schlüsselprotein in vielen zellulären Mechanismen dar. So konnte die Beteiligung von FAK in der Regulation der Zellmigration an verschiedenen Zellarten gezeigt werden. Die Überexpression von FRNK, der C-terminalen Domäne von FAK, ist in der Lage die *in vitro* Migration von GMZ wie auch die Neointimabildung in einem Schweinemodell zur Entwicklung der Restenose zu verhindern. FAK stellt somit ein vielversprechendes Ziel für die Inhibition der Restenoseentwicklung nach PTCA dar.

Der letzte Teil der Arbeit konzentrierte sich auf die Identifikation von Bindungspartnern der N-terminalen Domäne von FAK mit Hilfe eines bakteriellen „two hybrid“ Systems. Es wurde als ein möglicher Bindungspartner ein 17,9 kDa grosses Protein gefunden. Das humane Homolog ist als AGS4 bezeichnet und stellt einen GTPase Aktivator dar. Es zeigte sich, dass es in der Lage ist, mit der N-terminalen Domäne von FAK zu interagieren, und dass es stark in hämatopoetischen Zellen exprimiert wird.

Zusammenfassend kann man sagen, dass unsere Ergebnisse FAK als ein vielversprechendes Ziel für die Inhibition der GMZ Migration erscheinen lassen. Das Vorliegen verschiedener induzierter Signalwege kann durch die Rolle der EZM Proteine und der Wachstumsfaktoren in der Zellmigration erklärt werden. Das Ziel dieser Studie war die Signalkaskaden, die zu einer GMZ Migration und somit zu einer Restenose führen, zu unterbrechen. Die Ergebnisse zeigen, dass α_V Integrine und Signalkaskaden, die FAK vermittelt sind, wichtig für die Zellmigration sind. Die Unterbrechung dieser FAK vermittelten Signalwege, sei es durch einen pharmakologischen Inhibitor oder durch die Überexpression von FRNK führte zu einer Inhibition der Migration.

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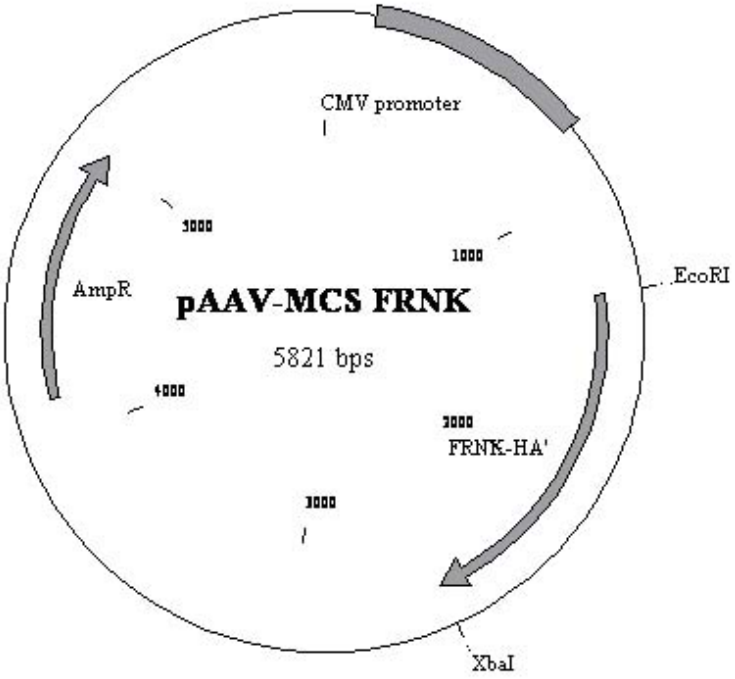
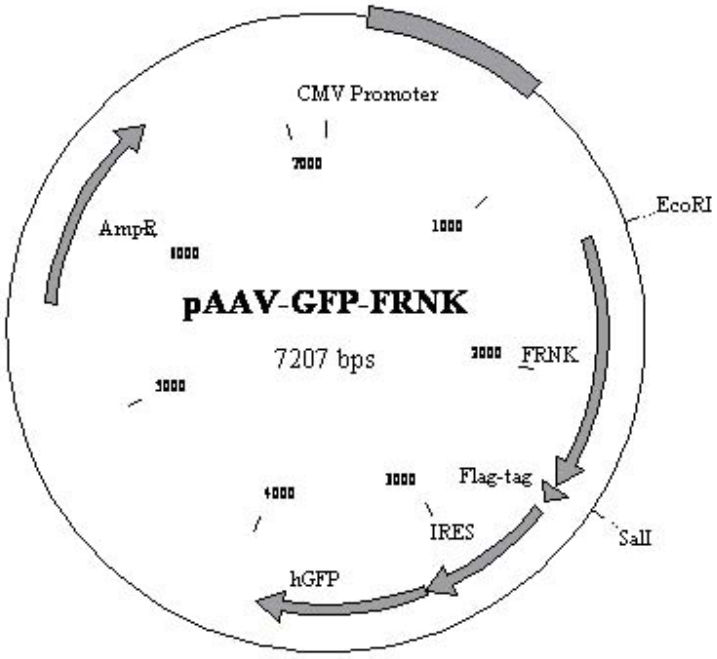
11. Appendix

11.1 Plasmid cards

pAAV-MCS-FRNK



pAAV-GFP-FRNK



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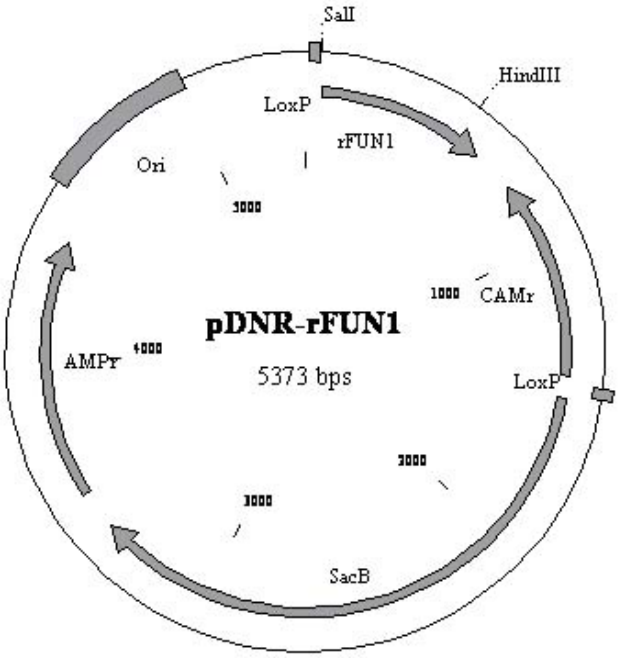

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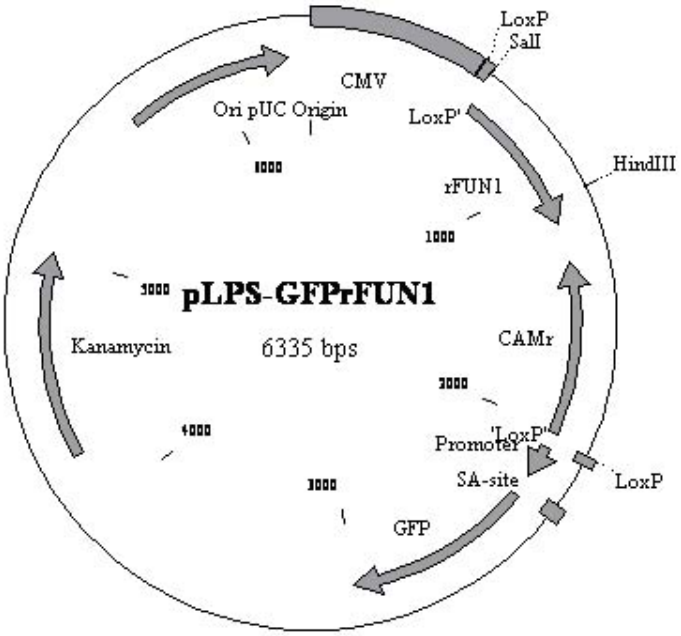

11.2 Abbreviations

%	Percentage
aa	Amino acid
AAV	Adeno- associated virus
Ab	Antibodies
APS	Ammonium per sulphate
bp	Base pair
BSA	Bovine serum albumin
Cam	Chloramphenicol
°C	Celsius
CFU	Colony forming unit
CN	Collagen
CS	Calf serum
CTCK	LB agar plate containing Cam, tet, carbenicillin & kan
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribo nucleic acid
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EEL	External elastic lamina
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERM	Ezrin/Radixin/Moesin
et al	Et altera
FACS	Fluorescence activated cell sorter
FA	Focal adhesion
FAK	Focal adhesion kinase
FAT domain	Focal adhesion targeting domain
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FIF200	a 200 kDa FAK family interacting protein
Fig	Figure
FN	Fibronectin
FRNK	FAK related non kinase
GFP	Green fluorescent protein
hCASMC	Human coronary artery smooth muscle cell
hr	Hour
HSPG	Heparan sulfate proteoglycan
ICAM-1	Intracellular adhesion molecule-1
IEL	Internal elastic lamina
IgA	Immunoglobulin A
IgCAMs	Immunoglobulin superfamily-related cell adhesion molecules
IgG	Immunogloubulin
ILGF1	Insulin like growth factor 1
IP	Immunoprecipitation
ITR	Inverted terminal repeats
Kan	Kanamycin
kDa	Kilodalton
LN	Laminin

LB	Luria-Bertani
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
MFI	Mean fluorescence intensity
MMPs	Matrix metalloproteinases
MT	Membrane-type MMPs
O.D	Optical density
ON	Osteopontin
p.Tyr	Phosphotyrosine
PBS	Phosphate buffer saline
PDGF-BB	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PFA	Paraformaldehyde
PI3 kinase	Phosphatidylinositol 3 kinase
PL	Poly-L-lysine
PRO	Proline rich sequences
PTCA	Percutaneous transluminal coronary angioplasty
PyK2	Proline-rich tyrosine kinase
RASMC	Rat artery smooth muscle cells
RGD	Arg-Gly-Asp
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis.
SDS	Sodium Dodecyl Sulphate
SFK	Src family kinase
SMC	Smooth muscle cell
SM	Smooth muscle
SPARC	Secreted protein, acidic and rich in cysteine
SUS	Suspension
TAE buffer	Tris acetic acid buffer
TBS	Tris buffer saline
TCK	LB agar plate containing tet, Cam, Kan
TE buffer	Tris/EDTA buffer
TGF-β	Transforming growth factor-β
TSP	Thrombospondin
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VN	Vitronectin
VSMC	Vascular smooth muscle cell
VSM	Vascular smooth muscle
WCL	Whole cell lysate

11.3 Publications

1. **Varadarajulu, J.,** Laser, M., Hupp, M., Wu, R. and Hauck, C.R. (2005) Targeting of αv integrins interferes with FAK activation and smooth muscle cell migration and invasion. *Biochem Biophys Res Commun*, **331**, 404-412.
2. Wu, R., Laser, M., Han, H., **Varadarajulu, J.,** Schuh, K., Hallhuber, M., Hu, K., Ertl, G., Hauck, CR., and Ritter, O. (2006) Fibroblast migration after myocardial infarction is regulated by transient SPARC expression. *J Mol Med* (In press).
3. **Varadarajulu, J.,** Laser, M., Strotmann, J., Joner, M. and Hauck, C.R. Adeno-associated virus mediated overexpression of FRNK interferes with SMC migration *in vitro* and neointima formation *in vivo.* (Manuscript in preparation).

11.4 Poster presentation

Adhesion meeting-podosomes-invadopodia-focal adhesions-Munich (Germany), April 28-30, 2005.

11.5 Curriculum vitae

Name: Jeeva Varadarajulu
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Education:

1989-1994	Government high school, Maganurpatti, Krishnagiri (Dt), Tamilnadu, India.
1994-1996	Mary immaculate girls higher secondary school, Tirupattur, Vellore (Dt), Tamilnadu, India.
1996-1999	Bachelor of Science (Microbiology), Sri Ramakrishna College of Arts and Science for women, Affiliated to Bharathiar university, Coimbatore, Coimbatore (Dt), Tamilnadu, India.
1999-2001	Master of Science (Applied Microbiology), K.S.R college of Arts & Science, Affiliated to Periyar University, Tiruchengode, Namakkal (Dt), Tamilnadu, India.
Since 2002 January	PhD student at the Center for Infectious Diseases, University of Würzburg, Würzburg, Germany. Supervisor: PD Dr.C.R.Hauck.