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Role of extracellular matrix in vascular smooth muscle cell behaviour and the identification of novel markers of cell phenotype.

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ROLE OF EXTRACELLULAR MATRIX IN VASCULAR SMOOTH MUSCLE CELL BEHAVIOUR AND THE IDENTIFICATION OF NOVEL MARKERS OF CELL PHENOTYPE.

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A thesis submitted for the degree of
Doctor of Philosophy
in the
Open University

Date of submission: September 1996
Date of award: 2 December 1996

ProQuest Number: C606757

ProQuest C606757

Section I ABSTRACT.

Increased synthesis and deposition of extracellular matrix proteins in the blood vessel wall are implicated in vascular disorders such as atherosclerosis, restenosis and hypertension. (Liau and Chan 1989). The increase in extracellular matrix proteins can be mostly attributed to smooth muscle cells (SMC's) within vascular lesions (Mecham *et al* 1987). It is presumed that modulation of SMC's from their normally quiescent, contractile phenotype to a proliferative synthetic phenotype results in increased synthesis of extracellular matrix proteins. Studies have also demonstrated that signals elicited from the extracellular matrix (ECM) may play a role in the regulation of this SMC phenotypic modulation (Yamamoto *et al* 1993; Hedin *et al* 1988). The mechanism by which ECM can alter the phenotypic state of SMC's is not well understood but clearly involves the induction of intracellular signals as a consequence of ECM ligand - cell surface integrin binding. These signals must subsequently exert downstream molecular events altering gene expression and ultimately cell phenotype.

The research project presented in this thesis examined the influence various extracellular matrix substrates have on vascular SMC behaviour *in vitro*. Initial observations demonstrated that SMC's cultured on different matrix substrates exhibit distinct morphological growth patterns. Functional differences in SMC proliferation and migration rates were also observed in response to seeding on different ECM surfaces.

Analysis of the expression levels of known SMC phenotypic protein markers between SMC's cultured on different matrix substrates did not reveal any significant differences in protein expression. Slight upregulation of Myosin Light Chain Kinase (MLCK)-210 kd isoform was observed in SMC's cultured on cellular fibronectin, Collagen III and Vitronectin substrates. The Meta-vinculin protein was upregulated in SMC's cultured on fibronectin coated substrates.

In order to identify altered gene expression patterns induced by ECM adhesion, SMC populations cultured on fibronectin coated plastic and SMC's grown on uncoated plastic were selected for analysis by the differential display technique. Differential display is a

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recently developed PCR based technique that allows the identification of differentially expressed genes between related cell populations (Liang and Pardee 1992). Much effort was spent optimising the differential display procedure to overcome such limitations as primer redundancy and high false positive selection rates. However, as a result of the persistence of false positives only one gene, meta-vinculin was confirmed as being differentially expressed between SMC populations cultured on fibronectin coated and uncoated tissue culture plastic surfaces.

It was concluded that the failure to identify a significant number of fibronectin modulated genes was probably a result of limitations in the differential display procedure as carried out in this study, and may also possibly be due to the existence of only very minor differences in phenotype between SMC's grown on plastic with or without fibronectin precoating.

Section II CONTENTS.

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Section III Abbreviations.

αSM	alpha Smooth Muscle specific isoform.
APS	Ammonium Persulphate.
bFGF	basic Fibroblast Growth Factor.
CDK	Cyclin Dependent Kinase.
c-FHC	complex - Familial Hypercholesterolemic.
CKI	Cyclin Dependent Kinase Inhibitor.
CMV	Cytomegalovirus.
CPM	Counts Per Minute
DAG	1,2 diacylglycerol.
DNA	Deoxyribonucleic Acid.
ECL	Enhanced Chemiluminesence.
ECM	Extracellular Matrix.
EDTA	Ethylenediamine-tetracetic acid.
EGF	Endothelial Growth Factor.
F-actin	Filamentous actin.
FAK	Focal Adhesion Kinase.
FCS	Fetal Calf Serum.
GRB2	Growth factor receptor binding protein 2.
GTP	Guanosine triphosphate.
HBSS	Hanks Balanced Salt Solution.
HGF	Hepatocyte Growth Factor (Scatter factor).
HUASMC's	Human Umbilical Artery Smooth Muscle Cells.
H:W	Ham's F12/Waymouth medium (50:50 mix).
IFN-γ	Interferon gamma.

IGF-1	Insulin-like Growth factor 1.
IL-1	Interleukin 1.
IL-6	Interleukin 6.
IP$_3$	Inositol 1,4,5 triphosphate.
LDL	Low Density Lipoproteins.
MAP	Mitogen activated protein.
M-CSF	Macrophage Colony Stimulating Factor.
MLCK	Myosin Light Chain Kinase.
NF-kB	Nuclear Factor kappa B.
PBS	Phosphate Buffered Saline.
PCNA	Proliferating Cell Nuclear Antigen.
PCR	Polymerase Chain Reaction.
PDGF	Platelet Derived Growth Factors.
PI-3 Kinase	PhosphatidylInositol 3-Kinase
PIP$_2$	Phosphatidylinositol 4, 5 bisphosphate.
PLC	Phospholipase C.
PKC	Protein Kinase C.
PTCA	Percutaneous Transluminal Coronary Angioplasty.
PTK	Protein tyrosine kinase.
PTP	Protein tyrosine phosphatase.
RGD	Arginine-Glycine-Asparagene cell binding domain.
RNA	Ribonucleic Acid.
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate.
SH2	src Homology 2 domain.

SMC's	Smooth Muscle Cells.
SSC	Sodium chloride (3M), Sodium citrate (0.3M) solution.
TEMED	Tetramethylethylenediamine.
TF	Tissue factor.
TFPI	Tissue Factor Pathway Inhibitor.
TGF-β	Transforming Growth Factor - beta.
TNFα	Tumour Necrosis Factor alpha.
TPA	12-O- tetradecanoyl-phorbal-13-acetate.
VLDL	Very Low Density Lipoproteins.
X-Gal	O-Nitrophenyl-β-D-Galactopyranoside (X-Gal).

Section IV INTRODUCTION.

IV.1 INCIDENCE OF ATHEROSCLEROSIS AND RESTENOSIS.

Atherosclerosis and its complications, such as myocardial infarction, stroke, and peripheral vascular disease, remain the major causes of morbidity and mortality in the western world. Atherosclerosis is a slowly progressive disease, which begins in early childhood, this together with its multifactorial nature has presented a major hurdle towards elucidation of the etiology and pathogenesis of the disease. However, in the past two decades cellular and molecular approaches to the study of the cells of the vascular wall have begun to provide numerous insights into the mechanisms involved in the pathogenesis of atherosclerosis (Munro and Cotran 1988).

Analysis of the cellular constituents in human atherosclerotic lesions have revealed an altered vascular histological pattern characterized by the presence of increased numbers of inflammatory and smooth muscle cells (SMC's). Such a pattern is similar to those observed at the sites of tissue injury. The disease was first likened to inflammation by Rayer in 1823, this concept was developed through the years culminating in the response to injury hypothesis which proposes that injury to the endothelium is the initiating factor in atherosclerosis. Injury to the endothelium in atherosclerosis is thought to arise by several mechanisms in response to chronically elevated serum levels of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) a condition known as hypercholesterolemia. Such injury has been found to increase endothelial plasma membrane viscosity which may promote the attachment of monocytes and platelets thereby initiating an inflammatory reaction (Ross 1986).

The above theory has claimed universal acceptance because it is supported by the identification of smoking, a high cholesterol diet, and genetic disorders impairing cholesterol metabolism all of which promote hypercholesterolemia, as major risk factors predisposing to atherosclerosis (Lusis and Sparkes 1989). The importance of endothelial injury is further exemplified by the related condition known as restenosis which develops following the balloon angioplasty procedure, carried out to dilate occluded atherosclerotic vessels. The ballooning

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procedure results in physical injury to the endothelium and the vessel wall and in 30-40% of cases this injury initiates an inflammatory response similar to that observed in the original atherosclerotic lesion which subsequently promotes excessive smooth muscle cell proliferation which is thought to contribute to reocclusion of the treated vessel (Shirotani *et al* 1993).

IV.2 PATHOGENESIS OF ATHEROSCLEROSIS.

Endothelial cells serve as a barrier between the blood supply and body tissues. Under normal conditions the endothelial cells perform a variety of critical functions including the transfer of metabolites between blood and tissues, the maintanence of thromboresistance and the regulation of vascular tone. During inflammatory reactions however, endothelial cells can modulate their behaviour to promote monocyte and lymphocyte adherance and subsequent leucocyte infiltration into tissue. Similarly during development endothelial cells become responsive to a variety of growth factor and cytokines and thereby regulate vascular growth (Munro and Cotran 1988).

It has been established from *in vitro* and *in vivo* studies that if endothelial cells are bathed in chronically elevated levels of LDL and VLDL, rapid cholesterol exchange will occur with the plasma membranes of the endothelial cells, leading to subtle alterations in cholesterol, phospholipid, and protein ratios, subsequently altering the viscosity of the plasma membranes of these cells and promoting increased attachment of monocytes to the vessel wall. (Alderson *et al* 1986; Edemann *et al* 1987). After monocytes have attached they release a variety of factors promoting endothelial dysfunction contributing to the localization of monocytes subendothelially. The adhered macrophages can damage the surrounding endothelium by releasing factors toxic for endothelial cells such as superoxide anions and oxidized LDL (Cathcart *et al* 1988). These and other macrophage derived growth factors and cytokines may also alter endothelial functions including, the loss of endothelial cell derived antithrombotic substances such as prostacyclin and heparin. This provides an opportunity for platelet adhesion to areas of connective tissue, thrombin and macrophages exposed by endothelial cell death, the adhered platelets subsequently degranulate releasing additional growth factors and cytokines into the area (Ross 1986).

These early events in atherosclerosis are responsible for the development of the initial atherosclerotic lesions, known as "fatty streaks", - these being macroscopically distinguishable,

cushion-like, whiteish, subendothelial intimal areas that are comprised of aggregations of lipid-rich macrophages and T-lymphocytes (Wick *et al* 1995). The development of fatty streaks occurs early in life. Fatty streaks can be found in the coronary arteries of half of the autopsy specimens from children aged 10-14 (Stary 1989). Most of these early lesions especially those in the aorta either disappear or remain harmless (Munro and Cotran 1988). In predisposed individuals, however, lesions at particular locations, quite often in coronary arteries are exposed to the continued release of activated endothelial as well as platelet and macrophage derived factors which include; b-thromboglobulin, vasoconstrictive amines, arachidonic acid metabolites, PDGF, EGF, FGF, TGFb, IL-1, IFN-γ and other less well characterized mitogens which can augment the initial inflammatory processes. The chronic generation of such a plethora of factors initiates phenotypic modulation of local SMC's from their normal contractile phenotype into a synthetic phenotype. This transformation into a synthetic phenotype is characterized by the induction of vascular SMC migration from their normal location in the vessel media towards the intima, where they undergo enhanced cell proliferation and overexpress a considerable amount of extracellular matrix macromolecules (ECM) contributing to the subendothelial formation of what is known as the neointima (Thyberg *et al* 1990) **(FIGIV.1)**.

FIGIV.1 - Initial Lesion Development.
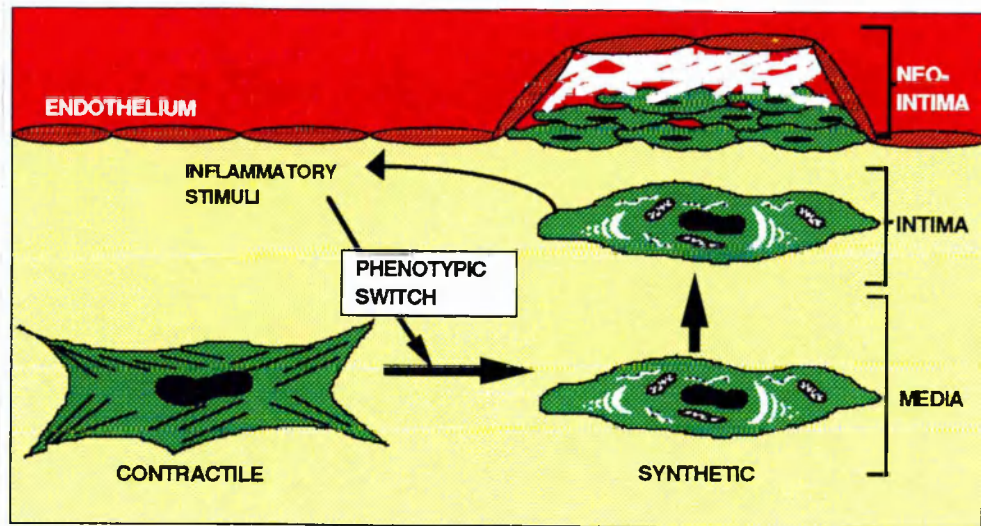
Progressive lipid accumulation by macrophages and SMC's also takes place resulting in foam cell development in these regions. Such events contribute to the progression of fatty streaks into the advanced atherosclerotic lesion known as the atheromatous plaque (Ross 1986; Clinton and Libby 1992). Although atherosclerotic lesions can be observed throughout the circulatory system, certain areas of the arterial tree are particularly prone to the development of lesions such as the aortic arch, the branching sites of larger vessels, as well as the carotid and coronary arteries (Wick et al 1995). These sites are typically subject to incresed haemodynamic stress which is thought to promote atherosclerotic lesion development (Sterpetti *et al* 1994).

The atheromatous plaque exhibits histologic variability, but a typical cellular plaque consists of a fibrous cap, composed mostly of smooth muscle cells, T lymphocytes and ECM molecules, a deeper necrotic core which contains cellular debris, extracellular lipid droplets, cholesterol crystals, and calcium deposits. This necrotic core often contains numerous large foam cells of both macrophage and SMC origin. Monocytes and macrophages are located at the margins of the lipid core. Around the periphery of lesions, evidence of neovascularization from the direction of the adventitia can sometimes be observed which may provide additional routes for

inflammatory cell infiltration thereby further contributing to plaque development (Munro and

Cotran 1988) **(FIG.IV.2)**.

FIG.IV.2 The Atherosclerotic Plaque.

CAP

Lipid co
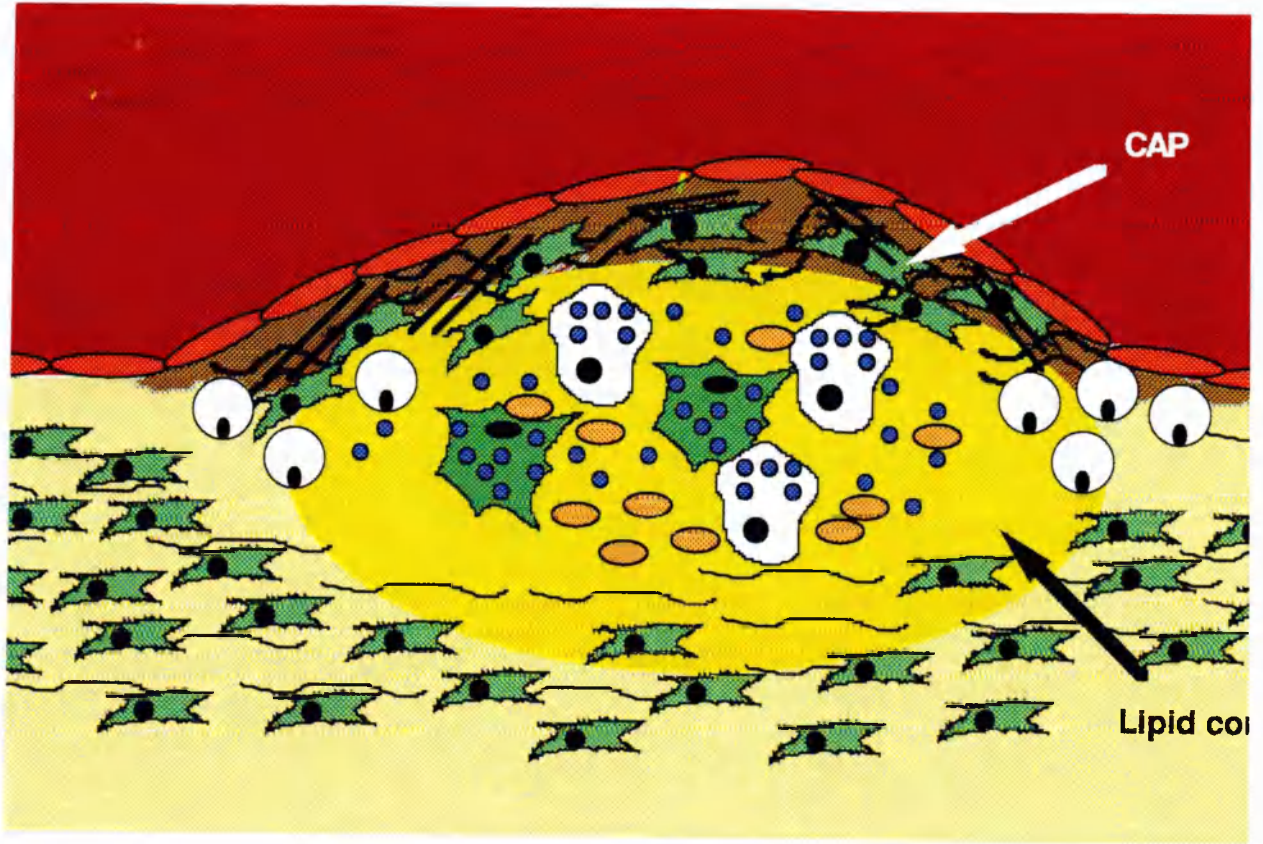
Endothelial cells -

Smooth Muscle Cells -

Monocytes -

Macrophage derived foam cells -

Smooth muscle derived foam cells -

Lipid droplets -

Cholesterol crystals -

ECM fibrils -

Recent pathological studies investigating disease progression in humans has revealed that if atherosclerotic plaques remain intact, the advanced lesion may only slightly occlude blood flow and may be of no significant consequence to the health of the affected individual. Data has shown that most of the sudden deaths from myocardial infarct are due to ruptures, particularly in the margins of the fibrous cap where there are more macrophages and possibly also more matrix metalloproteinase activity relative to ECM deposition taking place (Libby 1995). Cap rupture is often followed by haemorrhage into the plaque, thrombosis and occlussion of the artery (Davies and Thomas 1984). It therefore appears that the mechanical strength of the cap tissue is a critical determinant of morbidity induced by the atherosclerotic lesion. The mechanical strength of the plaque cap depends on the amount, and organization of the connective tissue matrix proteins. The principle proteinaceous constituent of this ECM include the interstitial collagens and elastin. Vascular SMC's are responsible for the synthesis and assembly of the bulk of both the collagenous and non collagenous macromolecules within such vessels (Libby 1995). Therefore, a loss of SMC synthetic activity would be expected in time to lead to degeneration and fragmentation of the connective tissue. Indeed histological examinations have revealed that unstable plaques prone to rupture contain a high macrophage to SMC ratio, whereas the more stable plaques contain a higher percentage of SMC's (Davies *et al* 1993).

IV.3   PATHOGENESIS OF RESTENOSIS.

Transluminal angioplasty was first introduced in 1964 as a non-surgical treatment for the dilation of arteries narrowed by atherosclerosis (Dotter and Judkins 1964). Percutaneous Transluminal Coronary Angioplasty (PTCA) is now the preferred treatment for re-dilation of occluded coronary arteries. The procedure utilises a balloon catheter that is positioned at the site of arterial stenosis. Balloon inflation causes endothelial denudation, disruption of the atherosclerotic plaque, dehiscence of the intima and plaque from the underlying media, and stretching and tearing of the media and adventitia, with resultant vessel dilation (Steele *et al* 1985). The chief limitation of successful PTCA is reocurrence of the stenosis, or restenosis. Restenosis is most commonly defined as; greater than a 50 % narrowing of the diameter of the lumen at the site of a previously successful PTCA. Restenosis occurs in about one third of patients in whom a narrowed artery has been dilated and in about 60% of those in whom a chronically occluded artery has been dilated. It typically occurs one to three months after PTCA (Landau *et al* 1994).

The process of restenosis is initiated by endothelial and deep vessel injury resulting from the ballooning procedure. This leads to platelet aggregation, thrombus formation, inflammation, and activation of macrophages and smooth muscle cells. These events induce the production and release of growth factors and cytokines, which results in the transformation of SMC's into a synthetic phenotype and subsequent migration of SMC's from the arterial media to the intima where they proliferate and produce extracellular matrix, thereby resulting in a stenosis of the vessel lumen (Shirotani *et al* 1993).

Studies carried out on the rat carotid balloon injury model have facillitated the kinetic analysis of the vascular SMC response. These studies have led to the description of four waves defining the SMC response to vascular injury (Friedman et al 1977; Clowes and Schwartz 1985; Hanke *et al* 1990). The first wave consists of medial SMC proliferation and is initiated 24 hours

after vessel injury. The release of bFGF from injured SMC's is thought to be a driving force behind this proliferation. (Linder and Reidy 1991; Olson *et al* 1992). The migration of SMC's across the internal elastic lamina to form the intima constitutes the "second wave" (Clowes *et al* 1983). The time period covering SMC migration is unclear. Several molecules in addition to PDGF including TGFβ, bFGF and angiotensin II may contribute to stimulating SMC migration. Once smooth muscle cells arrive in the intima of the rat artery, they may replicate for weeks to months. This replication is called the "third wave". The neointima can be stimulated to show a further increase in replication as a result of increased responsiveness to mitogens, this is known as the "fourth wave" (Schwartz *et al* 1995).

Although restenosis and atherosclerosis are initiated by common inflammatory mechanisms the difference in time course of disease progression and the processes ultimately leading to morbidity indicate the importance that separate approaches need to be taken towards the development of therapeutic treatments for each condition. For instance it may not be beneficial to target the prevention of SMC phenotypic modulation, migration and proliferation as a therapeutic intervention against primary atherosclerosis since these events can occur as early as the age of 5 years, long before atherosclerosis becomes a clinical problem. In contrast SMC migration, proliferation and phenotypic modulation are directly involved in the development of restenosis and they may be very appropriate targets for therapeutic intervention. Restenosis is responsible for the requirement of 185,000 additional coronary angioplasty operations every year in the United States alone (Landau *et al* 1994). This condition has also recently been identified as a characteristic feature within vessels of transplanted organs as well as in saphenous vein and coronary bypass grafts, brought about by the induction of immune responses elicited in response to foreign antigens or injury caused by surgery (Cox *et al* 1991). Inhibition of SMC phenotypic modulation therefore has important therapeutic potential for the prevention of restenosis.

IV.4 PHENOTYPIC MODULATION OF SMOOTH MUSCLE CELLS.

It has become increasingly evident that vascular SMC's occur in at least two distinct states, referred to as the contractile and synthetic phenotypes (Thyberg *et al* 1990). Contractile SMC's are so named because they have abundant myofilaments and will contract in response to chemical and mechanical stimuli. They are the normal SMC type found in adults. Synthetic SMC's obtained their name because of the extensive distribution of synthetic organelles including a prominent rough endoplasmic reticulum and Golgi complex within their cytoplasm. Synthetic SMC's are characteristic of developing fetal arteries as well as representing the predominant SMC phenotype found within atherosclerotic and restenoic lesions (Campbell *et al* 1988).

Thus it can be assumed that the normal development of an artery involves SMC differentiation from a synthetic state into a contractile state. Reversal of this process, "phenotypic modulation", occurs in situations such as arterial repair associated with the development of atherosclerosis and restenosis (Thyberg *et al* 1990).

The above observations suggest that mechanisms governing normal cell differentiation may play a major role in lesion development. The mechanisms that control the process of SMC differentiation are largely unknown, but are thought to involve the induction of intracellular signalling pathways initiated by hormone release as well as signals arising from the surrounding extracellular matrix and mesenchymal cell population. The generation of these signals is believed to regulate gene expression through transcriptional control involving the activity of DNA binding proteins as well as through post-transcriptional modifications, thereby regulating the type of proteins produced which subsequently determines cell phenotype (Alberts *et al* 1989). Elucidation of these molecular mechanisms may identify therapeutic targets beneficial in the prevention of lesion development.

A variety of factors have been identified which induce SMC phenotypic modulation including native and oxidized LDL (Rahm *et al* 1992), extracellular matrix components,

fibronectin (Hedin *et al* 1988), and type I collagen (Yamamoto *et al* 1993). A variety of inflammatory cytokines and growth factors are thought to act synergistically in promoting the synthetic phenotype. These factors include PDGF which, in addition to promoting SMC migration and proliferation, can also promote phenotypic modulation via the regulation of fibronectin dependent adhesion (Fujio *et al* 1993). TGF-β which can influence multiple SMC functions including the regulation of SMC proliferation and the induction of extracellular matrix production (Lawrence *et al* 1994). Macrophage derived prostaglandins and leukotrienes have also been shown to promote the synthetic phenotype (Sjolund *et al* 1984; Palmberg 1989). Thus in the reponse to injury hypothesis (Ross 1986), alterations in the extracellular signals as a result of endothelial injury would promote SMC phenotypic modulation **(FIG.IV.3.)**.

FIG.IV.3 - The Response to Injury hypothesis.

Isolated contractile SMC's revert back into the synthetic phenotype when they are established in cell culture. This transition has facillitated the identification of protein markers that are specific for the synthetic phenotype. Those identified so far include decreased expression of cytoskeletal proteins such as α SM actin (Liau *et al* 1990; Piallat *et al* 1993), smooth muscle myosin heavy chains and increased expression of non-muscle myosin heavy chains (Aikawa *et al* 1993; Grainger *et al* 1991); decreased expression of desmin (Osborn *et al* 1987); and induced expression of low levels of cytokeratins (Bader *et al* 1988). The expression of the cytoskeletal associated protein metavinculin, which normally localizes at focal adhesion sites decreases (Glukhova *et al* 1986). The expression of the actin and calmodulin binding proteins calponin and caldesmon are also altered during phenotypic modulation with cessation of calponin expression (Gimona *et al* 1990) and a switch in expression from the high molecular weight form of caldesmon to the low molecular weight form (Ueki *et al* 1987). Decreased expression of the actin binding high molecular weight α SM tropomyosin isoforms can also be observed in synthetic SMC's (Chamley-Campbell *et al* 1977). Up-regulation in the expression of osteopontin and matrix gla proteins can also be observed, these proteins normally play a role in bone mineralization and therefore may be responsible for the calcification commonly observed within atherosclerotic and restenoic lesions (Giachelli *et al* 1993; Shanahan *et al* 1993).

The characterization of protein markers specific for the SMC synthetic phenotype may allow the identification of such cells prior to lesion development as well as provide insights into the altered functions of these cells. This information could prove useful in disease prognosis and therapy. It has also been demonstrated that spontaneous phenotypic modulation of isolated SMC's is prevented if the cells are grown in co-culture with a confluent monolayer of endothelial cells (Campbell *et al* 1981; Campbell *et al* 1986). These results suggest that the endothelial cells release a factor or factors that help maintain SMC's in the contractile phenotype; loss of these factors during endothelial injury or following endothelial denudation would therefore assist phenotypic modulation.

IV.5 PROLIFERATION OF SMOOTH MUSCLE CELLS.

The significance of SMC proliferation in the development of atherosclerotic lesions has recently led to some controversy. Cell kinetic studies measuring either the frequency of SMC's able to incorporate labelled thymidine *ex vivo* or the number of SMC's identified as replicative on the basis of staining for a replicative marker such as the proliferating cell nuclear antigen (PCNA) (Gordon *et al* 1990; O'Brien *et al* 1993) indicates that SMC proliferation in atherosclerotic and restenotic lesions occurs infrequently or at low levels. These observations have led to the suggestion that the lumen narrowing associated with atherosclerotic and restenotic lesion formation may result mainly from vascular remodelling, elastic recoil, thrombosis and ECM deposition rather than SMC proliferation (Schwartz *et al* 1995; O'Brien *et al* 1993). Evidence supporting a role for SMC proliferation in atherosclerotic lesion formation includes observations that lesions are monoclonal or oligoclonal (Benditt and Benditt 1973). This implies that SMC proliferation must occur during the formation of the lesion and that the initial group of cells giving rise to the lesion must be very small (Schwartz *et al* 1995). Also an expansion in intimal smooth muscle mass has been described in various human lesions (Velican and Velican 1976; Sims *et al* 1989.). All of this evidence suggests that SMC proliferation must occur very early in lesion development either at a very low rate over several years or at a high rate in an episodic fashion (Schwartz *et al* 1995).

The importance of SMC proliferation in the development of atherosclerosis and restenosis is further emphasized by the observation that a major consequence of SMC phenotypic modulation observed is an increase in the rate of cell proliferation (Clowes and Schwartz 1985). Induction of this proliferation is mediated through the activation of cell surface receptors by inflammatory growth factors and cytokines, released during early lesion development. Studies have revealed that phenotypically modified SMC's exhibit an increased capacity to respond to such factors through the expression of novel or increased numbers of growth factor receptors. For

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example during transformation into the synthetic phenotype the transcription of PDGF and EGF receptors is upregulated (Pickering *et al* 1993; Rahm *et al* 1992). Synthetic SMC's also express the macrophage colony stimulating factor (M-CSF) receptor (c-*fms*), which is not normally expressed in contractile SMC's.

Activation of these growth factor receptors initiates the transduction of intracellular signals from the cell membrane into the cell nucleus, subsequently altering the balance of cell cycle control elements promoting the initiation of cell cycle progression required for cell proliferation. The progression of all eukaryotic cells through the cell cycle is governed by the sequential formation, activation, and subsequent inactivation of a series of cyclin cyclin-dependent kinase (CDK) complexes (Hunter 1993). Specific cyclins interact with specific CDK's and these different complexes each regulate progression through particular stages of the cell cycle. Thus progression from G1 to S phase involves CDK4/cyclin D complexes; S phase, CDK2/cyclin A, and M phase CDC 2/cyclin B (Nasmyth and Hunt 1993). The control of cell cycle progression is probably mediated by the cyclin dependent kinase inhibitors (CKI's) which tightly regulate the activity of cyclin/CDK complexes. Evidence exists suggesting that expression of CKI's is positively regulated by tumour suppressor proteins such as p53 (El-Diery *et al* 1993). This observation has led to the hypothesis that loss of tumour suppressor gene expression may subsequently decrease CKI gene expression, increasing the activity of G1 cyclin/CDK complexes that are required for progression into S phase. This hypothesis would account for the induction of uncontrolled cell growth associated with loss of tumour suppressor function (Hunter 1993). Such a theory is also relevant to the initiation of SMC proliferation accompanying restenosis as recent evidence indicates that induction of latent CMV infection within SMC's in response to the angioplasty procedure initiates the production of proteins that bind to and inactivate p53. This would result in decreased CKI gene expression, thereby inducing uncontrolled cell growth (Speir *et al* 1994). This mechanism also provides an explanation for the observation that virus infection is another possible risk factor contributing to the development of atherosclerosis (Legrand *et al* 1991).

Studies have also implicated a role for mitogen stimulated proto-oncogene expression in the control of signal transduction pathways leading to cell division, both c-*myb* and c-*myc* mRNA expression are upregulated during serum stimulation of SMC's (Rosenberg *et al* 1992; Biro *et al* 1993).

Many factors that are mitogenic for SMC's have been identified; these include: PDGF (Hedin *et al* 1988; Clemmons 1984); IGF-1 (Ferns *et al* 1991); IL-1 (Libby *et al* 1988; Porreca *et al* 1993); IL-6 (Seino *et al* 1994); EGF (Scott-Burden et al 1989; Pickering et al 1993); FGF (Schollman *et al* 1992; Salmivirta *et al* 1992); Thrombospondin (Majack *et al* 1988). TGF-β has been reported to both stimulate (Chen *et al* 1987) and inhibit SMC proliferation (Owens *et al* 1988; Grainger *et al* 1994). The mechanism for the inhibition of proliferation may be mediated by induction of CKI's (Hunter 1993). Various vasoactive hormones such as epinephrine/norepinephrine, serotonin, substance P and substance K as well as endothelin have been demonstrated to have mitogenic effects on SMC's (Thyberg *et al* 1990). Prostaglandins and leukotrienes can also stimulate SMC proliferation (Hajjar and Pomerantz 1992). All of these factors can be found at significantly higher concentrations than normal within atherosclerotic and restenoic lesions.

IV.6 MIGRATION OF SMOOTH MUSCLE CELLS.

The formation of a neointima within vessels is a critical event in the pathogenesis of atherosclerosis and restenosis (Clowes and Shwartz 1985). Neointimal formation results from the accumulation of SMC's on the intimal surface of vessels. This accumulation is due to a combination of enhanced proliferation and directed migration of SMC's from the media to the intima (Bornfeldt *et al* 1994). Both of these activities can be stimulated by a number of cytokines and growth factors produced within the lesion, often by modulated SMC's themselves, giving rise to autocrine stimulation, or they may be derived from circulating cells and platelets.

Chemotactic factors so far identified for SMC's include PDGF, the most potent chemotactic factor identified to date (Ferns *et al* 1990), EGF (Higashiyama *et al* 1993), IGF-1 (Bornfeldt *et al* 1994), IL-1 (Thiery and Boyer 1992), TGF-β (Bell and Madri 1989), fibrinogen (Naito *et al* 1989), and oxidized LDL (Autio *et al* 1990). The precise mechanisms responsible for growth factor induced chemotaxis of SMC's is not clear. However, evidence that most of the receptors for the above chemotactic factors belong to the protein tyrosine kinase receptor family leads to reasonable speculation that the induction of intracellular signals by tyrosine kinase activity is fundamental to initiation of SMC motility (Shimokado *et al* 1994).

Ligand binding to tyrosine kinase receptors stimulates receptor dimerization and tyrosine autophosphorylation, this subsequently leads to activation of phospholipase C (PLC), probably through the association of SH2 domains in PLC with specific phosphorylated tyrosine residues in the carboxyl terminal portion of the tyrosine kinase receptor. PLC activation mediates the hydrolysis of the plasma membrane phospholipid, phosphatidylinositol 4, 5 bisphosphate (PIP_2) into two products with intracellular second-messenger functions; inositol 1,4,5 trisphosphate (IP_3), which induces the release of calcium ions from internal stores and 1,2 diacylglycerol (DAG), which serves as a cofactor in the activation of protein kinase C (PkC) (Clyman et al 1994). The

phosphorylated tyrosine residues in the carboxyl terminal portion of the receptor can also interact again through SH2 domains with other intracellular proteins such as; GTPase activating protein, PI-3- kinase, Nck, GRB2, syp and the GTP binding proteins rac and rho, generating a variety of intracellular signalling cascades.

PhosphatidylInositol 3-kinase (PI 3-K) is an intracellular enzyme that phosphorylates the D-3 position of the inositol ring of phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate (Kapeller and Cantley 1994). PI 3-K is composed of two subunits, p110 and p85. The p110 subunit is the catalytic subunit that contains a lipid kinase domain. The p85 subunit can interact with the cytopasmic domains of receptors (Pawson 1995). p85 also contains proline rich sequences capable of mediating the binding of proteins containing SH3 domains such as the intracellular tyrosine kinases *abl, lck, fyn,* and *lyn.* The p85 subunit itself contains an SH3 domain, and *in vitro* studies have demonstrated SH3-dependent binding of p85 to proline rich sequences in the motor protein dynamin (Gout *et al* 1993). PI 3-K has also recently been demonstrated to play a role in the regulation of β1 integrin activity (Shimizu *et al* 1995). Thus, the p85 subunit of PI 3-K contains several structural motifs that allow PI 3-K to be coupled to a multitude of cell surface and intracellular molecules; for this reason it appears that a wide variety of cellular functions including mitogenesis and migration may be dependent on PI 3-K activity.

The transduction of these signals to the genes controlling cell motility are believed to induce cytoskeletal reorganization but the precise molecular mechanisms are unknown. However, it has been postulated that the induction of profilin release and a decrease in the expression of actin binding proteins alters F-actin polymerization and cross-linking respectively, thereby promoting changes in cell shape and subsequent migration (Machesky and Pollard 1993; Lee *et al* 1993).

Extracellular matrix plays a major role in controlling the migration of most cell types. Studies *in vitro* have revealed that SMC migration is enhanced on surfaces coated with gelatin (Autio *et al* 1990), fibronectin (Higashiyama *et al* 1993), type IV collagen (Dimilla *et al* 1993)

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and type V collagen (Shimokado *et al* 1994). The activity of the SMC chemotactic factors TGF-β, EGF and to some extent PDGF are mediated through modulation of extracellular matrix; TGF-β is thought to promote SMC migration by increasing the expression of various extracellular matrix components such as types I, III and V collagens (Lawrence *et al* 1994; Ritzenhaler *et al* 1993), fibronectin (Ignotz and Massaque 1986), elastin (Liu and Davidson 1988) and proteoglycans (Chen *et al* 1987). TGF-β also increases the expression of integrin receptors on the SMC surface that mediate cellular interactions with the extracellular matrix. In addition this cytokine represses the synthesis of extracellular matrix degrading proteases and induces the expression of inhibitors for such proteases all of which can regulate cell migration (Streuli *et al* 1993). The binding of EGF to its receptor and subsequent stimulation of SMC migration is enhanced when EGF is bound to heparan sulphate proteoglycans present in the extracellular matrix (Higashiyama *et al* 1993). One of the many effects PDGF confers upon SMC's is reorganization of integrin distribution, this was found to reduce the attachment strength of cells to fibronectin, thereby assisting migration (Fujio *et al* 1993).

For the majority of cells to migrate *in vivo* they need to be able to degrade surrounding extracellular matrix barriers in order to reach their target destinations. Proteolytic enzymes from all four classes of proteases have been implicated in this extracellular matrix turnover. Two main groups of enzymes; the plasmin/plasminogen activator system of serine proteases and the matrix metalloproteinases most likely play a role in assisting SMC migration through the vessel media (Grobmyer *et al* 1993). Plasmin can degrade extracellular matrix components directly (Liotta *et al* 1981) or through the activation of matrix metalloproteinases (Kleiner and Stevenson 1993). Plasmin is formed by the actions of tissue-type and urokinase-type plasminogen activators, the release of plasminogen activators from macrophages, endothelial and SMC's within atherosclerotic and restenoic lesions maybe an important regulator of SMC migration (Grobmyer *et al* 1993). Evidence also suggests that degradation of fibronectin, collagen and elastin by the action of

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proteolytic enzymes generates fragments of these matrix molecules that can act as chemotactic factors further assisting cell migration through the matrix (Wong and Wahl 1990).

The elucidation of the intracellular signalling mechanisms controlling SMC migration may identify therapeutic targets that can inhibit this migration, but it is clear from the literature that there are many factors within lesions that contribute to SMC migration, acting through mechanisms which are not fully understood. Further research into all such mechanisms will be required before efficient therapeutic strategies directed against SMC migration can be developed.

IV.7 EXTRACELLULAR MATRIX INDUCED MODULATION OF SMC BEHAVIOUR.

The vascular extracellular matrix (ECM) is a reinforced composite of collagen and elastic fibres embedded in a viscoelastic gel consisting of proteogycans, hyaluron, glycoproteins, and water. These components interact to form a biomechanically active polymer network that imparts tensile strength, elastic recoil, compressibility, and viscoelasticity to the vascular wall. In addition this network interacts with vascular cells and participates in the regulation of cell adhesion, migration and proliferation during vascular development and disease (Fuster *et al* 1996).

The pathogenesis of atherosclerosis and restenosis includes the abnormal production of extracellular matrix (ECM) proteins by neointimal SMC's (Liptay *et al* 1993). Several factors secreted by inflammatory macrophages within lesions may be responsible for stimulating ECM synthesis; TGF-β has been shown to stimulate the production of fibronectin (Ignotz *et al* 1987) and collagen (Lawrence *et al* 1994; Ritzentaler *et al* 1993); IGF stimulates the synthesis of type I collagen (McCarthy *et al* 1989). IL-1 and TNF also induce type I collagen synthesis (Kahari *et al* 1987; Goldring and Krane 1987), PDGF, EGF and IFN-γ have all been demonstrated to stimulate fibronectin synthesis (Blatti *et al* 1988, Francesci *et al* 1987). Thus macrophages may contribute to the formation and progression of atherosclerotic and restenoic lesions by inducing neointimal ECM production possibly via the induction of SMC phenotypic modulation. This is supported by *in vitro* studies which demonstrate that SMC's transformed into the synthetic phenotype synthesize 25-30 times more collagen than contractile cells. Non-collagen protein synthesis increased only 5-6 fold indicating a specific stimulation of collagen synthesis. Phenotypic modulation was also associated with an alteration in the relative proportions of types I and III collagens synthesized, with contractile SMC's synthesizing 78% type I collagen and 17% type III collagen, and synthetic cells synthesizing 90 % type I collagen and 5% type III collagen (Ang *et al*

1990; Liau and Chan 1989). A similar enrichment of type I collagen has been reported to occur *in vivo* within atherosclerotic lesions (Hanson and Bentley 1983).

The accumulation of ECM components within lesions not only contributes to vessel stenosis by physically retarding blood flow, it is also known to have a significant influence on regulating local SMC behaviour. Studies investigating the phenotypic modulation of SMC's *in vitro* have revealed that the matrix components fibronectin and collagen promote transition of cells into the synthetic phenotype (Hedin *et al* 1988). In contrast elastin and laminin maintained the cells in the contractile phenotype (Yamamoto *et al* 1993; Hedin *et al* 1988). The ability of ECM to induce changes in cell differentiation and/or gene expression of a variety of cells has been well documented. Fibronectin decreases the expression of α-SM actin in SMC's (Hedin *et al* 1990). In fibroblasts fibronectin peptides induced collagenase and stromelysin gene expression (Werb *et al* 1989), and a variety of genes involved in inflammation are induced as a result of monocyte adherence to ECM (Sporn *et al* 1990).

Cells respond to ECM through the interaction of cell surface adhesion receptors known as integrins, with specific recognition domains within matrix such as the arginine-glycine-aspartate (RGD) sequence which functions as a cell attachment site for a number of different ECM proteins, including fibronectin, thrombospondin, fibrinogen, von Willebrand factor and vitronectin (Kreis and Vale 1993). Studies have revealed that these interactions are not specific, individual integrins can often bind more than one ECM ligand. Equally, individual ECM ligands are recognised by more than one integrin (Hynes 1992).

The precise mechanisms whereby signals from the ECM are transduced via integrins to the intracellular machinery that controls SMC growth, migration, and differentiation, remains poorly defined (Juliano and Haskill 1993). However, it has been demonstrated that all integrins are alpha/beta heterodimers and their ligation by ECM ligands is followed by cytoskeletal reorganization such as the formation of focal adhesions. It seems reasonable that appropriate cell shape and cytoskeletal organization might regulate the biosynthetic capabilities of the SMC's and

thus contribute to cell growth or differentiation. The formation of focal adhesions has provided a model to explain ECM signalling to the cytoskeleton. Focal adhesions are found primarily at cell borders where bundles of actin filaments terminate, and contain a large number of different proteins that link the cytoplasmic domains of integrin receptors to the actin filament system. The formation of focal adhesions is regulated in migrating and mitotic cells indicating their importance in such functions. Current evidence overwhelmingly points to the integrin β-subunit cytoplasmic domain as the structure that targets integrins to focal adhesions (Sastry and Horwitz 1993). Indeed it was recently reported by Carol Otley that the β subunits contain binding sites for many of the focal adhesion associated proteins such as α-actinin, talin and focal adhesion kinase (FAK) (BSCB newsletter June 1994).

Integrin stimulated tyrosine phosphorylation of FAK has been observed in fibroblasts adhered to fibronectin. Since FAK is known to be a substrate for *src* family kinases (Kanner *et al* 1990), it is tempting to postulate that an interplay of src and FAK may be directly involved in integrin mediated signal transduction from the ECM. It has been hypothesized that integrin clustering induced by ECM ligand binding induces the cross-phosphorylation of adjacent FAK molecules, which could subsequently alter the phosphorylation and binding states of the other cytoskeletal associated proteins within focal adhesions including, paxillin, tensin, vinculin, talin and actinin as well as the GTP binding protein rho (Kanner *et al* 1990). Such events promote cytoskeletal reorganization that accompanies the induction of SMC proliferation and migration (Damsky and Werb 1992). It also seems likely that integrin-mediated activation of FAK is an early step in the signal transduction cascade that is responsible for the ECM induced SMC phenotypic modulation thought to occur within atherosclerotic and restenotic lesions **(FIG.IV.4)**.

FIG.IV.4 ECM Induced Cell Signalling.
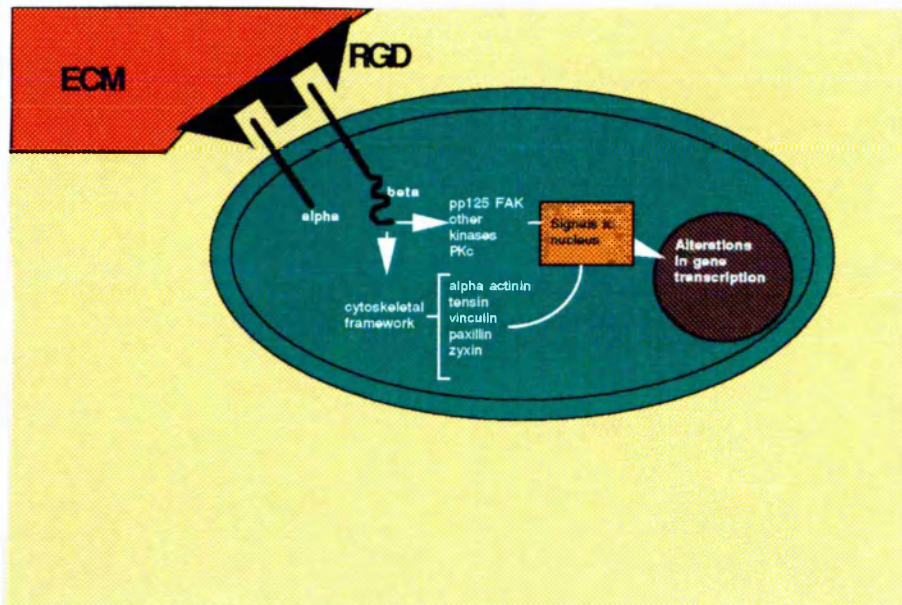
Events downstream of FAK that are responsible for the regulation of gene expression remain almost totally unknown. However, it has been demonstrated that translocation of enhanced levels of Nuclear Factor kappa B (NF-kB) into the nucleus occurs after adhesion of human monocytes. This translocation is thought to be mediated by integrin dependent kinase events and would explain the altered gene expression induced upon monocyte adhesion (Griffin *et al* 1993). It is tempting to place the increased tyrosine kinase activity of FAK in a cause and effect relationship with integrin induced gene expression, within SMC's by a similar mechanism. At this point, however, there is no current direct evidence that would implicate FAK activation with integrin induced gene expression (Juliano and Haskill 1993).

An alternative hypothesis on the mechanisms of integrin mediated signalling is based on the capacity of integrins to act as true receptors giving rise directly to biochemical signals within the cell. Supporting evidence for such a role comes from the observation that the $\beta 1$ integrin is tyrosine phosphorylated in transformed fibroblasts and this may account for the inability of these cells to reorganize their cytoskeleton in response to ECM signals (Damsky and Werb 1992). It is

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likely that these two views on the mechanisms of integrin-mediated signalling will turn out to be complementary rather than mutually exclusive.

Such signalling events, induced by the ECM which subsequently alter cell behaviour are termed outside-in signalling. There is also evidence that inside-out signalling can occur which ultimately controls how a cell behaves toward signals generated by the ECM. These signals arising from the cytoplasm probably act by modulating the affinity and/or specificity of integrins for their ECM ligands. These changes in affinity appear to arise from conformational changes in the integrin receptor (Sastry and Horwitz 1993). For instance the integrin $\beta 1$ subunit cytoplasmic domain becomes phosphorylated during mitosis. This modification reduces the affinity of the $\alpha 5$ $\beta 1$ receptor for fibronectin and could provide an inside-out signal that accounts for cell rounding associated with the progression of mitosis (Damsky and Werb 1992). A similar mechanism reducing affinity with ECM may also promote migration (Dimilla *et al* 1993). It is possible that the induction of proliferation and migration by phenotypically modified SMC's is mediated by these inside-out signals.

There is also evidence that the ECM can influence SMC behaviour indirectly by regulating the concentration and delivery of growth factors within lesions. As stated previously heparan sulphate proteoglycans, many of which are constituents of ECM, can function as modulators of growth factors including the SMC mitogenic factors FGF and EGF (Ruoslahti and Yamaguchi 1991). The binding of FGF to proteoglycans deposited on the cell surface is thought to alter the conformation of the growth factor facilitating the interaction with its specific cell surface receptor (Yayon *et al* 1991). The binding of growth factors to the ECM provides a reservoir of growth factor activity that could be released upon ECM degradation induced by the synthesis and release of ECM degrading enzymes (Ruoslahti and Yamaguchi 1991). The synthesis of such proteolytic enzymes by SMC's and macrophages is commonly observed within atherosclerotic and restenoic lesions (Liptay *et al* 1993). The degradation products of fibronectin, elastin and collagen resulting from ECM proteolysis have chemotactic properties that may contribute to the migration of SMC's

towards the vessel intima as well as promote macrophage influx into these areas, thereby contributing further to lesion development (Liptay *et al* 1993 and Wong and Wahl 1990).

It is clear that the ECM can modulate cell behaviour through multiple mechanisms. The construction of peptide fragments and monoclonal antibodies that can block or induce integrin receptors on SMC's may help elucidate some of the mechanisms that are involved in the development of atherosclerosis and restenosis.

The starting point of the work carried out in this study shall concentrate on investigating the influence ECM has on vascular SMC behaviour, in particular the role fibronectin plays in modulating SMC behaviour. Fibronectin is a 500Kd multifunctional adhesive glycoprotein found in the blood plasma and extracellular matrices. It consists of two subunit chains linked by disulphide bonds close to their carboxy terminal ends (Potts and Campbell 1994).

Fibronectin can bind to Smooth muscle cells, fibroblasts, monocytes and many other cell types. Fibronectin can also bind to collagens, heparin, and fibrin and so mediate the attachment and spreading of cells on a variety of substrates (Hynes 1985; Ruoslahti *et al* 1985; Yamada *et al* 1985). Fibronectin has been demonstrated to regulate the adhesion and migration of activated cells during acute and chronic inflammation (Springer 1990; Molossi *et al* 1995), wound healing (Grinnel and Phan 1983), tissue regeneration (Springer 1990) and tumour metastasis (McCarthy *et al* 1985).

Multiple cell binding sites have been characterised within the fibronectin molecule, including the RGD sequence, located within the central region of fibronectin and the CS1 sequence, at the carboxy-terminal end (Ruoslahti *et al* 1981; Yamada 1991). The primary cell surface receptors that recognise these sites are the $\alpha5\beta1$ integrin that recognises the RGD site and the $\alpha4\beta1$ integrin that binds the CS1 site.

The cell's response to fibronectin can be regulated by patterns of alternative splicing of the fibronectin transcript. Alternative splicing of fibronectin can involve the CS1 site and

generates a number of functionally different fibronectin isoforms (Kornblihtt *et al* 1985; Ffrench-constant 1995). The addition of type III repeats EIIIA and EIIIB by alternative splicing distinguishes cellular forms of fibronectin from plasma fibronectin (Hershberger and Culp 1990). Previous studies suggest that EIIIA and EIIIB containing fibronectin mRNAs predominate in the early embryo, whereas in the adult most of the normal tissue fibronectin lacks these domains (Oyama *et al* 1989; Ffrench-Constant and Hynes 1989; Pagani *et al* 1991). A recent study also revealed that alternatively spliced forms of fibronectin EIIIB and EIIIA were upregulated at 24-48 hours following balloon injury of rabbit aorta and illiac arteries (Bauters *et al* 1994). The appearance of the specific isoform containing the EIIIA domain has been described both in balloon injured intima from rat aorta and in atherosclerotic plaques from human arteries (Glukhova *et al* 1989). In addition to type III alternatively spliced fibronectins other fibronectin isoforms may be upregulated early in atherosclerotic lesions (Orekhov *et al* 1984; Shekonin *et al* 1987).

It has also recently been demonstrated that SMC attatchment to fibronectin resulted in activation of the p50/p65 heterodimeric form of NF-kB. NF-kB is a transcription factor that plays an important role in the inducible regulation of a variety of genes involved in the inflammatory and proliferative responses of cells. Using immunofluoresence and immunohistochemical techniques, activated NF-kB was detected in the fibrotic thickened intima/media and atheromatous areas of the atherosclerotic plaque. Activation of NF-kB was identified in SMC's, macrophages and endothelial cells. Little or no activated NF-kB was detected in vessels lacking atherosclerosis (Brand *et al* 1996). The evidence described above, together with the observation that fibronectin promotes modulation of cultured rat arterial SMC's from a contractile to a synthetic phenotype (Hedin *et al* 1988), suggests that altered expression of fibronectin plays a significant role in the pathogenesis of atherosclerosis and angioplasty induced restenosis.

IV.8 FUTURE PROSPECTS FOR THERAPY.

It appears that a critical step in the development of all atherosclerotic and restenotic lesions is the phenotypic modulation of SMC's into the synthetic phenotype (Thyberg *et al* 1990). This is a process that has attracted most attention with regards to the development of an effective therapeutic strategy against atherosclerosis and restenosis. However as stated earlier, recent pathological evidence indicates that SMC phenotypic modulation within advanced atherosclerotic lesions may promote a beneficial clinical outcome, whereas in restenosis this phenotypic modulation has a deleterious effect (Davies *et al* 1993). It would, however, appear that therapy targeted against SMC phenotypic modulation would inhibit initial lesion development of both disorders. The prevention of the early atherosclerotic lesions (fatty streaks) by targeting SMC phenotypic modulation may not be practical, as these lesions develop in all children by the age of 5 and have no serious consequences towards health until much later in life. Such therapy would however prove useful against the development of restenosis.

Recent insights into the biological mechanisms responsible for the development of atherosclerotic and restenoic lesions have identified targets for potential therapeutic exploitation. Platelets have a key role in atherosclerosis, restenosis, thrombosis and acute coronary syndromes. Platelets contribute towards lesion development, by releasing growth factors from their alpha granules upon attachment to the vessel wall. Therapeutic manipulation of platelet function has focussed principally on the use of aspirin and dipyridamole which, despite their relatively weak antiplatelet action, have proved to be effective in promoting the antithrombotic functions of the endothelium. However, the use of these drugs in human clinical trials has so far been unsuccessful (Harker 1989). More recently the platelet glycoprotein IIb/IIIa receptor has been identified as the pivotal mediator of platelet aggregation, making it a logical target for the control of the platelet reponse to vascular injury (Lefkovits *et al* 1995). Monoclonal antibodies against platelet receptors have been demonstrated to effectively eliminate platelet aggregation in animal studies (Harker

1989). Clinical trials have taken place to determine the efficacy of the antithrombotic drug Reopro,which is based the chimeric monoclonal antibody Fab fragment (c7E3). Reopro acts by blocking the IIb/IIIa receptor on platelets; initial results from clinical trials indicate that when administered as a bolus and 12 hour infusion Reopro reduces the incidence of myocardial infarction, restenosis and death in a significant number of individuals six months after angioplasty (Topol *et al* 1994).

The anticoagulant heparin has also been shown to effectively inhibit SMC proliferation *in vitro* as well as prevent intimal hyperplasia in animals. (Clowes and Clowes 1985; Reilly *et al* 1989). Nevertheless, heparin is a potent stimulator of ECM production, a function thought to be related to its anticoagulant activity, and heparin may promote initial lesion development by binding growth factors. Thus, although heparin has not been shown to be effective in preventing restenosis in humans ongoing trials with genetically engineered nonanticoagulant heparin administered at later stages in lesion development may prove to be effective (Forrester *et al* 1991).

Tissue factor (TF) is a cell surface glycoprotein that interacts with clotting factor VII/VIIIa initiating the extrinsic pathway of coagulation. In atherosclerotic plaques TF can be found on SMC and macrophage surfaces and large amounts are trapped in the necrotic core. Induction of TF following PTCA can be found in the intima and adventitia of injured vessels suggesting a possible involvement in the pathogenesis of restenosis. Tissue factor pathway inhibitor (TFPI) is an endogenous inhibitor of TF. TFPI administered in the presence of the thrombin inhibitor, Hirudin, was found to reduce the mean ratio of neointimal formation from 0.68 to 0.48 in rat carotids. The efficacy of this therapy is currently being studied in other animal models (Creasey 1995). Such anticoagulant therapy may prove useful at limiting stenosis resulting from plaque rupture. Such therapies targeted against the late stages of atherosclerosis could yield a beneficial outcome. Similarly research into the processes that govern atherosclerotic plaque stability and rupture could yield essential information that may lead to control over this clinically significant event. Studies into the architecture of atherosclerotic plaques have provided insights

into the mechanisms that govern plaque stability such as the balance of ECM synthesis and matrix metalloproteinase activity (Galis *et al* 1994). It can be envisaged that any therapy which promotes a shift towards increased ECM deposition and decreased matrix degrading activity may increase the tensile strength of the plaque cap thereby promoting plaque stability. Results from recent lipid lowering trials suggest one method by which plaque stabilization may be achieved. Lipid lowering treatment led to reductions in clinical events without substantial change in the degree of luminal stenosis. This could reflect a stabilization of the lesion (Blankenhorn and Hodis 1994; Brown *et al* 1993).

Because SMC migration and proliferation are critical events in both restenosis and atherosclerosis, the targeting of signalling pathways and elements that control them may yield exciting results. For example antisense oligonucleotides to a series of proto-oncogenes expressed in cells including c-*myc*, c-*fos* or c-*myb*, or even to cell cycle control proteins and other molecules involved in mitogenic signalling, and possibly also to some of the elements involved in SMC contraction such as smooth muscle myosin, smooth muscle Myosin Light Chain Kinase (MLCK) and caldesmon. Indeed antisense oligonucleotides targeted against *c-myc* and *c-myb* have proved effective in the inhibition of SMC migration and proliferation both *in vitro* and *in vivo* (Biro *et al* 1993; Simons *et al* 1992; Bennett *et al* 1994). In addition efforts have been undertaken to use antisense oligonucleotides directed against mRNA's encoding several cell cycle regulatory proteins as a therapy to prevent restenosis following balloon injury of rat carotid arteries. Liposomes and a balloon catheter were used to deliver antisense oligonucleotides targeted against cdc2 K and cyclin B. Cyclin B and cdc2 K oligonucleotides reduced neointimal formation by up to 50%; when used together the inhibitory effect was greater (Milner 1995).

Recent progress in identifying many of the signalling pathways involved in cell migration, have identified intracellular targets for the inhibition of the induction of SMC migration and therefore neointimal formation. Two tyrosine kinase inhibitors, methyl 2,5-dihydroxycinnamate and genestein inhibit *in vitro* SMC migration (Shimokado *et al* 1994). Agonists against known

SMC migration factors have only had limited success *in vivo* (Ross 1986). This probably reflects the complex variety of stimuli responsible for the induction of SMC migration *in vivo*.

It has been reported that the generation of superoxide anions and oxidized LDL play important roles in the induction of atherosclerotic and restenotic lesions. This has led to the indication that antioxidant therapy may combat lesion development. Antioxidants have been demonstrated to reduce intimal thickening following angioplasty in animals (Freyschuss *et al* 1993). The benefit of such treatment in humans has proven effective in human trials (Tomeo *et al* 1995; Odeh and Cornish 1995).

The upregulated expression of receptors including PDGF and EGF on phenotypically modified SMC's has provided the opportunity for using cytotoxic agents that specifically target proliferating SMC's. Targeted delivery of cytotoxins to specific cell surface receptors may be accomplished with recombinant fusion proteins (Pickering *et al* 1993). Restenosis would be particularly amenable to cytotoxic therapy since the location of the disease is predictable. There have been no clinical trials of cytotoxic agents in restenosis thus far.

Anti-inflammatory agents could inhibit the accumulation and activation of inflammatory cells within atherosclerotic and restenoic lesions. This would subsequently result in decreased expression of growth factors responsible for SMC phenotypic modulation and lesion development. Corticosteroids have been shown to inhibit SMC proliferation in animals (Liu *et al* 1989), but the administration of steroids one week after coronary angioplasty did not reduce the rate of restenosis (Pepine *et al* 1990). The potential value of other anti-inflammatory agents also remain unresolved (Forrester *et al* 1991).

The administration of growth factor and cytokine agonists could inhibit SMC modulation, proliferation and migration. TGF-β has been shown to increase ECM production and integrin receptor expression. In rat and porcine models of restenosis, TGFβ expression is increased within 2-6 hours following injury and may remain elevated for as long as 2 years (Majesky *et al* 1991; Miano *et al* 1993). The multifunctional nature of TGFβ and its association with restenosis make

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this cytokine an attractive therapeutic target for the prevention of restenosis. The role of such growth factors in the development of atherosclerotic and restenotic lesions are inherently complex, and invariably depend on a network of positive and negative signals acting in concert, rather than through a single mediator. The characterization of these networks and the functions that they promote will stimulate the development of many new therapeutic approaches aimed at preventing the development of atherosclerosis and restenosis.

Evidence that viral infection is responsible for the loss in tumour suppressor function within lesions highlights the potential of antiviral therapies in the inhibition of restenosis (Speir *et al* 1994).

The failure to observe any beneficial effects in humans with drugs previously successful at inhibiting restenosis in animal models raises the possibility that current animal models in use, such as the widely studied ballooned rat carotid model may not be suitable for studying the human disease (Forrester *et al* 1991). The rat balloon carotid model may poorly mimic restenosis in response to angioplasty performed on human vessels. This may be due to differences in the pathogenesis of the disease between animals and humans. Unlike the rat carotid artery, human arteries spontaneously develop a neointima after birth that increases rapidly until 6 months of age (Velican *et al* 1976; Sims *et al* 1989). Therefore, in the case of the rat carotid balloon injury model the formation of a new intima may poorly replicate the reponse to injury when an intima or even an atherosclerotic lesion may already exist. It follows that balloon injury of normal vessels from such animal models may result in a type of vessel injury very different from injury initiated by angioplasty carried out on human vessels that contain pre-existing atherosclerotic lesions. Such disparities may account for the limited success of clinical trials involving therapies that have proved successful in animal models (O'Brien *et al* 1993). Therefore it would seem that the development of new models may be required to test novel therapeutic applications against the disease. Recently the use of the c - FHC (complex - Familial Hypercholesterolemic) pig has been suggested as a solution to these problems. This is the only animal model that exhibits eccentric,

stenotic coronory atherosclerotic lesions with cellular composition and calcification similar to humans (Prescott *et al* 1991). Future studies will show whether this animal provides a useful model of the human restenosis and atherosclerosis conditions.

A variety of ECM molecules have been implicated in the modulation of SMC behaviour. Some including fibronectin, laminin, and collagen affect phenotype switching (Yamamoto *et al* 1993; Hedin *et al* 1988), others such as thrombospondin and vitronectin can influence the proliferation and migration of SMC's (Majack *et al* 1988). The work reported in this thesis is an examination of the effects of a range of ECM molecules on the behaviour and gene expression in human vascular SMC's.

Section V AIM OF PROJECT.

The aim of this PhD research project was to characterise the role that ECM molecules have on modulating the phenotype of human vascular SMC's *in vitro* and to identify any novel markers that may be associated with such alterations in phenotype.

The influence ECM molecules have on SMC behaviour was initially characterised by analysing alterations in the morphological growth patterns between SMC's cultured on different matrix substrates. It has previously been reported that SMC growth morphology may be indicative of their phenotypic state (Shackelton *et al* 1995). Changes in the functional aspects of SMC behaviour as induced by ECM adhesion was investigated by performing SMC migration and proliferation assays on various ECM coated substrates.

Immunocytochemical and immunoblotting studies were carried out on SMC samples cultured on different ECM substrates in order to identify any clear phenotypic differences between these populations. Antibodies specific for previously identified as well as potential candidates of SMC phenotypic marker proteins were used for these studies.

The differential display technique was applied to reveal any differences that exist at the molecular level of gene expression between SMC's cultured on different matrix substrates. However, before such analysis was initiated the differential display procedure was first optimized to allow for a more successful analysis of separate populations. Various improvements to the technique were developed and differential display analysis was performed on SMC's cultured on fibronectin coated surfaces compared with SMC's cultured on uncoated tissue culture plastic.

The identification of genes differentially expressed as a result of fibronectin adhesion may help elucidate the influence ECM has on cell phenotype; also for any genes identified as differentially expressed between distinct SMC populations their usefulness as SMC phenotypic markers could be determined by performing *in situ* hybridisation studies on normal and diseased vessel sections.

Section VI

<u>MATERIALS</u>

<u>AND</u>

<u>METHODS.</u>

VI.1. Materials (Results *Chapter 1.*):

Fresh umbilical artery tissue

Tissue culture flasks ($25mm^2$, $75mm^2$, $175mm^2$ - Nunclon)

Fibronectin pre-coated tissue culture flasks ($75mm^2$, $175mm^2$ - Falcon)

Tissue culture 96 well microtitre plate

37^oC Incubater -humidified and flushed with 7% Co_2 (LEEC).

Ham's F12 medium (Gibco BRL)

Waymouth medium (Gibco BRL)

Fetal Calf Serum (FCS) (Gibco BRL)

Glutamine (Gibco BRL)

Penicillin/Streptomycin (Gibco BRL)

0.25% Trypsin / 0.05% EDTA solution (Gibco BRL)

Econospin Centrifuge (Sorvall)

Hanks Balanced Salt Solution (HBSS)

Phosphate Buffered Saline (PBS-a) -Dulbecco tablets (Oxoid)

Deionised water

Plasma fibronectin (Sigma)

Cellular fibronectin (Sigma)

Collagen I (Sigma)

Collagen III (Sigma)

Collagen IV (Sigma)

Laminin (Sigma)

Vitronectin (Sigma)

Matrigel (Becton Dickinson)

Hoechst 33258 (Sigma)

Cytofluor 2350 plate reader (Millipore)

20x SSC (BDH)

SDS (Bio Rad)

Transwell inserts (Costar)

Low melting point agarose (Gibco BRL)

Protein assay reagent (Bio Rad)

Thermo max microplate reader (Molecular devices)

PDGF BB (Promega)

HGF (R & D systems)

Basic FGF (Sigma)

Cotton wool buds (Costar)

Coomassie fixative stain **(appendix I)**

Methanol (Fisons)

Acetic acid (BDH)

Xylene (BDH)

Hydromount (National diagnostics)

Axioplan microscope (Zeiss)

Camera FX-35DX (Nikon)

ChemoTx 96 well chemotaxis chamber (Neuroprobe)

VI.2. Methods (Results-*Chapter 1.*);

VI.3. Culture of Human SMC's.

Human umbilical artery vascular SMC's (HUASMC's) were derived from umbilical artery tissue by the explant method. Human umbilical artery tissue was dissected from fresh umbilical cords. Adventitial tissue was removed from the artery prior to explant of 1mm cross sections of arterial tissue in culture. SMC's were cultivated in a 50:50 mix of Ham's F12 medium (Gibco BRL) and Waymouth medium (Gibco BRL) supplemented with 16% (v/v) Fetal Calf Serum (FCS) (Gibco BRL), 2mM glutamine (Gibco BRL) and 50µg/ml penicillin/streptomycin (Gibco BRL) - (H:W 16% FCS). Cultures were incubated at 37°C in a 7%CO_2 (v/v) /93% (v/v) air environment. SMC's were routinely subcultured by treatment of confluent cultures with 0.25% trypsin / 0.05% EDTA solution (Imperial) and cultures were replated at $5.7x10^3$ cells per cm^2. Human adult FW aortic SMC's were a kind gift from Dr Cay Kielty University of Manchester. FW aortic SMC's were cultured using identical conditions to those used for HUASMC's.

All cells were cultured in standard tissue culture flasks (Nunclon) or microtitre plates (Nunclon) that were either uncoated or coated with equivalent concentrations (50µg/ml) of various extracellular matrix preparations.

Extracellular matrix substrates examined included;

> Plasma Fibronectin (Sigma)
>
> Cellular Fibronectin (Sigma)
>
> Collagen I (Sigma)
>
> Collagen III (Sigma)
>
> Collagen IV (Sigma)
>
> Laminin (Sigma)
>
> Vitronectin (Sigma)

Matrigel (Becton Dickinson).

These matrix substrates were chosen for analysis because they have previously been demonstrated to modulate SMC behaviour or have been implicated in the development of atherosclerosis and restenosis (Hedin *et al* 1990; Li *et al* 1994; Majack *et al* 1988; Salgar & Millis 1994; Thyberg *et al* 1990; Yamamoto *et al* 1993).

All proliferation and migration assays, RNA extraction and protein isolation performed on SMC's were carried out on cells between passages 5 to 10, 2 days prior to confluence following plating at 5.7×10^3 cells per cm^2. Similarly morphological analysis **(FIG's. VII.1 - VII.9)** were performed on SMC cultures 2 days prior to confluence.

VI.4. Extracellular matrix substrate preparation.

All matrix substrates for SMC culture were prepared and coated on to tissue culture grade surfaces in accordance with the manufacturer's instructions. In order to compare the influence various matrix substrates have on SMC behaviour *in vitro,* where possible matrix preparations were coated at equivalent concentrations of 50μg/ml.

Bovine plasma fibronectin (Sigma), 1 mg/ml stock solution was diluted 1:20 with phosphate buffered saline (PBS) to give a 50μg/ml solution. Plasma fibronectin solutions were coated on to tissue culture plastic by incubation at room temperature overnight. Excess fibronectin is removed by aspiration prior to cell seeding.

Human cellular fibronectin (Sigma), 500μg lyophilised derived from human foreskin was dissolved in 10 mls of PBS to provide a 50μg/ml solution. Cellular fibronectin solutions were coated on to tissue culture plastic by incubation at room temperature overnight.

Collagen type I derived from calf skin (Sigma) was used undiluted as a 0.1% (w/v) solution in 0.1 molar acetic acid.

Collagen type III (Sigma), 1mg lyophilised derived from calf skin was dissolved in 20ml of 0.25% (v/v) acetic acid solution. to give a 50μg/ml solution.

Collagen type IV, 1mg lyophilised derived from human placenta was dissolved in 20ml of 0.25% (v/v) acetic acid solution to give a 50μg/ml solution.

All collagen solutions were coated on to tissue culture plastic at 4°C overnight. Excess collagen was removed by aspiration. Surfaces were left to air dry for several hours at room temperature and were washed twice with PBS prior to cell seeding.

Vitronectin, 0.05mg lyophilised derived from human plasma was dissolved in 1ml of sterile deionised water to provide a 50μg/ml solution. Vitronectin was coated on to tissue

culture plastic for a 2 hour incubation at 37°C. Excess vitronectin was removed by aspiration prior to cell seeding.

Thrombospondin, 20µg lyophilised derived from human platelets was dissolved in 400µl of sterile deionised water to give a 50µg/ml solution. Thrombospondin was coated on to tissue culture surfaces overnight at room temperature. Excess thrombospondin was removed by aspiration prior to cell seeding.

Laminin, 500µg derived from human placenta was thawed overnight at 4°C and diluted in 10ml PBS to give a 50µg/ml solution. Laminin was incubated to cell culture surfaces for 2 hours at room temperature.

Matrigel (growth factor depleted) (Becton Dickinson) was thawed overnight at 4°C and coated on to tissue culture plastic undiluted at a density of $2ml/cm^2$ in a 37°C incubator for 1 hour prior to cell seeding.

VI.5. SMC Proliferation.

SMC proliferation was assayed by measuring the amount of DNA present in each well of microtitre plates at sequential time points following cell seeding. The most reliable method of determining a proliferative or anti-proliferative response of cells in culture is the analysis of cell number. As DNA content per cell remains relatively constant, oscillating only by a factor of 2 during S phase of the cell cycle, the absolute amount of DNA can be used as a reliable indicator of cell number. DNA content can be quantified through the use of DNA binding dyes, therefore the application of such DNA binding dyes can be utilised to measure cell proliferation.

The SMC proliferation assay used in these studies is based on the binding of the fluorescent dye; bisBenzimide (Hoechst 33258) to double stranded DNA. When the dye intercalates into the DNA double helix the emission characteristics change from that of the free dye. Hence the emission at 460nm following excitation at 360nm, is directly proportional to the amount of dye bound to double stranded DNA. Fluorescence staining intensity was measured using the Cytofluor 2350 plate reader (Millipore) and from reference to a DNA standard curve, the DNA content of SMC cultures could be directly quantified.

SMC proliferation assay:

1.   HUASMC's (passages 5-10) were seeded at a density of 3×10^3 cells per well in standard 96 well microtitre plates. Cells were cultivated in H:W 16% FCS and supplemented every 48 hours with fresh medium. SMC proliferation was assessed in a series of 5 replicate wells that were either uncoated or coated with the various matrix components described on p 53 for the exception of Matrigel which was excluded from proliferation analysis as a result of interference with Hoechst binding.

2. At sequential time intervals following cell seeding (24; 72; 120; 168 & 216 hours), culture medium was aspirated. Cells were washed twice in Hanks Balanced Salt Solution (HBSS) (Gibco BRL). Excess HBSS was removed and cells were stored at -20°C ready for subsequent DNA analysis.

3. Immediately prior to DNA analysis, plates were thawed and dried in a 37°C incubator.

4. For DNA concentration / Hoechst fluorescence staining intensity calibration curve, DNA standards were added to the outside wells of a microtitre plate; (500; 250; 125; 62.5 and 31.25 ng per well).

5. Cells and DNA standards were solubilized and stained in 1xSSC (sodium chloride 3M sodium citrate 0.3M) solution (BDH), containing 0.05% (w/v) SDS (Bio Rad) and 0.5 µg/ml Hoechst 33258 (Sigma). Samples were incubated in this solution for 30 minutes in the dark.

6. Fluorescence emission was measured on the Cytofluor at 460nm following excitation at 355nm.

7. Data was exported into Microsoft Excel. Using an Excel macro program, fluorescent staining intensity was evaluated on the DNA standard calibration curve to calculate the mean DNA content in ng per well for each culture condition.

VI 6. SMC Migration

Migration of SMC's was assayed by a modification of the Boyden's chamber method using Transwell inserts (Costar) **(FIG.VI.1.).**

(FIG.VI.1.) Costar Transwell Insert:
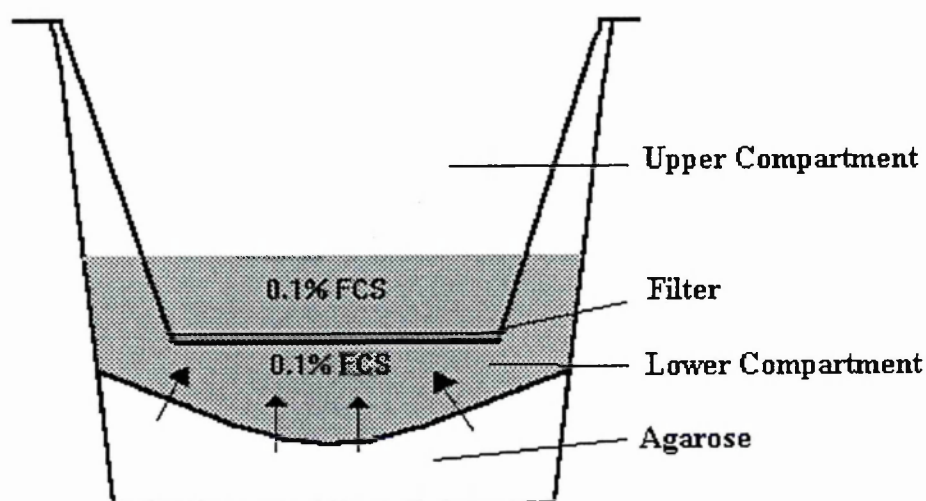
Initially SMC migration was stimulated by seeding cells in the upper compartment in 0.1% (v/v) FCS supplemented H:W medium with 16% (v/v) FCS H:W medium added to the lower compartment to provide a positive chemotactic gradient of serum factors that would encourage cells to migrate from the upper surface to the lower side of the filter. However, from carrying out protein assays on the medium from the upper compartment at regular time intervals following the addition of 0.1% (v/v) and 16% (v/v) FCS H:W media to the upper and lower

transwell compartments, it became apparent that any chemotactic gradient initially set up was lost within 30 mins.

To quantify serum content of medium in the upper transwell compartment, a variation of the Bradford protein assay was performed:

Bradford Protein assay (Bio - Rad):

1. Bio Rad protein assay reagent was diluted 1 in 10 using deionised water.

2. 10μl of each unknown (medium sample from either the upper or lower transwell compartment taken at various time points) was added to triplicate wells of a 96 well microtitre plate.

3. 10μl of serum standards; 16; 8; 4; 2; 1; 0.5; 0.25; 0.125; 0.06% (v/v) FCS H:W medium was also added to triplicate wells of a 96 well microtitre plate.

4. 100μl of diluted Bio Rad protein assay reagent was added to each well.

5. Optical density from each well was measured on the plate reader at wavelength 650 nm.

6. From reference to the standard curve the serum content of medium samples obtained from the upper and lower transwell compartments could be calculated.

In order to provide longer lasting gradients; neat FCS was embedded in a 1 % (w/v) agarose gel. An equal volume of FCS (Gibco BRL) was added to an equal volume of molten 2 % (w/v) agarose (Gibco BRL). The agarose serum blend was mixed briefly and using an eppendorf pippette 200μl cast into the lower transwell compartment as a ring. This ring formation allows the upper transwell insert and filter to fit into the lower compartment un-obstructed.

In order to compare SMC migration rates through uncoated and plasma fibronectin coated transwell filters the rates of SMC adhesion to both of these surfaces must first be taken into account to ensure that equal numbers of SMC's adhere to both uncoated and plasma fibronectin coated transwell filters.

SMC adhesion was assayed by quantifying the total DNA content using the Hoechst assay as described for SMC proliferation assays **(Section VI.5.)**. SMC's were seeded at a density of 1×10^4 cells per well in uncoated and plasma fibronectin coated microtitre plates. At various time points following cell seeding; (30; 120; 240; 360; 480; 600 & 720 minutes), plates were removed from the incubator and mean DNA content per well was measured.

Transwell Migration Assay:

1.   Following 5 hours preincubation in 16% (v/v) FCS H:W, SMC's on uncoated filters were washed x2 HBSS and replaced in a transwell compartment containing 1% (w/v) agarose/neat FCS gel and 750µl (v/v) 0.1% FCS H:W added to the lower compartment and 100µl (v/v) 0.1% FCS H:W was added to the SMC's in the upper compartments. At the same time SMC's were seeded onto fibronectin coated filters with 0.1% FCS (v/v) H:W in the upper and lower compartments and the 1% (w/v) agarose gel serum mix also in the lower compartment.

2.   SMC's in transwell inserts were incubated at 37°C in 93% (v/v) air/7% (v/v) CO_2 environment for the required time intervals (6, 12 and 24 hours).

3.   Once incubation was completed, transwell inserts were removed and washed x3 HBSS.

4.   Non-migrated cells were removed from the upper surface of the filter by scraping with cotton wool buds.

5.   Inserts were again washed x3 HBSS and stained for 10 minutes in Coomassie blue fixative stain **(appendix I)**.

6.   Inserts were washed x2 HBSS.

7.   Filters were removed from transwell inserts by dissolving the adhesive with Xylene (BDH.).

8.   Filters were subsequently mounted under glass coverslips with DPX (BDH) mountant.

9.   The number of SMC's per high power field (x100 magnification) were counted under a Zeiss Axioplan microscope.

Applying the same method, SMC migration in response to various specific chemotactic factors was investigated. Human recombinant PDGF BB (Platelet Derived Growth Factor - Promega) and HGF (Hepatocyte Growth Factor - R&D systems) were added at increasing doses to the agarose gel and set in the lower compartment.

Although the transwell SMC migration assay proved successful, transwell migration experiments were laborious and time consuming to prepare particularly when investigating a number of different conditions. The 96 well plate format chemotaxis chamber ChemoTx (Neuroprobe) **(FIG.VI.2.)** has the advantage over transwells in that a number of different chemotactic conditions can be investigated simultaneously. Also the measurement of SMC migration is probably more accurate as the number of migrated cells is quantified automatically by the measurement of optical density using the Thermo max microplate reader (Molecular Devices).

(FIG.VI.2.) Neuroprobe 96 well Chemotaxis Chamber.
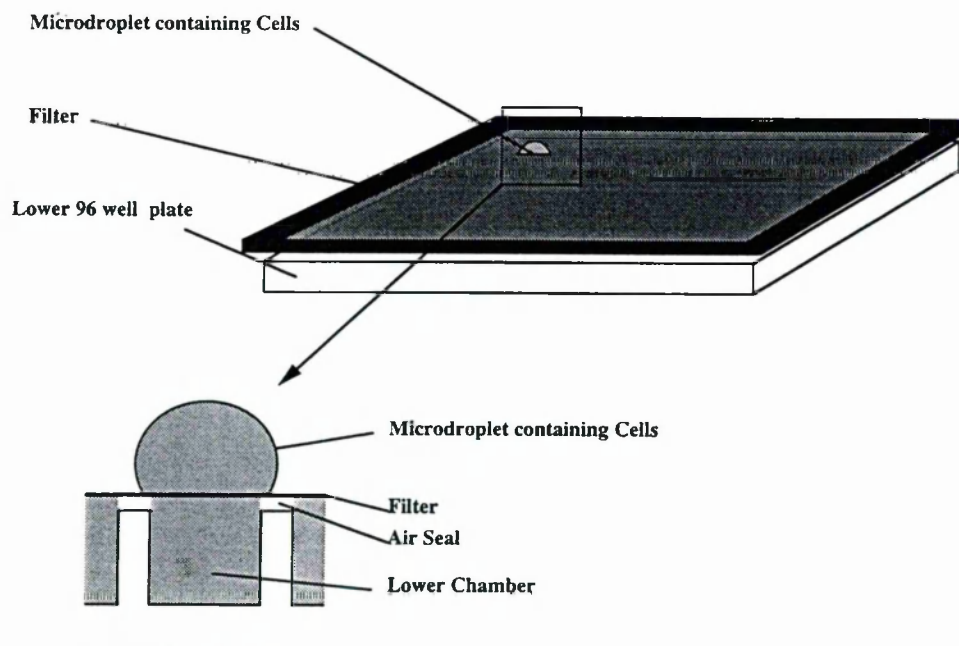
The Neuroprobe chemotaxis chambers were used to measure SMC migration rates through various matrix coated filters in response to a range of potential chemotactic factors.

Neuroprobe 96 well chemotaxis chamber (ChemoTx) migration assay:

1. Neuroprobe filters 8μm pore size were coated with the matrix substrates; plasma fibronectin, collagen's I, III, IV and laminin as previously described for coating other cell culture surfaces.

2. Chemotactic stimuli including; human recombinant PDGF BB (Promega), HGF (R & D systems) and basic FGF (Sigma) were diluted in 0.1% (v/v) FCS H:W to provide a 30ng/ml concentration. Each chemotactic preparation was dispensed as 29μl aliquots into the wells of the lower chamber in a series of 5 replicated wells. A negative control consisting of 0.1% (v/v) FCS H:W media containing no additional chemotactic factors was also added to 5 replicate wells for each filter.

3. Filters were washed x2 HBSS and air dried prior to positioning on top of the bottom 96 well plate containing the chemotactic stimuli. An aqueous seal forms between the filter surface and the walls of each well of the bottom plate.

4. 1x10^4 HUASMC's were added to the filter surface over each well in a 25μl droplet.

5. The top lid was set in place and chambers were incubated at 37°C in a 7% (v/v) CO$_2$/93% (v/v) air environment for 6 hours.

6. Following incubation chambers were removed from the incubator and dismantled. Filters were washed x2 HBSS.

7. Filters were stained and fixed in Coomassie blue fixative stain for 10 minutes.

8. Filters were placed in destain solution **(appendix I)** for 3 minutes and then allowed to air dry for 10 minutes.

9. Filters were then loaded into the Thermo max microplate reader to obtain mean absorbency values for total SMC adhesion to the filter for each chemotactic condition.

10. The filter is withdrawn and all non-migrated cells are removed from the upper surface of the filter using moist tissues.

11. The filter is inserted once again into the plate reader to obtain absorbency values for migrated cells only.

12. From O.D. values corresponding to the total cell adherence to the filter and for migrated cells only. The percentage of adhered SMC's that have migrated to the lower side of the filter under each condition could be calculated. Migration results from each matrix coated filter were expressed as fold increase over the negative control present on the same matrix coated filter. The fold increase of percentage SMC migration over control values for each chemotactic condition can then be compared between different matrix coated filters.

VI.7. Appendix I

Hoechst stain;

Hoechst 33258 (50 µg/ml stock) (Sigma)	50µl
20xSSC (BDH)	5ml
SDS (20% w/v solution) (Bio Rad)	50µl

-Make up with deionised water, shake and use immediately.

Destain Solution;

Methanol (Fisons)	20% (v/v)
Acetic acid (BDH)	7% (v/v)

-Make up solution with deionised water, shake and store at room temperature.

Coomassie brilliant blue fixative stain;

Coomassie brilliant blue (Sigma)	0.25%(w/v)
Methanol (Fisons)	50%(v/v)
Acetic acid (BDH)	10%(v/v)

-Make up solution with deionised water, stir and store at room temperature.

VI.8. Materials (Results - *Chapter 2.*):

FW human aorta cells (Dr C Kielty, University of Manchester)

Matrisperse (Becton Dickinson)

Matrigel (Becton Dickinson)

SMC Differentiation medium (Becton Dickinson)

Econospin Centrifuge (Sorvall)

50 ml tube (Falcon)

1XSDS sample buffer **(appendix II)**

Sapheneous vein tissue

Aorta tissue

Umbilical artery tissue

Mortar and pestle

Sonicator (Branson)

Modular Mini - PROTEAN II electrophoresis equipment (Bio Rad);

> Casting stand
>
> Gel clamp
>
> Inner glass plates (7.3 x 10.2 cm)
>
> Outer glass plates (8.3 x 10.2 cm)
>
> 1mm spacers
>
> 15 tooth teflon comb
>
> Buffer tank and lid

Power pack Ps 500X (Hoeffer Scientific Instruments)

Bis Acrylamide 30% w/v (37.5:1) (Anachem)

Deionised water

Tris.HCl 1.5M pH8.8 (Sigma) **(appendix II)**

Tris HCl 0.5M pH6.8 (Sigma) **(appendix II)**

SDS (Bio Rad) 10% solution

Tetramethylethylenediamine (TEMED) (Bio Rad)

Ammonium persulphate (APS) (Bio Rad) 10% solution

Methanol (Fisons) 50% solution

Postslip filter paper (Hollingsworth & Vose)

1xSDS running buffer **(appendix II)**

Prestained molecular weight standards (Sigma)

100°C water bath

Coomassie blue fixative stain **(appendix I)**

Destain **(appendix I)**

Gel dryer (Hoeffer Scientific Instruments)

Transfer cassette and electrophoresis unit (Hoeffer Scientific Instruments)

Transfer buffer **(appendix II)**

Nitro-cellulose membrane (Hybond-ECL - Amersham)

Marvel (Premeir beverages) 5% solution in PBS-Tween

PBS - Tween **(appendix II)**

Orbital shaker (Stovall)

Square petri dishes (100mm^2) (Sterilin)

Monoclonal anti SM-α Actin antibody (Mouse IgG2a) (Sigma)

Monoclonal anti Vimentin antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti Tropomyosin antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti Desmin antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti β-Tubulin antibody(Mouse IgG$_1$) (Sigma)

Monoclonal anti Collagen I antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti Cytokeratin 8 antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti Vinculin/meta-vinculin antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti Myosin light chain kinase antibody (Mouse IgG2b) (Sigma)

Monoclonal anti Cellular fibronectin antibody(Mouse IgM) (Sigma)

Monoclonal anti Collagen IV antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti Thrombospondin antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti Smooth muscle myosin antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti Laminin antibody (Mouse IgG$_1$) (Sigma)

Monoclonal anti Caldesmon antibody (Mouse IgG$_1$) (Sigma)

Anti-mouse IgG$_1$ FITC conjugate antibody (Sigma)

Anti-mouse horse radish peroxidase conjugate antibody (Sigma)

Saran wrap (Dow)

Hyperfilm ECL autoradiographic film (Amersham)

Hyperfilm cassette (Amersham)

Curix 60 developer (AGFA)

Camera FM2 (Nikon)

VI.9. Methods (Results *Chapter 2.*);

VI.10 Immunoblotting Analysis;

Protein isolation; Cultured SMC samples were trypsinized from matrix substrates and cell pellets were obtained by centrifugation at 1,500 rpm for 5 minutes. Pellets were washed x2 PBS and centrifuged prior to protein extraction. SMC's cultured in Matrigel were recovered using Matrisperse (Becton Dickinson) and the following protocol to depolymerise Matrigel;

1. Cell culture medium was aspirated from SMC cultures in Matrigel and washed x3 with HBSS.

2. Matrisperse solution was added to SMC cultures in Matrigel (2ml per 35mm dish)

3. The cell/gel layer was collected into an ice cold 50ml Falcon tube on ice.

4. Each culture dish was rinsed with an additional 2ml of Matrisperse and transferred to Falcon tube.

5. The Falcon tube was inverted several times and incubated on ice for 1 hour or until the Matrigel has completely depolymerized.

6. The cells were centrifuged at 500-800 rpm for 5 minutes at 4°C.

7. Cell pellets were washed x2 by gentle resuspension in ice cold PBS. Cells were briefly centrifuged once again, excess PBS was aspirated prior to protein isolation.

Total cellular proteins were extracted from all cultured SMC samples by solubilization in 1xSDS sample buffer **(appendix II)**. Approximately 1×10^6 SMC's were solubilized in 100μl of 1xSDS sample buffer.

Fresh human tissue samples from; Sapheneous vein, aorta and umbilical artery were rapidly frozen in liquid nitrogen. Frozen tissue samples were ground using a mortar and pestle to obtain a powder. Tissue powder was dissolved in 1xSDS sample buffer.

All protein samples were sonicated in a Branson sonifier 250 to disperse protein aggregates.

Electrophoretic separation of proteins; Proteins were separated by one dimensional gel electrophoresis in vertical slab 10x7 cm minigels. All protein gels were carried out using Bio Rad glass plates (10.2 x 7.3 cm and 10.2 x 8.3 cm) and Bio Rad minigel cassettes and casting equipment. Protein gels and electrophoresis equipment were prepared as follows;

1. Wash glass plates thoroughly in deionised water and air dry. Wipe plates with absolute methanol immediately prior to use.

2. Assemble one 10.2 x 8.3 cm glass plate and one 10.2 x 7.3 cm glass plate as a sandwich between two 1mm spacers.

3. Lock the sandwich into the casting stand.

4. Prepare the separating gel solution as follows to give a 7.5% (w/v) acrylamide gel;

Bis Acrylamide stock 30% w/v (37.5:1)	5.62ml
Deionised water	9.08ml
Tris.HCL 1.5M pH 8.8	10ml
10% (w/v) SDS	200µl
TEMED	20µl
10% APS	100µl

5. Apply 4ml of the separating gel solution to the glass plate sandwich.

6. Overlay top of separating gel with an even layer of 50% methanol, 200µl per gel. Allow gel to polymerize for 45 minutes.

7. Wash off 50% (v/v) methanol layer using copious amounts of deionised water. Remove excess H_2O using filter paper inserted between glass plate sandwich.

8. Prepare stacking gel solution as follows;

Bis Acrylamide stock 30% w/v (37.5:1)	2.6ml

Deionised water	12.2ml
Tris.HCL 0.5M pH 6.8	5ml
10% (w/v) SDS	200μl
TEMED	20μl
10% (v/v) APS	100μl

9.   Using a Pasteur pipette apply the stacking gel solution until the height of the solution in the sandwich reaches the top of the smaller glass plate.

10.   Insert a 1mm 15 tooth teflon comb into the layer of the stacking gel solution. If necessary add additional stacking gel to completely fill the spaces in the comb.

11.   Allow stacking gel solution to polymerize for 45 minutes at room temperature.

12.   Carefully remove teflon comb from the gel.

13.   Remove glass plate gel sandwich from casting rack and attach to the upper compartment of the gel running apparatus.

14.   Place upper buffer chamber containing gel into the lower buffer chamber. Fill upper chamber with 1xSDS electrophoresis running buffer **(appendix II)** so that the sample wells of the stacking gel are filled with buffer. Also partially fill the lower buffer chamber with 1xSDS electrophoresis running buffer.

15.   All protein samples and molecular weight markers (Sigma) are solubilized and denatured by heating at 100°C for 2 minutes. Place samples on ice ready for loading onto the gel.

16.   Using an Eppendorf pipetman the required amount of protein samples were loaded onto the gel. Also load 5μl of molecular weight standards. Add to empty wells at the extreme end of each gel 10μl of 1xSDS Sample buffer.

17. Fill the remainder of the upper and lower chamber with 1xSDS electophoresis buffer. Connect power supply and run at 20 mA of constant current per slab gel.

18. After the proteins have migrated the required distance through the gel, disconnect power supply and carefully remove gels from glass sandwich for either immunoblotting to membrane or protein staining.

Protein Staining; For detection of total protein bands, transfer gel into a suitable receptacle and cover with Coomassie blue fixitive stain. Agitate slowly overnight at room temperature. After staining is complete pour off stain and add destaining solution, swirl briefly and replace with fresh destain and agitate slowly for 4-6 hours. After destaining, the gels were washed in a destain/0.5% (v/v) glycerol solution for 10 minutes then rinsed in deionised water and dried between two dialysis sheets in gel dryer (Hoeffer Scientific Instruments) to maintain a permanent record.

Immunoblotting; Immunoblotting was performed to electrophoretically transfer protein from the gel to a nitro-cellulose membrane for subsequent analysis (Towbin *et al* 1979). Blotting was carried out in a tank of transfer buffer **(appendix II)** with the gel in a vertical orientation, completely submerged in transfer buffer between two large electrode panels as described;

1. Transfer sandwich is assembled in a tray filled with transfer buffer using a plastic transfer cassette and 2 sponge sheets.

2. On bottom half of plastic transfer cassette place one sheet of sponge, prewet with transfer buffer 2 sheets of postslip filter paper (9x14cm) (Hollingsworth & Vose) and place on top of sponge.

3. Place gel on top of filter paper, remove any air bubbles that develop between gel and filter paper.

4. Cut Hybond-ECL nitro-cellulose membrane (Amersham) to same size of gel plus an additional 1mm on each edge. Prewet in transfer buffer and apply to top side of the gel. Remove any air bubbles between the gel and membrane.

5. Prewet another 2 pieces of postslip filter paper and place on the anode (upper) side of the membrane ensuring all air bubbles are removed. Place another sponge sheet on top of this filter paper.

6. Complete assembly of transfer sandwich apparatus by locking top half of transfer cassette into place.

7. Fill transfer electrophoresis tank (Hoeffer scientific instruments) with transfer buffer and place transfer cassette containing sandwich into tank in correct orientation. (i.e. membrane side of sandwich adjacent to anode cathode).

8. Electrophoretically transfer proteins from gel to membrane at a constant current of 80mA overnight.

Immunoprobing with conjugated secondary antibody; After the electrophoretic transfer of proteins to the membrane is complete the power supply is turned off. The membrane is removed from the transfer cassette to a dish ready for the immunoprobing procedure as described below;

1. Place membrane in a dish with PBS-tween **(appendix II)** and wash for 5 minutes with agitation on an orbital shaker (Stovall) at room temperature.

2. Pour off PBS-tween and replenish with 100ml blocking buffer; 5% (w/v) Marvel (Premier beverages) in PBS-tween and incubate for 30 mins at room temperature with constant agitation.

3. Protein specific primary antibody was diluted in PBS-tween to obtain ideal working concentration (1 in 10,000 - for all antibodies used in this study). Blocking buffer was removed and replaced with diluted primary antibody (10ml per membrane). Membranes were incubated with primary antibody for 1 hour at room temperature on shaker.

4. Following primary antibody incubation, membranes were washed x3 PBS-tween at room temperature on shaker (5 mins per wash).

5. Membranes were then incubated with suitable primary antibody-relevant anti horse radish peroxidase conjugated second antibody diluted 1 in 10,000 with PBS-tween. Secondary antibodies were also incubated with membranes (10ml per membrane) for 1 hour on shaker.

6. Following secondary antibody incubation, membranes were washed x5 PBS-tween (5 mins per wash).

7. Antibody staining was analysed using the ECL detection system and reagents (Amersham). Following the final membrane wash in PBS-tween, membranes were incubated in a 50:50 mix of ECL reagents 1 and 2 for 1 minute.

8. Membranes were blotted briefly to remove excess ECL reagents and were exposed to autoradiographic hyperfilm ECL (Amersham) in autoradiographic hyperfilm cassettes (Amersham) for variable exposure times. Autoradiographic film was developed in the automatic Curix 60 developer (AGFA).

8.   Membranes were blotted briefly to remove excess ECL reagents and were exposed to autoradiographic hyperfilm ECL (Amersham) in autoradiographic hyperfilm cassettes (Amersham) for variable exposure times. Autoradiographic film was developed in the automatic Curix 60 developer (AGFA).

Rat Balloon Carotid Injury;

Male Wister rats were anesthesized with sodium pentobarbiturate (50 mg/kg). A 2F Fogarty arterial embolectomy catheter (Baxter, USA) was introduced into the right common carotid artery (RCCA) from external carotid artery, swelled with saline and pulled three times to denude endothelial cells throughout the RCCA. 14 days following surgery, rats were killed by bleeding under diethylether anesthesia. The RCCA was removed, washed with ice-cold saline The tissue was then separated into neointima and media, freezed on dry ice, and stored at -40 C until used. The normal left common carotid artery was also removed and stored. Protein was extracted from these tissue samples by solubilization in 1x SDSSB.

VI.11 Immunofluorescence:

HUASMC's were cultured on glass chamber slides (Nunclon) at a seeding density of 1x 10^4 cells per chamber. Individual chambers were pre-coated with various matrix substrates as previously described. Once SMC's within chambers were approximately 90% confluent they were fixed and proteins were stained by dual antibody fluorescence according to the following protocol;

1. Flick off culture medium from SMC cultures in chambers and wash x3 PBS.

2. Add Methanol (Fisons): Acetone (BDH) 50:50 mix at -20°C to half fill chambers.

3. Flick off and fill chambers with Methanol:Acetone 50:50 mix -20°C and incubate at room temperature for 1min.

4. Flick off and wash x2 PBS.

5. Add protein specific primary antibody at required dilution in PBS. Incubate cells with primary antibody for 1 hour at room temperature.

6. Wash x2 PBS.

7. Add diluted (1:100 in PBS) Fluorescein Isothiocyanate (FITC) conjugated secondary antibody of relevant species to primary antibody used. Incubate cell with secondary antibody for 1 hour at room temperature.

8. Wash cell x3 PBS and flick to dry.

9. Remove chambers and rubber seal from glass slide. Mount cells under glass coverslips (BDH) using; Hydromount mountant (National Diagnostics).

10. Analyse immunofluorescent staining using the appropriate wavelength filters to detect peak light emission at 520 nm on the Ziess Axioplan microscope

Incubation with FITC conjugated antibody and no primary antibody was performed as a control to monitor non specific fluoresence. A constant 1 minute exposure time was used when taking photographs of immunofluorescent staining.

VI.12 Appendix II

1x SDS sample buffer;

Glycerol (BDH)	10g
β-Mercaptoethanol (Sigma)	5ml
SDS (20% solution) (Bio Rad)	25ml
Tris (1M pH 6.8) (Sigma)	6.26ml
Bromophenol Blue (0.2% stock solution) (Sigma)	1ml

- Make up to 100ml with deionised water and stir to dissolve. Store at room temperature.

Running buffer (x10);

Tris Base (Sigma)	75.75g
Glycine (BDH)	360.5g
SDS (Bio Rad)	25g

- Make up to 2litres with deionised water. Store at room temperature, dilute 1/10 before use

Tris pH 8.8 (1.5M);

Tris base	181.65g

- Dissolve in 950ml with deionised water adjust pH to 8.8 using concentrated HCL (BDH).

Make up to 1 litre with deionised water, store at room temperature.

Tris pH 6.8 (0.5M);

Tris base	30.27g

- Dissolve in 200ml with deionised water adjust pH to 6.8 using concentrated HCL (BDH).

Make up to 250ml with deionised water, store at room temperature.

Transfer blot buffer;

Tris HCL (Sigma)	18g
Glycine (BDH)	84g
Methanol (Fisons)	1200ml

-Make upto 6 litres with deionised water.

SDS stock (20% solution w/v);

SDS (Bio Rad)	20g

-Make upto 100ml with deionised water.

Ammonium persulphate (10% solution w/v);

Ammonium persulphate	0.1g

-Add 900μl of deionised water and shake to dissolve, store at -20°C.

Destain/Glycerol solution;

Methanol (Fisons)	20% v/v
Acetic acid (BDH)	7% v/v
Glycerol (BDH)	0.5% v/v

-Make up solution with deionised water, stir and store at room temperature.

VI.13. Materials (Results *Chapter 3.*):

RNAzolB (TEL-TEST)

Hanks Balanced Salt Solution (HBSS) (Gibco BRL)

Chloroform (Fisons)

SpeedVac DNA 100 (Savant)

Diethyl pyrocarbonate (DEPC) (BDH)

MessageClean Kit (Genhunter), components; 10x reaction buffer, Dnase I

Phenol:Chloroform 3:1 (Sigma/Fisons)

3M sodium acetate (BDH)

1% agarose gel **(appendix III)** (Gibco BRL)

1X TBE buffer **(appendix III)**

RNA Map Kit (Genhunter), components;

      5X Reverse Transcription Buffer

      MMLV Reverse Transcriptase (100 u/μl)

      dNTP (250μM)

      T12MG, T12MA, T12MT, T12MC (all at 10μM)

      10X PCR Buffer

      dNTP (25μM)

      AP-1 (2μM), 5'-AGCCAGCGAA-3'

      AP-2 (2μm), 5'-GACCGCTTGT-3'

      AP-3 (2μm), 5'-AGGTGACCGT-3'

      AP-4 (2μm), 5'-GGTACTCCAC-3'

      AP-5 (2μm), 5'-GTTGCGATCC-3'

      Control RNA (0.1 μg/μl)

      Glycogen (10mg/ml)

^{35}[S]-dATP (Amersham)

RNA Image Kit (Genhunter), components;

    5X Reverse Transcription Buffer

    MMLV Reverse Transcriptase (100 u/μl)

    dNTP (250μM)

    H-T$_{11}$G, H-T$_{11}$A, H-T$_{11}$C (all at 2μM)

    10X PCR Buffer

    dNTP (25μM)

    H-AP1, H-AP2, H-AP3, H-AP4, H-AP5, H-AP6, H-AP7, H-AP8 (all at 2μM)

    Control RNA (1μg/μl)

    Glycogen (10mg/ml)

^{33}[P]-dATP (Amersham)

Taq DNA Polymerase (Perkin Elmer)

Omnigene Thermocycler (Hybaid)

Thermowell thin-wall 96 well plate Model H (Costar)

6% Bis acrylamide (37.5:1) (Anachem)

Tetramethylethylenediamine (TEMED) (Bio Rad)

Ammonium persulphate (APS) (Bio Rad) 10% solution

42mm X 32mm glass plate

42mm X 39mm glass plate

Gel slick (AT biochem)

0.4mm spacer x2 (BRL)

24 well sharkstooth comb x2 (BRL)

SE1160 Slab Gel Dryer (Hoeffer Scientific Instruments).

3000Xi power pack (Bio Rad)

3mm Chromatography paper (Whatman)

Saran wrap (Dow)

Hyperfilm Cassette (Amersham)

Hyperfilm MP Autoradiographic film (Amersham)

2M Sodium acetate (BDH)

TA Cloning Kit, components;

      Amplification primer #1

      Amplification primer #2

      T4 DNA Ligase

      pCRII vector

      Control DNA template

      10X PCR Buffer

      10X Ligation Buffer

      100mM dNTPs

      TE buffer

      SOC medium **(appendix III)**

      β-mercaptoethanol

      INV αF competent cells

Microflow hood (envair)

Luria-Bertani (LB) Medium **(appendix III)**

bacto-tryptone (Difco)

bacto-yeast extract (Difco)

Sodium chloride (Sigma)

Agar (Lab M)

Carbenicillin (Sigma)

42°C water bath (Grant)

Petri dishes 90mm diameter (Sterilin)

O-Nitrophenyl-β-D-Galactopyranoside (X-Gal) (Sigma)

14 ml tubes (Falcon)

Microbiological spreader (L.I.P.)

Innoculation hoops (Starstedt)

RNase A (Promega)

10X React 3 (Gibco BRL)

ECOR I (Gibco BRL)

1.8% agarose gel **(appendix III)**

Qiagen Plasmid Midi Kit (QIAGEN), components;

       QIAGEN-tip 100

       Buffer P1

       Buffer P2

       Buffer P3

       Buffer QBT

       Buffer QC

       Buffer QF

Hybaid Recovery Plasmid Midi Prep Kit (Hybaid), components;

       Pre-lysis buffer

       Alkaline lysis solution

       Neutralising solution

       Binding buffer

       Wash solution

       Spin filters

       Catch tubes

Sequanase DNA sequencing Kit version 2.0, components;

       Reaction buffer

Termination mixtures

Labelling mix

Sequenase polymerase

Pyrophosphatase

Glycerol enzyme dilution buffer

Stop solution

60°C water bath (Grant)

2N Sodium hydroxide (BDH)

1M Sodium acetate (BDH)

T7 primer; 5'-CGACTCACTATAGGGCGAATTG-3'

SP6 primer; 5'-CGCCAAGCTATTTAGGTGACAC-3'

QIAquick Gel Extraction Kit, components;

QIAquick spin columns

Buffer QX1

Buffer PE

2 ml collection tubes

50°C hot block (Teeke)

10mM Tris-HCL pH 8.5 (Sigma) **(appendix III)**

*redi*prime DNA labelling system, components;

Labelling mix **(appendix III)**

Control DNA

95°C hot block (Teeke)

^{32}[P] dCTP (redivue) (Amersham)

37°C hot block (Teeke)

0.2M EDTA (Sigma)

Nick Spin Column (Pharmacia Biotech)

TE buffer pH 7.4 **(appendix III)**

Centrifuge CPK R (Beckman)

mRNA Isolation Kit (Stratagene), components;

       Denaturing solution

       β-mercaptoethanol

       Oligo (dT) cellulose

       High-salt buffer

       Low salt buffer

       Elution buffer

       NaCl (5M)

       Sodium acetate (3M)

       Glycogen (20mg/ml)

       Transfer pipets

       Push columns

       Syringes (10 ml)

1.5% agarose/formaldehyde gel **(appendix III)**

Formaldehyde (37% solution) (Sigma)

10X MOPS **(appendix III)**

Formamide (Sigma) -deionised

Mixed bed Resin (Sigma)

65°C hot block (Teeke)

RNA size markers 0.16-1.77 Kb (Gibco BRL)

RNA size markers 0.24-9.5 Kb (Gibco BRL)

Formaldehyde gel loading buffer **(appendix III)**

Power pack Ps 500X (Hoeffer Scientific Instruments)

20X SSC (BDH)

Hybond membrane (Amersham)

Ethidium Bromide 1% solution **(appendix III)**

U.V. Crosslinker (Stratagene)

Rapid Hyb Buffer (Amersham)

65°C oven (Hybaid)

Oven flasks (Hybaid)

2X SSC/0.1% (w/v) SDS

1X SSC/0.1% (w/v) SDS

0.1X SSC/0.1% (w/v) SDS

Hyperfilm ECL autoradiographic film (Amersham)

Hyperfilm cassette (Amersham)

Curix 60 developer (AGFA)

Camera FM2 (Nikon).

VI.14 Methods (Results *Chapter 3.*).

VI.15 RNA ISOLATION;

Total RNA was isolated from tissue samples and cultured cell populations by a variation of the guanidinium thiocyanate method introduced by (Chomczynski & Sacchi 1987);

1. Ground tissue powder obtained from vascular tissue samples (see methods **VI.10.**), Cultured cell samples were washed x2 HBSS (Gibco BRL) and then solubilized with RNAzol B (5mls RNAzol B per confluent T175 flask of cells).

2. 100μl chloroform (Fisons) was added per 1 ml of homogenate. The mixture was shaken vigorously for 15 seconds and incubated on ice for 5 minutes.

3. The suspension was centrifuged at 12,000 g for 15 minutes at 4°C.

4. An upper aqueous phase that develops was transferred to a clean tube and an equal volume of isopropanol was added. Samples were incubated at 4°C for 15 minutes.

5. Samples were then centrifuged at 12,000 g for 15 minutes at 4°C.

6. Supernatant was removed and the RNA pellet was washed once with 75% ethanol by vortexing and subsequent centrifugation at 7,500g for 8 minutes at 4°C.

7. The supernatant was removed, and the pellet was dried under vacuum for 3 minutes.

8. The RNA pellet was dissolved in an appropriate volume of DEPC treated water.

9. RNA quantity and purity were assessed by reading absorbency values at wavelengths 260nm and 280nm on a spectrophotometer (Beckman).

VI.16 mRNA Differential Display;

Removal of contaminating genomic DNA; Total RNA was isolated using the RNAzol B method described above from HUASMC populations (passage 8) cultured on plastic and plasma fibronectin substrates.

Untreated RNA samples were amplified for original differential display experiments **(FIGs IX.1 a-d)**. However, in subsequent experiments RNA samples were subjected to DNase I treatment in order to remove contaminating DNA. DNase I treatment was carried out using the reagents and recommended protocol supplied with the MessageClean kit (Genhunter);

1. DNase I digestion, the following components were added in order;

Total RNA	50μl (50μg/μl)
10x Reaction buffer	5.7 μl
Dnase I	1μl

Reactions were mixed well and incubated for 30 minutes at 37°C.

2. To each sample 40μl of phenol/chloroform (3:1) was added. Samples were then vortexed for 30 seconds and incubated for 10 minutes on ice.

3. Samples were centrifuged at 12,000g for 5 minutes at 4°C

4. The upper phase was removed to a clean tube. 5μl of 3M sodium acetate and 200μl of 100% ethanol were added to each sample and allowed to incubate for 1 hour at -70°C.

5. Samples were centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 0.5ml 70% ethanol.

8. RNA was redissolved in 10μl of DEPC treated water.

9. RNA concentration was quantitated by reading absorbance at 260nm on spectrophotometer.

10. RNA integrity was assessed before use by running 1μg on a 1% agarose (Gibco BRL) mini gel made up in 1x TBE buffer prepared with sterile DEPC treated water.

10. RNA samples were stored as 1μg/μl aliquots at -70°C. Immediately prior to differential display analysis RNA samples were diluted in DEPC treated water to give a concentration of (0.1μg/μl).

Reverse transcription; Initially RNA was reverse transcribed and PCR amplified using the primers and reagents supplied in the RNAmap kit (Genhunter) and a ^{35}S radiolabelled dATP nucleotide. Later differential display experiments were performed using modified primers and reagents supplied with the RNAimage kit (Genhunter) together with ^{33}P radiolabelled dATP. The following reverse transcription and PCR protocol was performed when using both kit reagents or different radiolabelled nucleotides;

The following components from the RNAmap kit were thawed on ice. Four reverse transcriptase reactions were set up as described;

dH$_2$O	9.4μl
5x RT buffer	4.0μl
dNTP (250μM)	1.6μl
Total RNA (0.1μg/μl)	2.0μl
TM$_{12}$T, A ,C or G (one for each RT reaction)	2.0μl

The following components from the RNAimage kit were thawed on ice. Three reverse transcriptase reactions were set up as described;

dH$_2$O	9.4μl
5x RT buffer	4.0μl
dNTP (250μM)	1.6μl
Total RNA (0.1μg/μl)	2.0μl
H-T$_{11}$C, G orA (one for each rt reaction)	2.0μl

Reverse transcriptase samples were placed in a thermocycler (Hybaid) and incubated at 65°C for 5 minutes 37°C for 60 minutes and 75°C for 5 minutes. After 10 minutes at 37°C, 1μl MMLV reverse transcriptase was added to each tube, and mixed quickly by "finger tipping" incubation was then continued. Once reverse transcription completed RT reaction tubes were placed on ice ready for PCR step.

PCR amplification; The components used below were thawed on ice. A Master mix was prepared for each anchored and arbitrary primer combination. For samples run in triplicate, 6 PCR reactions were required for each primer combination (3 PCR reactions per population);

	1 reaction (μl)	Master mix (x6.5) (μl)
dH$_2$O	10	65
10x PCR buffer	2	13
dNTP (25μM)	1.6	10.4
H-AP/AP	2	13
H-T$_{11}$C, G or A/TM$_{12}$T, C, G or A	2	13
^{35}SdATP/^{33}PdATP	0.2	1.3
Ampli Taq (Perkin Elmer)	0.2	1.3

Master mixes were centrifuged briefly and kept on ice. 2μl of each RT reaction was aliquoted into individual wells of a multiwell plate (Costar) in a series of triplicates. The plate was briefly centrifuged. 18μl of each master mix was added to each relevant RT reaction (master mix containing H-T$_{11}$C is added to RT reaction performed using H-T$_{11}$C). Each PCR reaction was overlaid with mineral oil and briefly centrifuged. The Multiwell plate containing the reactions was placed in the PCR machine and incubated at 99°C - 30 seconds; 40°C - 2 minutes, 72°C - 30seconds for 40 cycles and 72°C for 5 minutes.

6% Denaturing polyacrylamide gel electrophoresis;

A 6% denaturing polyacrylamide gel for the separation of amplified differential display products was prepared as follows; One side of a 42mm x 32mm glass plate was coated with gel slick (AT Biochem) and formed a sandwich with a 42mm x 39mm glass plate. The glass plates were separated by two 0.4mm thick spacers. The bottom and sides of the sandwich were sealed with tape.

Acrylamide gel solution was prepared by adding to 100ml of 6% bis acrylamide (37.5:1) (Anachem); 10% APS-266.6µl (Sigma) and TEMED-53.3µl (Bio Rad). The solution was briefly mixed and poured into the glass sandwich using a 50ml syringe. Two inverted 24 well sharkstooth combs were inserted into the upper side of the gel. The polyacrylamide gel was left to polymerize at room temperature for one hour.

Once gel polymerized the bottom tape on the glass sandwich was cut open and the sandwich was attached firmly to the S2 electrophoresis unit (BRL). 1x TBE running buffer was added to the upper and lower reservoirs of the electrophoresis unit. The gel was pre-run at 60 watts constant power for 30 minutes. Once the gel is pre-run the inverted sharkstooth combs were removed and the resultant trough formed was flushed with running buffer to remove settled urea. The sharkstooth combs were inserted into the gel in the correct orientation, each tooth was embedded 1mm into the gel.

Differential display PCR samples were prepared for electrophoresis as follows; 3.5µl of each PCR sample was added to 2µl of loading dye in a multiwell plate. The plate was incubated at 80°C for two minutes. After incubation samples (2.5µl) were immediately loaded on to the polyacrylamide gel. Identical duplicate or triplicate reactions were loaded side by side, adjacent to equivalent PCR reactions from the comparative population. Samples were electrophoresed for 4 hours at 60 watts constant power (with voltage not to exceed 2000) until the Xylene dye reaches the bottom of the gel.

Once electrophoresis complete the power supply was switched off and the 42mm x 32mm glass plate removed from the sandwich. The acrylamide gel was then blotted onto

3mm filter paper (Whatman). The gel was covered with saran wrap (Dow) and dried at 80°C for 1 hour 30 minutes in a slab gel dryer (Hoeffer Scientific Instruments). Stratagene labels were placed over each corner of the gel to allow orientation of autoradiograph film. Differential display gels were left to expose to autoradiographic film in cassettes for 24-72 hours at room temperature.

Reamplification of cDNA probe;

After developing autoradiographic film, the gel was orientated with the autoradiographic film. Any bands that appeared reproducibly differentially expressed between HUASMC populations cultured on Plasma fibronectin and uncoated plastic were cut out of the gel using a clean razor blade. DNA was extracted from the gel as follows;

1. The gel slice along with the 3mm paper (on which the gel was dried) was soaked in 100μl of deionised water for 10 minutes.

2. The tube containing gel slice with cap tightly closed (sealed with parafilm) was placed in a boiling water bath for 15 minutes.

3. Sample tubes were then centrifuged at 12,000g for 2 minutes at room temperature to collect condensation, and pellet the gel and paper debris.

4. The supernatant was transferred to a new microfuge tube, to which was added 10μl of 2M sodium acetate, 5μl of glycogen (10mg/ml stock) and 450μl of 100% ethanol. These samples were left to incubate at -70°C for 30 minutes.

5. Samples were centrifuged at 12,000g for 10 minutes at 4°C to pellet DNA.

6. The supernatant was removed and the pellet rinsed with 200μl ice-cold 85% ethanol. Samples were centrifuge briefly at 12,000g and residual ethanol was removed.

7. The pellet was dissolved in 10μl of deionised water and 4 μl was used for PCR reamplification. The remainder of sample was stored at -20°C.

8. Reamplification PCR was carried out using the same primer set and PCR conditions as used for differential display reactions from where the band was detected, with the

exception that dNTP 250μM stock was used instead of 25μM stock. Also a 40μl reaction is recommended;

Deionised water	20.4μl
10x PCR buffer	4μl
dNTP (250μM)	3.2μl
Relevant $T_{12}M$ or $H-T_{11}$ anchored primer	4μl
cDNA template from differential display gel	4μl
AmpliTaq (Perkin-Elmer)	0.4μl

Each PCR reaction was overlaid with mineral oil and briefly centrifuged, reactions were incubated in the omnigene PCR machine at 99°C - 30 seconds; 40°C - 2 minutes, 72°C - 30 seconds for 40 cycles and 72°C for 5 minutes.

Ligation and cloning of reamplified differential display products

Reamplified differential display products were ligated into the TA cloning vector pCR II (Invitrogen) and cloned into competent INVαF' cells to allow sequencing using primers homologous to the Sp6 and T7 promoter sites. The TA cloning system (Invitrogen) is established as a universal method for the direct cloning of PCR amplified nucleic acid (Mead *et al* 1991).

Vector ligation and transformation reactions were performed using the reagents and protocol supplied with the TA cloning kit. Ligation reactions were performed by adding the following components in order;

Deionised water	5ml
Ligation buffer	1ml
pCR II vector	2ml
Differential display product	1ml
T4 DNA ligase	1ml

Ligation reactions were incubated overnight at 14°C.

Transformation of competent *E. coli* cells;

LB agar plates were prepared prior to transformation; The L B and agar preparation **(appendix III)** were heated in a microwave oven to dissolve. Molten agar was left to cool at room temperature for 30 minutes. The antibiotic Carbenicillin (Sigma) was added to give a final concentration of 50 μg/ml and then the molten LB agar was poured into 90 mm diameter dishes (Sterilin). LB agar plates were left to set for 1 hour and stored at 4°C. Immediately before transformation of *E. coli* cells, agar plates were dried in a 37°C incubator for 30 minutes. To aid selection of positive inserts O-Nitrophenyl-β-D-Galactopyranoside (X-gal) (25ml of 40ng/ml stock) (Sigma) was added to each plate and dispersed over the entire surface using a disposable microbiological spreader (L.I.P).

INVαF' *E.-coli* competent cells were transformed with differential display reamplified PCR products as described;

1. An appropriate number of vials of competent "one shot" cells (Invitrogen) were thawed on ice.

2. 2μl of 0.5 M β-Mercaptoethanol was aliquoted into each vial of "one shot" cells and mixed by stirring with a pipette tip.

3. 2μl of each ligation reaction was aliquoted into a vial of competent cells and stirred gentl with pipette tip to mix.

4. The vials were incubated on ice for 30 minutes.

5. Vials of competent cells were heat shocked for exactly 30 seconds in a 42°C water bath.

6. The vials were then placed on ice for 2 minutes.

7. 450μl of SOC medium **(appendix III)** was added to each vial.

8. Vials were incubated in a 37°C incubator "shaking" at 225 rpm for exactly 1 hour. After incubation vials with the transformed cells were placed on ice.

9. 50μl and 200μl aliquots from each transformation vial was plated on an LB agar plate containing 50 μg/ml carbenicillin and X-Gal.

10. Plates were incubated at 37°C for at least 18 hours and were then shifted to +4°C for 2-3 hours for colour development.

Mini prep purification and analysis of cloned DNA;

Small scale plasmid DNA purification was performed using a modification of the method introduced by Ish-Horwicz & Burke (1981).

1. Positive colonies (white) were toothpicked and inoculated on LB-agar master plates and into 2ml L-broth 50μg/ml carbenicillin cultures. Cultures were incubated overnight in the 37°C shaker.

2. 2ml cultures were dispensed into sterile 2ml eppendorf tubes. Cultures were centrifuged at 12,000g for 2 minutes at room temperature.

3. Supernatants were removed and cell pellets were incubated on ice for 15 minutes.

4. 100μl chilled solution I **(appendix III)** was added to each cell pellet. Samples were then vortexed and incubated at room temperature for 5 minutes.

5. Next 200μl solution II **(appendix III)** was added, samples were vortexed and incubated on ice for 5 minutes.

6. 150μl chilled solution III **(appendix III)** was added to each sample, which were then once again vortexed and incubated on ice for 5 minutes.

7. Samples were centrifuged for 2 minutes at 12,000g at room temperature.

8. The clear supernatant was removed to a clean eppendorf to which was added 1/2 volume phenol/chloroform. Samples were vortexed and centrifuged at 12,000g for 2 minutes at room temperature.

9. The top aqueous layer was removed to a clean eppendorf. 900μl 100% ethanol was added and samples were vortexed and incubated at room temperature for 2 minutes.

10. Samples were centrifuge at 12,000g for 2 minutes at room temperature.

11. The supernatant was then removed. To the DNA pellet 1.4ml 70% ethanol was added, samples were vortexed.

12. Each sample was centrifuged for 2 minutes at room temperature, the supernatant was removed and the DNA pellet was allowed to air dry.

13. DNA pellets were resuspended in T.E. pH 7.4 and 1µl RNase A was added.

14. Vector DNA and insert was digested using ECOR1 restriction enzyme as follows;

Add in order;	Miniprep DNA	5µl
	React 3 buffer	1µl
	ECOR1	1µl
	deionised H_2O	3µl

Digests were incubated at 37°C for 2 hours.

15. Cloned inserts were analysed to determine whether they correspond with the expected size of the differential display products. This was determined by running 9µl of plasmid digest on a 1.8 % agarose gel with appropriate molecular weight markers.

Preparation of plasmid DNA for sequencing.

Colonies from the master plate that contain the correct size insert (as determined by digestion of corresponding miniprep DNA) were innoculated into 20ml cultures of LB medium and incubated overnight in the 37°C shaker. Purification of plasmid DNA in preparation for sequencing was achieved using two methods. Initially the QIAGEN plasmid midi protocol and later the Hybaid Recovery plasmid midi prep kit were employed. Both protocols are outlined below;

QIAGEN Plasmid Midi Protocol;

1. INVαF' *E. coli* cells were harvested from 20ml L-broth cultures by centrifugation at 4°C for 15 minutes at 6,000 g. Supernatant was removed.

2. 4 ml of buffer P2 was added to each sample, mixed gently, and incubated at room temperature for 5 minutes.

3. 4 ml of chilled buffer P3 was then added to samples, mixed immediately but gently, and incubated at room temperature for 5 minutes.

4. Samples were centrifuged at 15,000 g for 30 minutes at 4°C. Supernatant was removed.

5. A QIAGEN-tip 100 was equilibrated by applying 4ml buffer QBT. The collumn was allowed to empty by gravity flow.

6. The supernatant from step 4 was applied onto the Qiagen-tip and allowed to enter the resin by gravity flow.

7. The Qiagen-tip was washed with 2 x 10ml of buffer QC.

8. DNA was eluted from the Qiagen-tip with 5ml buffer QC.

9. Eluted DNA was precipitated with 0.7 volumes of isopropanol at room temperature.

10. Samples were centrifuged at 8,000 g for 30 minutes at 4°C.

11. DNA pellets were washed with 5ml of cold 70% ethanol,and left to air dry for 5 minutes. DNA was redissolved in a suitable volume of deionised water (for automated sequencing Charing Cross/Durham) or TE pH 7.4 (for manual sequencing).

Hybaid Recovery Plasmid Midi Prep Kit Protocol;

The Hybaid Recovery Kit was employed in preference to the QIAGEN Midi Kit in response to the simpler handling of large sample numbers that the kit provided.

1. INVαF' *E. coli* cells were harvested from 20ml L-broth cultures by centrifugation at 4°C for 5 minutes at 4,000 g.

2. Supernatant was decanted and cells were resuspended in 500µl of deionised water and then transferred to a 2ml microcentrifuge tube.

3. Samples were centrifuged for 30-60 seconds, the supernatant was removed, samples were briefly centrifuged again and residual liquid removed.

4. 200µl pre-lysis solution was added to each solution and mixed by vortexing or pippetting up and down until the cells are completely suspended.

5. 400μl alkaline lysis solution was added to samples which were gently inverted 15 times. Samples were incubated at room temperature for 5 minutes.

6. 300μl ice cold neutralising solution was added to samples and vortexed for 5 seconds at full speed.

7. Samples were placed on ice for 5 minutes and centrifuged at 14,000 g for 5 minutes at room temperature. Supernatant was transferred to a clean 2ml tube.

8. To the supernatants 900μl of binding buffer (redissolved at 60°C) was added and sample tubes were inverted gently 15 times to ensure a uniform mixture for efficient DNA binding to the binding buffer.

9. Binding buffer/DNA complex was allowed to settle at room temperature for 1-4 minutes. Upper supernatant was removed and discarded. The binding buffer/DNA mix was transferred to a spin filter.

10. Samples were centrifuged at 14,000 g for 5 minutes at room temperature, supernatant was decanted from the catch tube. 500μl wash solution was added to the spin filter which was then centrifuged at 14,000 g for 2 minutes.

11. The catch tube was emptied and the spin filter was centrifuged again at 14,000 g for 5 minutes to dry the filter contents. The filter was transferred to a new catch tube.

12. DNA was eluted from the spin filter in 100μl of deionised water, water was applied to filter by gently stirring binding mix with a pipette tip.

13. The spin filter placed within a catch tube was centrifuged at 14,000 g for 5 minutes at room temperature. The spin filter was discarded and eluted plasmid DNA stored at 4°C or -20°C.

DNA purified using QIAGEN and Hybaid protocols were diluted to a concentration of 250ng/ml in deionised water and despatched for automated sequencing either at the; Advanced Biotechnology Centre, Charing Cross and Westminster Medical School or Department of Biological Sciences, University of Durham.

Sequencing of differential display products;

QIAGEN purified differential display fragments; 19.1, 19.2 and 31.1 were sequenced manually using the SEQUENASE DNA sequencing kit, version 2.0 (USB) and the following protocol that represents a variation of the dideoxy Sanger method (Sanger *et al* 1977);

1. To 20μl of QIAGEN purified DNA (250 ng/μl),10μl of deionised water and 3μl of 2N sodium hydroxide were added. Samples were then incubated at room temperature for 5 minutes.

2. 120μl chilled ethanol and 15μl 1M sodium acetate pH 5 were added to samples and left to incubate at room temperature for 5 minutes.

3. Samples were centrifuged at 14,000 g for 5 minutes at room temperature and supernatant was removed.

4. The DNA pellet was washed in 70% ethanol and then centrifuged at 14,000 g for 5 minutes at room temperature. The supernatant was removed and the pellet was left to air dry for 4 minutes.

5. To this denatured DNA; 1μl stock primer (6 pmole) (T7 or SP6) and 2μl reaction buffer were added.

6. DNA was resuspended and annealed at 65°C for 2 minutes.

7. DNA was left to cool at room temperature to 35°C over 15-30 minutes and then chilled on ice.

8. 2.5μl of termination mixes were aliquoted into each well of a microtray; A, G, C, T.

9. Termination mixes were prewarmed at 37°C.

10. Labelling mix was diluted 1:5.

11. Sequenase was diluted 1:8 in enzyme dilution buffer; 1μl Sequenase added to; 0.5μl Pyrophosphate and 6.5μl buffer.

12. To ice cold annealed DNA mix (10μl); 1μl DDT 0.1M, 2μl diluted labelling mix, 0.5μl ^{35}S dATP, 2μl diluted sequenase were added. Samples were mixed and incubated at room temperature for 2-5 minutes.

13. 3.5μl of labelling reaction was transferred to each termination well, mixed and incubated for 5 minutes at 37°C.

14. 4μl stop solution was added to each sample. Samples were heated to 75-100°C for 2 minutes immediately before loading onto a sequencing gel.

15. A 6% DNA sequencing gel was prepared and loaded as previously described for differential display analysis. The sequencing gel was run at 40 watts constant power for approximately 3 hours.

16. Sequencing gels were fixed by gently pouring over gel fixitive solution **(appendix III)** and leaving for 5 minutes. This fixitive step was repeated once and the gel was then dried and placed in a cassette with autoradiographic film for exposure, as previously described for differential display gels.

Purification of differential display products;

Reamplified DNA from differential display gels were gel purified using the QIAquick gel extraction kit and reagents (QIAGEN).

1. 30μl of reamplified differential display product was electrophoresed on a 1.8% agarose gel.

2. DNA band was excised from the gel with a clean scalpel.

3. The gel slice was weighed and 3 volumes of buffer QX1 was added to one volume of gel.

4. Samples were incubated at 50°C for 10 minutes. Dissolution of the gel was aided by flicking and inverting the tube every 2-3 minutes.

5. A QIAquick spin column was inserted into a 2ml collection tube.

6. Sample was loaded into the spin column and centrifuged for 60 seconds at 14,000 g. Elutant was discarded.

7. 0.75ml buffer PE was added to spin column. Columns were centrifuged at 14,000g for 60 seconds and elutant was discarded. Centrifugation was repeated and any remaining elutant was also discarded.

8. QIAquick columns were inserted into clean 1.5 ml microfuge tube.

9. DNA was eluted by adding 50µl 10mM Tris-HCL pH 8.5 and centrifuged for 60 seconds at 14,000 g

Radiolabelling of purified differential display products.

Purified differential display products were random primed labelled using the protocol and reagents supplied in the Rediprime kit (Amersham). This labelling method was originally based on the procedure developed by Feinberg and Vogelstein 1984;

1. The DNA to be labelled was diluted to a concentration of 25ng in 45µl of sterile water.

2. DNA was denatured at 95°C for 5 minutes and centrifuged briefly.

3. The denatured DNA was added to the labelling mix which was reconstituted by gently flicking the tube until the blue colour was evenly distributed. Labelling mix and DNA was centrifuged briefly.

4. 5µl of Redivue ^{32}P CTP (Amersham) was added to labelling preparation and mixed by gently pipetting up and down 4 to 5 times. Samples were centrifuged briefly.

5. Labelling reactions were incubated at 37°C for 10 minutes.

6. The reaction was stopped by adding 5µl of 0.2M EDTA.

The labelled reaction mixture was passed through a NICK Spin Column (Pharmacia) to separate unincorporated ^{32}P - labelled nucleotides from ^{32}P labelled nick translated DNA fragments. NICK Spin Columns were prepared and used as follows;

1. The columns were inverted several times to resuspend the gel, then placed upright to allow the gel to settle.

2. The top cap and bottom cap were removed. The column was allowed to drain. To remove any air bubbles the column was tapped gently.

3. Columns were placed in centrifuge tubes and 1 ml of equilibration buffer (TE pH 7.4 - **appendix III**) was added. Columns were allowed to drain.

4. A further 2 ml of equilibration buffer were added to columns and allowed to drain.

5. Using a counterbalance, columns were centrifuged for 4 minutes at approximately 500 x g in a swinging-bucket rotor centrifuge (Beckman). The column was removed from the supernatant and the eluate discarded.

6. The column was placed in an upright position and the labelled probe was applied to the centre of the flat gel surface.

7. An uncapped 1.5 ml microcentrifuge tube was placed inside the centrifuge tube. The loaded column was then placed inside the centrifuge tube, with the tip of the column inside the microcentrifuge tube. The sample was eluted by centrifugation for 4 minutes at 500 x g.

The microcentrifuge tube containing the labelled purified probe was removed from the centrifuge tube.

8. The labelled DNA probe was denatured by heating to 95-100°C for 5 minutes, then chilled on ice.

Isolation of mRNA samples.

Polyadenylated mRNA from HUASMC's cultured on plastic or fibronectin coated plastic were isolated using the Messenger RNA Isolation Kit from Stratagene. The procedure was carried out as follows;

1. HUASMC's were trypsinized from culture substrates as previously described (see methods **VI.3**.). Cells were collected by centrifugation.

2. Denaturing solution was prepared by adding 50μl of β- mercaptoethanol to 5 ml of room-temperature denaturing solution.

3. 5 ml of the prepared denaturing solution was added to the cell pellet. The cells lyse immediately and the DNA was sheared by passing the sample several times through a 21 - gauge needle.

4. 10 ml of room temperature elution buffer was added to the sample. Samples were mixed by inversion.

5. An RNase-free tube containing 1.3 ml of elution buffer was placed in a 68°C heating block.

6. Samples were centrifuged at 12,000 xg for 10 minutes at room temperature. Supernatant was removed.

7. The tube containing the oligo(dT) cellulose slurry was gently agitated to evenly resuspend the material. 5 ml of oligo(dT) cellulose slurry was transferred to a microcentrifuge tube for every sample to be processed.

8. The supernatant from step 6 was transferred to the oligo (dT) cellulose.

9. Gently rotate the tube for 15 minutes at room temperature, only fast enough to keep the cellulose in suspension.

10. The cellulose resin was pelleted by centrifuging the sample mix at 1,500 rpm for 4 minutes at room temperature. The supernatant was carefully removed and discarded.

11. The oligo (dT) cellulose pellet was resuspended in 5 ml high salt buffer. Repeat steps **10.** & **11.** two more times

12. After the third and final high-salt wash, the resin was pelleted, the high-salt buffer removed and the oligo (dT) cellulose pellet was subsequently resuspended in 5 ml of low salt buffer.

13. The sample was mixed and centrifuged at 1,500 rpm for 4 minutes at room temperature. The supernatant was removed and the oligo (dT) cellulose was resuspended in 5 ml of low-salt buffer.

14. A sterile 10 ml syringe was removed from the protective covering and the plunger partially pulled out. The syringe was replaced inside its wrapper to keep it RNase-free.

15. Using a transfer pipette a push column was filled with 2.5 ml from the 5 ml oligo (dT) cellulose suspension. The end of the syringe was attached to the push column.

16. The buffer was gently pushed through the column by depressing the plunger allowing the resin to pack into the uniform bed at the bottom of the column.

17. An additional 2.5 ml of oligo (dT) cellulose suspension was added to the column using the transfer pipette. The syringe was reattached and used to push all the remaining solution out of the column.

18. 400µl of 68°C elution buffer (from step 5) was added to the top of the resin and slowly pushed through the column using the syringe. Eluted RNA was collected in sterile RNase-free microcentrifuge tubes.

19. To precipitate RNA for concentration or storage, added to each sample was 1/10th volume of 3M sodium acetate and x2 volumes of 100% (v/v) ethanol. mRNA samples were stored at -70°C.

20. mRNA samples were resuspended by centrifugation at 12,000 g for 25 minutes at 4°C. Supernatant was aspirated off and RNA pellet resuspended in DEPC water to give desired concentration.

Northern blot procedure;

Electrophoretic separation and transfer of RNA to nylon membranes for analysis was achieved using a modification of the methods pioneered by (Alwine *et al* 1977 and Southern 1975).

1. A 1.5% agarose gel was prepared; 1.5g agarose was dissolved in 74 ml 1x TBE (prepared in DEPC water).

2. Molten agarose was allowed to cool down to 60°C prior to the addition of 17 ml formaldehyde 37% solution (Sigma).

3. 9 mls of 10x MOPS **(appendix III)** was added to agarose solution to give a final concentration of 1x.

4. Agarose solution was mixed gently ensuring no air bubbles develop.

5. 80 mls of agarose mix was poured into Horizon 11.14 RNase free electrophoretic chamber containing 14 tooth comb. Agarose mixture was left to set for 45 minutes - 1 hour.

6. Sample loading buffer was prepared as follows;

Formamide (deionised)	12.5µl
Formaldehyde	5µl
10 x MOPS	2.5µl
DEPC	2.5µl

Formaldehyde loading buffer **(appendix III)** 2.5µl

7. Loading buffer was briefly centrifuged and 25µl was added to 5µl of RNA samples (4µg/µl concentration) or 5µl of RNA size markers.

8. Samples were briefly centrifuged, and incubated at 65°C for 15 minutes.

9. Samples were then placed on ice, 30µl of sample (20µg RNA) was loaded per well on the northern gel.

10. RNA samples were electrophoresed through the gel at a constant 115 volts for 2 hours 30 minutes.

Separated RNA species were transferred by capillary action to a nylon membrane for further analysis (Southern 1975);

1. 20x SSC was poured into a tray.

2. A glass plate was placed on top of an eppendorf pipette lid to provide a gel support.

3. 2 pieces of 3mm paper wick were prepared, 3mm paper was prewet in 20xSSC and laid over glass plate.

4. Once electrophoresis complete the power pack was switched off and the gel removed from the electrophoresis chamber. Marker tracks were cut off the gel and soaked in DEPC water.

5. The remainder of the northern gel was inverted on to the 3mm paper wick.

6. The peripheral edges of the gel was surrounded with Saran wrap.

7. Hybond membrane (Amersham) was cut to just larger than the gel. The membrane was placed on the gel ensuring no air bubbles develop.

8. 2 pieces of 3mm paper, just larger than the membrane were soaked in 2xSSC. The filter paper was placed over the membrane ensuring no air bubbles develop.

9. A vertical stack of paper towels was placed on top of the 3mm paper, and then a sandwich box containing an even 1 kg weight was placed on the towels.

10. The blotting apparatus was left overnight.

- Marker lanes were stained in 250 ml DEPC water + 10µl 1% ethidium bromide. for 3 minutes, the gel was then rinsed with DEPC water overnight and photographed with a ruler adjacent to the gel to record distance markers had migrated through gel.

11. 3mm filter paper and paper towels were removed following overnight incubation, The location of gel wells was marked on Hybond membrane with pencil.

12. Membranes were placed on moist (DEPC water) 3mm paper. Membranes were placed RNA side up in crosslinking oven (Stratagene) and subjected to autocrosslink procedure.

13. Membranes were wrapped in saran wrap and stored at room temperature in a dry place ready for probe hybridisation reactions.

Probe hybridisation to northern blot;

Membranes were pre-hybridised in Rapid-hyb buffer (Amersham) at 65°C for 15 minutes. Membrane hybridisation reactions were performed in rotating flasks within a Hybaid oven. The radiolabelled probe was added to the Rapid-hyb prehybridisation buffer and allowed to hybridise to the membrane for 3-4 hours at 65°C. Following incubation of probe the hybridisation buffer was poured off and membranes were washed as follows;

20 minutes in 50ml 2 x SSC, 0.1% (w/v) SDS at room temperature.

2 x 15 minutes in 50 ml 1 x SSC, 0.1% (w/v) SDS at 65°C.

If membrane still contained widely dispersed radioactive label an additional wash in 0.1 x SSC, 0.1% (w/v) SDS at 65°C was performed.

VI.17 Appendix III

10X TBE

Tris base (Sigma)	108.8g
Boric acid (Sigma)	55.6g
EDTA disodium salt (Sigma)	9.3g

-Dissolve in 1 litre deionised water. For 1X TBE dilute 10X TBE 1 in 10 with deionised water.

1% ethidium bromide solution

Dissolve 1g ethidium bromide (Electran) (BDH) in 100 ml deionised water.

1% agarose minigel;

Add 25 ml of 1X TBE to 0.25g agarose (Gibco BRL). Microwave to dissolve, let cool and add 1µl 1% ethidium bromide solution. Gently mix solution and pour into Horizon minigel casting equipment (BRL).

1.8% agarose minigel;

Add 25 ml of 1X TBE to 0.45g agarose (Gibco BRL). Microwave to dissolve, let cool and add 1µl 1% ethidium bromide solution. Gently mix solution and pour into Horizon minigel casting equipment (BRL).

LB Medium

bacto-tryptone (DIFCO)	10g
bacto-yeast extract (DIFCO)	5g
NaCl (Sigma)	10g

-Add 950 ml deionised water and stir to dissolve. Adjust the pH to 7.0 with 5 N Sodium

Hydroxide (BDH). Make volume up to 1 litre with deionised water. Sterilise by autoclaving for 20

minutes at 15 lb/sq. in. on liquid cycle.

Lb-agar

bacto-tryptone (DIFCO)   10g

bacto-yeast extract (DIFCO)   5g

NaCl (Sigma)   10g

-Add 950 ml deionised water and stir to dissolve. Adjust the pH to 7.0 with 5 N Sodium

Hydroxide. Make volume up to 1 litre with deionised water. Add 15g agar (LAB M). Sterilise by

autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.

SOC medium (Invitrogen)

Tryptone   2%

Yeast extract   0.5%

NaCl   10mM

Kcl   2.5mM

MgSO4   10mM

glucose   20mM

TE buffer

Tris Hcl pH 7.4(Sigma)   10mM

EDTA pH 8.0(Sigma)   1mM

Rediprime labelling mix

Each aliquot of labelling mix contains the following in a stabilized form;

- A buffered solution of dATP, dGTP, dTTP, exonuclease free Klenow enzyme and random primers (9 mers).

10X MOPS

MOPS (Sigma)	41.2g
3M Sodium Acetate (BDH)	16.6 ml
500mM EDTA disodium salt (Sigma)	20 ml

-Make up to 1 litre with DEPC treated water and stir to dissolve. Sterilize by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.

1.5% agarose/formaldehyde gel

Add 74 ml 1X TBE to 1.5g agarose, dissolve in microwave. Molten agarose is cooled down to 60°C prior to the addition of 17 ml fomaldehyde (37% solution) (Sigma) and 9 ml of 10X MOPS. Agarose solution is mixed gently and poured into Rnase free Horizon 11:14 casting equipment.

Formaldehyde gel-loading buffer

50% glycerol (Fisons)

1mM EDTA (pH 8.0) (Sigma)

0.25% bromophenol blue (Sigma)

0.25% xylene cyanol FF (Sigma)

Solution I

Glucose(BDH)	9g
2M Tris HCL(Sigma)	12.5 ml

0.2M EDTA disodium salt (Sigma)   50 ml

-Make up to 1 litre with deionised water. Store at 4°C

Solution II

2N Sodium Hydroxide (BDH)   20 ml

10% SDS (Bio Rad)   100 ml

-Make up to 1 litre with deionised water and store at room temperature.

Solution III

5M Potassium acetate (BDH)   147.21g

Glacial acetic acid (BDH)   57.2 m

-Make up to 500 ml with deionised water adjust pH to 4.8 and store at 4°C.

Sequencing gel fixitive

Methanol (Fisons)   200 ml

Acetic acid (BDH)   200 ml

-Make up to 2 litres with deionised water.

RESULTS

Chapter 1 (Section VII).

Morphological and Functional Analysis of Vascular Smooth Muscle Cells Cultured on Different ECM Substrates.

VII.1. Introduction - Vascular SMC behaviour in culture.

A number of previous studies characterising the behaviour of vascular SMC's within an *in vitro* culture system, suggest that SMC's freshly isolated from normal vascular tissue samples undergo spontaneous modulation from a contractile phenotype to a synthetic phenotype. This transition in phenotype is characterised by the reorganisation of the cytoskeleton that includes the loss of myofilament bundles and formation of actin containing stress fibres and radiating networks of microtublules and intermediate filaments. An increase in the amount of rough endoplasmic reticulum and golgi apparatus is also observed (Thyberg *et al* 1990). In parallel with such changes in the contractile and secretory apparatus a definite shift in the expression of SMC specific isoforms of contractile and cytoskeletal proteins to non-muscle isoforms can be observed. For example during the first few days in culture decreased expression of smooth muscle α-actin is accompanied by increased expression of β - actin (Glukhova *et al* 1986). Similarly the expression of SM - Myosin heavy chain is downregulated, while the expression of non-muscle forms of myosin increase (Kawamoto & Adelstein 1991; Rovner *et al* 1986). In addition overall RNA and protein synthesis together with cell proliferation are also activated upon isolation of SMC's in culture. Moreover the synthesis and secretion of extracellular matrix molecules such as; Collagen I; Elastin; Fibronectin; Thrombospondin and proteoglycans is also enhanced. (Ang *et al* 1990; Liau & Chan 1989).

The apparent "dedifferentiation" of SMC's induced by cell culture correlates with the activation of synthetic and secretory functions, a process closely resembling the phenotypic modulation of SMC's from a contractile to a synthetic phenotype that accompanies the initial development of atherosclerotic and restenotic lesions (Thyberg *et al* 1990). This spontaneous phenotypic modulation is thought to occur in response to the presence of serum derived

growth factors and cytokines as well as an altered extracellular matrix environment that exist in culture conditions. Thus all SMC's isolated in culture undergo modulation becoming representative of the synthetic phenotype.

Further studies characterising the progression of vascular SMC's *in vitro* demonstrate that when SMC's are grown on ordinary uncoated tissue culture surfaces, they form a preconfluent monolayer; subsequently regions of this monolayer develop characteristic multicellular foci that have been described as "hill and valley" structures. Observations by Thomas-Salgar and Millis 1994 and Li *et al* 1994 indicate that these structures generate functionally differentiated nodules that consist of quiescent SMC's embedded in a carbohydrate rich extracellular matrix (Brennan *et al* 1982; Li *et al* 1994; Salgar & Millis 1994). SMC's within nodules contain myofilaments and express proteins including clusterin that appear to be related to cell differentiation (Diemer *et al* 1982; Li *et al* 1994). These characteristics indicate that SMC's within such nodules maybe regaining previously lost differentiated contractile phenotypic properties.

It has been postulated that the formation of nodular structures is regulated by the presence of extracellular matrix (ECM) and some studies have demonstrated that fibronectin delays the onset of nodule formation and maintains cells in a synthetic phenotype (Brennan *et al* 1982; Hedin & Thyberg 1987). Therefore the analysis of vascular SMC growth morphology *in vitro*, particularly the transition from a monolayer to a nodular morphology, may be useful for studying regulators of SMC phenotypic modulation. To assess the role that ECM has on modulating human SMC phenotype *in vitro* my studies have included examining alterations in the growth morphology of human umbilical artery SMC's induced by culture on various matrix coated substrates.

The initiation of SMC proliferation and migration by various inflammatory stimuli has been proposed as the underlying events responsible for the formation of the neointima and subsequent vessel occlusion that characterises restenosis and atherosclerosis (Ross 1986). In

order to investigate the role that ECM molecules play in regulating mitogenic and chemotactic responses of SMC's, the studies described in this thesis have included the development of *in vitro* SMC proliferation and migration assays. These assays were designed to allow comparison of SMC proliferation and migration responses to various mitogenic and chemotactic stimuli between SMC's cultured on different extracellular matrix substrates.

The morphological growth patterns of vascular SMC's cultured on each matrix substrates together with growth patterns on ordinary uncoated tissue culture grade plastic (Nunclon) were assessed by light microscopy. Photomicrographs (X40 magnification) recorded SMC growth morphology **(FIG's. VII.1- VII.9.)**. Each photomicrograph is a representative example from at least 3 separate plating studies of SMC morphology on each substrate.

It was observed that SMC's cultured on uncoated tissue culture plastic form the characteristic "hills and valley" growth morphology with the majority of cells becoming rapidly incorporated within nodular structures **(FIG. VII.1)**. SMC's cultured on collagen I, plasma and cellular fibronectin grow in a clear monolayer pattern, the cells also appear elongated and orientate parallel to one another **(FIG. VII.2 - VII.4)**. SMC's cultured on collagen types III and IV, laminin and vitronectin also grow in a monolayer like fashion, however, localised areas of multicellular aggregation progressively develop on these substrates and a proportion of cells become incorporated into nodular like structures **(FIG. VII.5 - VII.8)**. All SMC's cultured on Matrigel in serum free medium rapidly form nodular structures **(FIG. VII.9)**. SMC cultures on Matrigel in medium supplemented with serum also form this nodular growth pattern.

(FIG.VII.1.) SMC's cultured on tissue culture grade plastic:
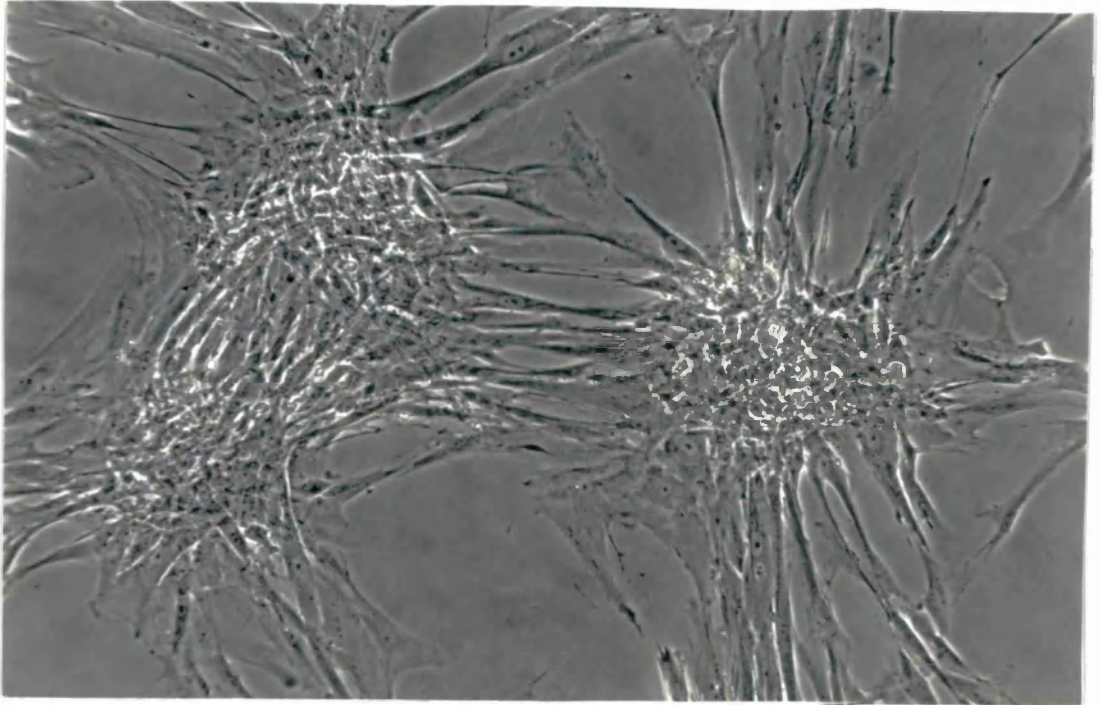
(FIG.VII.2.) SMC's cultured on plasma fibronectin:
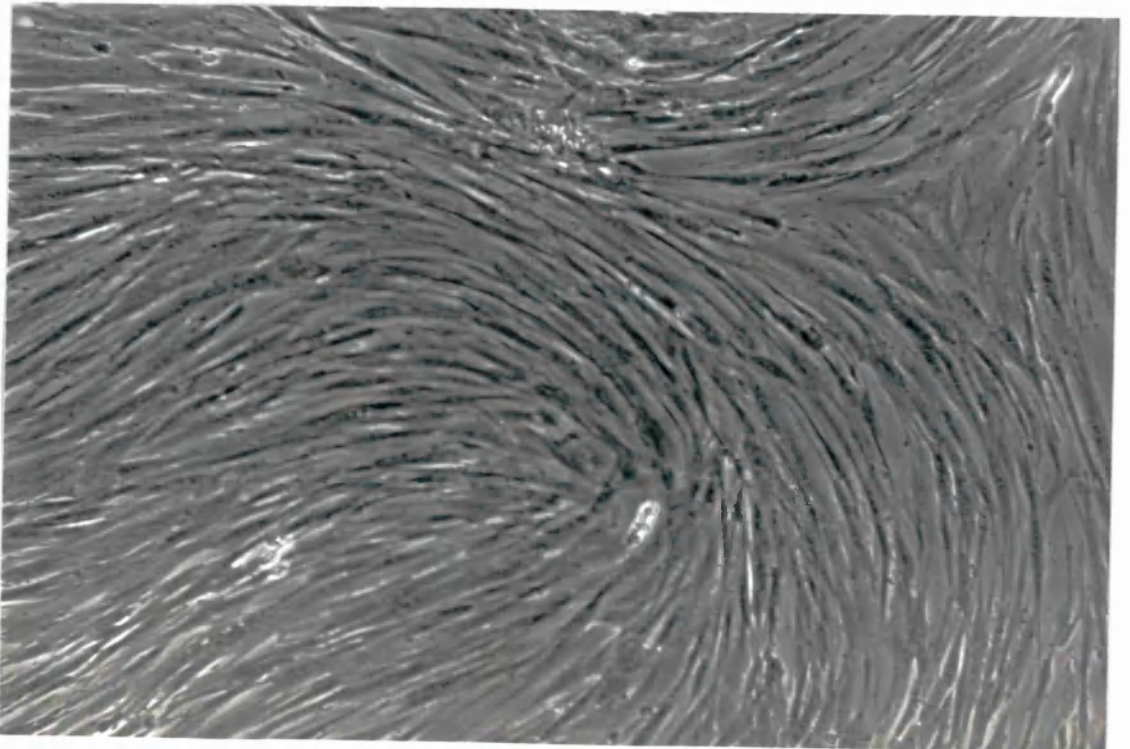
(FIG.VII.3.) SMC's cultured on cellular fibronectin:
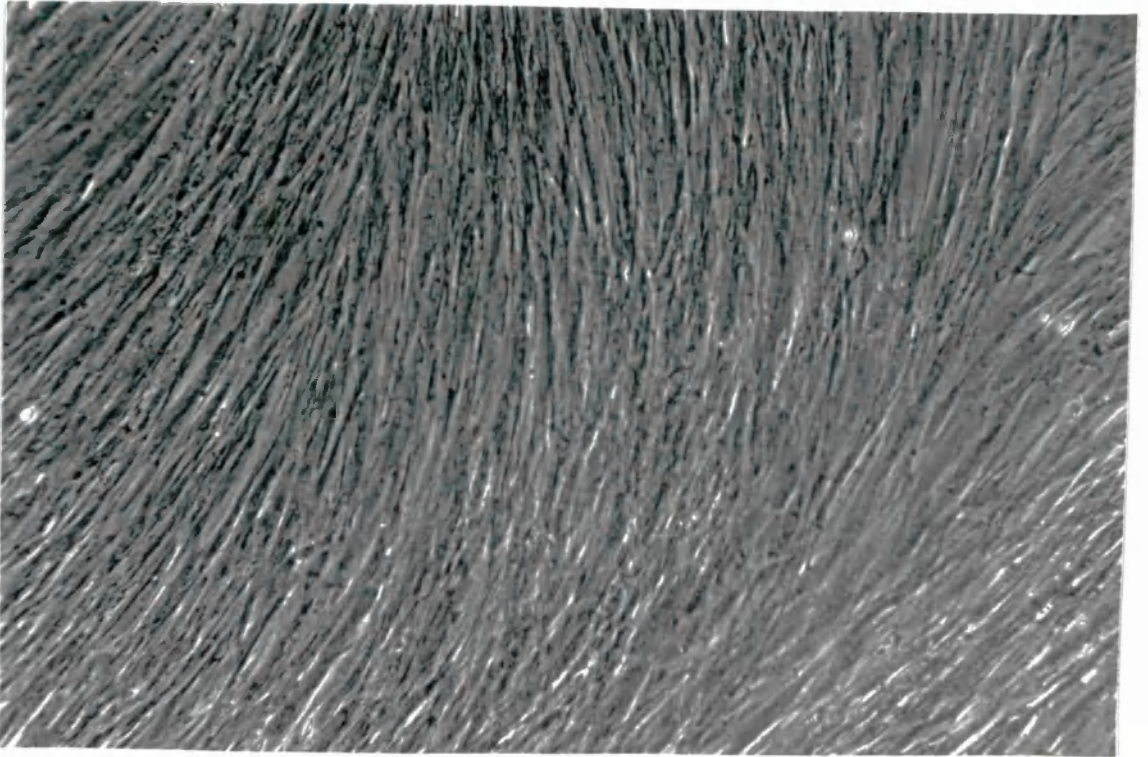
(FIG.VII.4.) SMC's cultured on collagen I:
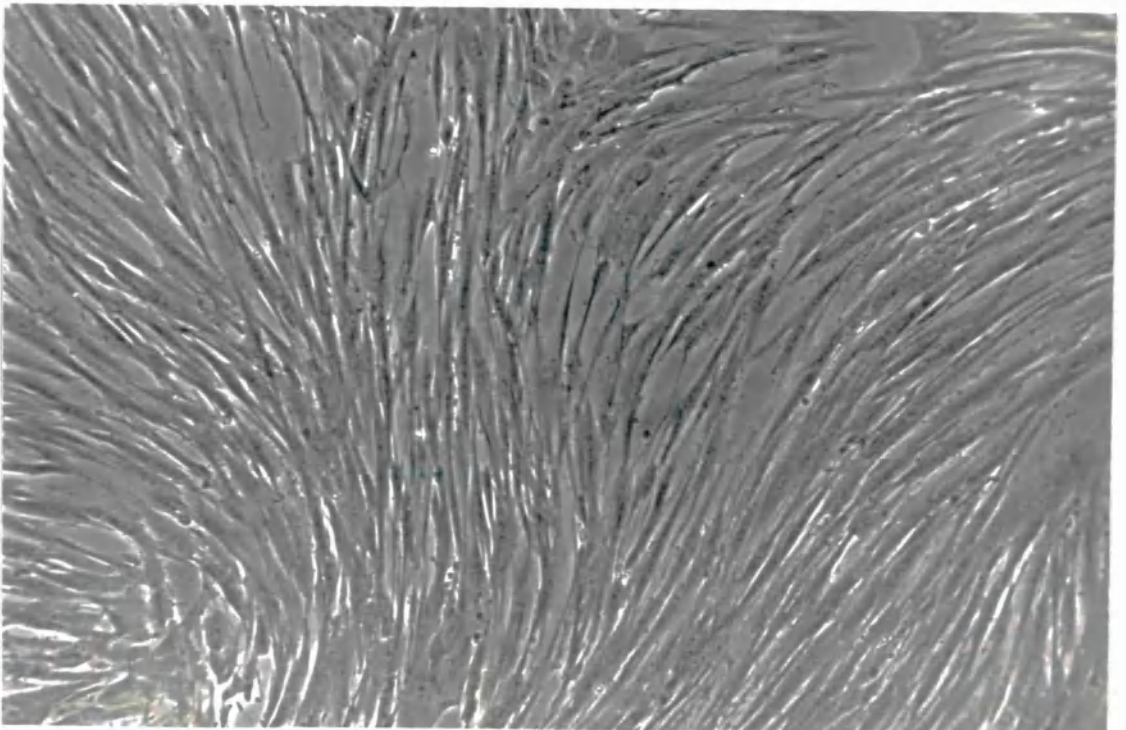
(FIG.VII.5.) SMC's cultured on collagen III:
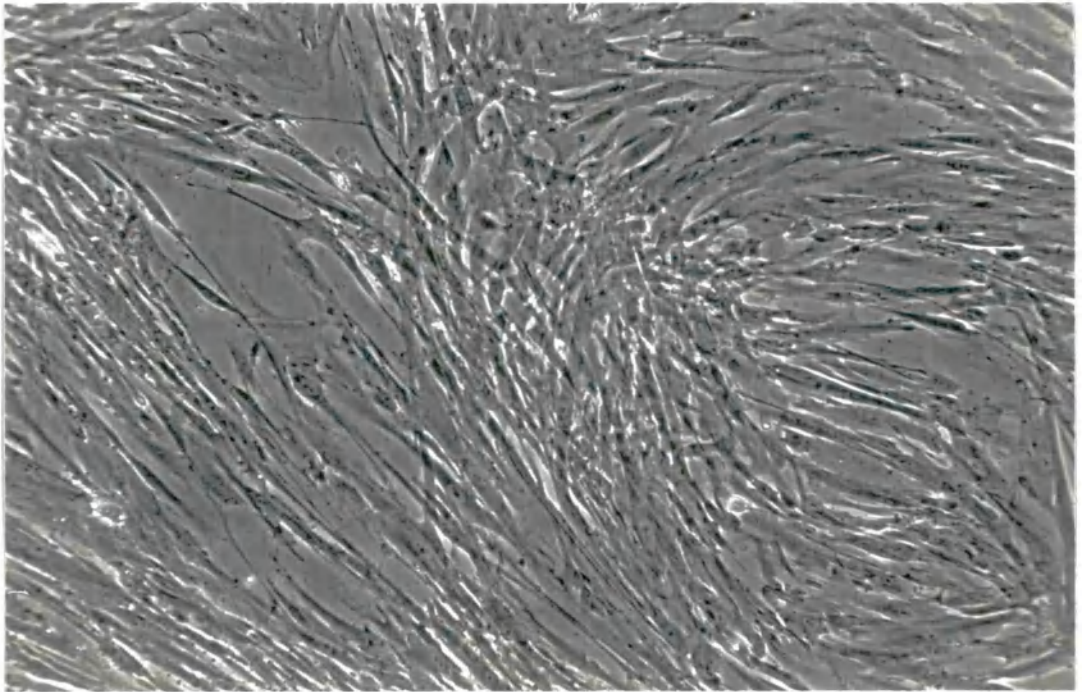
(FIG.VII.6.) SMC's cultured on collagen IV:
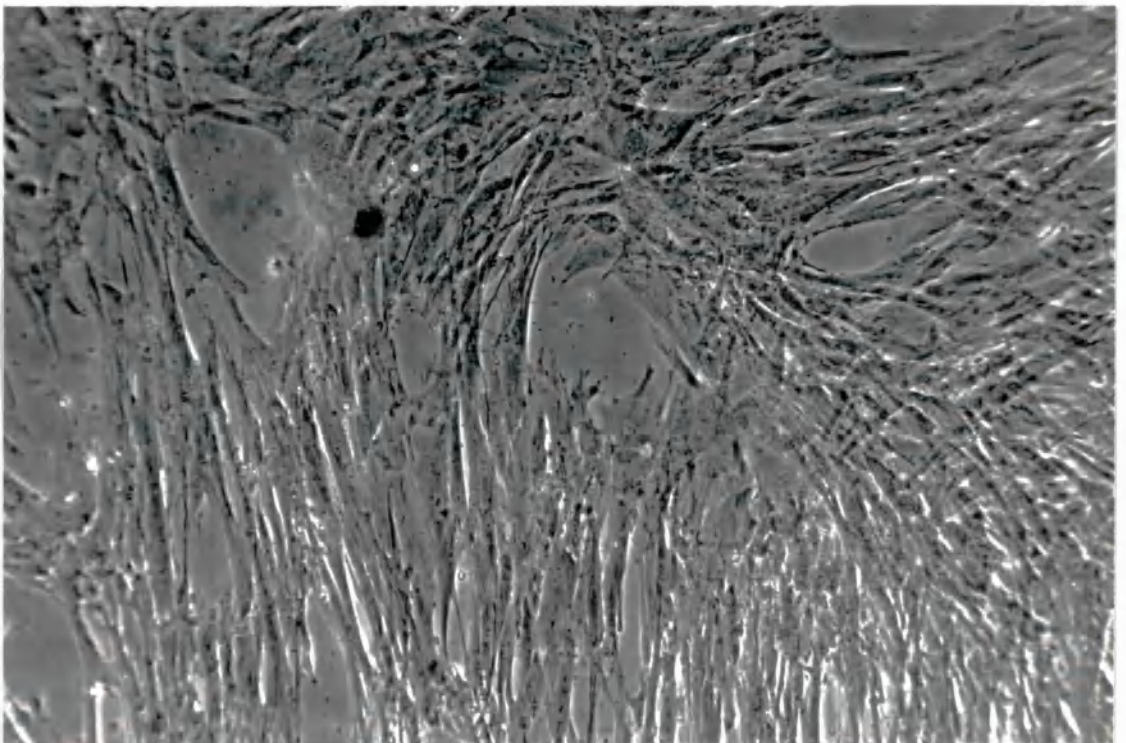
(FIG.VII.7.) SMC's cultured on laminin:
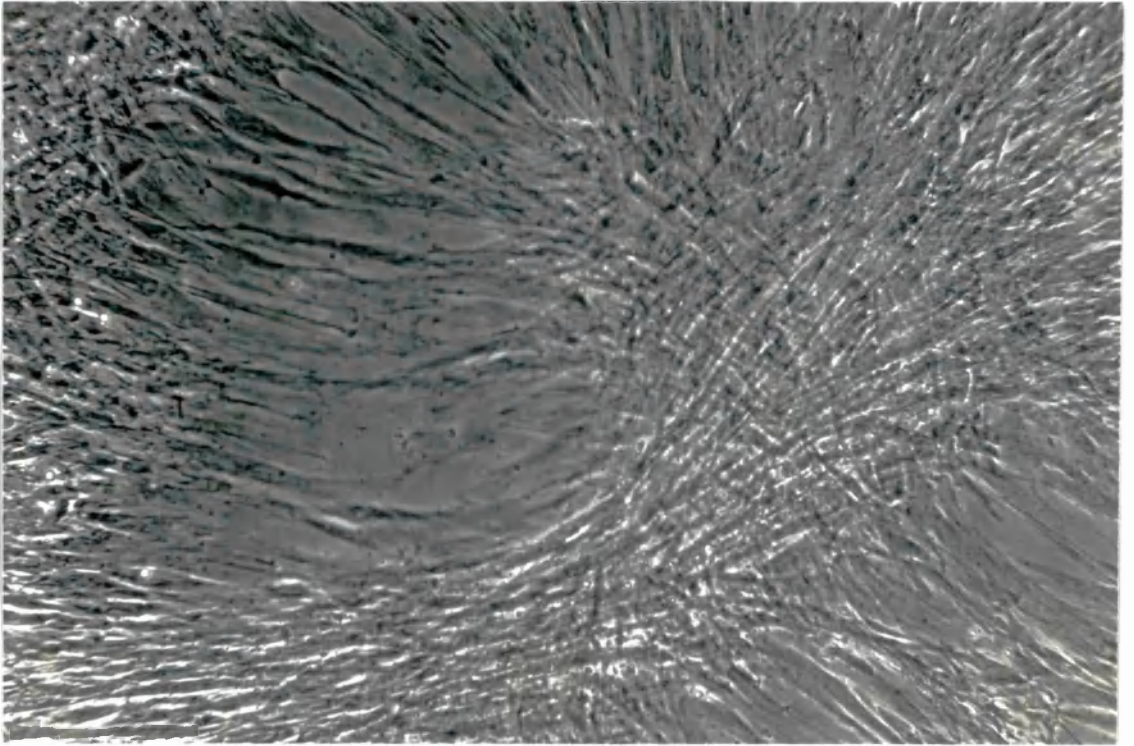
(FIG.VII.8.) SMC's cultured on vitronectin:
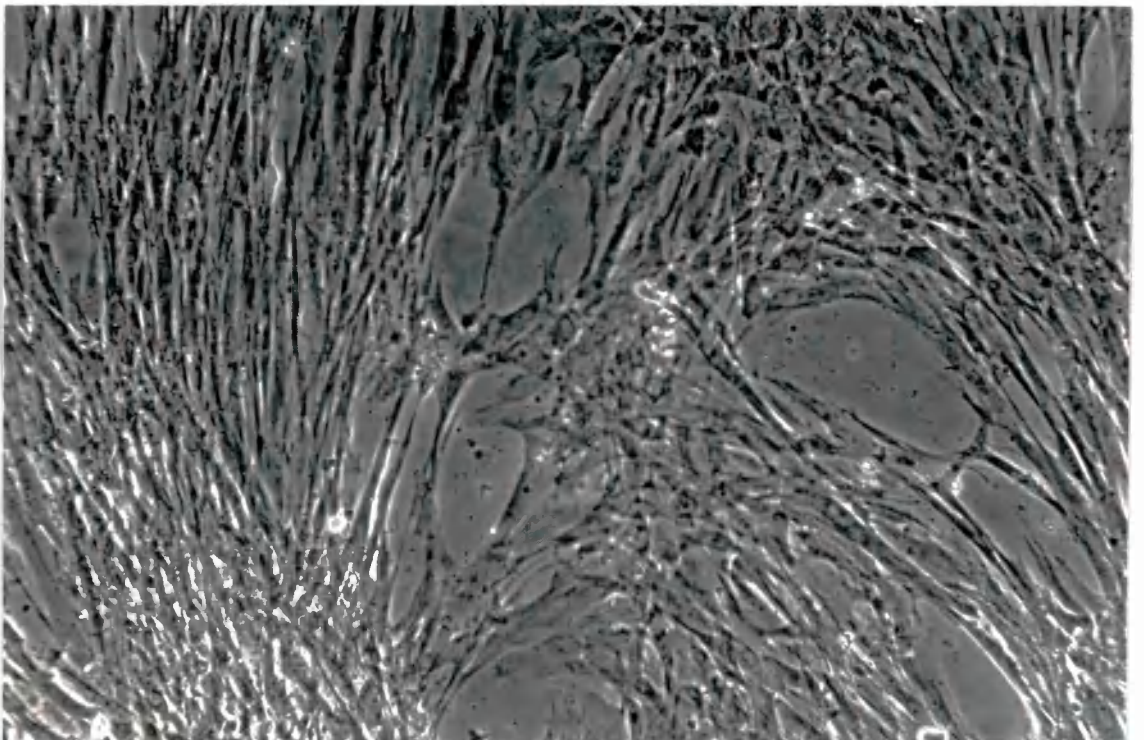
(FIG.VII.9.) SMC's cultured on Matrigel:
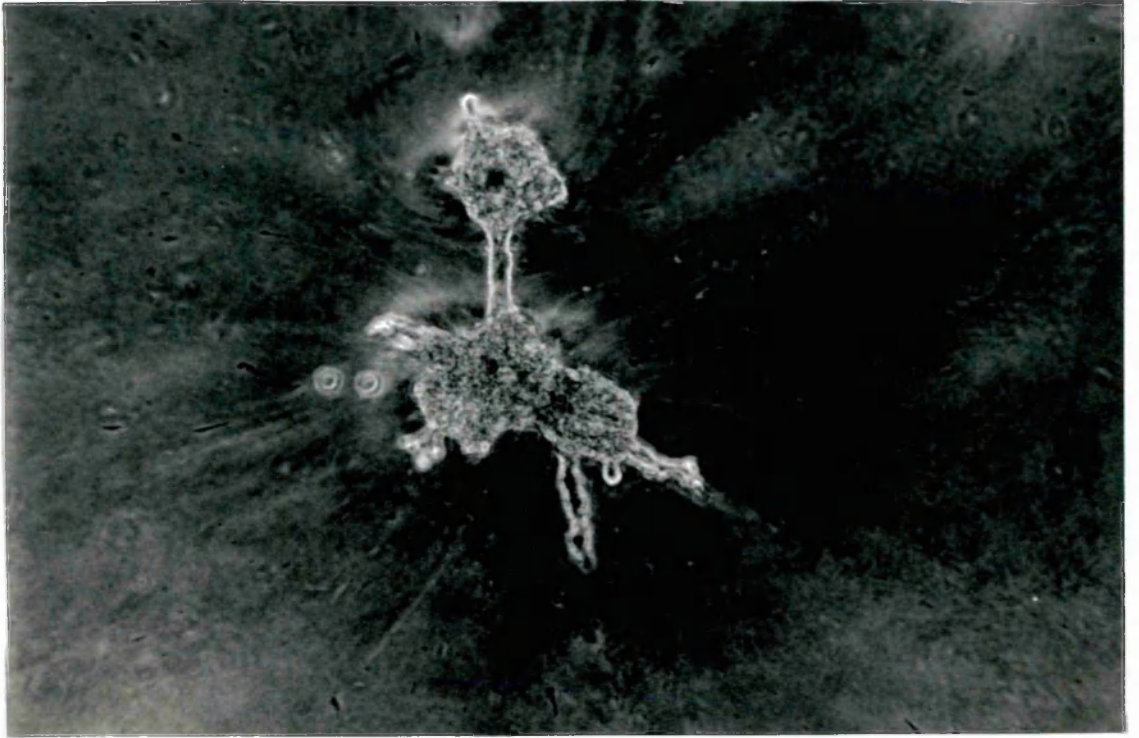
Applying the Hoechst DNA stain proliferation assay, growth curves of HUASMC's cultured on different matrix substrates were obtained. **(FIG.VII.10)**, displays typical growth curves for HUASMC's (passage 5) proliferation on plastic and plasma fibronectin coated surfaces in response to H:W 16%FCS medium. These results demonstrate enhanced proliferation rates in response to H:W 16%FCS by HUASMC's cultured on plasma fibronectin when compared with identical cells cultured on uncoated tissue culture plastic

(FIG.VII.10.)
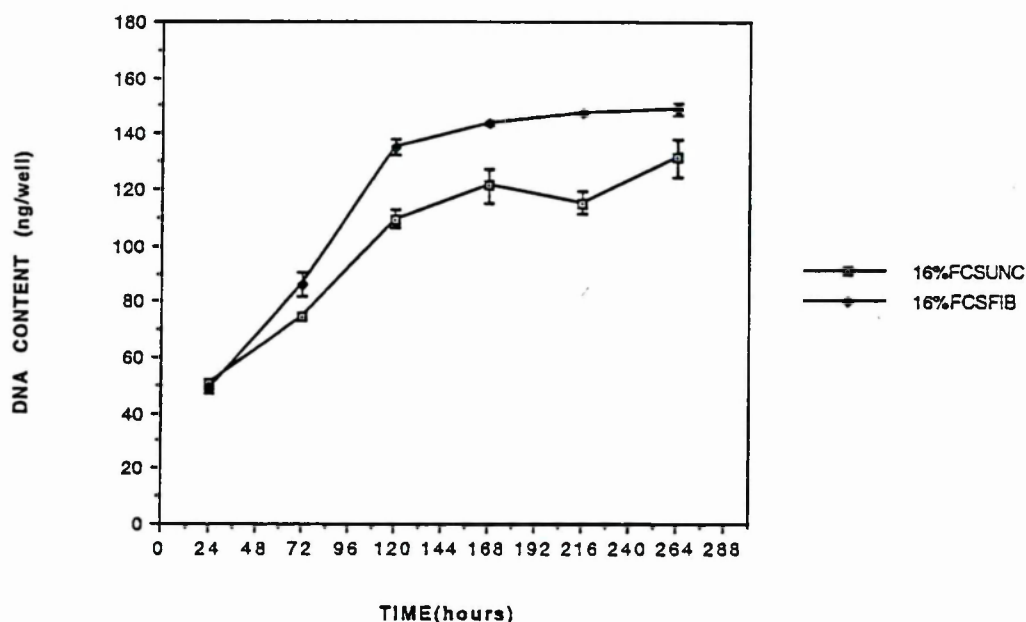
(FIG.VII.10) HUASMC Proliferation on Fibronectin Coated and Uncoated Plastic in response to H:W 16% FCS. This is a representative result from 3 experiments. Error bars are SEM. Data points at 24 hrs are not significantly different between fibronectin and plastic populations all subsequent data points are however significantly different between populations ($P < 0.05$) as determined by the student's t test.

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Similar studies on SMC cultures that have not been supplemented with fresh medium

reveal that SMC's cultured on fibronectin reach considerably higher cell densities than cells on

plastic. Also the lifespan of the cells was significantly extended by growth on fibronectin
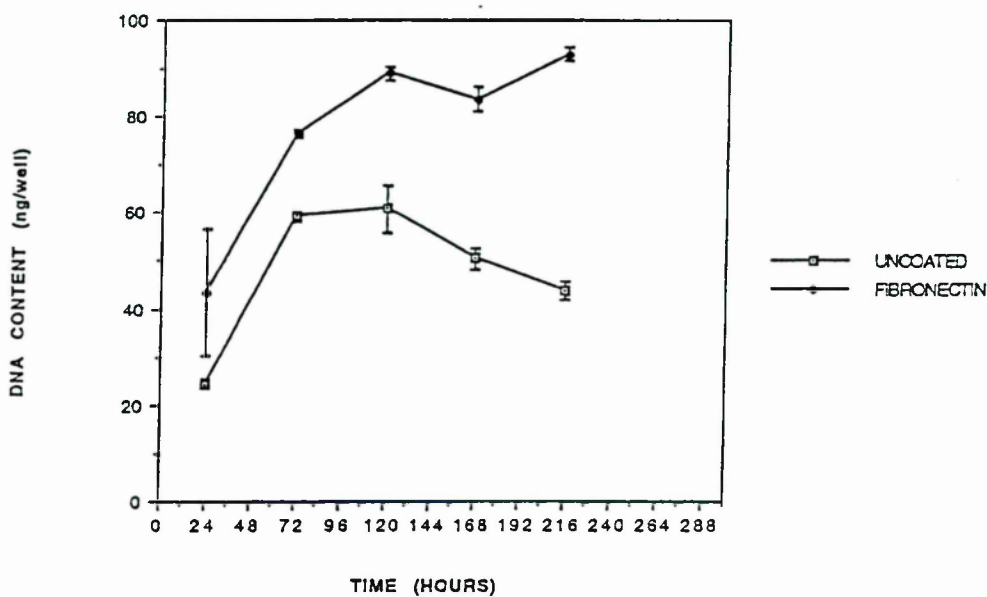
(FIG.VII.11).

(FIG. VII.11) HUASMC Proliferation on Fibronectin and Uncoated Plastic in Response to H:W 16% FCS (no medium change). This is a representative result from 3 experiments. Error bars are SEM. Data points are significantly different between plastic and fibronectin SMC populations (P < 0.05) as determined by the student's t test.

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Growth curves obtained from later passage HUASMC's (passage 10) were calculated to demonstrate more clearly the different rates of proliferation in response to H:W 16%FCS exhibited by SMC's cultured on different matrix substrates. **(FIG.VII.12.).**

(FIG.VII.12.).
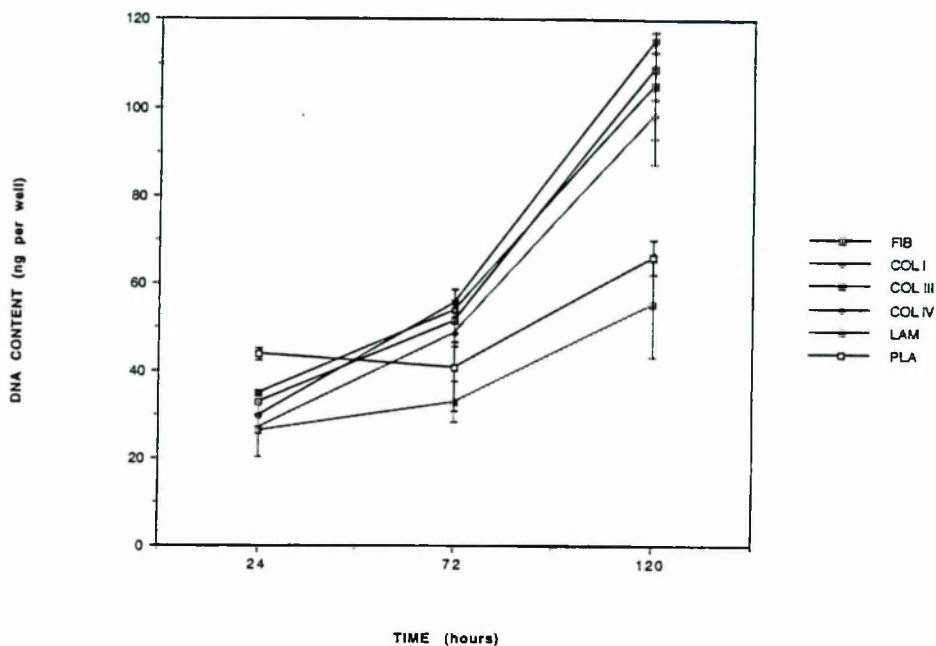
(FIG. VII.12.) HUASMC (passage 10) Proliferation on Plasma Fibronectin, Collagen's I, III, IV, Laminin and Plastic Substrates in Response to H:W 16% FCS. This is a representative result from 3 experiments. Error bars are SEM. All data points are not significantly different between various matrix cultured SMC populations with the exception that at 120 hours , the DNA content of SMC's cultured on plastic are significantly less than fibronectin, Collagen's I, III & IV cultured SMC's (P < 0.05). Also at this same time point the DNA content of SMC's cultured on Laminin is significantly less than that of SMC's on plastic (P < 0.05) as determined by the student's t test.

Protein assays demonstrated that any chemotactic gradient initially set up within Costar Transwell inserts was lost within half an hour as indicated by the fact that the serum concentration in the upper compartment increased from 0.1% to 8% FCS **(FIG.VII.13.)**.

(FIG.VII.13.).
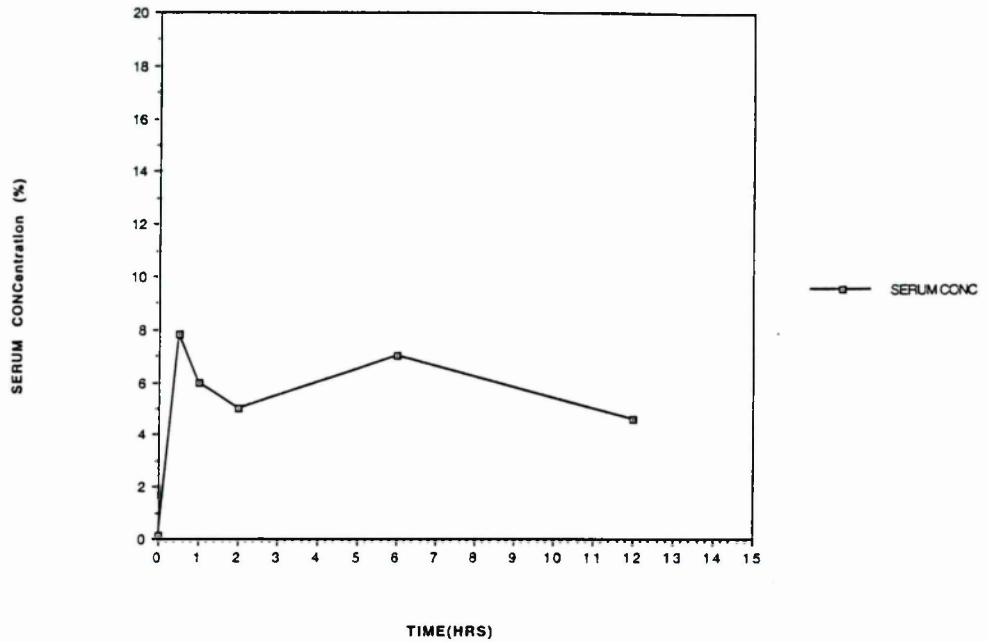
(FIG.VII.13.) Increase in Serum Concentration in the Upper Transwell Compartment Over Time.

To provide longer lasting gradients neat FCS was embedded in a 1% agarose gel that was cast in the lower compartment of the transwell. This agarose gel resulted in slow release of FCS out of the agarose providing a chemotactic gradient between the lower and upper transwell compartments that was maintained up to 24 hours.(FIG.VII.14.).

(FIG.VII.14.).
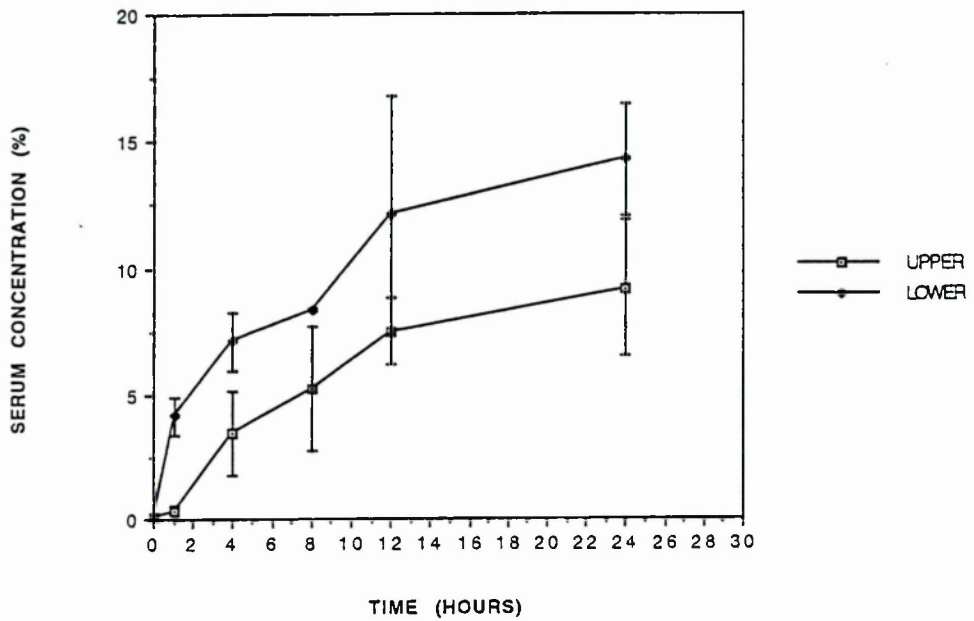
(FIG.VII.14) Increase in Serum Concentration in the Upper and Lower Transwell Compartments Over Time. This is a representative result from 2 experiments. Error bars are SEM. Data points at 1, 4 and 8 hours are significantly different between the upper and lower transwell ($P < 0.05$) as determined by the student's t test. All subsequent data points are not significantly different.

In order to compare SMC migration through fibronectin and uncoated transwell filters, HUASMC adhesion rates to fibronectin and uncoated tissue culture surfaces were assessed by DNA quantification at sequential time points following SMC seeding in uncoated and plasma fibronectin coated microtitre plate wells. These adhesion assays demonstrated that HUASMC's adhere much more rapidly to fibronectin coated surfaces than to uncoated surfaces. Also SMC adhesion was significantly enhanced in the presence of FCS.**(FIG.VII.15.).**

(FIG.VII.15.).
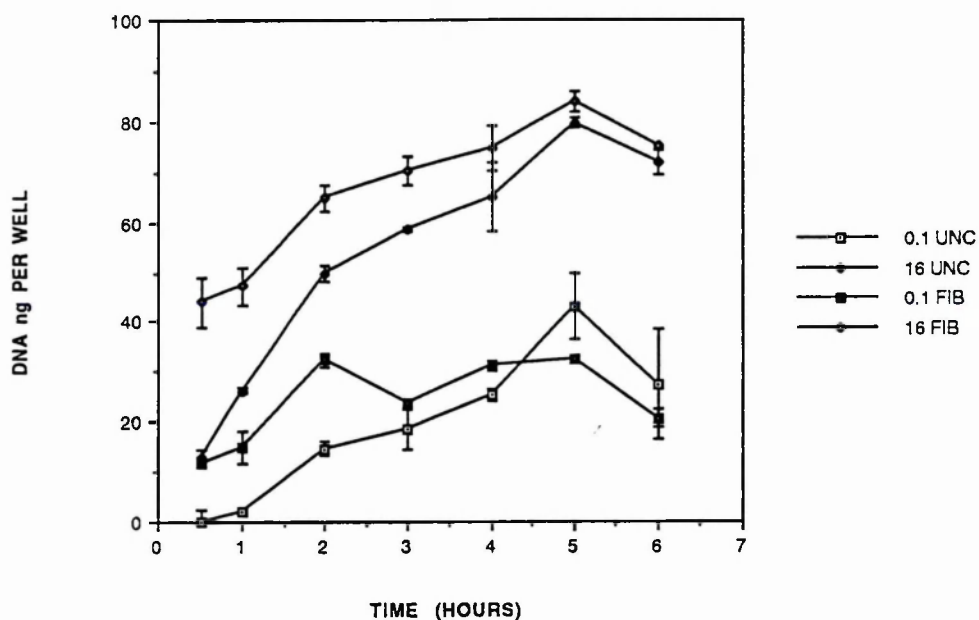
(FIG.VII.15.) HUASMC Adhesion to Fibronectin Coated and Uncoated Surfaces in the Presence of Either H:W 16%FCS or H:W 0.1% FCS. This is a representative result from 3 experiments. Error bars are SEM. Data points are significantly different between plastic and fibronectin SMC populations (P < 0.05) as determined by the student's t test.

Transwell SMC migration assay's demonstrated that SMC migration rates in response to a positive gradient of FCS are significantly greater on plasma fibronectin than on uncoated tissue culture surfaces **(FIG.VII.16.)**.

(FIG.VII.16.).
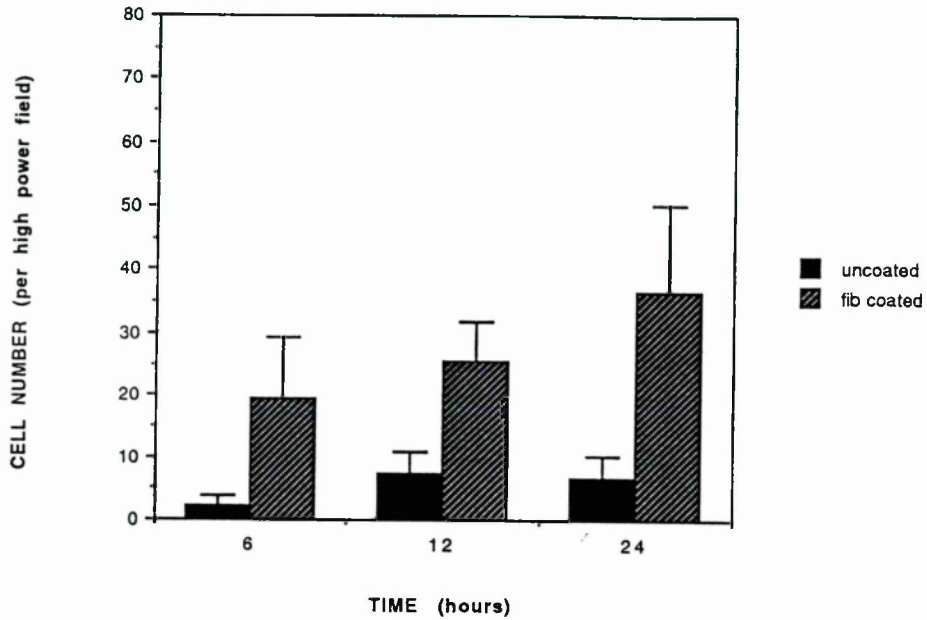
(FIG. VII.16). HUASMC Migration Through Fibronectin Coated and Uncoated Transwell Filters in Response to FCS. This is a representative result from 3 experiments. Error bars are SEM. Data points are significantly different between plastic and fibronectin SMC populations (P < 0.05) as determined by the student's t test.

A dose response of SMC migration towards increasing concentrations of PDGF BB and HGF was demonstrated through fibronectin coated transwell filters (FIG's. VII.17 & .18).
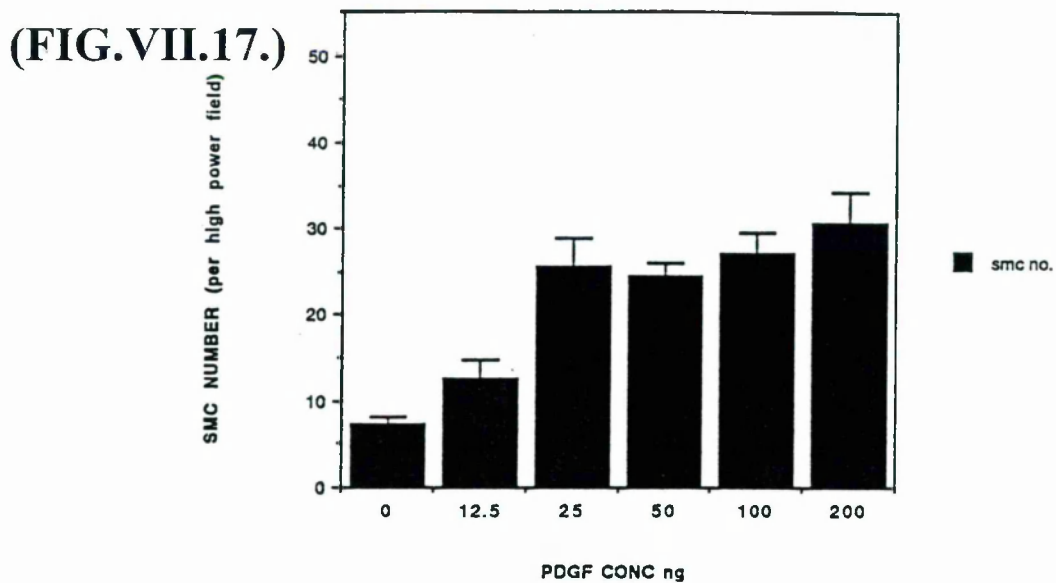
(FIG.VII.17.)

(FIG.VII.17.) HUASMC Migration Through Fibronectin Coated Filters in Response to PDGF BB. This is a representative result from several experiments. Error bars are SEM. Values are significantly different between 12.5 & 25ng concentrations, all subsequent values are not significantly different (P < 0.05) as determined by the student's t test.
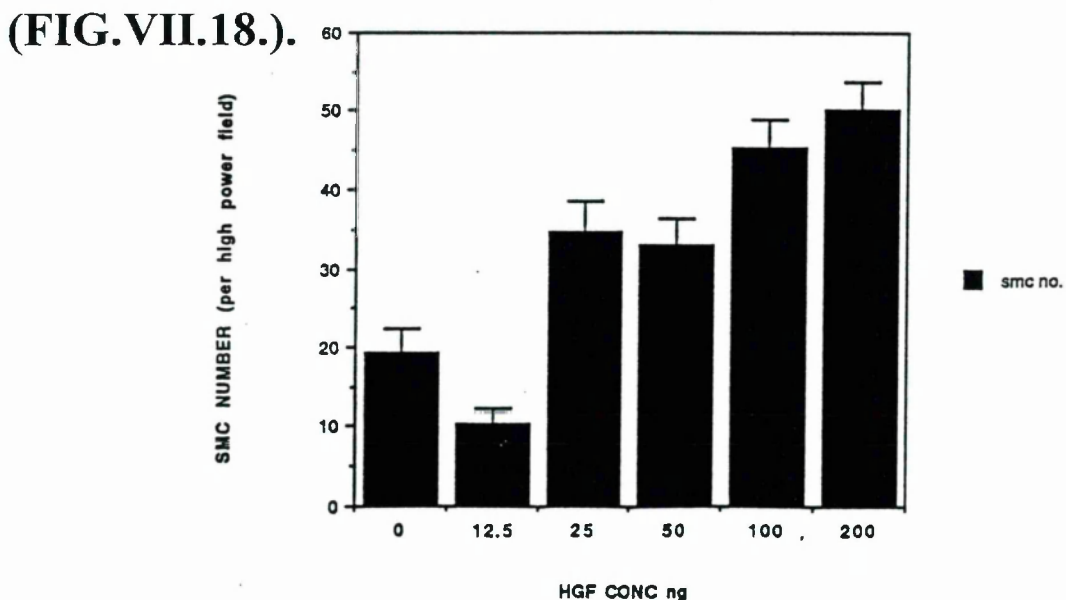
(FIG.VII.18.).

(FIG. VII.18.) HUASMC Migration Through Fibronectin Coated Filters in Response to HGF. This is a representative result from several experiments. Error bars are SEM. Values are significantly different between 12.5 & 25ng concentrations, all subsequent values are not significantly different (P < 0.05) as determined by the student's t test.

SMC migration rates in response to FCS differed through various matrix coated

Neuroprobe 96 well migration chamber filters **(FIG. VII.19.).**

(FIG.VII.19.).
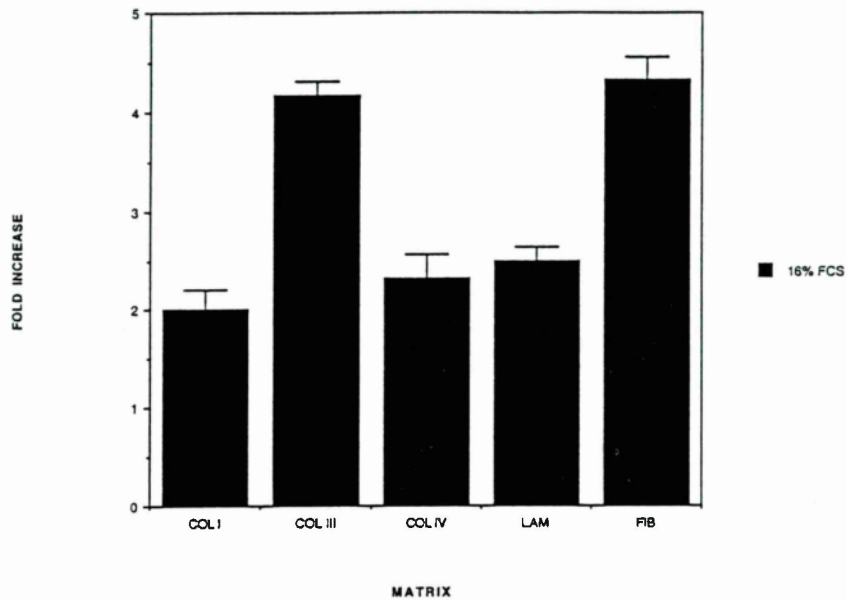
(FIG.VII.19.) HUASMC Migration Through Various Matrix Coated Filters in Response to H:W 16% FCS. This is a representative result from 3 experiments. Error bars are SEM. Migration values on collagen III and fibronectin are significantly different from values on Collagens I & IV and laminin ($P < 0.05$) as determined by the student's t test.

Migration in response to specific factors also varies depending on what type of matrix the SMC's are cultured on **(FIG's; VII.20-.22.)**.

(FIG.VII.20.).
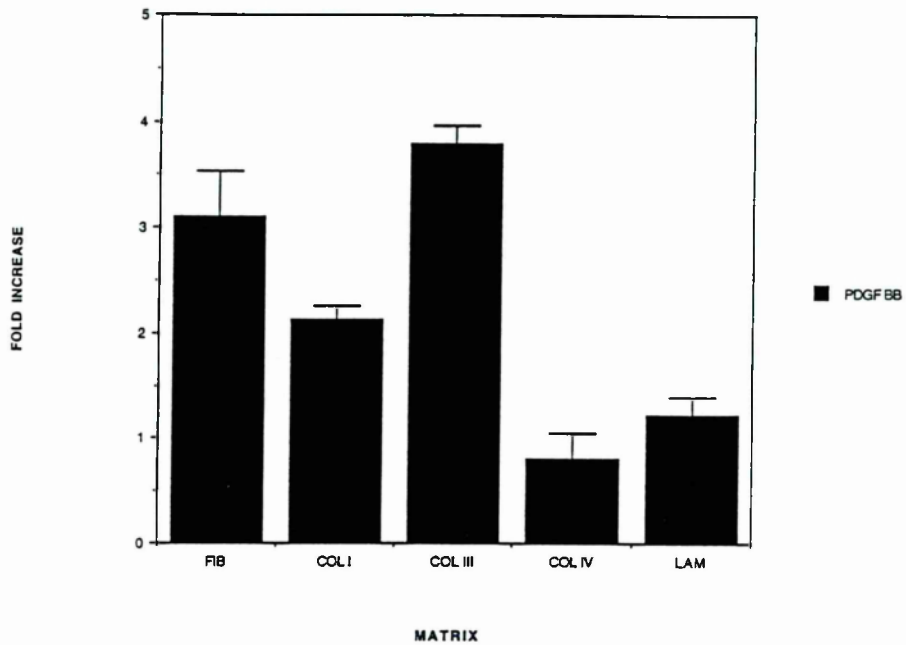
(FIG.VII.20.) HUASMC Migration Through Various Matrix Coated Filters in Response to 30ng/ml PDGF BB. This is a representative result from 3 experiments. Error bars are SEM. Migration values on collagen III and Fibronectin are significantly different from values on Collagen I also these values are all significantly different from migration on collagen IV and laminin (P < 0.05) as determined by the student's t test.

(FIG.VII.21.).
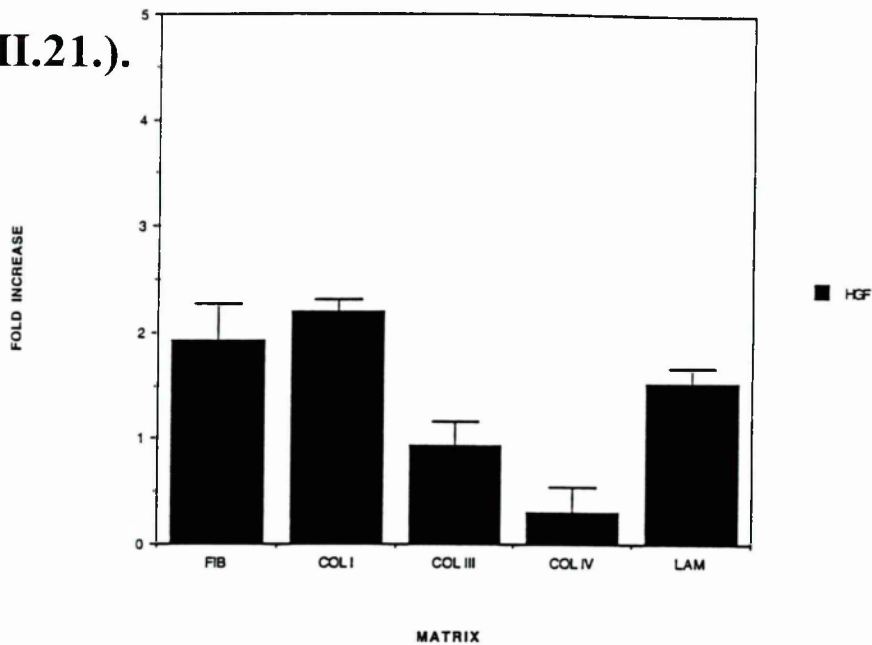
(FIG.VII.21.) HUASMC Migration Through Various Matrix Coated Filters in Response to 30ng/ml HGF. This is a representative result from 3 experiments. Error bars are SEM. Migration values on collagen I, Fibronectin and laminin are significantly different from values on Collagen types III & IV (P < 0.05) as determined by the student's t test

(FIG.VII.22.).
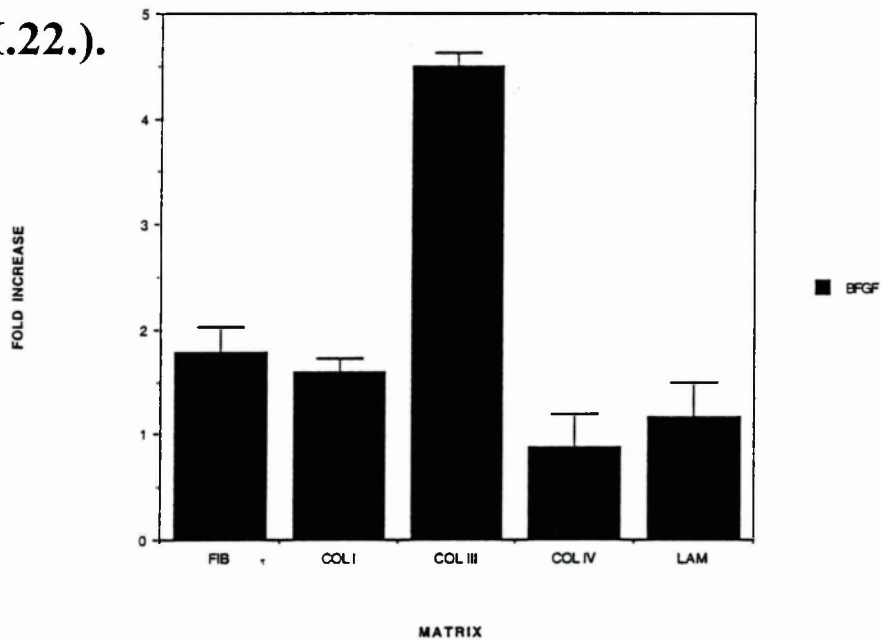
(FIG.VII.22) HUASMC Migration Through Various Matrix Coated Filters in Response to 30ng/ml bFGF. . This is a representative result from 3 experiments. Error bars are SEM. Migration values on collagen III are significantly greater than values on all other matrix substrates. Migration values on Fibronectin and collagen are significantly greater than values on Collagen IV (P < 0.05) as determined by the student's t test.

Chapter 1. Results - Summary;

In accordance with previous morphological studies of vascular SMC's in culture by Thomas-Salgar & Millis (1994) and Li *et al* (1994), it could be inferred that the distinct morphological patterns exhibited by culturing HUASMC's on different matrix coated substrates provide an indication that the ECM molecules analysed in this study play a role in the modulation of human SMC phenotype.

SMC's show an enhanced proliferation rate in response to serum when HUASMC's are cultured on the matrix substrates; Plasma fibronectin and collagens I, III and IV compared with SMC's cultured on plastic or laminin.

Protein assays carried out on samples from both Transwell compartments indicate that to obtain a long lasting chemotactic gradient between the upper and lower Transwell compartments, the chemotactic source must be embedded in a low melting point agarose gel within the lower compartment.

Adhesion assays demonstrate that SMC's adhere much more rapidly to fibronectin coated surfaces than to uncoated surfaces. It was concluded from adhesion assays that to compare HUASMC migration through fibronectin and uncoated transwell filters, a 5 hour preincubation step in H:W 16%FCS was required for HUASMC's seeded on to uncoated transwell filters prior to the initiation of the migration assay. This preincubation should ensure relatively equal numbers of HUASMC adhesion to plasma fibronectin and uncoated transwell filters.

SMC migration towards a positive gradient of FCS or PDGF BB and HGF was considerably enhanced over fibronectin coated filters when compared to migration through uncoated filters. SMC migration rates towards FCS were approximately two-fold greater on plasma fibronectin and collagen III coated filters compared with migration on collagen I, IV and laminin. Migration in response to specific growth factors also varied depending on the matrix substrate. PDGF BB induces significant levels of migration through plasma fibronectin, collagen

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III, and collagen I coated filters, whereas little or no migration was observed through collagen IV, and laminin. Basic FGF induced a high level of migration through collagen III, low levels of migration through fibronectin and collagen I, and once again no migration was observed through laminin or collagen IV. HGF induced significant levels of SMC migration through collagen I, plasma fibronectin and laminin, whereas no migration towards HGF could be observed through collagen IV and collagen III.

The demonstration from the SMC proliferation and migration assays that matrix molecules can alter the mitogenic and chemotactic responses of vascular SMC's to various stimuli provide further evidence of the importance that ECM may play in the control of vascular SMC behaviour that is associated with the pathogenesis of early atherosclerotic and restenotic lesion progression.

Chapter .2. (Section VIII).

Phenotypic characterisation of SMC's

cultured on different ECM substrates.

VIII.1 Introduction - Markers of SMC phenotype.

The transformation of vascular SMC's from a contractile to a synthetic phenotype that is associated with atherosclerotic or restenotic lesion development *in vivo* and also during the isolation of SMC's *in vitro*, is accompanied by alterations in the expression patterns of a number of proteins. Proteins for which expression is exclusive to a specific SMC phenotypic state are often referred to as SMC phenotypic markers. Many of the SMC phenotypic markers identified to date include cytoskeletal and ECM proteins. Such proteins are thought to play an integral role in the contractile and synthetic functions of SMC's and therefore are logical candidates for markers of contractile and synthetic SMC phenotypes.

The identification and subsequent analysis of these SMC phenotypic markers, provides a useful tool for studying the mechanisms that regulate SMC phenotypic modulation *in vitro* and *in vivo*. These markers may also prove useful for diagnostic and therapeutic purposes.

A number of SMC phenotypic markers have been described in the literature. The assignment of many of these markers to a synthetic or a contractile phenotype is somewhat controversial. This controversy arises from the identification of many of these phenotype markers through the use of poorly defined *in vivo* and *in vitro* models of SMC phenotypic modulation, as well as from the comparison of protein expression levels between neonatal and adult vascular tissue samples from disparate animal species. The reliability of these models to mimic the differences between the contractile and synthetic SMC phenotypic states that are associated with restenosis and atherogenesis have not been firmly established.

However, in association with the transformation from a contractile to a synthetic phenotype, the following "contractile phenotypic markers" are thought to decrease; SM–α actin (Owens *et al* 1986; Liau *et al* 1990); SM myosin heavy chains (Aikawa *et al* 1988; Kuro-o *et al* 1991); α-SM tropomyosin (Muthuchamy *et al* 1993); Desmin (Kocher *et al*

1986; Osborn *et al* 1987); Meta-vinculin (Glukhova *et al* 1986; Meyer *et al* 1994) Calponin (Dunband *et al* 1993); SM22α (Shanahan *et al* 1993) and Heavy chain caldesmon (Glukhova *et al* 1986; Ueki *et al* 1987).

The following "synthetic phenotypic markers" have been observed to increase upon the transition into the synthetic phenotype; Cytokeratin 8 (Jahn *et al* 1988); Collagen's, I, III, IV, V (Ang et al 1990; Hanson & Bentley 1983; Shekonin et al 1987); Fibronectin (Shekonin *et al* 1987; Bauters *et al* 1995); Osteopontin (Giachelli *et al* 1992); Caldesmon light chain (Glukhova *et al* 1986; Ueki *et al* 1986); Non-muscle myosin (Aikawa *et al* 1987) and Non-muscle β actin (Owens *et al* 1986).

A key to understanding the mechanisms that control SMC phenotypic modulation lie in the identification of the environmental signals and factors that govern the differentiated SMC phenotypic state. Inflammatory macrophages and injured endothelial cells found within atherosclerotic and restenotic lesions synthesize and release a large number of biologically active molecules including; growth factors, cytokines, proteolytic enzymes, prostaglandins and leukotrienes. Many of these substances have been proposed to play a role in the induction of SMC phenotypic modulation (Sjolund et al 1984; Palmberg et al 1989; Holycross et al 1992; Liptay *et* al 1993; Morisaka et al 1993; Majack 1987). It has been well established that the ECM within lesions has profound effects on SMC behaviour, including SMC migration and proliferation, and undoubtedly also plays a key role in the control of SMC phenotype. Indeed a number of *in vitro* studies that have been carried out mostly on rat SMC's, demonstrate that ECM molecules can regulate the SMC phenotypic modulation processes taking place *in vitro* (Hedin *et al* 1988; Li *et al* 1994). It has also been recently proposed that culturing human SMC's in the reconstituted basement membrane matrix preparation Matrigel (Becton Dickinson) supplemented with serum free SMC differentiation medium (Becton Dickinson) encourages the transition into a contractile phenotype (Grushkin-Lerner and Flaherty 1995).

In order to investigate further the role that ECM molecules have on the modulation of human SMC phenotype *in vitro*, monoclonal antibodies specific for previously identified and potential SMC phenotypic markers were utilised to carry out immunoblotting and immunocytochemical analysis of FW human adult aorta SMC's (gift from Dr C Kielty University of Manchester) and HUASMC's cultured on various matrix substrates. Protein samples were also obtained from the original umbilical artery tissue from where HUASMC's were isolated from and also from normal human sapheneous vein and aorta tissue. These normal tissue protein samples provide a positive control for the SMC contractile markers and a negative control for synthetic markers.

To ensure relatively equal protein loading between samples run on different tracks of a polyacrylamide gel, total protein was stained on duplicates of SDS-polyacrylamide gels that were used for immunoblotting. Protein staining was carried out using Coomassie blue fixitive stain as described in the Materials and Methods section **VI.10**. Relative protein loading between samples was assessed by observation. Sample loading was adjusted on subsequent gels to provide approximately equal loading between samples. Loading adjusted Coomassie stained gels containing separated protein samples obtained from normal vascular tissue and HUASMC's cultured on various matrix substrates are represented in **(FIGs;VIII.1. & VIII.2.)**.

(FIG.VIII.1.).
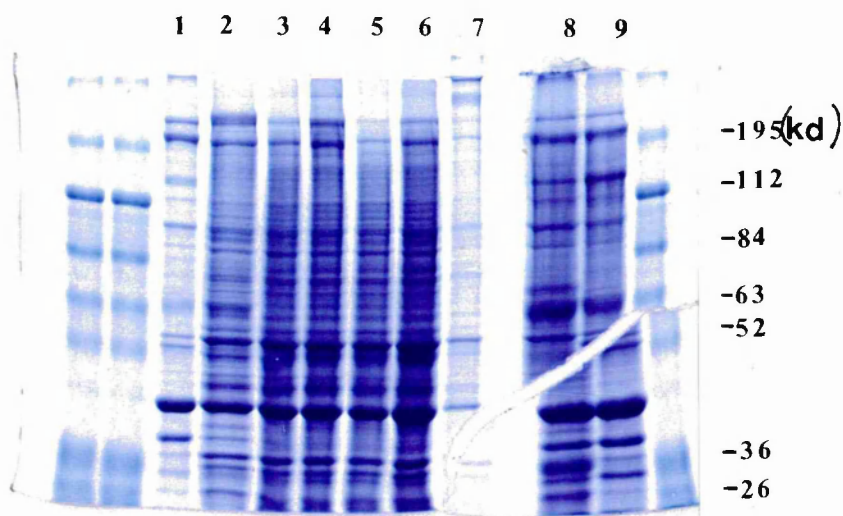
**(FIG.VIII.1.)** Coomassie blue stained gel of separated protein samples obtained from human SMC's or tissue; Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's-cultured; on plastic. **3**- on collagen I; **4**-on collagen III; **5**- on collagen IV; **6** on vitronectin 7- in Matrigel. **8**- Aorta tissue. **9**- Sapheneous vein tissue.
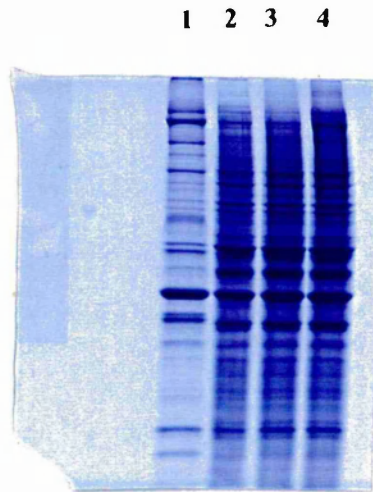
(FIG.VIII.2.).

(FIG.VIII.2.) Coomassie blue stained gel of separated protein samples isolated from human SMC's or tissue; Lanes; **1**- Umbilical artery tissue. **2**- HUASMC's cultured on; plastic; **3**- on plasma fibronectin and **4**-on cellular fibronectin.

Protein samples subjected to SDS-PAGE were transferred to nitro-cellulose membranes. Nitro-cellulose membranes were probed with monoclonal antibodies specific for various SMC phenotypic marker proteins. Photographs of the autoradiographs obtained from these immunoblots are represented in **(FIGs;VIII.3-VIII.20)**. Immunoblotting studies demonstrate that for most of the marker proteins studied no significant alterations in their levels can be observed between SMC's isolated in culture and growing on different matrix substrates. Slight alterations in the levels of meta-vinculin protein were observed with upregulation of the protein on plasma and cellular fibronectin relative to SMC's cultured on the other matrix substrates **(FIG. VIII.21 & VIII.22)**. Also the 210Kd isoform of |MLCK was found to be upregulated on cellular fibronectin, collagen III and vitronectin **(FIG.VIII.17 & VIII.18)**. Significant differences in the levels of synthetic and contractile marker can be observed between tissue samples and samples from SMC's isolated in culture.
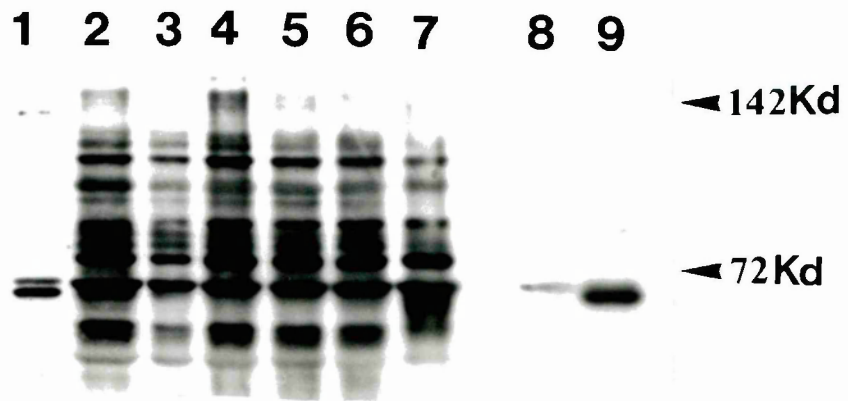
(FIG.VIII.3.).
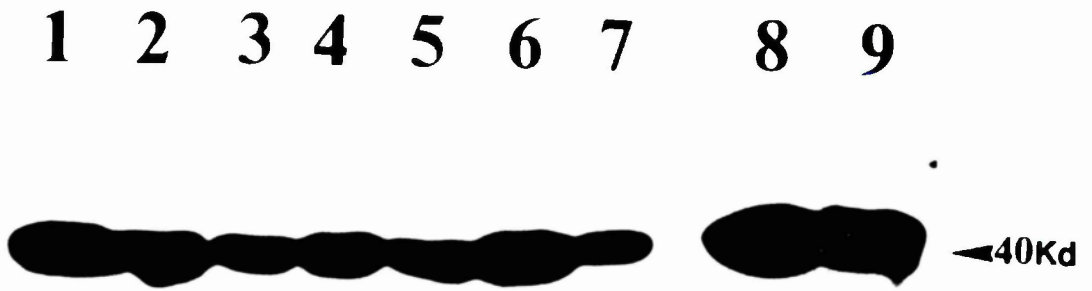
(FIG.VIII.4.).
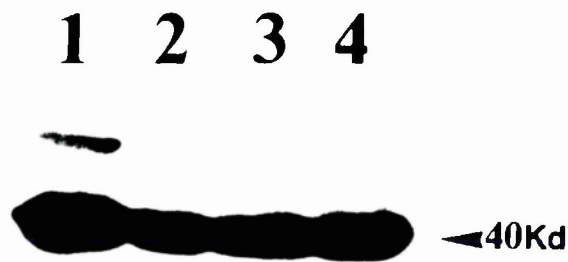
SM-α Actin. SDS-PAGE analysis of total protein extracts isolated from HUASMC's cultured on various matrix substrates and also from human vascular tissue samples. Immunoblots were performed with a monoclonal anti-SM-α Actin antibody (Sigma);
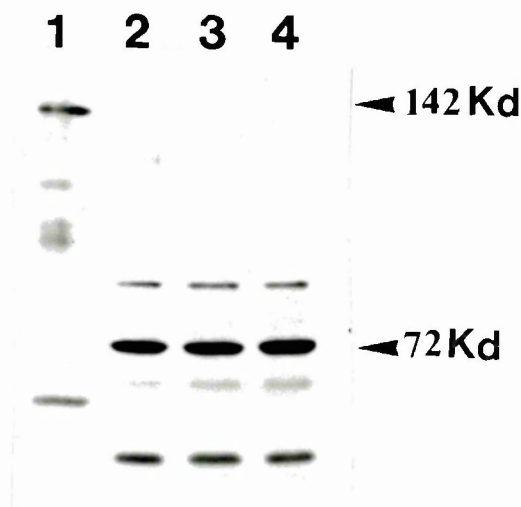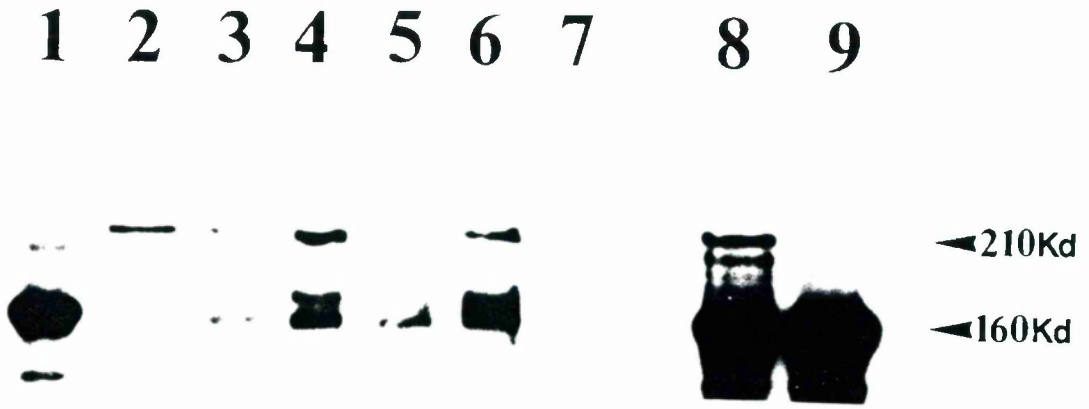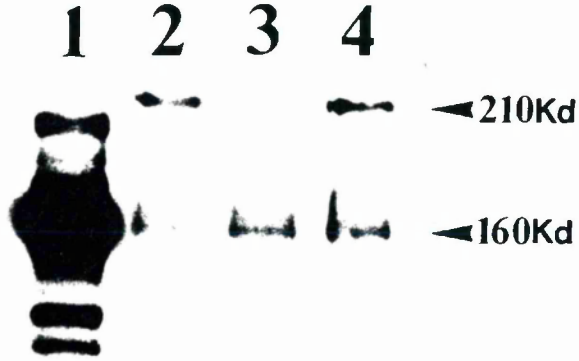
(**FIG.VIII.3.**); Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's-cultured; on plastic. **3**- on collagen I; **4**-on collagen III; **5**- on collagen IV; **6** on vitronectin **7**- in Matrigel. **8**- Aorta tissue. **9**- Sapheneous vein tissue.

(**FIG.VIII.4.**); Lanes; **1**- Umbilical artery tissue. **2**- HUASMC's cultured on; plastic; **3**- on plasma fibronectin and **4**-on cellular fibronectin.
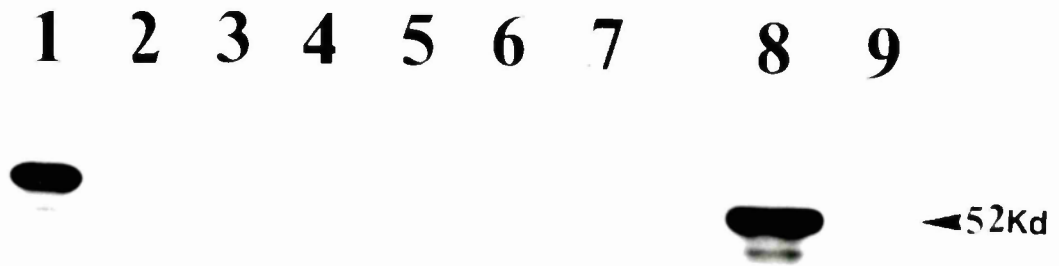
(FIG.VIII.5.).

(FIG.VIII.6.).
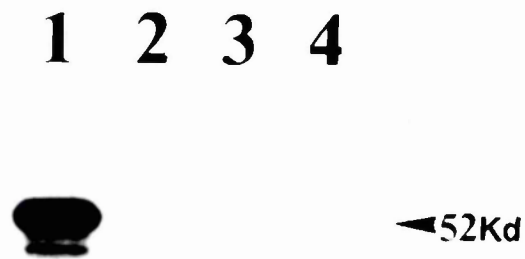
Desmin. SDS-PAGE analysis of total protein extracts isolated from HUASMC's cultured on various matrix substrates and also from human vascular tissue samples. Immunoblots were performed with a monoclonal anti-Desmin antibody (Sigma);

(**FIG.VIII.5.**); Lanes; 1- Umbilical artery 16 tissue; 2- HUA16SMC's-cultured; on plastic. 3- on collagen I; 4-on collagen III; 5- on collagen IV; 6 on vitronectin 7- in Matrigel. 8- Aorta tissue. 9- Sapheneous vein tissue.

(**FIG.VIII.6.**); Lanes; 1- Umbilical artery tissue. 2- HUASMC's cultured on; plastic; 3- on plasma fibronectin and 4-on cellular fibronectin.
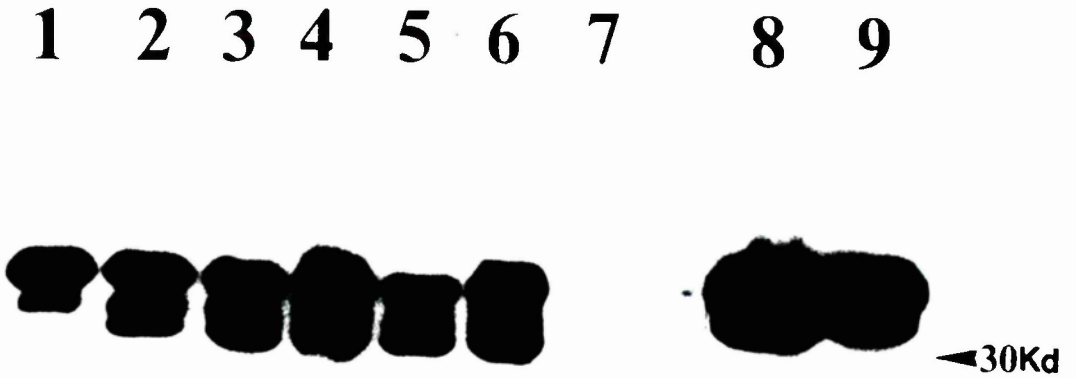
(FIG.VIII.7.).
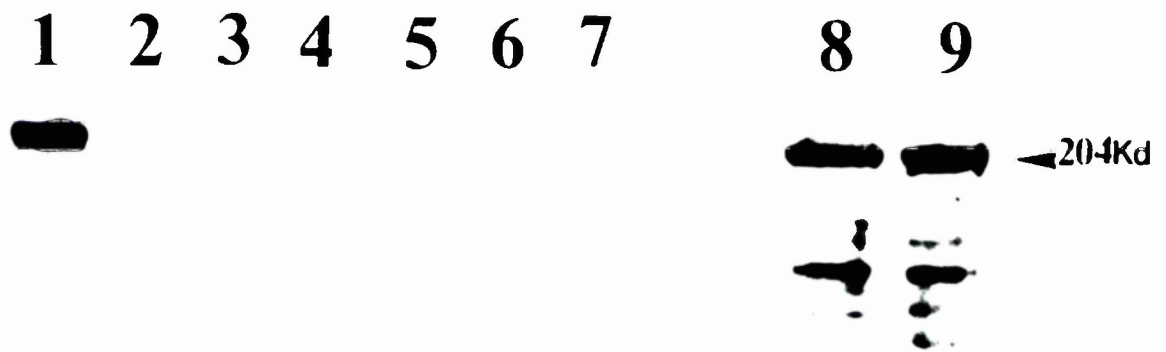
(FIG.VIII.8.).

SM-Myosin. SDS-PAGE analysis of total protein extracts isolated from HUASMC's

cultured on various matrix substrates and also from human vascular tissue samples.

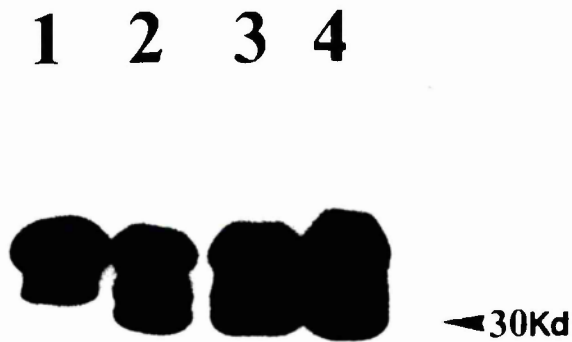Immunoblots were performed with a monoclonal anti-SM-Myosin antibody (Sigma);

(FIG.VIII.7.); Lanes; 1- Umbilical artery 16 tissue; 2- HUA16SMC's-cultured; on

plastic. 3- on collagen I; 4-on collagen III; 5- on collagen IV; 6 on vitronectin and 7- in

Matrigel. 8- Aorta tissue. 9- Sapheneous vein tissue.

(FIG.VIII.8.); Lanes; 1- Umbilical artery tissue. 2- HUASMC's cultured on, plastic,

3- on plasma fibronectin and 4-on cellular fibronectin.
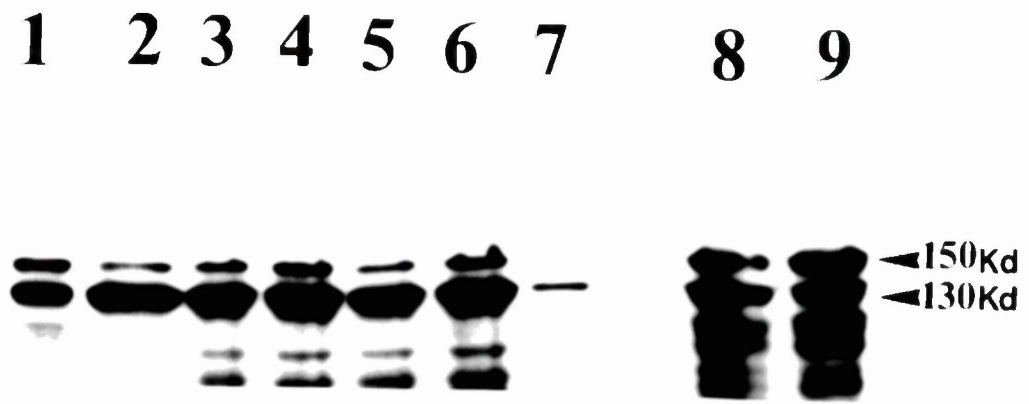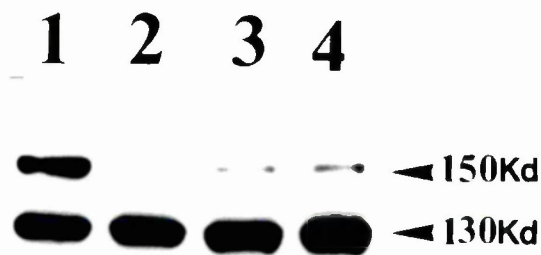
(FIG.VIII.9.).
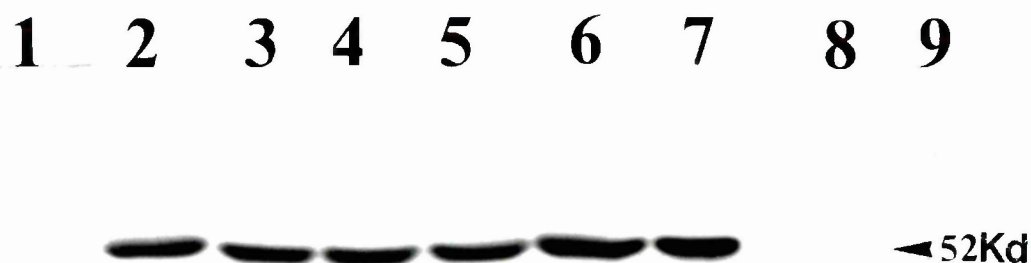
(FIG.VIII.10.).
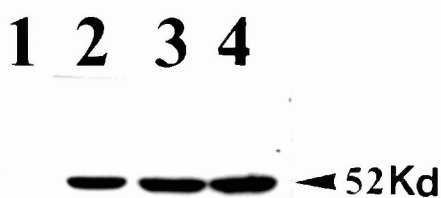
Cytokeratin 8. SDS-PAGE analysis of total protein extracts isolated from HUASMC's cultured on various matrix substrates and also from human vascular tissue samples. Immunoblots were performed with a monoclonal anti-Cytokeratin 8 antibody (Sigma);

(FIG.VIII.9.); Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's-cultured; on plastic. **3**- on collagen I; **4**-on collagen III; **5**- on collagen IV; **6** on vitronectin 7- in Matrigel. **8**- Aorta tissue. **9**- Sapheneous vein tissue.

(FIG.VIII.10.); Lanes; **1**- Umbilical artery tissue. **2**- HUASMC's cultured on; plastic; **3**- on plasma fibronectin and **4**-on cellular fibronectin.

(FIG.VIII.11.).
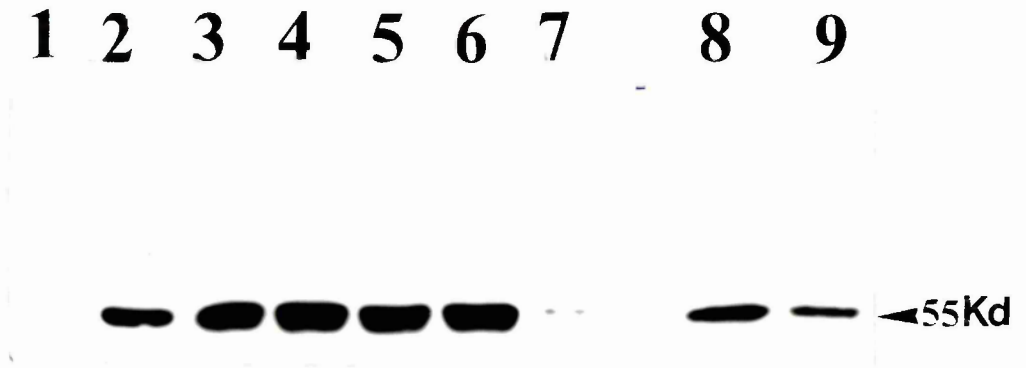
(FIG.VIII.12.).

β-**Tubulin.** SDS-PAGE analysis of total protein extracts isolated from HUASMC's cultured

on various matrix substrates and also from human vascular tissue samples. Immunoblots were

performed with a monoclonal anti-β-Tubulin antibody (Sigma);

(**FIG.VIII.11.**); Lanes; 1- Umbilical artery 16 tissue; 2- HUA16SMC's-cultured; on

plastic. 3- on collagen I; 4-on collagen III; 5- on collagen IV; 6 on vitronectin 7- in Matrigel.

8- Aorta tissue. 9- Sapheneous vein tissue.

(**FIG.VIII.12.**); Lanes; 1- Umbilical artery tissue. 2- HUASMC's cultured on; plastic;

3- on plasma fibronectin and 4-on cellular fibronectin.

(FIG.VIII.13.).

(FIG.VIII.14.).
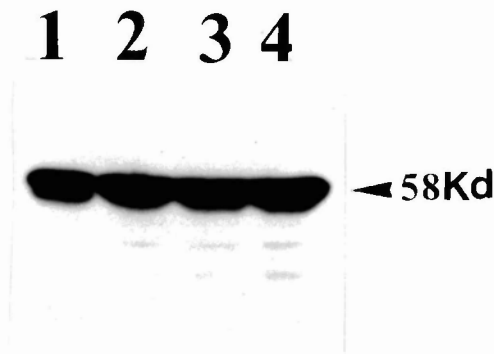
Vimentin. SDS-PAGE analysis of total protein extracts isolated from HUASMC's cultured on various matrix substrates and also from human vascular tissue samples. Immunoblots were performed with a monoclonal anti-Vimentin antibody (Sigma);

(FIG.VIII.13.); Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's-cultured; on plastic. **3**- on collagen I; **4**-on collagen III; **5**- on collagen IV; **6** on vitronectin **7**- in Matrigel. **8**- Aorta tissue. **9**- Sapheneous vein tissue.

(FIG.VIII.14.); Lanes; **1**- Umbilical artery tissue. **2**- HUASMC's cultured on; plastic; **3**- on plasma fibronectin and **4**-on cellular fibronectin.

(FIG.VIII.15.).

(FIG.VIII.16.).

Caldesmon. SDS-PAGE analysis of total protein extracts isolated from HUASMC's cultured on various matrix substrates and also from human vascular tissue samples. Immunoblots were performed with a monoclonal anti caldesmon antibody (Sigma);

(FIG.VIII.15.); Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's-cultured; on plastic. **3**- on collagen I; **4**-on collagen III; **5**- on collagen IV; **6** on vitronectin **7**- in Matrigel. **8**- Aorta tissue. **9**- Sapheneous vein tissue.

(FIG.VIII.16.); Lanes; **1**- Umbilical artery tissue. **2**- HUASMC's cultured on; plastic; **3**- on plasma fibronectin and **4**-on cellular fibronectin.

(FIG.VIII.17.).

(FIG.VIII.18.).

Myosin Light Chain Kinase. SDS-PAGE analysis of total protein extracts isolated from HUASMC's cultured on various matrix substrates and also from human vascular tissue samples. Immunoblots were performed with a monoclonal anti-Myosin Light Chain Kinase antibody (Sigma);

(**FIG.VIII.17.**); Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's-cultured; on plastic. **3**- on collagen I; **4**-on collagen III; **5**- on collagen IV; **6** on vitronectin **7**- in Matrigel. **8**- Aorta tissue. **9**- Sapheneous vein tissue.

(**FIG.VIII.18.**); Lanes; **1**- Umbilical artery tissue. **2**- HUASMC's cultured on; plastic; **3**- on plasma fibronectin and **4**-on cellular fibronectin.

(FIG.VIII.19.).

(FIG.VIII.20.).

Tropomyosin. SDS-PAGE analysis of total protein extracts isolated from HUASMC's cultured on various matrix substrates and also from human vascular tissue samples. Immunoblots were performed with a monoclonal anti-tropomyosin antibody (Sigma);

(**FIG.VIII.19.**); Lanes; 1- Umbilical artery 16 tissue; 2- HUA16SMC's-cultured; on plastic. 3- on collagen I; 4-on collagen III; 5- on collagen IV; 6 on vitronectin and 7- in Matrigel. 8-Aorta tissue. 9- Sapheneous vein tissue.

(**FIG.VIII.20.**); Lanes; 1- Umbilical artery tissue. 2- HUASMC's cultured on; plastic; 3- on plasma fibronectin and 4-on cellular fibronectin.

(FIG.VIII.21.).

(FIG.VIII.22.).

Vinculin/Meta-vinculin. SDS-PAGE analysis of total protein extracts isolated from HUASMC's cultured on various matrix substrates and also from human vascular tissue samples. Immunoblots were performed with a monoclonal anti-Vinculin/Meta-vinculin antibody (Sigma);

(FIG.VIII.21.); Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's-cultured; on plastic. **3**- on collagen I; **4**-on collagen III; **5**- on collagen IV; **6** on vitronectin and **7**- in Matrigel. **8**-Aorta tissue. **9**- Sapheneous vein tissue.

(FIG.VIII.22.); Lanes; **1**- Umbilical artery tissue. **2**- HUASMC's cultured on; plastic; **3**- on plasma fibronectin and **4**-on cellular fibronectin.

A summary of the analysis of SMC phenotypic marker protein expression between HUASMC's cultured on various matrix substrates and also investigation into the expression levels of these proteins in normal human vascular tissue samples is represented in **(Table VIII.1.)**.

The intensity of bands on immunoblots was scored as follows:

+++++   -Highly expressed.

++++   -Well expressed.

+++   -Moderately expressed.

++   -low level expression.

+   -Very low level expression.

-          -No detectable expression.

Table VIII.1 Phenotypic Analysis of SMC's Cultured on Various ECM Substrates.

	Plastic	Plasma Fibronectin	Cellular Fibronectin	Collagen I	Collagen III	Collagen IV	Vitronec-tin	Matrigel	Umbilical artery	Aorta	Saph. Vein
SM-actin	+++	++++	++++	++++	+++	++++	+++++	++++	+++++	+++++	+++++
Vimentin	++++	++++	+++	+++	+++	++++	++++	++++	++++	++++	+++
Cytokeratin 8	+++	+++	+++	+++	+++	+++	+++	+++	++++	-	+
β-tubulin	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++
Desmin	-	-	-	-	-	-	-	-	+	++++	+
SM-myosin	-	-	-	-	-	-	-	-	++++	+++++	+++++
Tropomyosin 30kd	+++	+++	+++	+++	+++	+++	+++	+++	++++	+++	+++
Vinculin	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++
Meta-vinculin	+++	++++	+++	+++	+++	+++	+++	+++	+++	+++	+++
MLCK 210kd	+++	++	+++	++	+++	+	++	++	-	+++	-
MLCK 160kd	+	+	+	++	+	+	++	+	+++++	+++++	+++++
Caldesmon 70kd	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-
Caldesmon 150kd	-	-	-	-	-	-	-	-	++	-	+

Protein samples were obtained from HUASMC's and also from FW human aorta

SMC's that were cultured in Matrigel and SMC "differentiation medium" (Becton

Dickinson). These culture conditions are marketed by Becton Dickinson as a procedure for

gradually promoting conversion of cultured rat and human aorta SMC's into the contractile

phenotype. Protein was extracted from HUASMC's and FWSMC's cultured on fibronectin

coated flasks (Falcon) supplemented with H:W 16%FCS as a control for the synthetic

phenotype. Protein was also extracted from the same cell types at sequential time points (24;

72; 144; 216; 312 hours) following seeding in Matrigel and growth in "differentiation

medium". These protein samples were also immunoblotted and probed with antibodies

specific for various SMC phenotype marker proteins.**(FIGs; VIII.25-.36.).** Protein loading

between different HUASMC samples and FW aorta SMC samples was indicated by

Coomassie blue stained SDS-PAGE gels **(FIGs; VIII.23 & VIII.24.)**.

(FIG.VIII.23.).
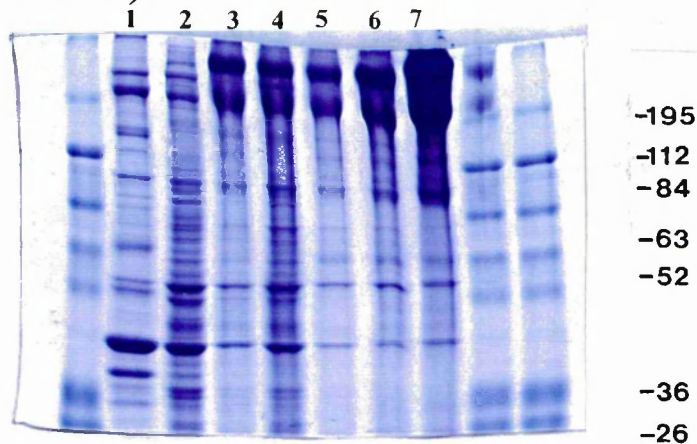
(FIG.VIII.23.). Coomassie blue stained gel of separated protein samples isolated from

human SMC's or tissue; Lanes; **1-** Umbilical artery 16 tissue; **2-** HUA16SMC's cultured on

plasma fibronectin; **3-** Matrigel (24hrs); **4-** Matrigel (72hrs); **5-** Matrigel (144hrs); **6-**

Matrigel (216hrs);**7.**-Matrigel (312hrs).

(FIG.VIII.24.).
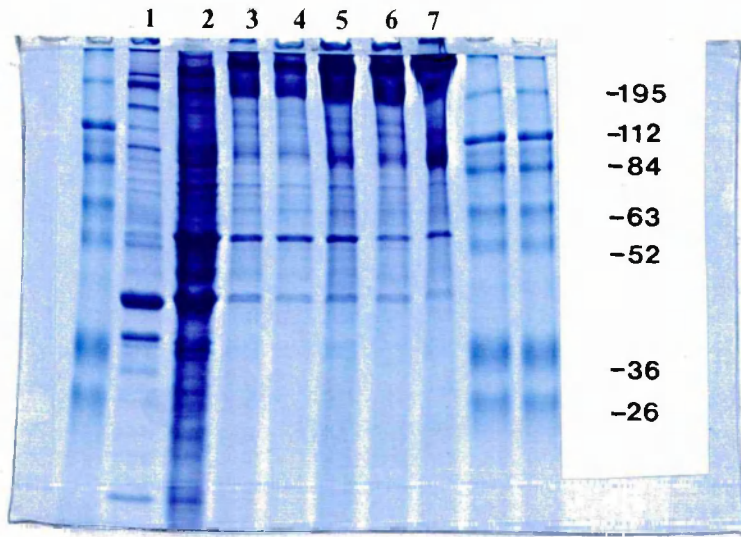
(FIG.VIII.24.). Coomassie blue stained gel of separated protein samples isolated from human SMC's or tissue; Lanes; 1- aorta tissue; 2- FW aorta SMC's cultured on plasma fibronectin; 3- Matrigel (24hrs); 4- Matrigel (72hrs); 5- Matrigel (144hrs); 6- Matrigel (216hrs);7.-Matrigel (312hrs).
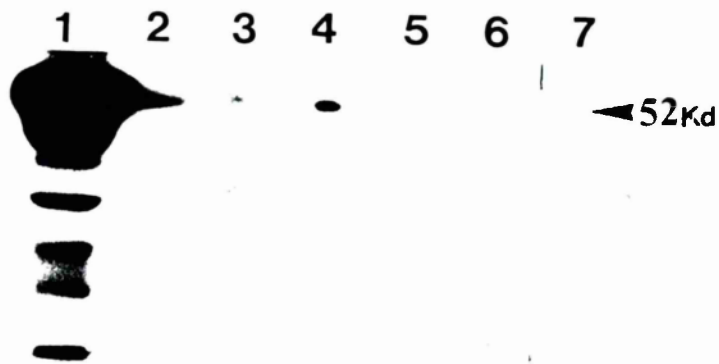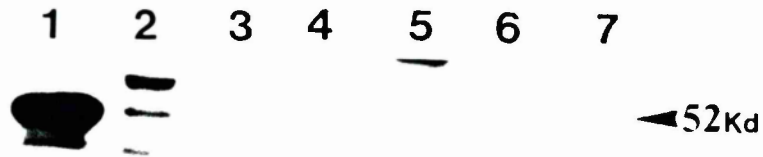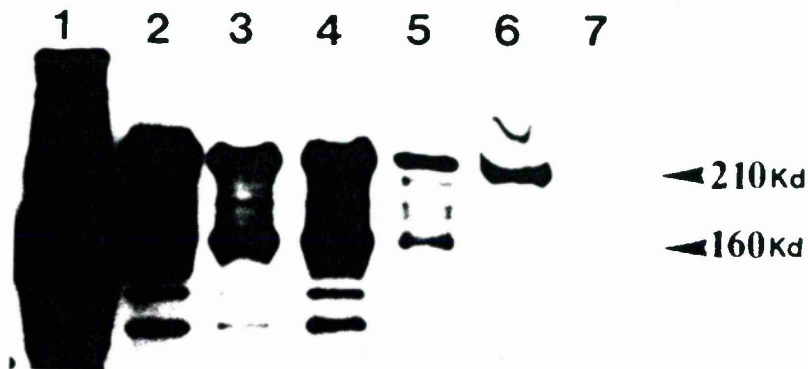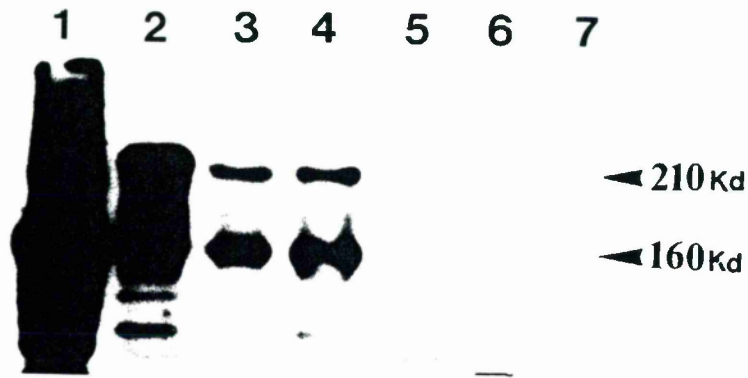
(FIG.VIII.25.).
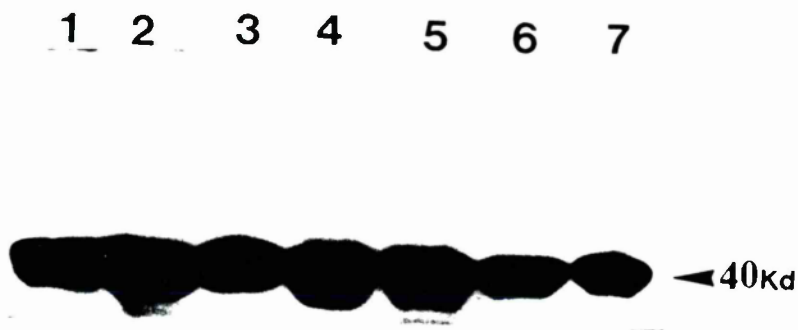
1 2 3 4 5 6 7

◄40Kd

SM-α Actin. SDS-PAGE analysis of HUASMC's cultured in Matrigel supplemented with differentiation medium (Becton Dickinson). Total protein extracts were isolated from HUASMC's at sequential time points following seeding in Matrigel. Immunoblots were performed with monoclonal anti-SM-α actin antibody (Sigma).

(FIG.VIII.25.); Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's cultured on plasma fibronectin; **3**- Matrigel (24hrs); **4**- Matrigel (72hrs); **5**- Matrigel (144hrs); **6**- Matrigel (216hrs);**7**.-Matrigel (312hrs).

(FIG.VIII.26.).
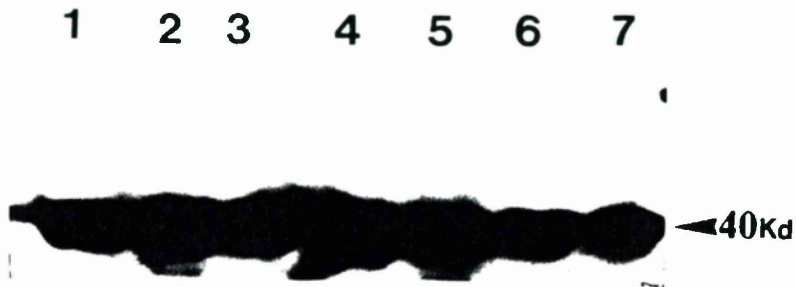
SM-α Actin. SDS-PAGE analysis of FW aorta SMC's cultured in Matrigel supplemented

with differentiation medium (Becton Dickinson). Total protein extracts were isolated from

FW SMC's at sequential time points following seeding in Matrigel. Immunoblots were

performed with monoclonal anti-SM-α actin antibody (Sigma).

   (FIG.VIII.26.); Lanes; **1-** Human aorta tissue; **2-** HUA16SMC's cultured on plasma

fibronectin; **3-** Matrigel (24hrs); **4-** Matrigel (72hrs); **5-** Matrigel (144hrs); **6-** Matrigel

(216hrs);**7.**-Matrigel (312hrs).

(FIG.VIII.27.).


```
    1   2   3   4   5   6   7

    ·  ▬                        ◄204Kd
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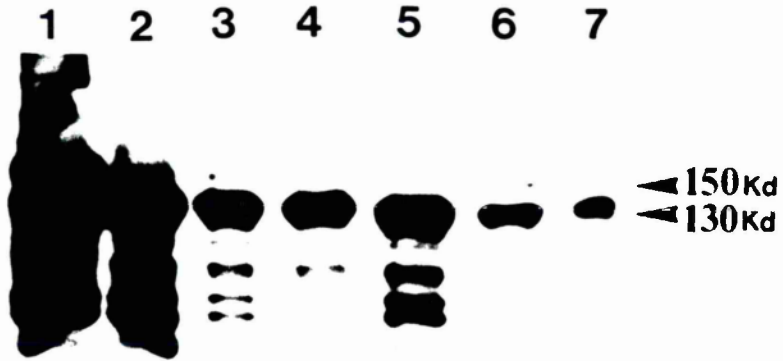
SM-Myosin. SDS-PAGE analysis of HUASMC's cultured in Matrigel supplemented with differentiation medium (Becton Dickinson). Total protein extracts were isolated from HUASMC's at sequential time points following seeding in Matrigel. Immunoblots were performed with monoclonal anti-SM myosin antibody (Sigma).

(**FIG.VIII.27.**); Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's cultured on plasma fibronectin; **3**- Matrigel (24hrs); **4**- Matrigel (72hrs); **5**- Matrigel (144hrs); **6**- Matrigel (216hrs);**7**.-Matrigel (312hrs).

(FIG.VIII.28.).
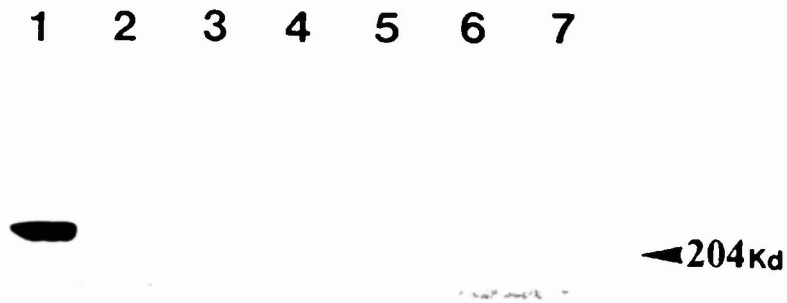
◄204Kd

SM-Myosin. SDS-PAGE analysis of FW aorta SMC's cultured in Matrigel supplemented

with differentiation medium (Becton Dickinson). Total protein extracts were isolated from

FW SMC's at sequential time points following seeding in Matrigel. Immunoblots were

performed with monoclonal anti-SM myosin antibody (Sigma).

(FIG.VIII.28.); Lanes; 1- Human aorta tissue; 2- HUA16SMC's cultured on plasma

fibronectin; 3- Matrigel (24hrs); 4- Matrigel (72hrs); 5- Matrigel (144hrs); 6- Matrigel
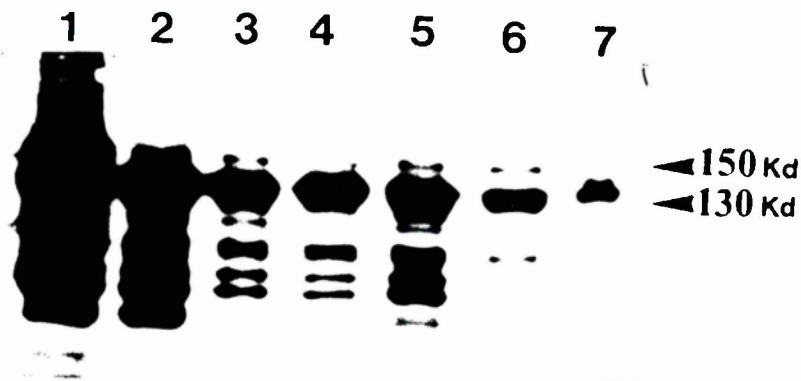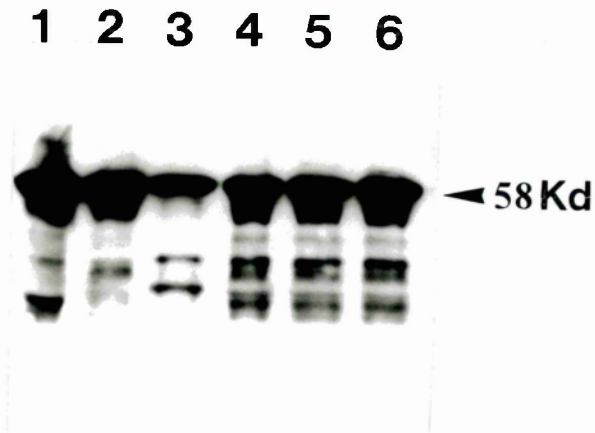
(216hrs);7.-Matrigel (312hrs).

(FIG.VIII.29.).

Desmin. SDS-PAGE analysis of HUASMC's cultured in Matrigel supplemented with differentiation medium (Becton Dickinson). Total protein extracts were isolated from HUASMC's at sequential time points following seeding in Matrigel. Immunoblots were performed with monoclonal anti-Desmin antibody (Sigma).

(**FIG.VII.29.**); Lanes; **1-** Umbilical artery 16 tissue; **2-** HUA16SMC's cultured on plasma fibronectin; **3-** Matrigel (24hrs); **4-** Matrigel (72hrs); **5-** Matrigel (144hrs); **6-** Matrigel (216hrs);**7.-**Matrigel (312hrs).

(FIG.VIII.30.).

Desmin. SDS-PAGE analysis of FW aorta SMC's cultured in Matrigel supplemented with differentiation medium (Becton Dickinson). Total protein extracts were isolated from FW SMC's at sequential time points following seeding in Matrigel. Immunoblots were performed with monoclonal anti-Desmin antibody (Sigma).

   (FIG.VIII.30.); Lanes; **1**- Human aorta tissue; **2**- HUA16SMC's cultured on plasma fibronectin; **3**- Matrigel (24hrs); **4**- Matrigel (72hrs); **5**- Matrigel (144hrs); **6**- Matrigel (216hrs);**7.**-Matrigel (312hrs).
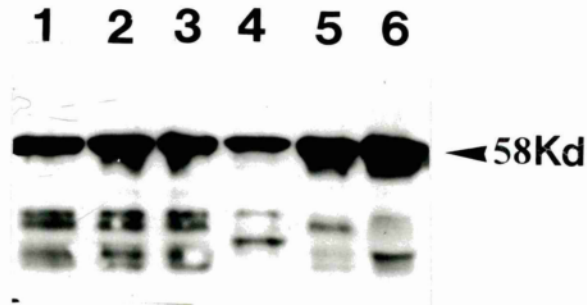
(FIG.VIII.31.).

Myosin Light Chain Kinase. SDS-PAGE analysis of HUASMC's cultured in Matrigel

supplemented with differentiation medium (Becton Dickinson). Total protein extracts were

isolated from HUASMC's at sequential time points following seeding in Matrigel

Immunoblots were performed with monoclonal anti-MLCK antibody (Sigma).

(FIG.VIII.31.); Lanes; **1-** Umbilical artery 16 tissue; **2-** HUA16SMC's cultured on

plasma fibronectin; **3-** Matrigel (24hrs), **4-** Matrigel (72hrs), **5-** Matrigel (144hrs), **6-**

Matrigel (216hrs);**7.-**Matrigel (312hrs).
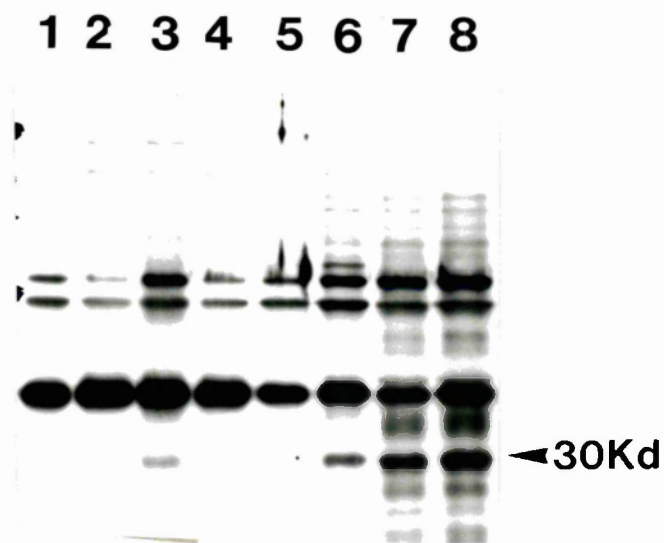
(FIG.VIII.32.).

Myosin Light Chain Kinase. SDS-PAGE analysis of FW aorta SMC's cultured in Matrigel supplemented with differentiation medium (Becton Dickinson). Total protein extracts were isolated from FW SMC's at sequential time points following seeding in Matrigel. Immunoblots were performed with monoclonal anti-MLCK antibody (Sigma).

(FIG.VIII.32.); Lanes; 1- Human aorta tissue; 2- HUA16SMC's cultured on plasma fibronectin; 3- Matrigel (24hrs); 4- Matrigel (72hrs); 5- Matrigel (144hrs); 6- Matrigel (216hrs);7.-Matrigel (312hrs).

(FIG.VIII.33.).

Vinculin/Meta-Vinculin. SDS-PAGE analysis of HUASMC's cultured in Matrigel supplemented with differentiation medium (Becton Dickinson). Total protein extracts were isolated from HUASMC's at sequential time points following seeding in Matrigel. Immunoblots were performed with monoclonal anti-Vinculin/Meta-vinculin antibody (Sigma).

(FIG.VIII.33.); Lanes; 1- Umbilical artery 16 tissue; 2- HUA16SMC's cultured on plasma fibronectin; 3- Matrigel (24hrs); 4- Matrigel (72hrs); 5- Matrigel (144hrs); 6- Matrigel (216hrs);7.-Matrigel (312hrs).

(FIG.VIII.34.).

Vinculin/Meta-Vinculin. SDS-PAGE analysis of FW aorta SMC's cultured in Matrigel

supplemented with differentiation medium (Becton Dickinson). Total protein extracts were

isolated from FW SMC's at sequential time points following seeding in Matrigel.

Immunoblots were performed with monoclonal anti-Vinculin/Meta-vinculin antibody

(Sigma).

(FIG.VIII.34.); Lanes; 1- Human aorta tissue; 2- HUA16SMC's cultured on plasma

fibronectin; 3- Matrigel (24hrs); 4- Matrigel (72hrs); 5- Matrigel (144hrs); 6- Matrigel

(216hrs);7.-Matrigel (312hrs).

(FIG.VIII.35.).

Vimentin. SDS-PAGE analysis of HUASMC's cultured in Matrigel supplemented with differentiation medium (Becton Dickinson). Total protein extracts were isolated from HUASMC's at sequential time points following seeding in Matrigel. Immunoblots were performed with monoclonal anti-Vimentin antibody (Sigma).

(**FIG.VIII.35.**); Lanes; **1**- Umbilical artery 16 tissue; **2**- HUA16SMC's cultured on plasma fibronectin; **3**- Matrigel (24hrs); **4**- Matrigel (72hrs); **5**- Matrigel (144hrs); **6**- Matrigel (216hrs).

(FIG.VIII.36.).

Vimentin: SDS-PAGE analysis of FW aorta SMC's cultured in Matrigel supplemented with differentiation medium (Becton Dickinson). Total protein extracts were isolated from FW SMC's at sequential time points following seeding in Matrigel. Immunoblots were performed with monoclonal anti-Vimentin antibody (Sigma).

    (FIG.VIII.36.): Lanes; **1**- Human aorta tissue; **2**- HUA16SMC's cultured on plasma fibronectin; **3**- Matrigel (24hrs); **4**- Matrigel (72hrs); **5**- Matrigel (144hrs); **6**- Matrigel (216hrs).

The relative levels of tropomyosin isoforms was analysed in protein samples obtained from cultured rat aortic SMC's, intimal, and medial layers of balloon injured (right) and normal (left) rat carotid tissue.

(VIII.37.).

(FIG.VIII.36.): Lanes; **1-** Right media; **2-** Left media; **3-** Right media + intima; **4-** Left media; **5-** Right media; **6-** Right intima; **7.-**Rat aortic SMC's cultured on fibronectin; **8 -** Rat aortic SMC's cultured on plastic.

Immunofluorescent characterisation of HUASMC's cultured on uncoated and plasma fibronectin coated surfaces was performed using SMC phenotype marker specific antibodies. This immunostaining was carried out to assess whether any alterations in the distribution of these protein markers could be associated with either a distinct nodular or monolayer growth morphology such as that exemplified by SMC's cultured on fibronectin coated and uncoated surfaces respectively. Immunofluorescent antibody staining of SMC's cultured on both substrates using those antibodies that resulted in clear staining patterns together with comparative phase photomicrographs at the same magnification on same field (x 400 magnification) are represented in **(FIGs; VII.38-.59)**. Immunofluorescent staining patterns obtained with the majority of monoclonal antibodies were not significantly different between SMC's cultured on plastic with SMC's cultured on plasma fibronectin, with the exception of MLCK which appears to have a higher degree of association with stress fibres in SMC's cultured on fibronectin **(FIG's; VIII.46 & VIII.47)**. Immunofluorescent staining with the caldesmon antibody localised to the cell nucleus and appears to be upregulated in SMC's cultured on fibronectin **(FIG's; VIII.56 & VIII.57)**. This nuclear localisation was somewhat suprising and may reflect non specific binding of the caldesmon antibody as previously indicated by the observation of numerous bands on the caldesmon probed immunoblot **(FIG.VIII.15)**.

(FIG.VIII.38a)
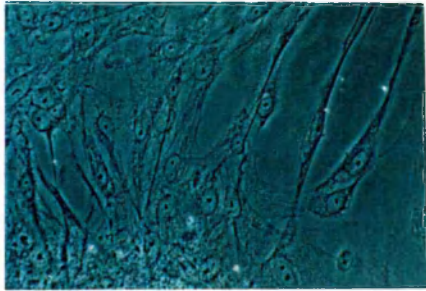
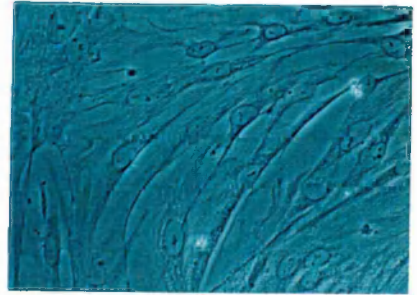# (FIG.VIII.39a)
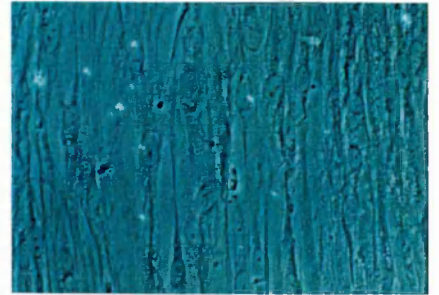
(FIG.VIII.38b)
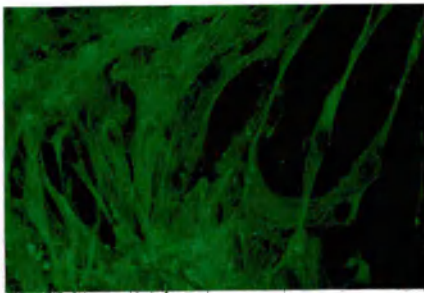
(FIG.VIII.39b)
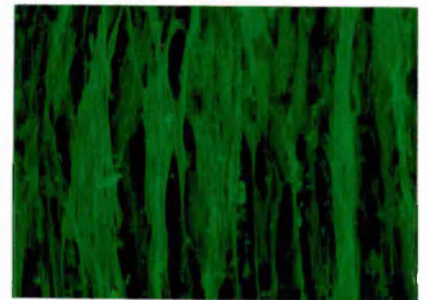
SM α-Actin. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.38 a & b.)** and also on plasma fibronectin **(FIG.VIII.39 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-SM α-actin antibody.
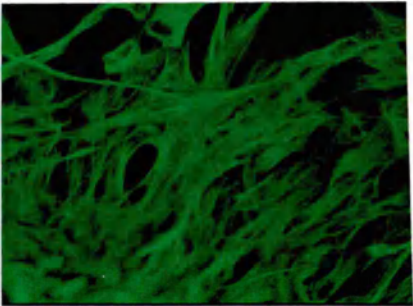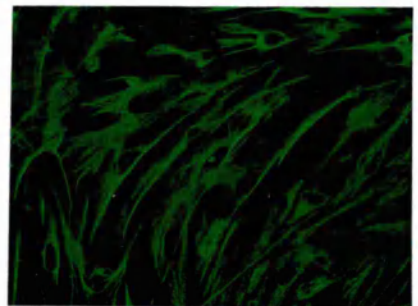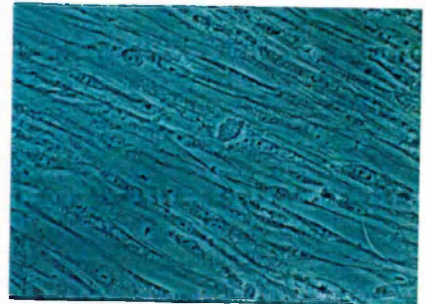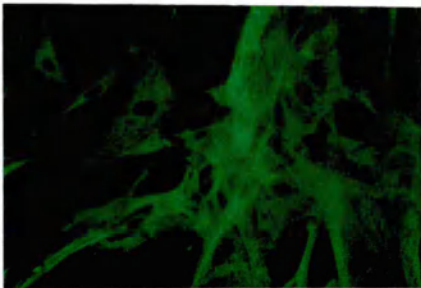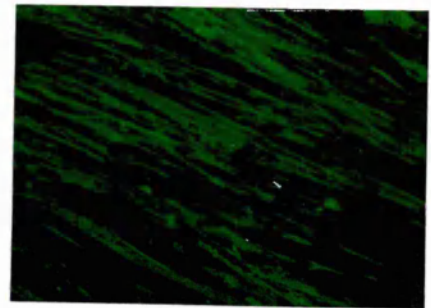
(FIG.VIII.40a)
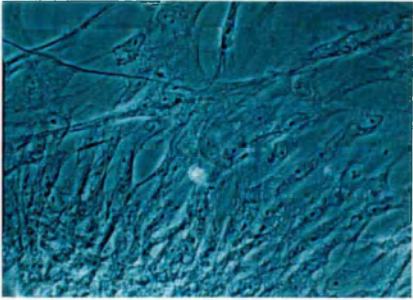
(FIG.VIII.41a)

(FIG.VIII.40b)

(FIG.VIII.41b)

Vimentin. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.40 a & b.)** and also on plasma fibronectin **(FIG.41 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-Vimentin antibody.

(FIG.VIII.42a)
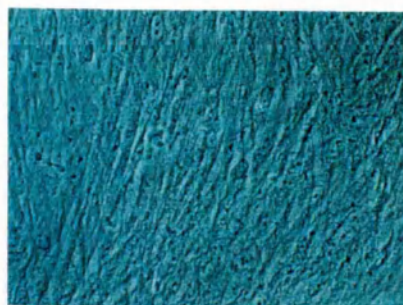

(FIG.VIII43a)

(FIG.VIII.42b)

(FIG.VIII.43b)

Vinculin/Meta-Vinculin. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.42 a & b.)** and also on plasma fibronectin **(FIG.VIII.43 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-Vinculin/Meta-vinculin antibody.
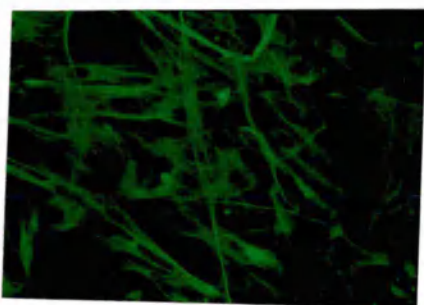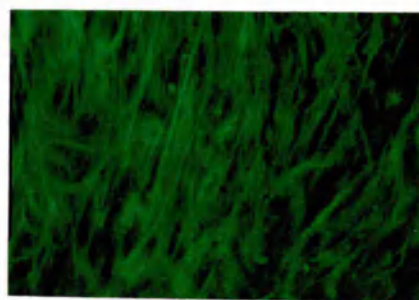
(FIG.VIII.44a)
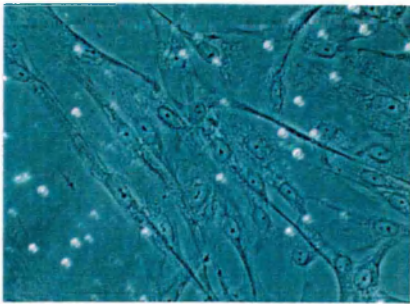

(FIG.VIII.45a)

(FIG.VIII.44b)
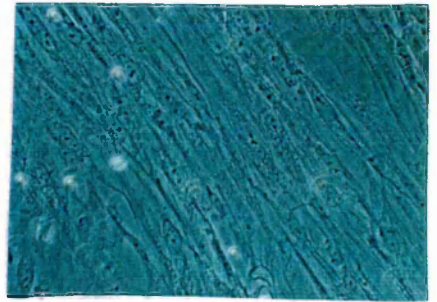

(FIG.VIII.45b)

Cytokeratin 8. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.44 a & b.)** and also on plasma fibronectin **(FIG.VIII.45 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-cytokeratin 8 antibody.
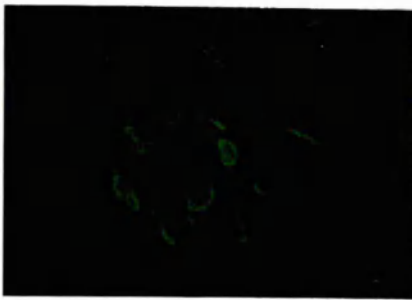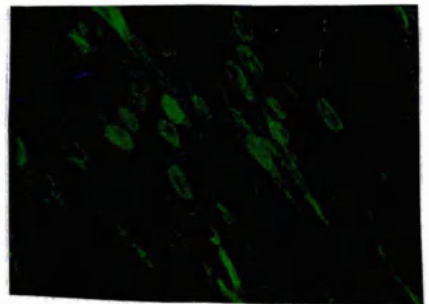
175

(FIG.VIII.46a)   **(FIG.VIII.47a)**

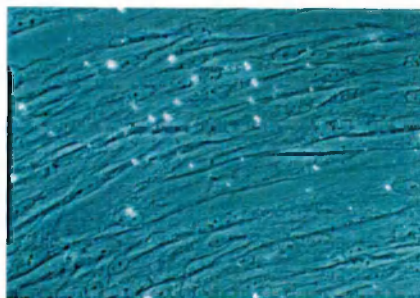**(FIG.VIII.46b)**   **(FIG.VIII.47b).**

Myosin Light Chain Kinase. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.46 a & b.)** and also on plasma fibronectin **(FIG.VIII.47 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-Myosin Light Chain Kinase antibody.

(FIG.VIII.48a)

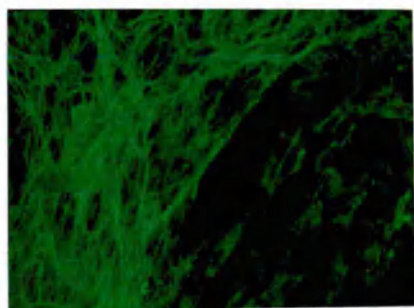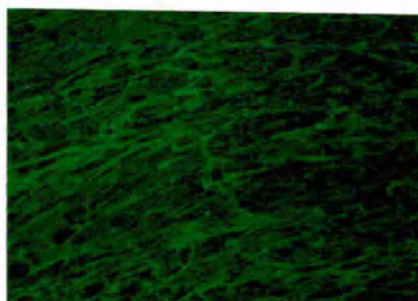**(FIG.VIII.49a)**

(FIG.VIII.48b)

(FIG.VIII.49b)

Cellular Fibronectin. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.48 a & b.)** and also on plasma fibronectin **(FIG.VIII.49 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-Cellular Fibronectin antibody.

(FIG.VIII.50a)

(FIG.VIII.51a)

(FIG.VIII.50b)

(FIG.VIII.51b)

Collagen I. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.50 a & b.)** and also on plasma fibronectin **(FIG.VIII.51 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-Collagen I antibody.

(FIG.VIII.52a)

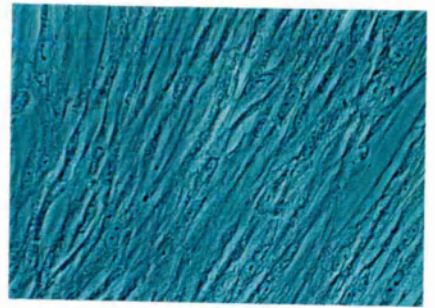

(FIG.VIII.53a)

(FIG.VIII.52b)
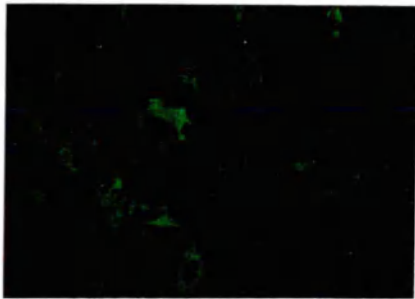
(FIG.VIII.53b)

Collagen IV Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.52 a & b.)** and also on plasma fibronectin **(FIG.VIII.53 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-Collagen IV antibody.

(FIG.VIII.54a)
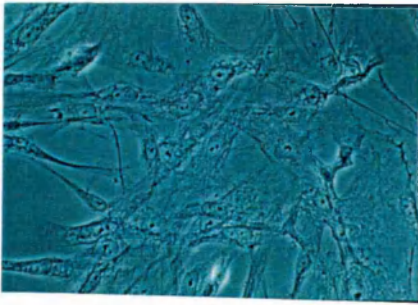
(FIG.VIII.55a)
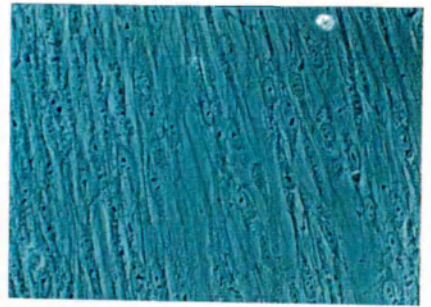
(FIG.VIII.54b)
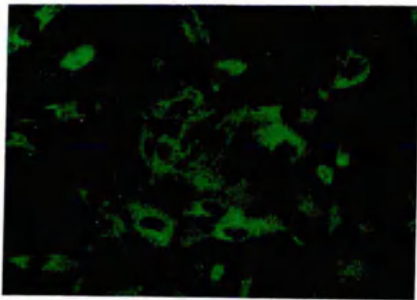
(FIG.VIII.55b)
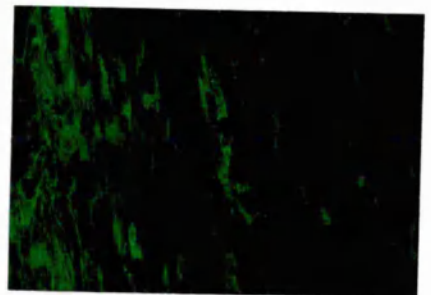
Thrombospondin. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.54 a & b.)** and also on plasma fibronectin **(FIG.VIII.55 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-Thrombospondin antibody.
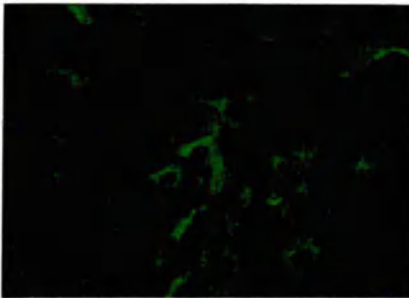
(FIG.VIII.56a)
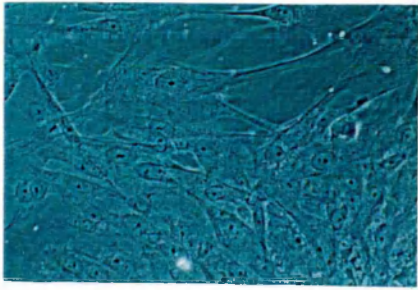
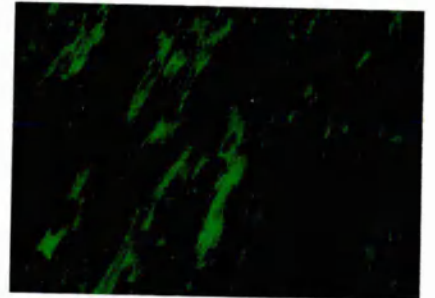

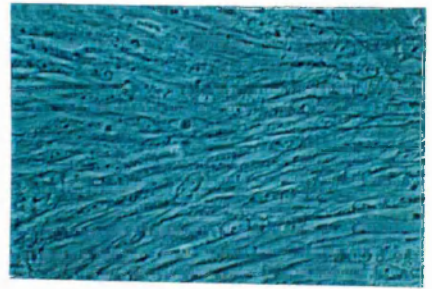**(FIG.VIII.57a)**

(FIG.VIII.56b)
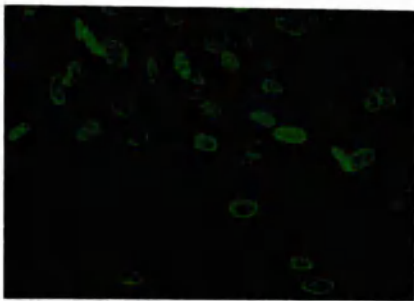
(FIG.VIII.57b)
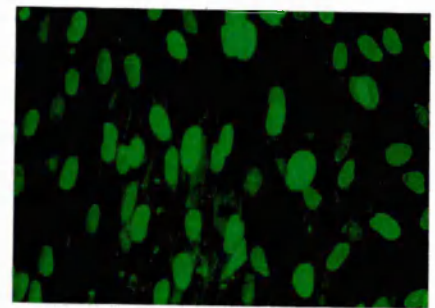
Caldesmon. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.56 a & b.)** and also on plasma fibronectin **(FIG.VIII.57 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti-Caldesmon antibody.

(FIG.VIII.58a)

(FIG.VIII.59a)
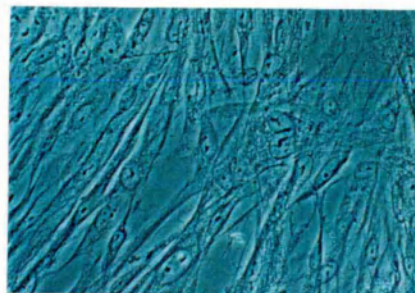
(FIG.VIII.58b)

(FIG.VIII.59b)
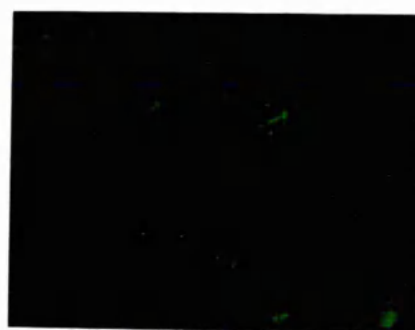
Tropomyosin. Phase contrast photomicrographs and immunofluorescent staining patterns of HUASMC's cultured on plastic **(FIG.VIII.58 a & b.)** and also on plasma fibronectin **(FIG.VIII.59 a & b.)** were obtained using FITC conjugated second antibody specific for a monoclonal anti Tropomyosin antibody.
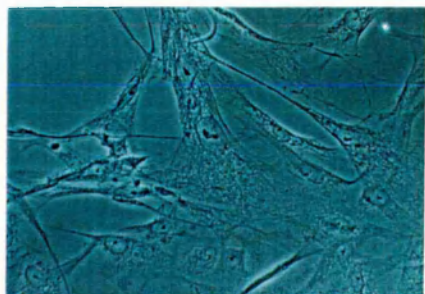
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Chapter 2. Results - Summary;

Immunoblotting of protein samples obtained from HUASMC's cultured on different matrix substrates using antibodies specific for SMC phenotypic marker proteins, basically confirms that all SMC's in culture, irrespective of what type of matrix they are cultured on represent the de-differentiated synthetic phenotype. Well established synthetic markers such as cytokeratin 8 and light chain caldesmon are expressed in the cultured cells but are absent or only present at very low levels in normal tissue samples. Conversely, well known contractile markers such as Desmin and SM-myosin are absent in the cultured cells but well expressed in the tissue. This analysis also seems to indicate that the phenotypic status of cultured HUASMC's is not drastically altered by ECM as determined by the phenotypic markers analysed in this study, since only slight differences in the protein levels of meta-vinculin and the 210Kd isoform of MLCK were observed. Meta-vinculin appears to be upregulated on fibronectin substrates and the 210Kd isoform of MLCK was upregulated in SMC's cultured on cellular fibronectin, collagen III, and vitronectin substrates.

Interesting results to arise from this marker analysis were the identification of a 30Kd low molecular weight isoform of tropomyosin and a 210Kd isoform of MLCK as potential markers for synthetic SMC's. The 30kd isoform of tropomyosin was well expressed in all cultured HUASMC samples but totally absent in all three tissue preparations. Also MLCK 210Kd isoform was present at high levels in all cultured HUASMC samples but at significantly lower levels in tissue samples.

Further characterisation of tropmyosin isoforms demonstrated that a low molecular weight isoform of tropomyosin was expressed exclusively in cultured rat vascular SMC's and balloon injured rat carotid tissue, but was absent in normal rat carotid tissue.

Immunoblotting with the SMC phenotype marker specific antibodies on protein samples obtained from FW aorta and HUA SMC samples that were cultured in differentiation

medium and Matrigel supplied by Becton Dickinson did not reveal any transition from a synthetic to a contractile phenotype. The expression of SMC contractile markers; Desmin and SM myosin heavy chain were not induced by culture in Matrigel.

SMC growth in Matrigel did induce down-regulation in the expression of MLCK isoforms, vinculin, and meta-vinculin. This evidence suggests that culturing HUASMC's and FW aorta SMC's in Matrigel supplemented with differentiation medium induces phenotypic alterations. These alterations in phenotype do not however, appear to correlate with a clear transition from a synthetic to a contractile phenotype.

Immunofluorescent studies carried out on HUASMC's cultured on uncoated and plasma fibronectin coated tissue culture surfaces revealed no clear differences between these two populations in either the levels or distribution of phenotype marker proteins; SM-α Actin; Vimentin; Cytokeratin 8; Vinculin; Fibronectin; Collagen I; Collagen IV; Thrombospondin and Tropomyosin. The association of Myosin light Chain Kinase (MLCK) antibody staining with fibrils did appear to be slightly upregulated in HUASMC's cultured on fibronectin. Also antibody staining to caldesmon was of greater intensity in HUASMC's cultured on plasma fibronectin.

Chapter.3.(Section IX)

Differential display analysis of SMC's cultured on fibronectin compared with plastic.

IX.1 Introduction - Differential display technique.

The differential display technique was first presented by Liang and Pardee in 1992. They described differential display as a method for the isolation of individual messenger RNAs (mRNAs) that are differentially expressed between various cell populations or upon altered conditions (Liang and Pardee 1992).

Differentially expressed mRNA species are identified by differential display by means of the polymerase chain reaction (PCR). The basic principle of the procedure is to systematically amplify up mRNA from separate populations by reverse transcriptase - PCR using a set of 3' anchored degenerate primers to drive the reverse transcriptase reaction. These anchored primers are then used in combination with a selection of rationally designed arbitrary primers for PCR amplification. A radioactive nucleotide is included in the PCR step so that amplified products can be run side by side on a sequencing gel and visualised by autoradiography. Bands from the gel that appear differentially expressed between populations can be cut out of the gel, cloned and sequenced and also used as a probe to confirm differential gene expression by northern blotting or to obtain full length sequence by screening a cDNA library.(see methods section **VI.16.**).

Differential display has the advantage over existing methods for the identification of differentially expressed genes such as subtractive hybridisation, in that it is simpler and faster to carry out, provides increased sensitivity and requires very little RNA (2μg per population) as starting material for the initial identification of potential differentially expressed products.

In order to further investigate the influence that SMC adhesion to ECM has on cell phenotype, differential display analysis was applied to RNA samples extracted from HUASMC's cultured on fibronectin and plastic. HUASMC's cultured on fibronectin and plastic were selected for comparison, not only because of the clear morphological and

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functional differences observed between these two populations that are indicative of an alteration in phenotype (*Chp. 1* **Results Section VII**), but also because large quantities of RNA can be obtained from SMC's cultured on these two substrates. Such large quantities of RNA are essential for subsequent northern blot analysis that is required to confirm differential display results.

Differential display analysis was undertaken with the aim of identifying SMC genes whose expression levels were modulated by adhesion to fibronectin *in vitro*. The identification of fibronectin modulated genes will help elucidate poorly understood downstream molecular events induced by ECM adhesion as well as the role fibronectin has in modulating SMC phenotype. Also subsequent investigation into whether any of these *in vitro* differentially expressed genes are also differentially expressed *in vivo* between contractile SMC's localised in normal vascular tissue with synthetic SMC's within lesions as indicated by *in situ* hybridisation studies, will help evaluate the *in vitro* model by confirming whether any of the *in vitro* differentially expressed genes represent markers of either a contractile or a synthetic phenotype.

At the time when this analysis was first carried out one major disadvantage of the differential display technique was that it was so new and remained to be optimised to overcome serious limitations such as redundancy and under representation of certain mRNA species and also a high false positive hit rate (Liang *et al* 1994). The following results section outlines results obtained from differential display analysis of fibronectin and plastic modulated HUASMC's **(FIGs; IX.1-IX.5)**. Also described are the further optimisation and alterations to the procedure that were carried out during the course of these studies.

Initial differential display experiments were performed on total mRNA samples from HUASMC's cultured on uncoated and plasma fibronectin coated tissue culture plastic. RNA samples were isolated using the RNAzolB reagent and protocol (see methods **VI.15.**) from HUASMC's cultured on both substrates. Represented in **(FIG. IX.1.)** is a preliminary differential display gel on which products from plastic and fibronectin modulated HUASMC's amplified using the same arbitrary and anchored primer combinations were run in adjacent tracks. This differential display analysis was performed using the four 14 mer oligo dt anchored primers degenerate in the penultimate base; $T_{12}MT$, $T_{12}MA$, $T_{12}MG$ and $T_{12}MC$ in conjunction with five 10 mer arbitrary primers; AP1-5 that were supplied in the RNA map kit (Genhunter). Differential display PCR was carried out as recommended by Genhunter using [35]S radiolabelled dATP (see methods **VI.16.**). Thirteen differentially expressed products were selected from this gel for further analysis **(FIG. IX.1a,b,c&d.)**. Differential display gels were electrophoresed at a constant power of 60 mA for 3 hours resulting in the separation of amplified products ranging in size from a 100bp to 600bp.

(FIG. IX.1a.).
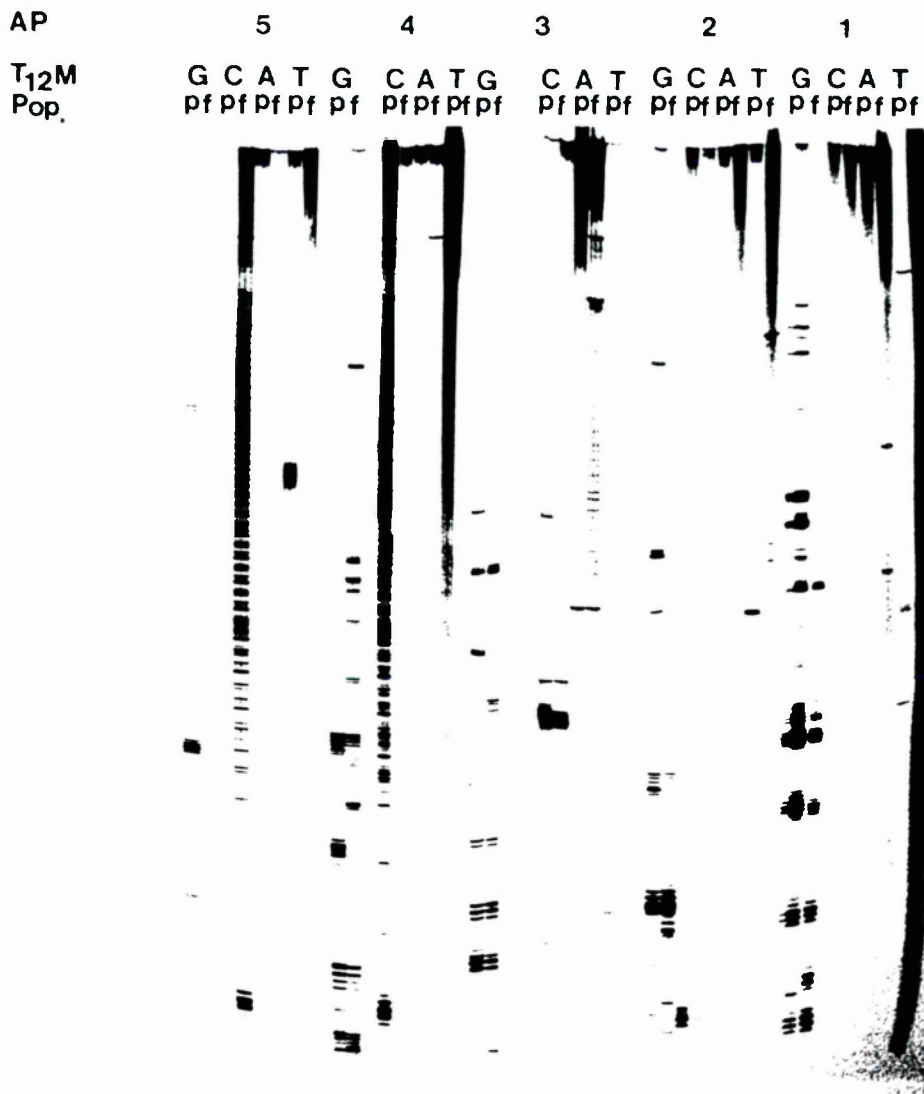
(FIG. IX.1a.) Differential display on fibronectin and plastic modulated HUASMC samples using arbitrary primers AP 1-5 in combination with anchored oligo dt primers $T_{12}MA$, $T_{12}MC$, $T_{12}MG$ and $T_{12}MT$.

(FIG.IX.1b.).

(FIG.IX.1c.).

(FIG.IX.1d.).

(FIG. IX.1 b,c & d.) Higher magnification of differential display performed with primers; $T_{12}MA$, C; G and A in conjunction with primers AP1-8. Differentially expressed bands are indicated by arrows (→).

The thirteen differentially expressed bands identified were cut out of the gel reamplified by PCR, using the relevant primer pairs, gel purified and sequenced manually or on an ABI automated sequencer (Advanced Biotechnology Centre - Charing Cross) (see methods **VI.16.**) **Table IX.1** lists the results of homology searches of the differential display sequences with genes submitted to the Genbank/EMBL gene database.

(Table. IX.1.).

CLONE NO.	SAMPLE	PRIMER COMBINATION	PRODUCT SIZE	DATA BASE SEARCH
7.1	Fibronectin	$AP1/T_{12}MG$	100bp	Novel
7.2	Fibronectin	$AP1/T_{12}MG$	100bp	Novel
19.1	Fibronectin	$AP3/T_{12}MA$	600bp	Novel
19.2	Fibronectin	$AP3/T_{12}MA$	400bp	Novel
31.1	Fibronectin	$AP4/T_{12}MG$	330bp	Novel
31.2	Fibronectin	$AP4/T_{12}MG$	200bp	ALU region
31.4	Fibronectin	$AP4/T_{12}MG$	150bp	Novel
32.1	Plastic	$AP4/T_{12}MG$	220bp	Novel
32.2	Plastic	$AP4/T_{12}MG$	120bp	Novel
32.3	Plastic	$AP4/T_{12}MG$	120bp	Novel
32.4	Plastic	$AP4/T_{12}MG$	110bp	Novel
40.1	Plastic	$AP5/T_{12}MG$	320bp	ALU region
40.4	Plastic	$AP5/T_{12}MG$	300bp	ADP/ATP translocase

The identification of a large number of novel sequences and sequences rich in alu repetitive regions indicated that amplification of contaminating genomic DNA present within the RNA samples under study may have taken place. To reduce the possibility of amplifying contaminating genomic DNA which may contribute to the selection of false positives, all subsequent differential display studies were performed on DNAse treated RNA samples. Also to further reduce the possibility of selecting false positives each PCR primer combination amplification reaction was carried out in duplicate. Only those bands that were reproducibly differentially expressed between populations were selected for further analysis.

Subsequent differential display amplification reactions were performed using three 17mer one base anchored oligo dt primers; H-T_{11}G, H-T_{11}C and H-T_{11}A in conjunction with rationally designed 13 mer arbitrary primers supplied in the RNAimage kit (Genhunter). The use of three one base anchored oligo dt primers cuts down the number of reverse transcription reactions needed for each RNA sample, and also minimises the redundancy and under-representation of certain RNA species due to the degeneracy of the primers. The introduction of a restriction site at the 5' ends on both the anchored and arbitrary primers makes the primers longer and more efficient in cDNA amplification while allowing the cloned cDNA to be more readily manipulated (Liang *et al* 1994). Also [33]P dATP was used as label for differential display PCR. [33]P was found to be a better alternative to [35]S labelled nucleotides which can undergo a high rate of decomposition when exposed to the high temperatures used in the PCR reaction often resulting in release of [35]S labelled gas and subsequent contamination of PCR blocks (Trentmann *et al* 1995).

(**Fig.IX.2a,b,c**). Represents a differential display gel performed using DNAse treated RNA samples amplified with the newly developed primers. Amplification reactions were performed in duplicate. Eight clear differentially expressed bands were identified between fibronectin and plastic modulated HUASMC populations.

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(FIG.IX.2a.).

(FIG. IX.2a.) Differential display on fibronectin and plastic modulated HUASMC samples using arbitrary primers H-AP 5-7 in combination with anchored oligo dt primers H-T$_{11}$G, H-T$_{11}$C and H-T$_{11}$A
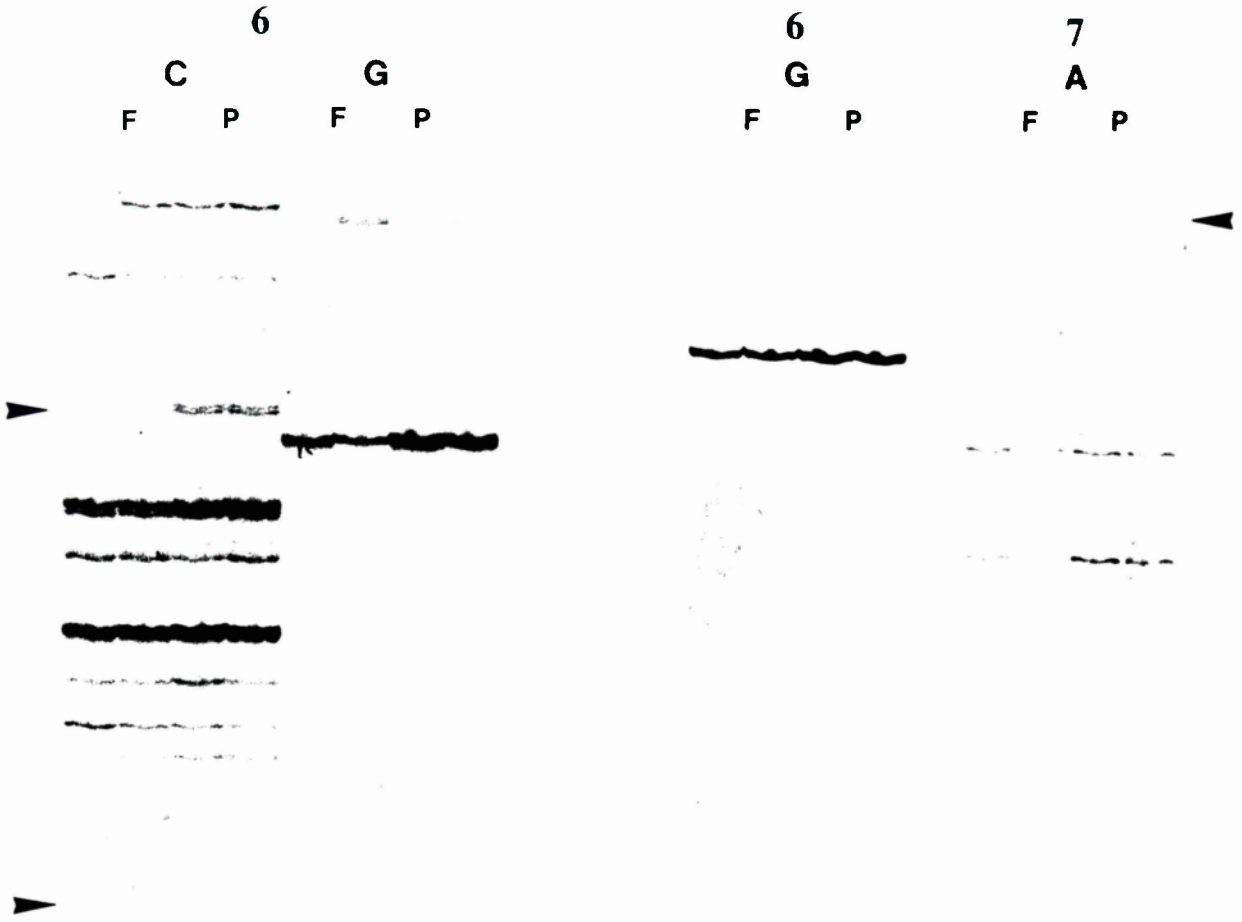
(FIG. IX.2 b. & c.) Higher magnification of differential display performed with primers; H-T₁₁C; G and A in conjunction with primers AP5-7. Differentially expressed bands are indicated by arrows (→).

(FIG. IX.2d.).

(FIG. IX.2d.). Higher magnification of differential display performed with primers. H-T$_{11}$C.

G and A in conjunction with primers AP5-7. Differentially expressed bands are indicated by

arrows(→).

In order to obtain an estimate of the number of PCR artefacts amplified by each primer combination used for differential display, the number of bands differing between identical duplicate reactions were counted. These results are presented in **(Table IX.2)**.

Table. IX.2. Number of different DNA bands amplified between identical differential display PCR amplification reactions, from gel presented in (FIG.IX.2a.).

PRIMER COMBINATION	RNA SAMPLE	NO. OF DIFFERENCES
H-AP5/H-T_{11}A	Fibronectin	4
H-AP5/H-T_{11}A	Plastic	5
H-AP5/H-T_{11}C	Fibronectin	6
H-AP5/H-T_{11}C	Plastic	3
H-AP5/H-T_{11}G	Fibronectin	28
H-AP5/H-T_{11}G	Plastic	27
H-AP6/H-T_{11}A	Fibronectin	4
H-AP6/H-T_{11}A	Plastic	5
H-AP6/H-T_{11}C	Fibronectin	11
H-AP6/H-T_{11}C	Plastic	14
H-AP6/H-T_{11}G	Fibronectin	-
H-AP6/H-T_{11}G	Plastic	20
H-AP7/H-T_{11}A	Fibronectin	13
H-AP7/H-T_{11}A	Plastic	7
H-AP7/H-T_{11}C	Fibronectin	2
H-AP7/H-T_{11}C	Plastic	2
H-AP7/H-T_{11}G	Fibronectin	25
H-AP7/H-T_{11}G	Plastic	12

From these values it seems that certain primer combinations, most notably H-AP5/H-T_{11}G, H-AP6/H-T_{11}G and H-AP7/H-T_{11}G, result in the amplification of many PCR artefacts, whereas other combinations result in very few. The amplification of such a large number of PCR artefacts could lead to the mistaken selection of artefacts as differentially expressed genes, thereby further contributing to the high false positive selection rate. To reduce this possibility it was decided that future differential display PCR amplification reactions would be carried out in triplicate and only those differences reproduced in at least 2 out of 3 corresponding pairs of PCR reactions would be selected for further analysis. Also only those differentially expressed bands greater in size than 150 base pairs were selected for further analysis to provide a greater rate of success during subsequent processing of the DNA such as reamplification and probe hybridisation reactions. **(FIGs IX.3a,b,c,d; IX.4a,b,c & IX.5a,b)**.

(FIG. IX.3a.).

(FIG. IX.3a.) Differential display on fibronectin and plastic modulated HUASMC samples using arbitrary primers H-AP 1 & 2 in combination with anchored oligo dt primers H-T$_{11}$G, H-T$_{11}$C and H-T$_{11}$A.
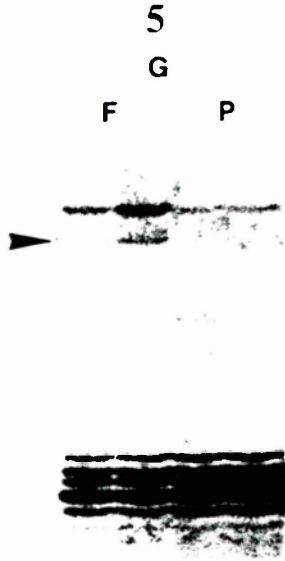
(FIG. IX.3b.).
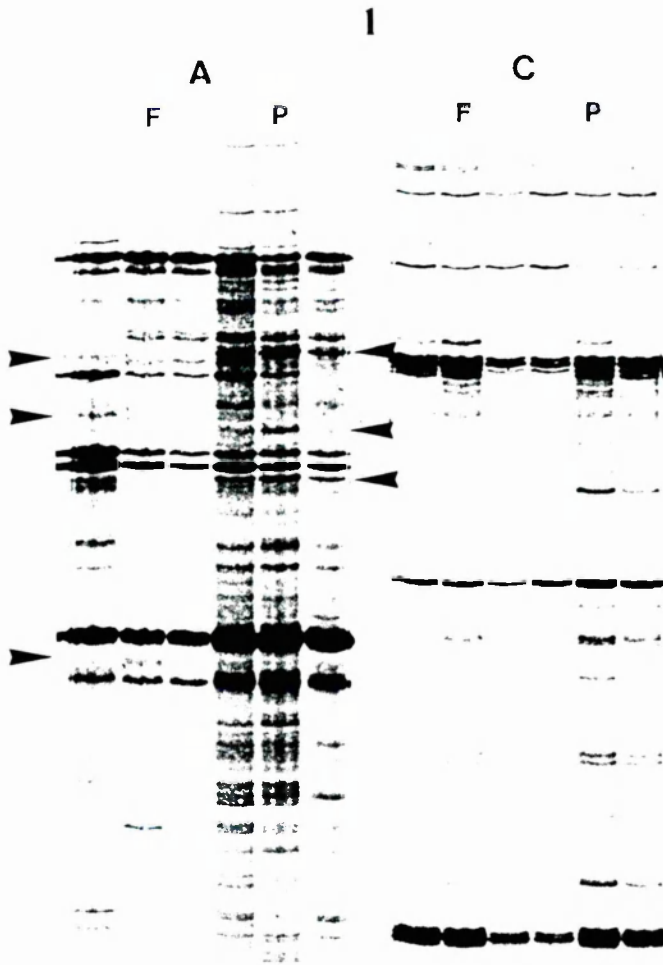
(FIG.IX.3c.).
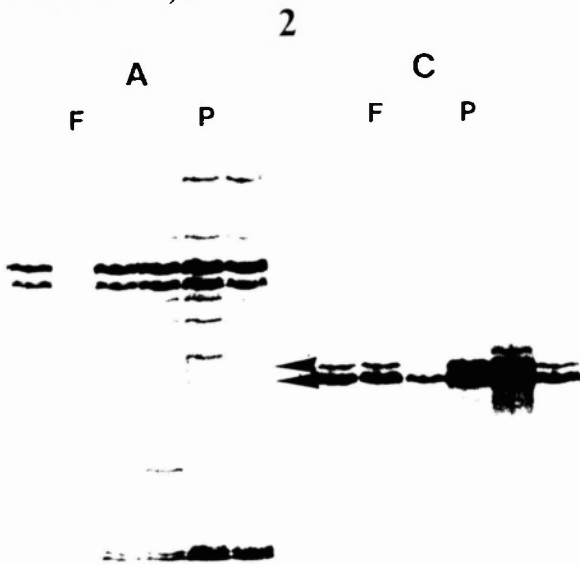
(FIG.IX.3d.).
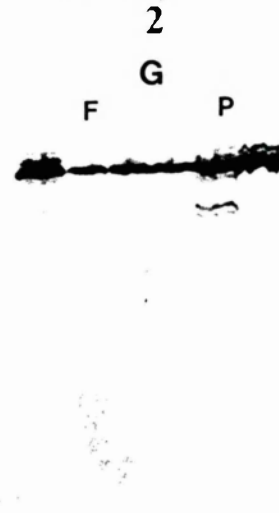
(FIG. IX.3b,c&d.) Higher magnification of differential display performed with primers; H-

$T_{11}C$; G and A in conjunction with primers AP 1 & 2. Differentially expressed bands are
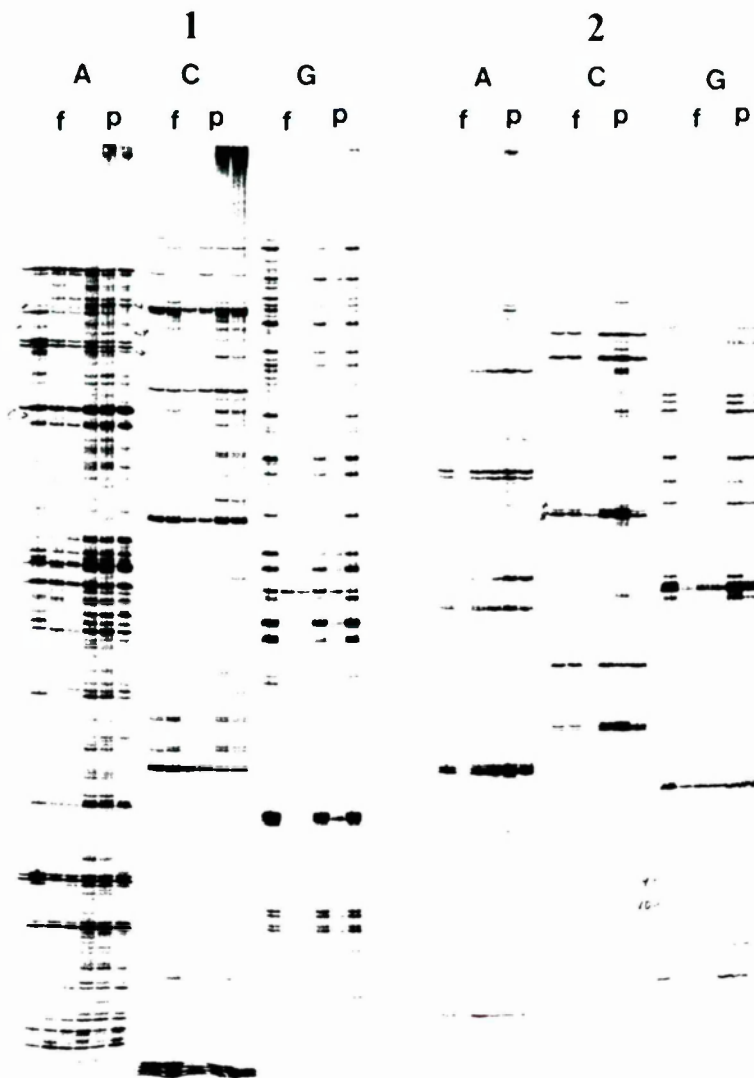
indicated by arrows (➙) .

(FIG.IX.4a.).
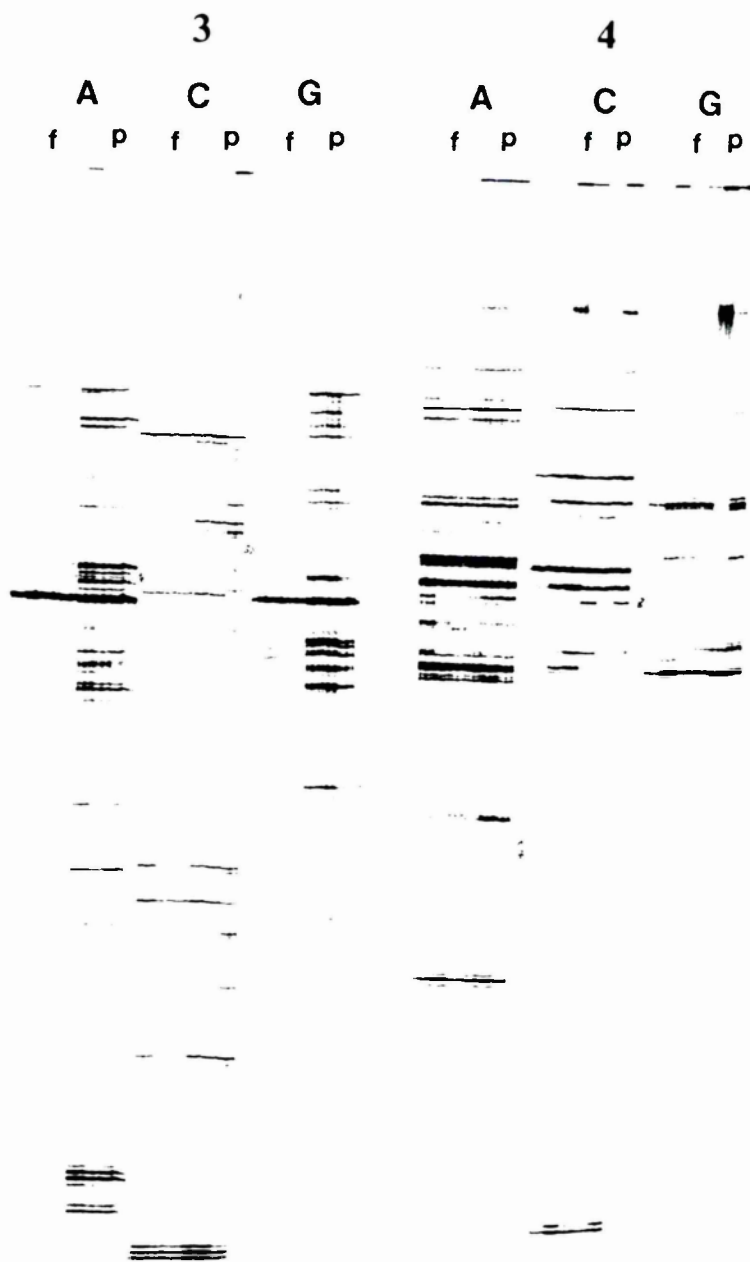
(FIG. IX.4a.) Differential display on fibronectin and plastic modulated HUASMC samples using arbitrary primers H-AP 3 & 4 in combination with anchored oligo dt primers H-T$_{11}$G, H-T$_{11}$C and H-T$_{11}$A.

(FIG.IX.4b.).
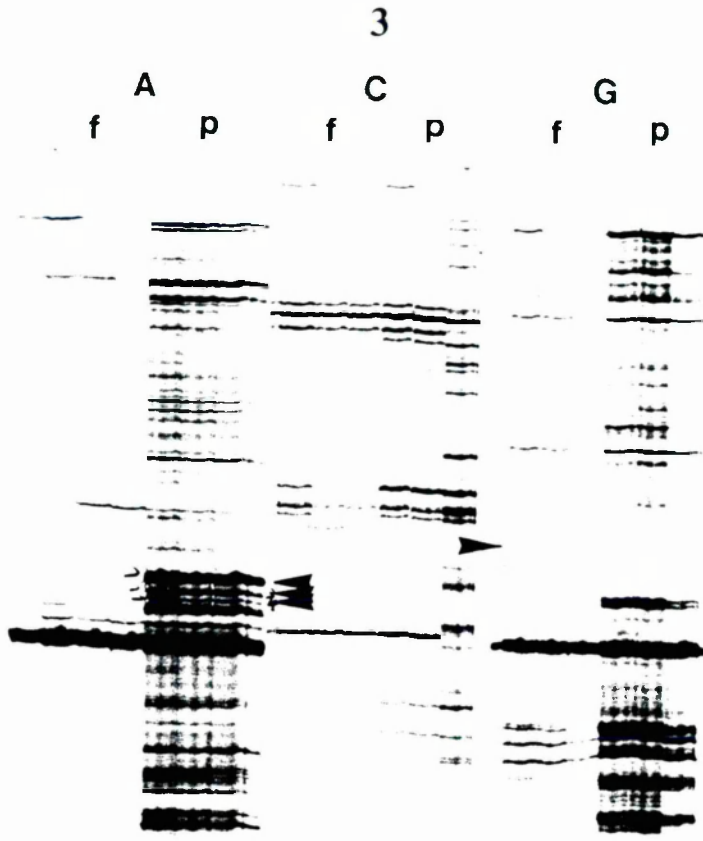
(FIG.IX.4c.).
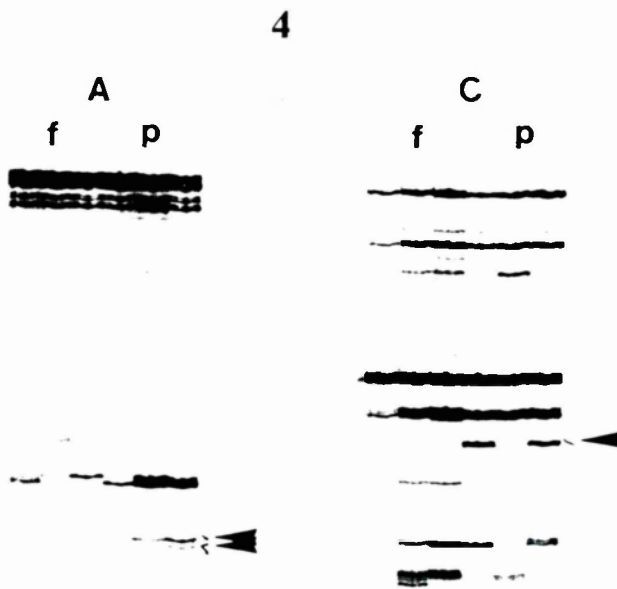
(FIG. IX.4b. & c.) Higher magnification of differential display performed with primers; H-T$_{11}$C; G and A in conjunction with primers AP 3 & 4. Differentially expressed bands are indicated by arrows (→).

(FIG. IX.5a.).
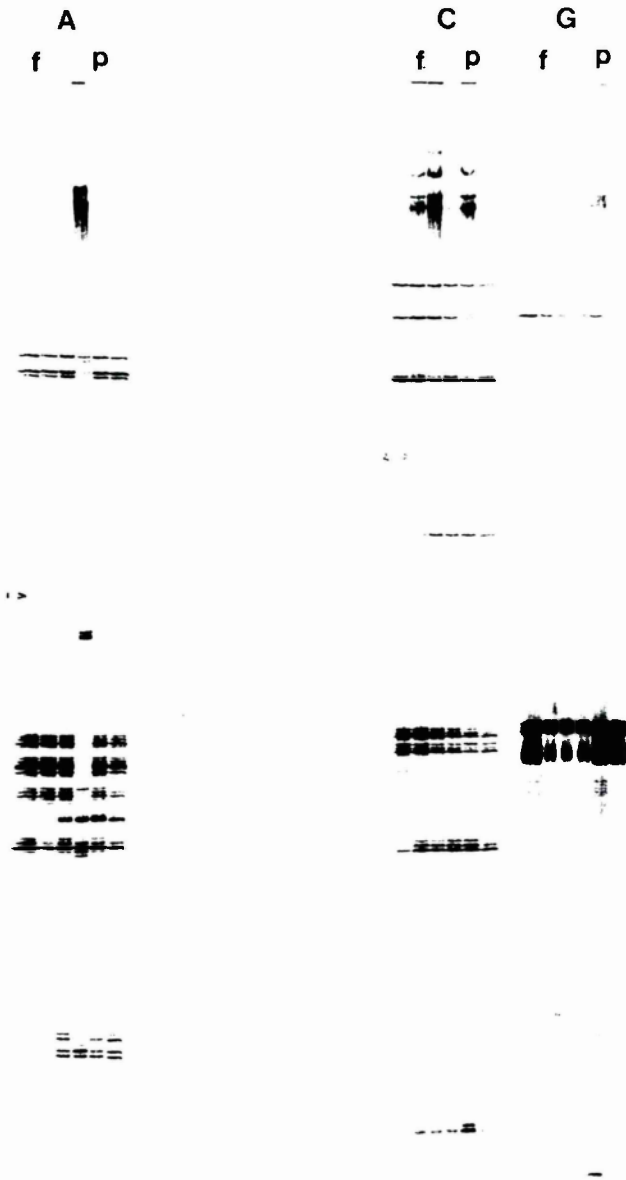
(FIG. IX.5a.) Differential display on fibronectin and plastic modulated HUASMC samples using arbitrary primer H-AP 8 in combination with anchored oligo dt primers H-T$_{11}$G, H-T$_{11}$C and H-T$_{11}$A.

(FIG.IX.5b.).

(FIG. IX.5b.) Higher magnification of differential display performed with primers; H-T$_{11}$ C; G and A in conjunction with primers AP 8. Differentially expressed bands are indicated by arrows (→).

From the differential display gels, **(FIGs IX.2a, IX.3a, IX.4a & IX.5a)** carried out using the modified primers and reagents supplied in the RNAimage kit (Genhunter) 27 differentially expressed bands were identified between plastic and fibronectin modulated HUASMC populations. 22 of these differential display fragments have been successfully cloned into the TA cloning vector and sequenced. Searches were carried out on the Genbank/EMBL gene database's to identify any homology that exists between these sequences and known genes. As indicated in **(TABLE IX.3)** nine sequences were found to be homologous with known genes; the remaining thirteen did not reveal any significant homology and so may therefore represent novel genes. It is also possible that the preferential amplification by differential display of DNA fragments from the 3'end of genes, may result in the identification of sequences from known genes that have not been entered into the data base as a result of their extreme 3' location.

TABLE IX.3. Summary of data base search on sequences identified as differentially expressed between fibronectin and plastic modulated SMC populations.

CLONE. N0.	SAMPLE	PRIMER COMBINATION	PRODUCT SIZE	DATA BASE SEARCH
A1.1	Fibronectin	H-AP5/H-T$_{11}$A	450bp	14 Iq JH
A3.1	Fibronectin	H-AP5/H-T$_{11}$G	150bp	Novel
A4.3	Fibronectin	H-AP6/H-T$_{11}$G	200bp	Novel
A5.1	Plastic	H-AP6/H-T$_{11}$G	200bp	Novel
A6.1	Plastic	H-AP6/H-T$_{11}$G	325bp	cSRP 72
B2.1	Fibronectin	H-AP1/H-T$_{11}$A	500bp	I.R.R. protein
B3.6	Fibronectin	H-AP1/H-T$_{11}$A	450bp	I-2-S'ase
B4.1	Plastic	H-AP1/H-T$_{11}$A	450bp	Novel
B5.1	Plastic	H-AP1/H-T$_{11}$A	450bp	Novel
B6.5	Fibronectin	H-AP1/H-T$_{11}$A	300bp	Vinculin/meta-vinculin
B7.1	Plastic	H-AP2/H-T$_{11}$A	250bp	Novel
B9.1	Fibronectin	H-AP2/H-T$_{11}$G	140bp	Novel
B10.2	Fibronectin	H-AP2/H-T$_{11}$G	140bp	Novel
C1.1	Fibronectin	H-AP3/H-T$_{11}$A	250bp	p54
C2.2	Plastic	H-AP3/H-T$_{11}$A	250bp	Pentaxin 3
C3.1	Plastic	H-AP3/H-T$_{11}$A	225bp	Pentaxin 3
C4.1	Plastic	H-AP3/H-T$_{11}$A	250bp	PTPase
C5.1	Fibronectin	H-AP3/H-T$_{11}$G	250bp	Novel
C6.1	Plastic	H-AP4/H-T$_{11}$A	100bp	Novel
C7.1	Plastic	H-AP4/H-T$_{11}$A	100bp	Novel
C8.1	Plastic	H-AP4/H-T$_{11}$C	225bp	Novel
D2.2	Fibronectin	H-AP8/H-T$_{11}$C	300bp	Cyt p450

As a result of the high number of false positives that are associated with differential display analysis it is important to confirm the differential expression of genes identified by differential display before studying them further. Confirmation of differential gene expression was carried out by screening northern blots containing RNA from HUASMC's cultured on fibronectin and plastic (see methods Section **VI.16.**). Northern blots were screened using ^{32}P CTP random primed labelled differentially expressed products as nucleic acid probes.

One important consideration that had to be taken into account before screening northerns was that differentially expressed bands cut out of a differential display gel may contain more than one product. Studies by Guimaraes *et al* 1995 and myself (results not shown) confirm this possibility does exist. Such a scenario may result in the amplification and sequencing of cDNA that may not necessarily represent the differentially expressed product observed on the gel. Thus using probes designed specifically to the sequenced differential display products listed in **(Table IX.3)** may result in additional false positives. To reduce the possibility of obtaining false positives on northerns as a result of using an incorrect probe, reamplified DNA from differentially expressed bands cut out of the differential display gel were random primed labelled with radioactive ^{32}P CTP (see methods Section VI.). This method should allow labelling of all the products contained within a band.

Northern blots probed with random primed labelled differential display fragments that resulted in clear transcripts are represented in **(FIGs IX.6-.14).** These figures also reveal transcript signals obtained by screening the same northerns with a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative mRNA levels of the housekeeping gene GAPDH between fibronectin and plastic modulated HUASMC RNA samples provides an indication of loading between both samples that was used as a reference to estimate the relative mRNA levels of differential display products between HUASMC's cultured on fibronectin and plastic. No significant differences in transcript levels were observed.

(FIG.IX.6.)

C1.1
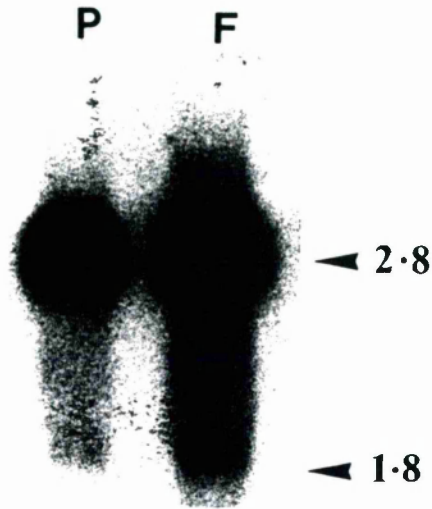
GAPDH
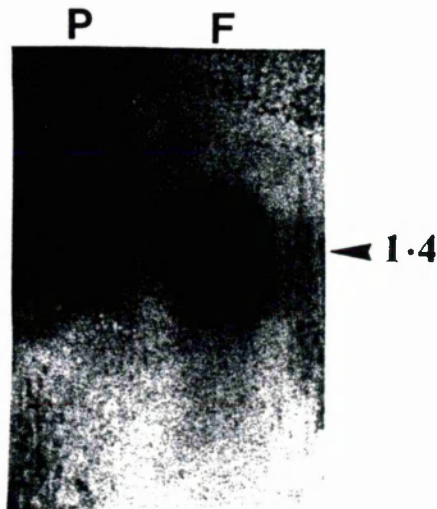
(FIG.IX.6.) C1.1 (p54)/GAPDH probe. Northern blot analysis on RNA samples obtained from HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic. This blot was probed with random primed labelled differential display product; C1.1 (originally found to be homologous with p54). and also a GAPDH probe.

(FIG.IX.7.)

(FIG.IX.7.) C2.2 (Pentaxin 3)/GAPDH probe. Northern blot analysis on RNA samples obtained from HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic. This blot was probed with random primed labelled differential display product; C2.2 (originally found to be homologous with pentaxin 3). and also a GAPDH probe.

(FIG.IX.8.)
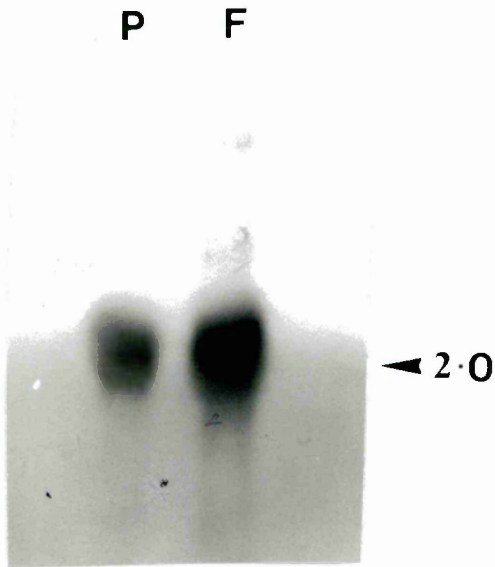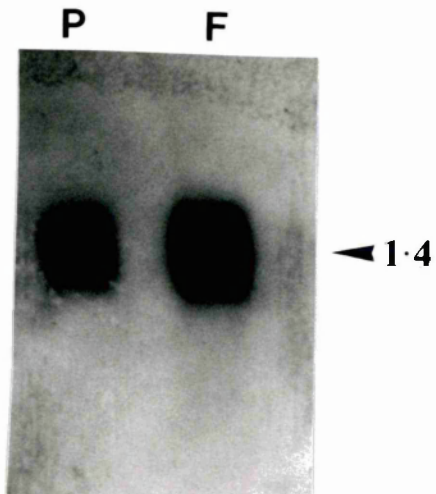
(FIG.IX.8.) C3.1 (Pentaxin 3)/GAPDH. Northern blot analysis on RNA samples obtained

from HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic.

This blot was probed with random primed labelled differential display product; C3.1 (also

originally found to be homologous with pentaxin 3). and a GAPDH probe.

(FIG.IX.9.)
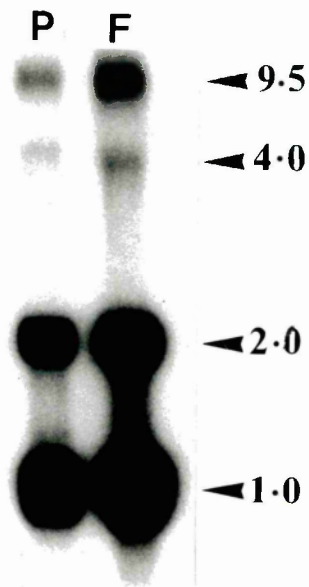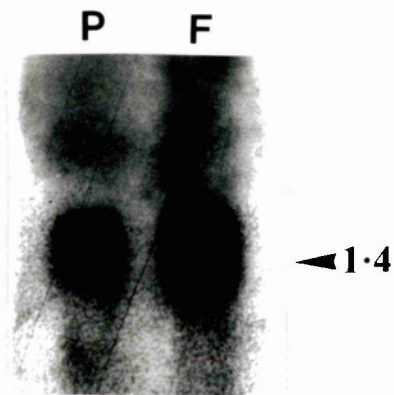
(FIG.IX.9.) B5.1/GAPDH probe. Northern blot analysis on RNA samples obtained from HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic. This blot was probed with random primed labelled differential display product; B5.1 (originally found to have no homology with previously identified genes). and also a GAPDH probe.

(FIG.IX.10.)

C4.1
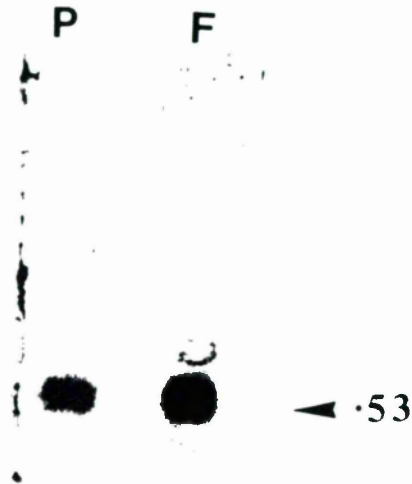
GAPDH
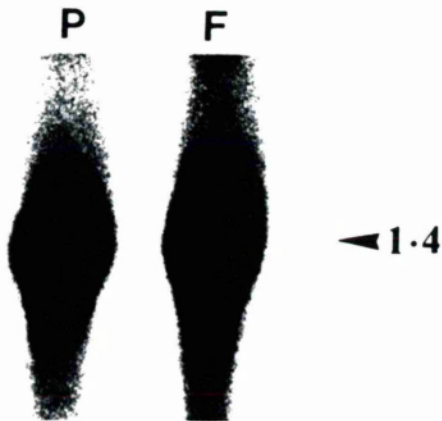
(FIG.IX.10.) C4.1 (PTPase)/GAPDH. Northern blot analysis on RNA samples obtained from HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic. This blot was probed with random primed labelled differential display product; C4.1 (originally found to be homologous with phosphotyrosyl protein phosphatase). and also a GAPDH probe.

(FIG.IX.11.)

B7.1
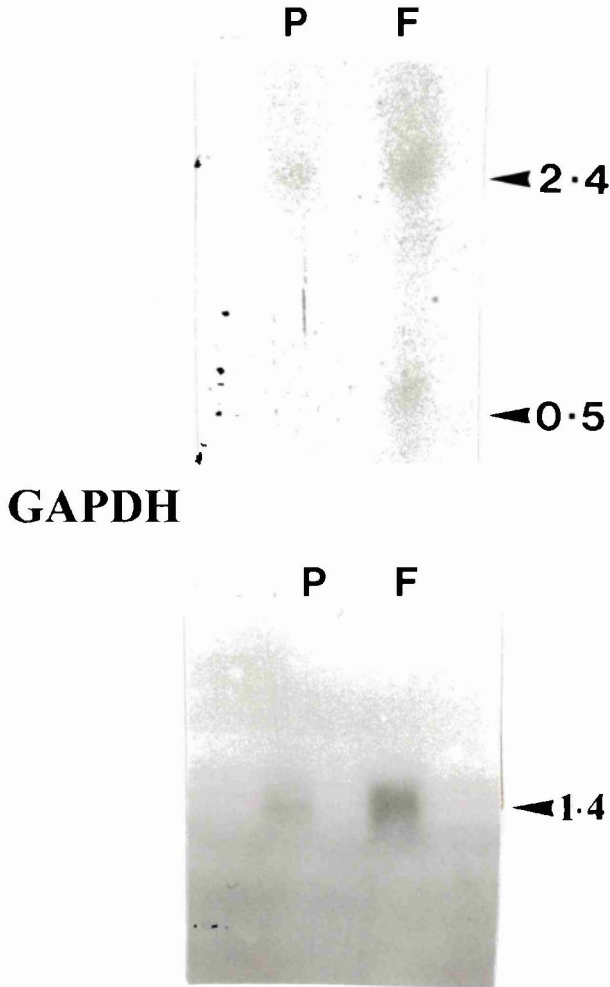
GAPDH

(FIG.IX.11.) B7.1/GAPDH. Northern blot analysis on RNA samples obtained from HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic. This blot was probed with random primed labelled differential display product; B7.1 (originally found to have no homology with previously identified genes). and also a GAPDH probe.

(FIG.IX.12.)
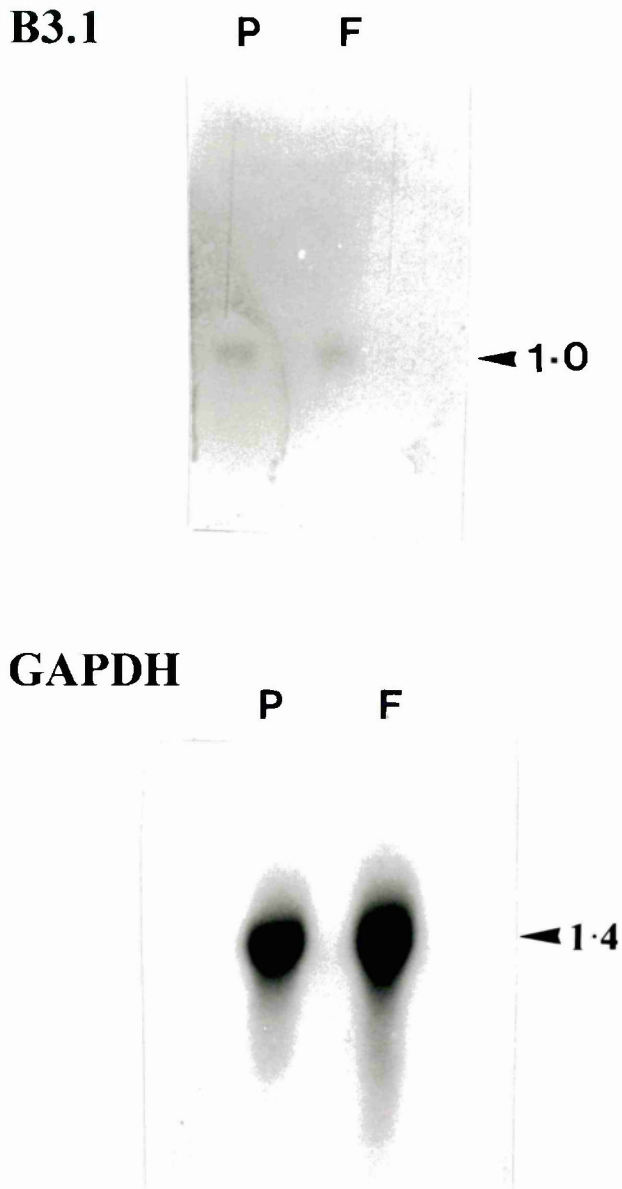
(FIG.IX.12.) B3.1 (Iduronate-2-sulphatase). Northern blot analysis on RNA samples obtained from HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic. This blot was probed with random primed labelled differential display product; B3.1 (originally found to be homologous with Iduronate-2-sulphatase). and also a GAPDH probe.

(FIG.IX.13.)
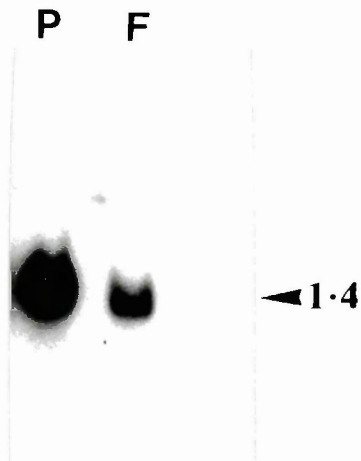
(FIG.IX.13.) **B4.1/GAPDH.** Northern blot analysis on RNA samples obtained from

HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic. This

blot was probed with random primed labelled differential display product; B4.1 (originally

found to have no homology with previously identified genes). and also a GAPDH probe.
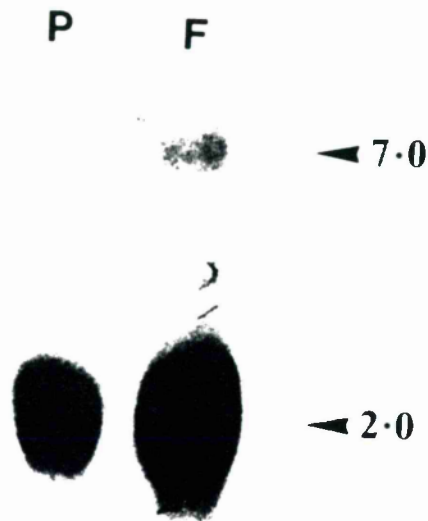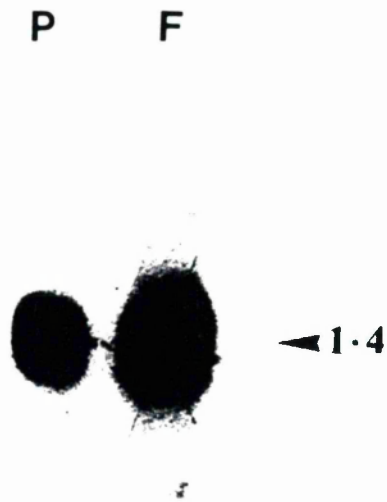
(FIG.IX.14.)
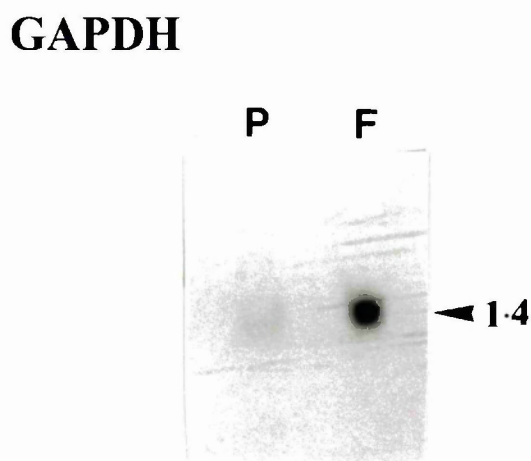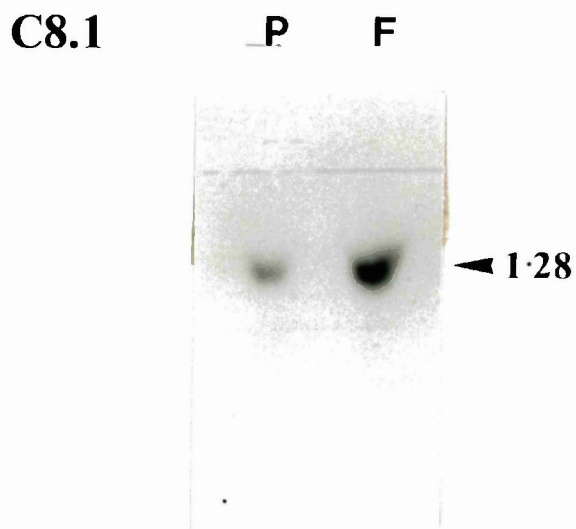
(FIG.IX.14.) **C8.1/GAPDH.** Northern blot analysis on RNA samples obtained from HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic. This blot was probed with random primed labelled differential display product; C8.1 (originally found to have no homology with previously identified genes). and also a GAPDH probe.

Differential display product B6.5 that was identified as being upregulated in fibronectin modulated HUASMC samples, failed to yield any transcript signal on a northern when used as a random primed labelled probe. B6.5 was found to be homologous to either vinculin or the meta-vinculin genes. Meta-vinculin is an alternatively spliced form of vinculin that contains an extra exon inserted at amino acid position 915. Vinculin and meta-vinculin run as 130Kd and 150Kd proteins respectively on SDS-polyacrylamide gels. Because my differential display product B6.5 was found to be homologous to the common 3' end of both vinculin and meta-vinculin **(FIG.IX.15.)** it could not be determined whether it was vinculin or meta-vinculin that was apparently upregulated on fibronectin modulated HUASMC's. **(FIG. IX.15.). Vinculin/Meta-vinculin;**

<u>VINCULIN/ META-VINCULIN:</u>
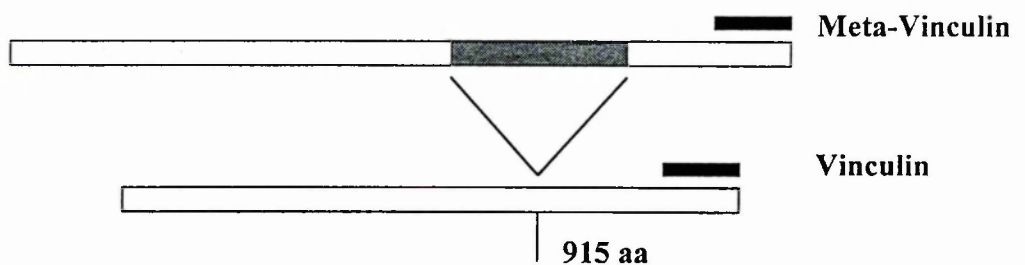
- B6.5 Differential Display fragment

Antibodies are, however, available that detect both vinculin and meta-vinculin. Such a monoclonal antibody from Sigma was used to carry out immunoblotting of protein samples obtained from HUASMC's cultured on plasma fibronectin coated and uncoated tissue culture plastic (see methods **IV.10**). **(FIG. IX.16.)**

(FIG.IX.16.).
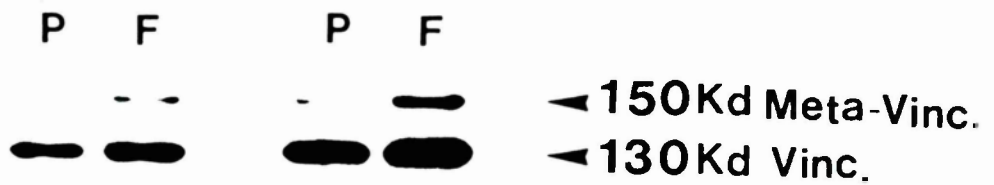
(FIG.IX.16.) SDS-PAGE analysis of total protein extracts isolated from HUASMC's on uncoated and plasma fibronectin coated tissue culture plastic. Separated protein samples were transferred to nitro-cellulose membranes and probed with a monoclonal anti-Vinculin/Meta-Vinculin antibody. Lanes **1**; HUASMC's cultured; on plastic - 5µl loading; **2**; on fibronectin - 5µl loading. **3**; on plastic - 10µl loading. **4**; on fibronectin - 10µl loading.

In order to characterise further some of the novel genes identified by differential display, their expression patterns between various cell types associated with vascular tissue and also between proliferating and quiescent SMC's were examined. The novel differential display products B4.1 and B5.1 were selected for further analysis as they had previously been demonstrated to provide clear signals on northern blots (FIGs. IX.9 & IX.13.). These differential display products were further characterised by using specific probes to screen northern blots containing RNA isolated from various cell sources; Motile lymphocytes (M MOLT 4) and non motile lymphocytes (NM MOLT 4); Endothelial cells (HUVEC's); Fibroblasts (lung MRC-5) and a myelomonocytic cell line (U937); (FIGs IX.17 & IX.19). These probes were also hybridised to blots containing RNA from HUASMC's at different stages in the cell cycle in response to the presence or absence of serum factors (FIGs. IX.18. & IX.19.). A probe specific for the 28S ribosomal RNA subunit was designed and used to provide an estimation of RNA loading between samples in the following blots. The analysis of 28S ribosomal RNA was preferred over GAPDH for these blots as transcripts obtained using B4.1 and B5.1 probes were similar in size to the GAPDH transcript.

(FIG.IX.17.).
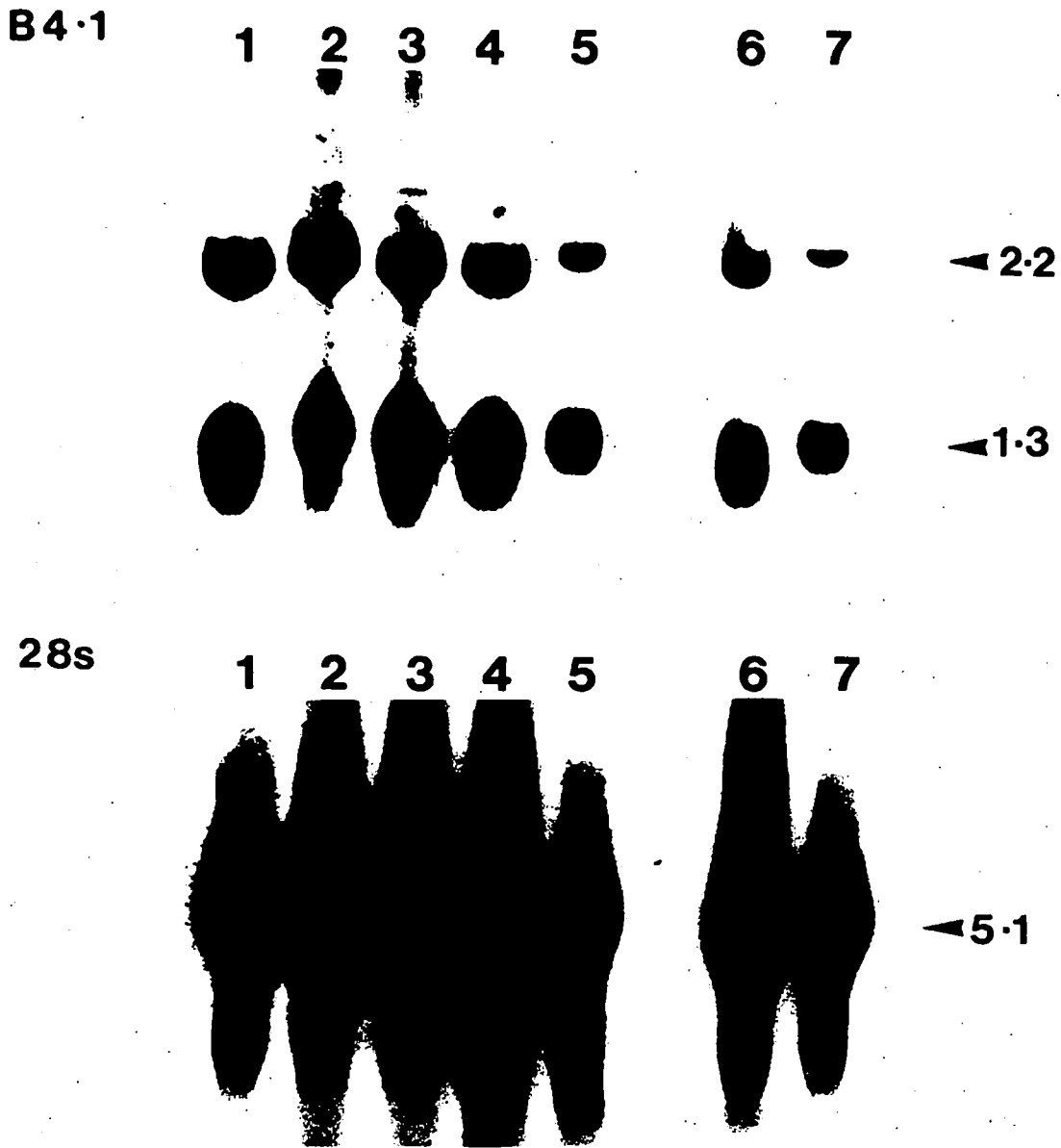
(FIG.IX.17.) B4.1/28S rRNA. Northern blot analysis on RNA samples obtained from various cell types. This blot was probed with a specific random primed labelled probe against the differential display product B4.1 (novel) and also with a probe specific for 28S ribosomal RNA to estimate loading between samples. Lanes; 1. Motile MOLT 4. 2. Non motile MOLT 4. 3.U937. 4. HUVEC's. 5. Fibroblasts. 6. HUASMC's on fibronectin. 7. HUASMC's on plastic.

(FIG.IX.18.).
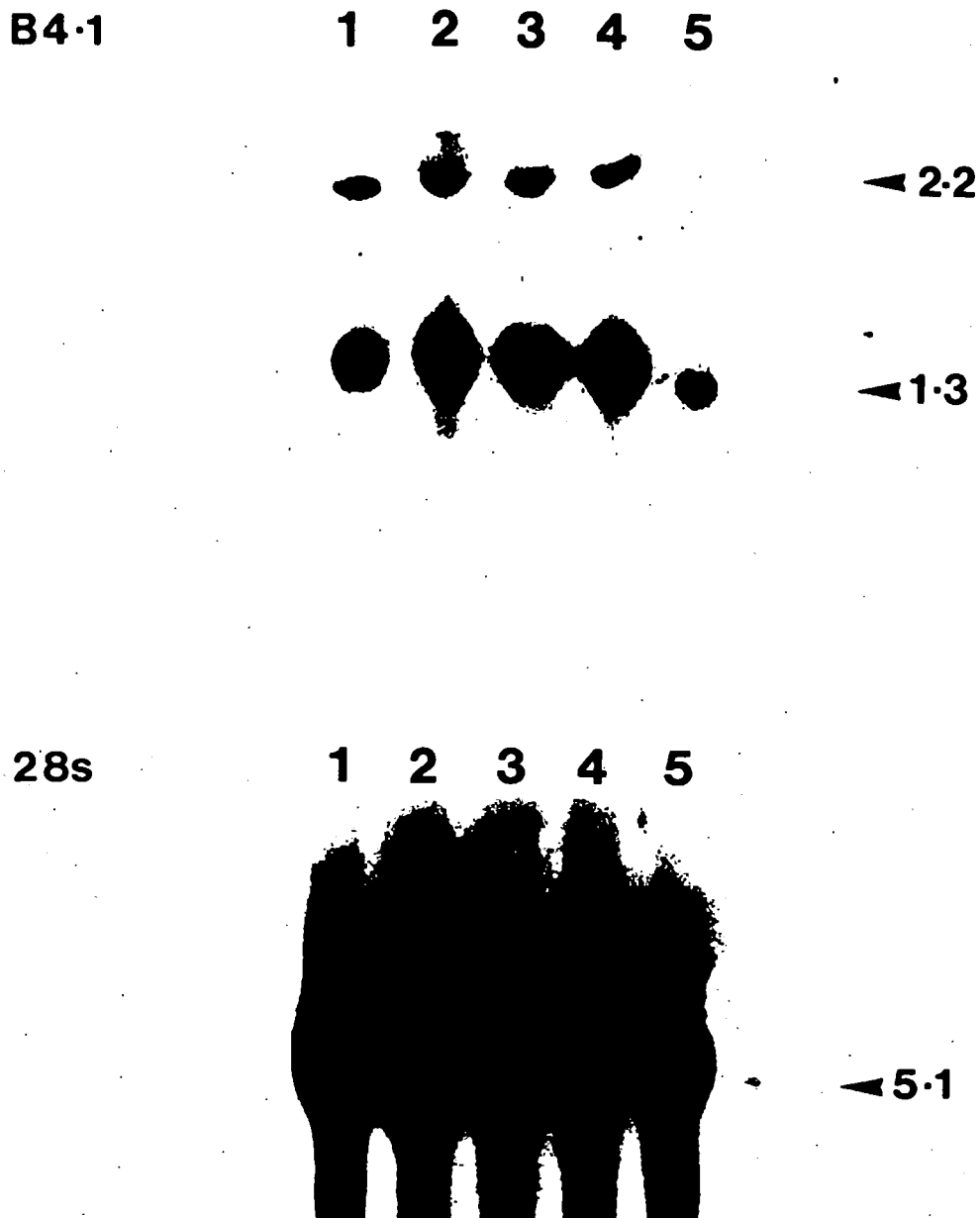
(FIG.IX.18.). B4.1/28S rRNA. Northern blot analysis on RNA samples obtained from proliferating HUASMC's and HUASMC's gradually entering quiescence as a result of serum deprivation. This blot was probed with B4.1 and 28S ribosomal RNA specific probes. Lanes; 1. - HUASMC's - 0 hours serum starvation. 2. 6 hours serum starvation. 3. 12 hours serum starvation. 4. 24 hours serum starvation. 5. 48 hours serum starvation.

(FIG.IX.19.).
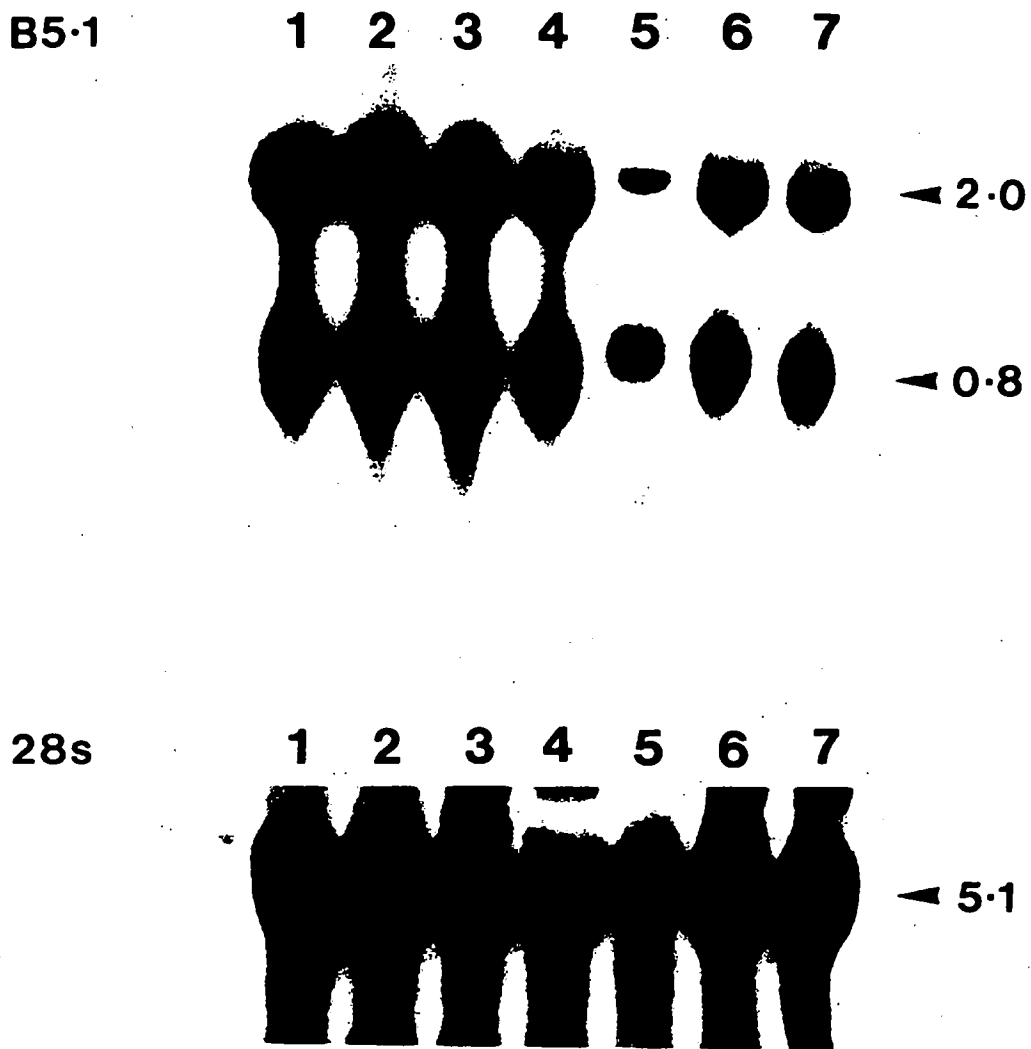
(FIG.IX.19.).B5.1/28S rRNA Northern blot analysis on RNA samples obtained from various cell types. This blot was probed with a specific random primed labelled probe against the differential display product B5.1 (novel) and also with a probe specific for 28S ribosomal RNA to estimate loading between samples. Lanes; 1. Motile MOLT 4. 2. Non motile MOLT 4. 3.U937. 4. HUVEC's. 5. Fibroblasts. 6. HUASMC's on fibronectin. 7. HUASMC's on plastic.

(FIG.IX.20.).

B5·1

```
        1   2   3   4   5
                            ◄2·0
                            ◄1·0
                            ◄0·8
```

28s

```
        1   2   3   4   5
                            ◄5·1
```
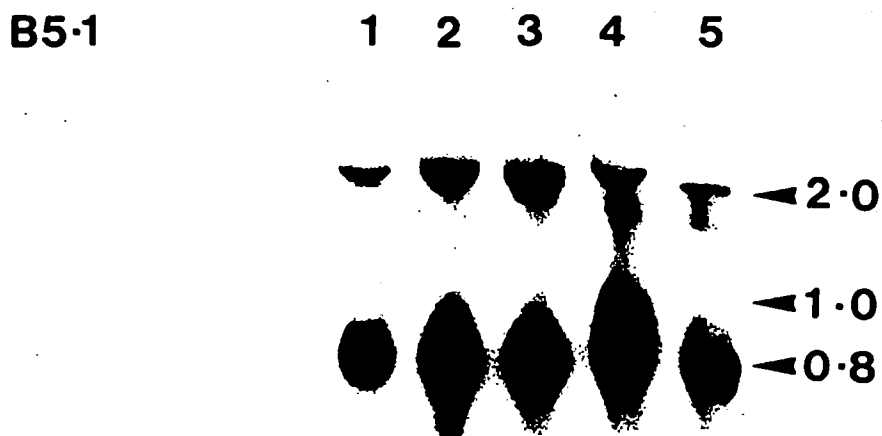
(FIG.IX.20.).B5.1/28S rRNA. Northern blot analysis on RNA samples obtained from proliferating HUASMC's and HUASMC's gradually entering quiescence as a result of serum deprivation. This blot was probed with B5.1 and 28S ribosomal RNA specific probes. Lanes; 1. - HUASMC's - 0 hours serum starvation. 2. 6 hours serum starvation. 3. 12 hours serum starvation. 4. 24 hours serum starvation. 5. 48 hours serum starvation.

Quantitation of transcript levels for B4.1 and B5.1 were obtained by analysing the
above northern blots using the Instant imager (Canberra Packard). The Instant imager uses a
microchannel array detector to allow high-resolution radioactive detection of blots. The
Instant imager software can then create image patterns of radioactivity directly from blots
enabling quantitation in counts per minute of specific transcripts. Transcript levels for B4.1,
B5.1 and also 28S ribosomal RNA present on identical blots were measured in counts per
minute. Ratios of B4.1 and B5.1 levels against corresponding 28S ribosomal RNA levels on
identical blots was calculated to obtain an accurate value of B4.1 and B5.1 mRNA levels
between samples. These ratio values are represented in **(FIGs.IX.21-IX.24)**.

(FIG.IX.21.).
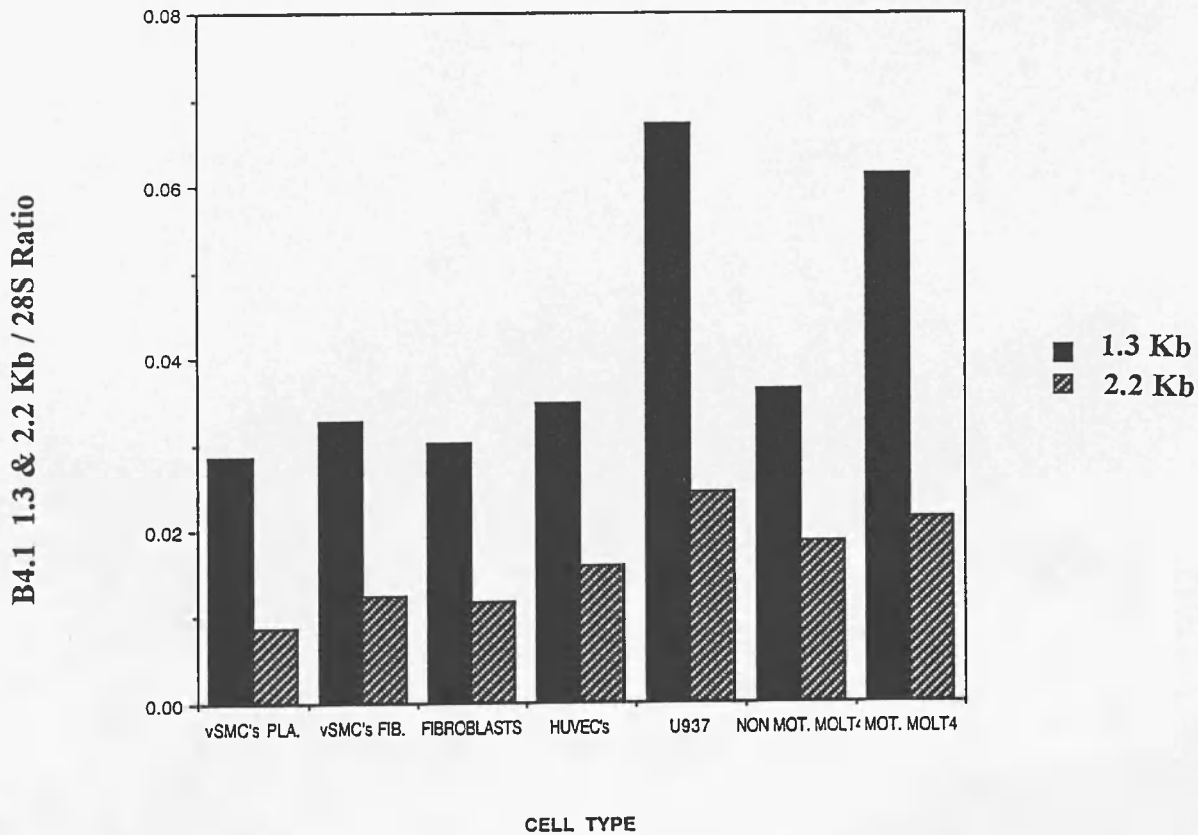
(FIG. IX.21.). Ratios of B4.1 mRNA transcript levels against 28S ribosomal RNA levels
between RNA samples from various cell populations. - From northern blot depicted in
(FIG.IX.17.)

(FIG.IX.22.).
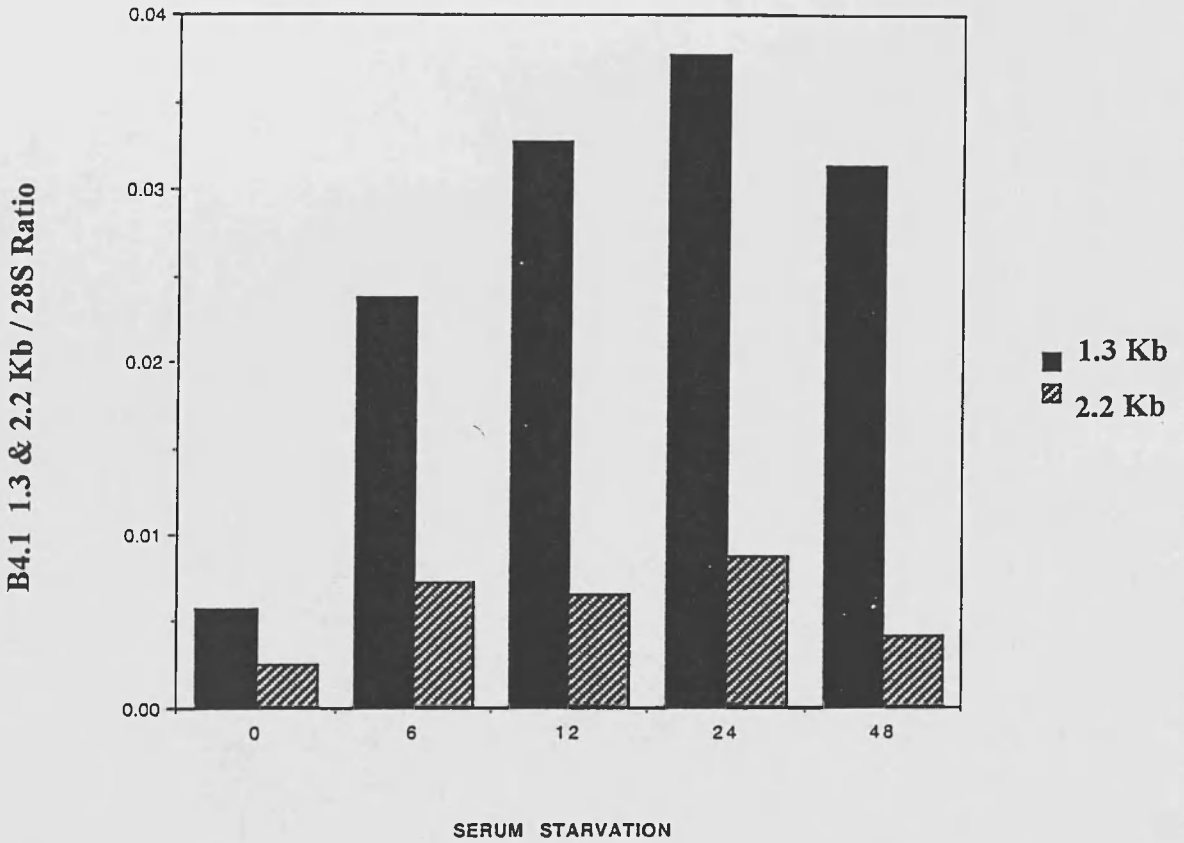
(FIG.IX.22.). Ratios of B4.1 mRNA levels against 28S ribosomal RNA levels between RNA

samples obtained from HUASMC's proliferating in the presence of serum and from

HUASMC's at increasing time points following serum starvation. From northern blot

depicted in (FIG.IX.18.).

(FIG.IX.23.).
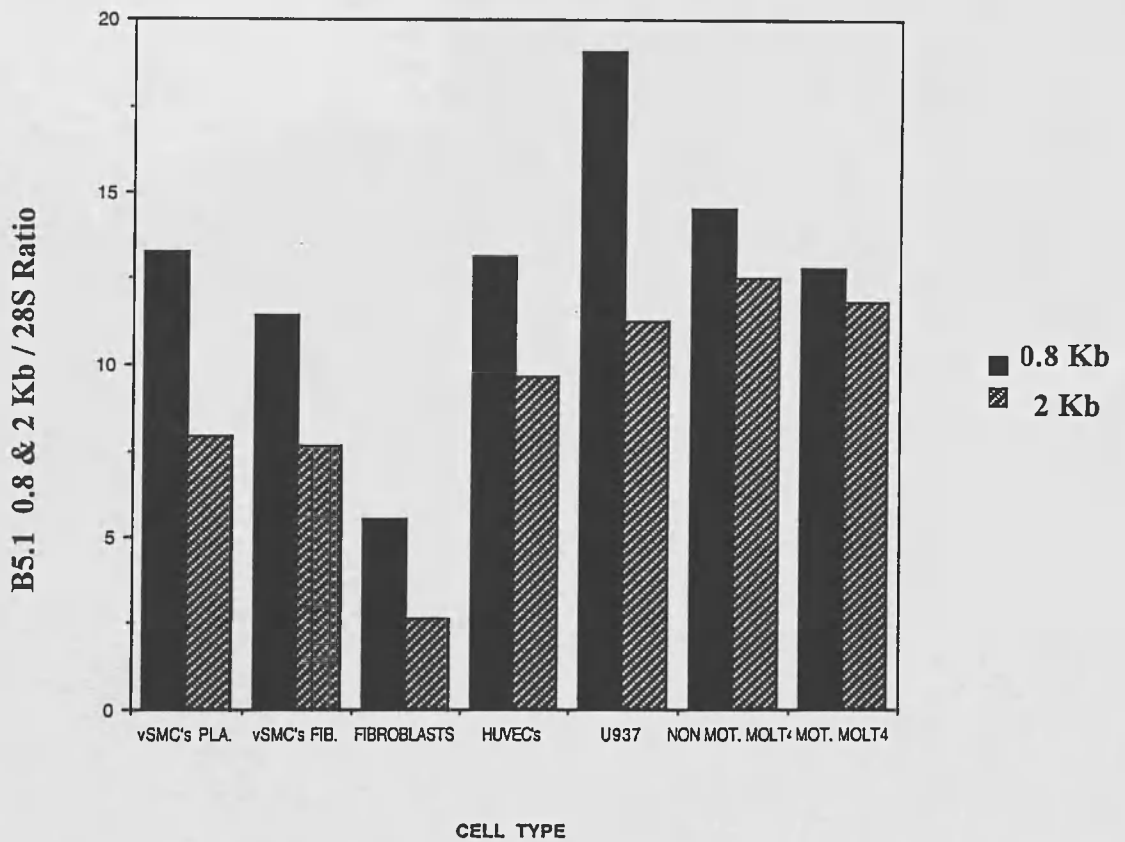
(FIG. IX.23.). Ratios of B5.1 mRNA transcript levels against 28S ribosomal RNA levels between RNA samples from various cell populations. From northern blot depicted in **(FIG.IX.19.)**.

(FIG.IX.24.).
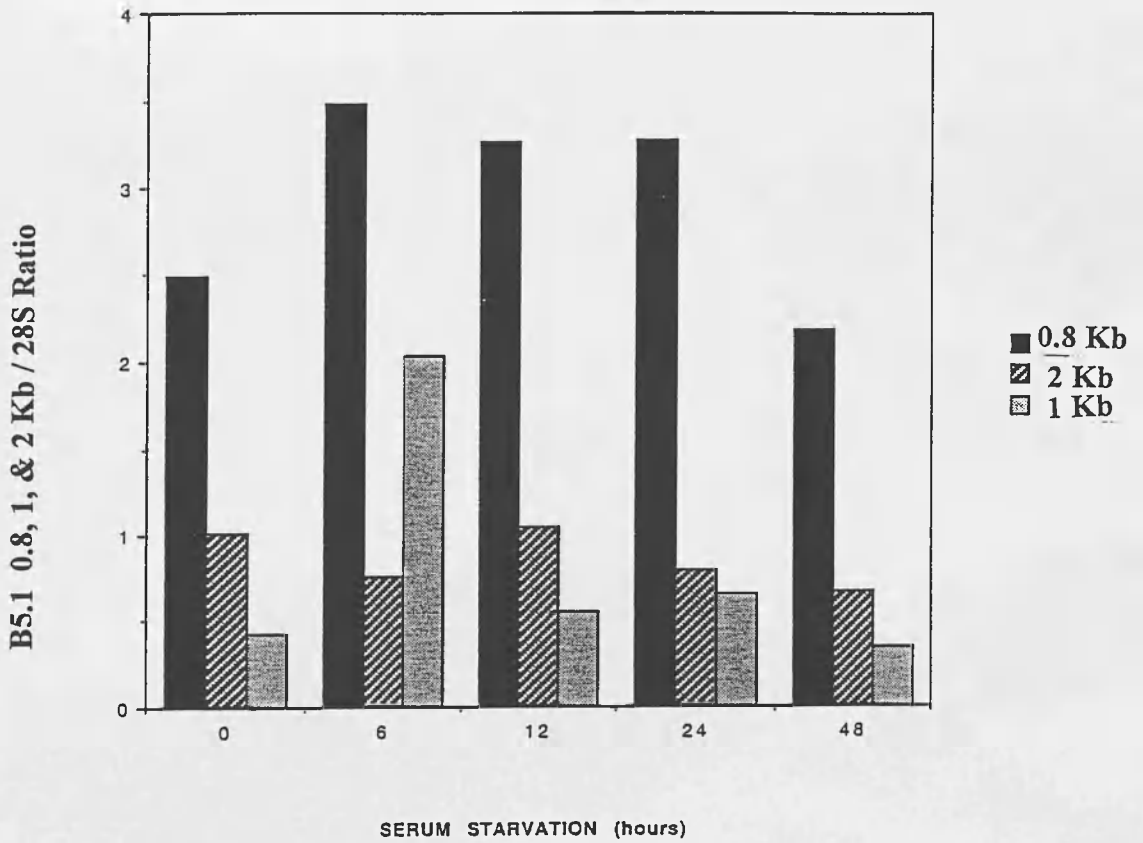
(FIG.IX.24.). Ratios of B5.1 mRNA levels against 28S ribosomal RNA levels between RNA

samples obtained from HUASMC's proliferating in the presence of serum and from

HUASMC's at increasing time points following serum starvation. From northern blot

depicted in **(FIG.IX.18.)**.

Chapter 3. - Results summary:

The mRNA differential display technique was employed as a strategy to identify genes differentially expressed by HUASMC's grown on fibronectin coated and uncoated plastic. Following initial differential display experiments several refinements and improvements to the technique were applied. These refinements included;

1. The use of DNAse treated RNA samples as templates for differential display reverse transcriptase PCR.

2. The use of ^{33}P dCTP as a label for differential display PCR.

3. The use of longer arbitrary and anchored primers for PCR.

4. The use of only three one base anchored oligo dT primers.

5. Each primer combination amplification reaction was carried out in triplicate.

6. Only differentially expressed bands greater than 150 base pairs were selected for further analysis.

7. The use of random primed labelled probes generated from differentially expressed bands to screen northerns.

From the 4 differential display gels run after modifications to the procedure had been implemented **(FIGs.IX.2a, IX.3a, IX.4a & IX.5a)**, incorporating 24 separate PCR primer combination amplification reactions. 27 differentially expressed bands were identified between the two populations. 22 of these differentially expressed bands were successfully reamplified, cloned and sequenced. Searches were carried out on the EMBL/Genbank gene database to identify any homology that exists between our sequences and known genes. Nine sequences were found to be homologous with known genes. The remaining thirteen sequences did not reveal any significant homology with known genes and so may therefore represent novel genes **(Table IX.3.)**.

Northern blots of RNA samples from HUASMCs cultured on plasma fibronectin and plastic substrates that had been probed with the random primed labelled differential display products; B3.6, B4.1, B5.1, B7.1, C1.1, C2.2, C3.1, C4.1, and C8.1, all revealed equal levels of mRNA between both SMC populations indicating that they represent false positives. (FIGs IX.16.-IX.14.). The possible reason for the persistence of false positive's and additional refinements to the differential display procedure that may eliminate this problem are proposed in the discussion; **Section X.**

No signals were obtained on northern blots when differential display fragments; A1.1, B2.1, B6.5, C5.1 and D2.2 were used as random primed labelled probes. The failure to observe any transcript with these probes may be due to low level expression of the homologous genes or a result of the small size of the probes used (200–400bp).

Immunoblot analysis on proteins extracted from HUASMC's cultured on plastic and plasma fibronectin using a monoclonal antibody specific for vinculin and meta-vinculin indicated that both vinculin but to a greater degree meta-vinculin proteins were present at higher levels in HUASMC's cultured on plasma fibronectin coated surfaces, thereby confirming the pattern of expression indicated by differential display product B6.5 (**FIG.IX.16.**).

Although the novel genes B4.1 and B5.1 identified by differential display proved to represent false positives in that they are equally expressed between HUASMC's cultured on plasma fibronectin and plastic, the fact that these two products produced highly expressed transcripts as shown by northern blotting indicates that they may still serve as markers for the highly proliferative synthetic phenotype exemplified by such HUASMC's in culture. To assess further the potential of these novel genes as useful phenotypic markers, their expression levels between various cell types associated with vascular tissue and expression in relation to HUASMC proliferation and quiescence was examined by northern blot (**FIGs. IX.17. - IX.20.**). Instant image analysis of relative B4.1 and B5.1 transcript levels indicated that transcripts from both

genes were well expressed in all cell types, although the smaller 1.3 Kb B4.1 transcript appeared to be greater in U937 and motile molt 4 cells compared to the other samples (**FIG.IX.21.**). The levels of the B5.1 transcripts appeared to be reduced in fibroblast samples when compared with the other cell types (**FIG.IX.23.**). The small 1.3Kb transcript identified with the B4.1 probe appears to be cell cycle regulated as the level of this transcript gradually increased as time following serum starvation increased (**FIG.IX.22.**). A transient 1Kb transcript identified with the B5.1 probe appears at 6 hours following serum starvation and then disappears by 12 hours following serum starvation (**FIG.IX.24**). All other transcripts identified with B4.1 and B5.1 probes do not appear to be regulated between proliferating HUASMC's and HUASMC's entering quiescence.

Section X DISCUSSION.

Knowledge accumulated over the last two decades has provided strong evidence implicating vascular SMC's as having an important role in the pathology of atherosclerosis and restenosis. It has been demonstrated that initial atherosclerotic or restenotic lesion development is accompanied by the induction of vascular SMC;

(a) Phenotypic modulation

(b) Migration

(C) Proliferation

These events are thought to be responsible for the formation of the neointima that contributes to the vessel occlusion associated with atherosclerosis and restenosis (Bornfeldt et al 1994). As discussed in section **IV.4** such alterations in vascular SMC behaviour are thought to be induced by the release of a variety of inflammatory mediators (Ross 1996). Previous studies also suggest that the interaction of SMC's with ECM may well be important in the regulation of SMC phenotypic modulation, proliferation, and migration (Hedin *et al* 1988; Yamamoto *et al* 1993).

The following section will discuss each of these three aspects of SMC behaviour in turn, emphasising the influence ECM has upon them. The results from research carried out on this area and presented within this thesis and their relevance to *in vivo* events associated with atherosclerosis and restenosis shall also be discussed.

X.1 Extracellular Matrix Induced Alteration in SMC Growth Morphology.

Vascular SMC's together with endothelial cells, are the principal cell types that constitute mammalian blood vessels. SMC's are responsible for maintaining vascular tone through their contractile activity and also participate in vascular growth and repair. During the normal development of the vascular system SMC's differentiate from a synthetic to a contractile phenotype. However, under pathological conditions such as atherosclerosis or restenosis, SMC's can de-differentiate and revert to the synthetic phenotype in a process widely termed phenotypic modulation.

Evidence from numerous studies suggests that phenotypic modulation of vascular SMC's also occurs spontaneously *in vitro* (Schwartz *et al* 1986; Campbell *et al* 1988). When cells are isolated from adult tissues they are initially in the contractile phenotype. Over a period of days they undergo conversion to the synthetic phenotype (Li *et al* 1994). A variety of factors have been identified which induce such phenotypic modulation including; native and oxidized LDL (Rahm *et al* 1992); extracellular matrix components, fibronectin (Hedin *et al* 1988) and type I collagen (Yamamoto *et al* 1993). Growing cells on fibronectin or collagen type I has been demonstrated to speed up the conversion into the synthetic phenotype whereas growth on laminin or the commercially prepared matrix substrate matrigel retards this conversion and maintains the cells in the contractile phenotype for much longer periods of time (Yamamoto *et al* 1993; Hedin *et al* 1988; Li *et al* 1994)). A number of inflammatory cytokines and growth factors present in culture medium are also thought to act synergistically in promoting the synthetic phenotype.

Thus the behaviour of SMC's in culture may be similar to that shown following the removal of or damage to the endothelium that occurs prior to atherosclerosis and restenosis. The absence of a functional endothelium *in vivo* as well as *in vitro* exposes SMC's to a

variety of serum derived growth factors and metabolites that can contribute to the induction of SMC phenotypic modulation.

Previous studies have demonstrated that as human SMC growth progresses through subculture the cells gradually lose many of their recently acquired synthetic characteristics. This progressive loss of synthetic phenotypic activity is associated with a morphological modulation characterised by a change from a substrate attached monolayer culture, to a nodular culture in which most of the cells are present within multicellular aggregations (nodules). After passaging, these vascular SMC's *in vitro* proliferate in a characteristic pattern, initially forming a pre-confluent monolayer that subsequently develops areas of multicellular foci that have been described as "hill and valley" structures (Ross 1986). These structures rapidly develop into morphologically distinct nodules that consist of quiescent SMC's embedded in a carbohydrate rich extracellular matrix (Diemer *et al* 1992). SMC's within these nodules have been described, contradictorily as being similar to both SMC's from atherosclerotic plaques (Diemer *et al* 1992) and differentiated quiescent SMC's representative of a contractile phenotype (Thomas-Salgar and Millis 1994). The stronger evidence favours the theory that SMC's within nodules exhibit more contractile phenotypic characteristics than their counterparts growing in a monolayer morphology. The results presented in this thesis provide further evidence supporting the suggestion that SMC's growing in a nodular morphology exhibit fewer synthetic phenotypic characteristics than SMC's within monolayer cultures.

Initial studies carried out for this thesis demonstrated that the formation of nodules during subculture can be inhibited by culturing HUASMC's on various ECM coated surfaces. HUASMC's cultured on ordinary tissue culture plastic, laminin coated plastic and in Matrigel form the characteristic hills and valley growth morphology that could be indicative of a gradual progression into the contractile phenotype (FIG's. VII.1, VII.7 & VII.9.). HUASMC's cultured on fibronectin and collagen I grow to form distinct monolayer cultures

where the cells appear much more elongated and orientate parallel to one another forming a monolayer when confluent, suggesting that their synthetic-like phenotypic characteristics are being maintained (**FIG's. VII.2-.4.**). HUASMC's cultured on collagen III, IV, and vitronectin also form monolayer-like cultures but not as distinct as those observed on fibronectin and collagen I substrates, as occasional areas of cellular aggregation can still be observed on these substrates (**FIG's. VII.5, VII.6 & VII.8**). This could indicate that SMC's grown on these substrates may develop a phenotype that is intermediate between the contractile-like one observed on plastic, laminin and matrigel with the synthetic-like phenotype conferred by growth on fibronectin and collagen I.

The demonstration that ECM substrates can influence the growth morphology of human SMC's cultured *in vitro* provides an indication that the phenotypic modulation of SMC's *in vitro* can be influenced by ECM. This observation contributes to the suggestion that the ECM environment may play a significant role in regulating SMC phenotypic modulation associated with human atherosclerosis and restenosis.

It has previously been indicated that synthesis of the secreted glycoprotein, clusterin, by cultured SMC's is associated with the process of nodule formation (Thomas-Salgar and Millis 1994). This raises the possibility that the effect of fibronectin on SMC cultures may be through the inhibition of clusterin secretion. This possibility was tested by treating fibronectin modulated SMC's with conditioned media from SMC's cultured on plastic, which presumably contains clusterin. Such treatment was unsuccessful in promoting nodule formation in fibronectin modulated SMC cultures. Also antibodies against clusterin did not inhibit either the rate or quantity of nodule formation in plastic cultured SMC's (results not shown).

X.2 Extracellular Matrix Induced Modulation of SMC Mitogenic and Chemotactic Responses.

Vascular SMC's play a major role in the formation of arterial intimal lesions in atherosclerosis and are a major component of the accumulated fibrocellular mass in the neointima associated with restenosis following balloon angioplasty (Ross 1986; McBride *et al* 1988). The control of intimal SMC proliferation and migration have been demonstrated to be critical determinants of vessel closure following vascular injury associated with coronary bypass surgery, PTCA and atherosclerosis (Vlasic *et al* 1993). Although some vascular SMC's reside in the tunica intima of normal human arteries that are susceptible to atherosclerosis, the majority of vascular SMC's reside in the tunica media where they are quiescent and express a variety of differentiation specific genes important for their function in the regulation of vessel tone and blood pressure (Pauly *et al* 1994).

The increased accumulation of vascular SMC's within intimal lesional areas is thought to arise as a consequence of SMC phenotypic modulation. SMC's that have changed to a synthetic phenotype demonstrate upregulated expression of a number of growth regulatory genes and cytokines. Furthermore synthetic SMC's can demonstrate an enhanced response in an autocrine manner to their own secreted products or paracrine manner to inflammatory stimuli of non-SMC origin via the upregulated expression of receptors such as PDGF or c-*fms*. Likewise synthetic SMC's may regulate their response to the altered ECM environment of the neointima *via* modified expression of cell surface integrins.

Such changes in SMC behaviour may be responsible for the increased responsiveness to various inflammatory stimuli that induces excessive SMC proliferation and migration thereby contributing to the development of the neointima associated with restenosis and atherosclerosis.

The role that various ECM substrates have on regulating the mitogenic and chemotactic behaviour of SMC's in response to a range of stimuli was investigated during this study. Several *in vitro* SMC proliferation and migration assays were set up to compare HUASMC proliferation and migration rates on various ECM coated substrates.

Hoechst DNA proliferation assays (**Section VI.4**) demonstrated that HUASMC's seeded on fibronectin, collagen I, collagen III, and collagen IV coated tissue culture plastic exhibit enhanced proliferation rates in response to serum when compared with HUASMC's cultured on uncoated tissue culture plastic or laminin coated plastic (**FIG's.VII.10-VII.12.**). Growth curves obtained from SMC cultures that were not supplemented with fresh medium suggest that SMC's cultured on fibronectin possess a reduced requirement for serum derived survival factors. SMC's grown on fibronectin in unsupplemented cultures reach considerably higher cell densities than cells on plastic. Also the lifespan of cells was extended by growth on fibronectin (**FIG.VII.11.**). These results indicate that the mitogenic response of vascular SMC's to serum derived factors can be altered by the type of ECM substrate on which the cells are cultured.

ECM may modulate the SMC mitogenic response in a number of ways. Cell surface integrin receptors can transduce signals that promote alterations in the cytoskeleton that can in turn directly influence cell adhesion and shape, properties that are tightly coupled with cell motility and proliferation. Integrins can also transduce signals co-operatively with growth factor receptors. It has been well documented that the majority of cells require anchorage as mediated by ECM-ligand interactions in order to allow the growth factor initiated progression into S phase of the cell cycle (Zhu *et al* 1996). Such co-operative signalling is therefore likely to result in the generation of a variety of intracellular signals that combine to influence the expression and function of the cell cycle regulatory elements thereby promoting cell proliferation.

The identification of the ECM derived signalling networks that regulate such mitogenic responses remain to be fully elucidated. However, recent insights implicating an association of integrin function with cyclin/CDK activity (Fang *et al* 1996; Meredith *et al* 1995), may help elucidate the mechanisms underlying extracellular control of mitogenesis and lead to novel therapeutic approaches to the control of SMC proliferation associated with atherosclerosis or restenosis.

ECM may contribute to the phenotypic modulation of SMC's. The subsequent transition into the synthetic phenotype is associated with increased cell proliferation. This upregulation in cell proliferation is likely to result from the combination of enhanced growth factor receptor expression CSF-1, PDGF, and EGF receptors (Pickering *et al* 1993; Rahm *et al* 1992; Inaba *et al* 1992), and increased expression of autocrine growth factors such as, PDGF and bFGF (Walker et al 1986;).

The ECM may also influence the cell response to certain growth factors, for example Heparan sulphate proteoglycans (HSPGs) can bind to bFGF protecting it from proteolytic degradation and providing a reservoir of active bFGF in the ECM. Also bFGF binding to its receptor is significantly enhanced when it is present as a complex with HSPG. It has been postulated that HSPG may increase bFGF receptor-ligand complex stability by conferring conformational alterations on the growth factor molecule (Nugent *et al* 1993). Similar interactions may take place between other growth factor and ECM molecules.

SMC migration on different matrix coated substrates was assayed by two methods. Both methods are modifications of the Boyden chamber and incorporate either Transwell inserts or ChemoTx 96 well chambers (**section VI.5**).

Migration assays performed on transwell inserts demonstrated that rates of SMC migration in response to a positive gradient of serum were significantly greater on plasma fibronectin than on uncoated polycarbonate filters (**FIG.VII.16.**). This result was further supported by time lapse video studies (results not shown) demonstrating increased SMC migration rates on

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fibronectin coated tissue culture plastic compared with uncoated plastic. Dose responses of positive migration towards gradients of PDGF BB and HGF were also observed through fibronectin coated transwell filters. No such responses were observed through uncoated filters (FIG's VII.17 & VII.18).

Migration assays performed using the ChemoTx migration chamber demonstrated that rates of migration towards serum were greatest on fibronectin and collagen III coated filters compared to migration on collagen IV, collagen I and laminin coated filters on which SMC's migration rates were about half fold less than those observed on fibronectin and collagen III (FIG.VII.19.). High levels of SMC migration in response to PDGF BB were observed through collagen III, collagen I and fibronectin coated filters. No migration in response to PDGF BB could be detected through laminin and collagen IV coated filters (FIG.VII.20.). HGF induced significant levels of migration through collagen I, fibronectin and laminin coated filters, whereas no migration towards HGF could be observed through collagen IV and collagen III (FIG.VII.21). Significant levels of SMC migration in response to bFGF could be observed on collagen III filters with relatively low levels of migration on fibronectin and collagen type I coated filters. Once again practically no migration was observed on laminin and collagen IV coated filters (FIG. VII.22).

Therefore it seems clear that SMC's on different ECM substrates produce differential migratory responses to chemokines. Results obtained from these SMC migration assays seem to indicate that the ECM substrates collagen IV and laminin do not effectively promote SMC migration to the various chemotactic stimuli investigated.

ECM may be able to modulate the motility of SMC's in a number of ways; as described in the previous section ECM-integrin interaction can contribute to SMC phenotypic modulation resulting in increased synthesis of autocrine chemotactic factors and expression of chemotactic factor receptors, also co-operative signalling between integrins and chemotactic factor receptors may take place contributing to SMC migration. Signalling

mediated via integrins has previously been described to influence cell migration directly. For a cell to migrate across a substratum under *in vitro* conditions they must first adhere to it with sufficient strength to allow subsequent spreading at the cell margin. One part of the cell margin must become specialized to extend outwards while the opposing side retracts and continued locomotion requires the spatial and temporal regulation of the development and breaking of cell-substratum contacts.

The most extensively studied and well characterised cell-substratum contacts are the focal adhesions (Lee *et al* 1993). Focal adhesions have been observed only under *in vitro* conditions. Thus their relevance to SMC behaviour *in vivo* is unclear. Focal adhesions do however, provide a model to explain the link between the ECM, cell surface integrins and the intracellular cytoskeleton. The type of ECM integrin interactions taking place between SMC's and the extracellular environment will govern the strength of cell anchorage and thus the propensity to migrate. Signalling events triggered by the clustering of integrin molecules at the sites of cell substratum contact are involved in cell motility. Integrin clustering can result in the phosphorylation of the molecular components within focal adhesions, this may lead to the induced levels of PIP_2 activity which in turn may lead to an increased rate of actin polymerization via the binding of PIP_2 to the actin binding proteins profilin and gelsolin (Wang *et al* 1993). Such integrin mediated alterations in cytoskeletal organisation that regulate actin filament dynamics can generate the necessary force required for cell motility. Also similar signalling through integrins can lead to the sequential development and breakdown of focal contacts between the cell surface and substratum, which is required to provide the necessary traction that enables the cell to migrate. Therefore ECM-integrin interactions have an essential involvement in the control of SMC migration.

The correlation of low migration and proliferation rates with "hills and Valley" SMC cultures, such as those on uncoated plastic or laminin coated substrates, provides further

evidence suggesting that SMC's within nodules exhibit less synthetic phenotypic

characteristics than SMC's growing in a monolayer morphology.

X.3 SMC Phenotypic Marker Analysis.

After establishing that morphological and functional differences do result from culturing SMC's on different ECM substrates, this project progressed to investigating whether such ECM substrates could induce alterations in SMC phenotype. Phenotypic characterisation of SMC's cultured on different ECM substrates was achieved by carrying out western blot analysis using antibodies that have previously been demonstrated to be or are possible candidates as SMC phenotypic markers. The expression of these markers was also examined in protein samples obtained from the same human umbilical artery tissue from which the cultured HUASMC's were isolated, as well as from human sapheneous vein and aorta tissue samples. These tissue samples provided a positive control for the contractile phenotypic markers.

The results of this marker analysis as summarised in **Table VIII.1.** indicates that all SMC populations in culture appear to be representative of the synthetic phenotype irrespective of the type of ECM on which they are cultured. Well established synthetic markers such as cytokeratin 8 and light chain caldesmon are well expressed in the cultured cells but absent in the normal tissue samples. Conversely, well known contractile markers such as desmin and SM-myosin are absent in the cultured cells but well expressed in tissue.

These results seem to indicate that the phenotypic status of cultured SMC's as determined by the markers used in this analysis, is not drastically altered by extracellular matrix, as only slight differences in the levels of meta-vinculin and the 210 Kd isoform of MLCK were observed. This result was perhaps not unexpected as many of the putative SMC differentiation markers identified to date have not previously been found to be re-expressed under culture conditions and the majority of the synthetic markers are characteristic for SMC's cultured under most conditions. Such observations provide support for the view that the contractile and synthetic SMC phenotypes do not represent a simple black and white

comparison but are rather a consequence of a continuum of phenotypic alterations resulting

in two distinct phenotypes (contractile and synthetic) at the opposing ends of this continuum.

So it is likely that the phenotypic markers analysed in this study are only exclusively

expressed at the extreme ends of the spectrum between contractile and synthetic SMC

phenotypes and do not pick up the more subtle alterations in phenotype such as those

possibly induced by ECM in culture. Indeed the failure to observe any clear differences in the

expression levels of the phenotypic marker proteins studied, indicate that under *in vitro*

conditions ECM does not have the power to completely shift the phenotype of HUASMC's

from a classic synthetic to a contractile phenotype or *vice versa*. It is probable, however, in

view of the alterations in growth morphology as well as proliferation and migration rates

conferred by ECM (section X.1 & .2), that ECM molecules may have the ability to shift the

phenotypic state of SMC's towards a slightly more contractile or synthetic phenotype.

The identification that fibronectin, collagen III and vitronectin can upregulate the

expression levels of the 210 Kd MLCK isoform is interesting with respect to SMC function

(FIG.VIII.16). MLCK is a Ca^{2+}/calmodulin dependent protein kinase that specifically

phosphorylates smooth muscle myosin regulatory light chain (Stull *et al* 1986). The

phosphorylation of the 20 Kd regulatory light chain of myosin by MLCK plays a key role in

the initiation of smooth muscle contraction. In smooth muscle, a rise in intracellular Ca^{2+}

levels results in the formation of regulatory calcium-calmodulin complexes that can interact

with the catalytic subunit of MLCK thereby activating the enzyme to phosphorylate serine at

position 19 on the regulatory light chain of myosin. Phosphorylation of serine 19 of myosin

light chain allows the myosin ATPase to be activated by actin and the muscle to contract

(Somlyo and Somlyo 1994). MLCK activity has also been demonstrated to play a role in the

modulation of skeletal muscle contraction, receptor capping, shape change and cell motility

as well as other functions (Sellers and Adlestein 1987). It therefore seems likely that MLCK

activity plays an important role in determining SMC behaviour that may be characteristic of

either a contractile or synthetic phenotype. However, the use of the 210 Kd MLCK isoform as a phenotypic marker is limited because it has yet to be fully characterised. Only when specific probes and antibodies are designed to the 210 Kd isoform can the value of 210 Kd MLCK as a SMC phenotypic marker be fully assessed *in vivo*.

The expression level of meta-vinculin was found to be upregulated in HUASMC cultures on plasma or cellular fibronectin (**FIG. VIII.21**). The potential use of meta-vinculin as a SMC phenotypic marker shall be further discussed in **section X.4**.

Another interesting result from this phenotypic analysis is the identification of a 30 Kd low molecular weight (non-muscle) isoform of tropomyosin as a potential marker for synthetic SMC's. This 30 kd isoform is well expressed in all cultured SMC's but absent in all three tissue preparations (**FIG. VIII.18 & VIII.19**). This is interesting because the low molecular weight 30 kd isoforms of tropomyosin (TM30pi and TM30nm) have not previously been proposed to be markers of synthetic SMC's. Western blot analysis using an anti-tropomyosin antibody was performed on protein samples obtained from intimal and medial layers from balloon injured and normal rat carotid tissue. A low molecular weight isoform of tropomyosin corresponding in size to the rat homologue (TM4) of the 30 kd human tropomyosins (TM30pi) was found to be well expressed in cultured rat cells and also in the intima of the ballooned tissue which contains synthetic SMC's, and less abundant in the vessel media which is composed predominantly of normal contractile SMC's. Also this isoform of tropomyosin was not found to be present at all in the normal rat carotid artery (**FIG. VIII.36**). This result provides strong evidence implicating a low molecular weight isoform of tropomyosin as a marker for the synthetic SMC phenotype.

The tropomyosins are a family of actin associated cytoskeletal proteins that are thought to play a structural and/or regulatory role in the organisation of microfilaments into bundles of stress fibres (Matsumura *et al* 1983). In skeletal and cardiac muscles, tropomyosin acts in concert with troponin, in regulating muscle contraction. The interaction of F-actin

with myosin and subsequent wave of muscle contraction is dependent on the conformation of the actin-bound troponin-tropomyosin complex, which is ultimately regulated by levels of intracellular calcium (Chalovich 1992). In smooth and non-muscle cells where the troponin complex is absent, the role of tropomyosin is less clear. A functional role for tropomyosin in non skeletal muscle tissues can however be inferred from its interaction with caldesmon, calponin and tropomodulin and also from studies implicating a possible role for tropomyosins in governing the stability of F-actin. (Matsumura and Matsumura 1985).

There appear to be two discrete classes of tropomyosins, a muscle type and a non-muscle type. Considerable interest in the function of non-muscle tropomyosins has been generated by observations that the abundance of non-muscle isoforms relative to muscle isoforms of tropomyosin increase upon cell transformation (Lin *et al* 1985). A common phenomenon associated with cell transformation is that at least one muscle tropomyosin isoform appears to decrease or disappear and in parallel the level of one non-muscle tropomyosin increases. This alteration in tropomyosin expression in transformed cells accompanies the reduction in the size and number of microfilament bundles and an increase in microfilaments associated as a meshwork structure. These observations have led to the postulation that a possible function of tropomyosins in non-skeletal muscle cells is in the regulation of actin stability.

The evidence suggesting that tropomyosin regulates actin stability originates from the observations that skeletal muscle tropomyosin binds strongly to F-actin, whereas the binding of non-muscle tropomyosin to actin is much weaker. This weaker binding of non-muscle tropomyosins can be attributed to the truncated length of these proteins. Although the overall structure of muscle and non-muscle tropomyosins are similar, non-muscle tropomyosins have a 37 amino acid residue deletion at the amino terminal end of the protein. As a result of this shorter length non-muscle tropomyosins bind only 6 actin monomers compared with the 7 monomers that muscle tropomyosin binds (Cote and Smillie 1981). Also in addition to the

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amino terminal deletion the sequence of the first 42 residues and last 27 residues of non-muscle tropomyosins differ from the corresponding skeletal muscle tropomyosin sequence (Cote and Smillie 1981). This variability contributes to the relatively poor head to tail polymerization of non-muscle tropomyosins when compared to their muscle specific counterparts. A reduction in head to tail polymerization is thought to have an effect on the co-operative binding potential of tropomyosin to actin and so also contributes to a decrease in binding affinity with F-actin.

Both muscle and non-muscle tropomyosins appear to decrease the binding of gelsolin to actin filaments and to diminish the ability of gelsolin-Ca^{2+} to fragment these filaments. Also tropomyosin binding to F-actin protects against the disassembly of F-actin by actin depolymerizing factor (ADF). It has been suggested that because non-muscle tropomyosins have lower binding affinity to F-actin they may be less effective at inhibiting gelsolin and ADF mediated destruction of actin filaments.

Therefore, the conversion to non-muscle tropomyosin during transformation may cause the transformed cell to rearrange the organisation of microfilaments, such as reducing the number and size of microfilament bundles (Lin et al 1984). The upregulated expression of non-muscle tropomyosins in transformed cells raises the question whether the expression of these isoforms is associated with proliferation and migration in other cell types. Indeed previous studies have revealed that antibodies directed against a non-muscle chicken tropomyosin isoform bound to stress fibres and membrane ruffles of migrating fibroblasts, whereas antibodies directed to muscle isoforms of tropomyosin stained only stress fibres.

The associated expression of non-muscle isoforms of tropomyosin with cell proliferation and migration, taken together with its exclusive expression in cultured SMC's and balloon injured rat carotid vessels, strongly suggest that it may be a potential candidate as a phenotypic marker characterising synthetic SMC's. Further work will be required to identify which particular tropomyosin isoform is modulated before subsequent *in situ*

hybridisation studies can be performed on diseased vessels that would confirm whether this tropomyosin isoform is a specific marker for synthetic SMC's

Immunocytochemical analysis performed on HUASMC populations cultured on either plasma fibronectin or uncoated tissue culture plastic, revealed no great differences in either the levels or distribution of the phenotype markers studied (**FIG's. VIII.37-58.**). The association of MLCK antibody staining with fibrils did appear to be slightly upregulated in HUASMC's cultured on fibronectin (**FIG. VIII.46b**). Also antibody staining to caldesmon localised to the cell nucleus was of greater intensity in HUASMC's cultured on plasma fibronectin (**FIG. VIII.56b.**). The slight upregulation of MLCK and caldesmon activity in SMC cultures on fibronectin could be linked with the increased motility of SMC's observed on this substrate.

Matrigel matrix is a soluble basement membrane extract of the Engelbreth-Holm-Swarm (EHS) tumour that gels at room temperature to form a matrix containing various basement membrane and ECM components (Kleinman *et al* 1986). The major components of Matrigel matrix are laminin, collagen IV, entactin, and heparan sulphate proteoglycan (Kleinman et al 1982; Bissel et al 1987). Growth factors, collagenases, plasminogen activators and other undefined components have also been reported in Matrigel matrix (Vukicevic et al 1992). Matrigel matrix has previously been used for attachment and to allow the differentiation of many normal and transformed cell type including, neurons, oligodendrocytes, hepatocytes, Sertoli cells, chick lens, melanoma cells, vascular endothelial cells, thyroid and hair follicle cells (Kleinman *et al* 1986; Hadley *et al* 1985; Kubota *et al* 1988).

It has previously been reported that the induction of the differentiated contractile phenotype can be achieved by culturing human aortic SMC's on growth factor-reduced Matrigel in serum free medium. This differentiation of SMC's was characterised by multilayered nodular morphology and a low proliferative index. Also immunocytochemical

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studies demonstrated expression of the cytoskeletal differentiation markers α smooth muscle actin, heavy chain myosin and caldesmon (Grushkin-Lerner and Flaherty 1995).

In this study both human aortic and HUASMC's were cultured on growth factor reduced Matrigel supplemented with serum free medium. Total protein samples were extracted from these cultures at sequential time points following cell seeding. Protein samples from these Matrigel cultures were separated by polyacrylamide gel electrophoresis together with protein from SMC's cultured on plasma fibronectin and protein isolated from human aorta and umbilical artery tissue. Immunoblotting studies were subsequently carried out on these samples using antibodies specific for known SMC phenotypic marker proteins (**FIG's. VIII.22-.35**). Immunoblotting was preferred to immunocytochemisty as a high degree of non-specific antibody binding to nodules was observed. Results from this immunoblotting analysis did not demonstrate a clear alteration in phenotype as a result of culturing either human aortic SMC's or HUASMC's on Matrigel. The expression of the classic contractile SMC markers, desmin and smooth muscle myosin heavy chain were not induced upon growth in Matrigel (**FIG's. VIII.26-VIII.29**). These results, therefore, do not correlate with the previous study by Grushkin-Lerner and Flaherty indicating SMC growth on Matrigel induces the transition into the contractile phenotype.

X.4 Differential Display Analysis of SMC's Cultured on Different ECM Coated Substrates.

It seems likely that the induction of SMC phenotypic modulation which precedes atherosclerosis and restenosis development results in the activation of a group of genes which promote SMC growth and migration and the inactivation of others that may be pro-differentiation. The phenotypic analysis of SMC's cultured on different ECM substrates using antibodies specific for known or candidate phenotypic marker proteins did not reveal any gross alteration in phenotype between SMC populations grown on each substrate, indicating that any alteration in phenotype induced by ECM *in vitro* is subtle. The aim of results *chapter 3.(section IX)* was to identify such subtle changes in SMC gene expression that are induced by ECM adhesion, and also subsequently discover whether they are associated with the SMC phenotypic modulation taking place *in vivo* that is involved with the development of atherosclerosis and restenosis.

The mRNA differential display technique was employed as a strategy to allow the phenotypic characterisation at the molecular level of SMC populations cultured on different ECM substrates. Differential display is a recently developed PCR based technique that was conceived to allow the identification and molecular cloning of differentially expressed genes between related cell populations. Compared with standard methods for the identification of differential gene expression such as subtractive and differential hybridization, differential display has the advantages in that it does not need polyA+ RNA and requires only small amounts of total RNA in each condition for screening, initial results can be obtained very rapidly, it is easy to compare gene expression in multiple conditions on a single gel with side by side analysis, and sensitivity is high since the differences are amplified by PCR.

This method should therefore prove effective for identifying potential subtle changes in SMC gene expression induced by ECM adhesion.

Differential display analysis was applied to HUASMC's cultured on plasma fibronectin coated plastic compared with SMC's grown on uncoated plastic. These two SMC populations were chosen for comparison not only because of the clear morphological and functional differences that exist between these two populations but also because large quantities of RNA could be obtained from cells cultured on these substrates which is important for running northern blots that are required to confirm differential display results.

It soon became apparent that one of the major pitfalls of differential display is the high incidence of false positives that can be obtained. Several modifications to the original differential display procedure, the results of which are represented in (FIG's.IX.1a-d.) were applied in an attempt to reduce the number of false positives. The most important of these modifications is probably the use of good quality uncontaminated RNA samples for RT-PCR template. Subsequent differential display experiments used RNA samples that are DNase treated. This prevents the amplification of any contaminating genomic DNA within RNA samples which may result in the misleading appearance of a differentially expressed band. The amplification of genomic DNA most probably took place during the first attempt on differential display as indicated by the amplification of products rich in alu repetitive sequences (Table IX.1).

Also from early differential display gels it was observed that certain primer combinations resulted in the amplification of a large number of PCR artefacts that also could be mistaken for differentially expressed genes. The differential display gel represented in (FIG's.IX.2a-d) contains samples that were run in duplicates. In certain supposedly "identical reactions" clear differences can be observed (Table IX.2). To reduce the possibility of selecting such artefacts it was decided to carry out each PCR primer combination amplification reaction in triplicate. Differential display gels represented in (FIG's.IX.3-IX.5)

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are examples of these later differential display experiments. The decision was made to select only those differences represented in at least two out of the three corresponding pairs of PCR reactions. Also only those differentially expressed bands greater in size than 150bp were selected for further analysis, this was to allow for a greater rate of success during subsequent processing of the DNA such as re-amplification, cloning and probe-hybridisation to northern blots.

A summary of the results from the modified differential display experiments are shown in **(Table IX.3)**. From four differential display gels incorporating twenty four separate PCR primer combination amplification reactions, twenty seven differentially expressed bands were identified between the two populations.

Twenty two of these differentially expressed bands were successfully cloned and sequenced. Searches were carried out on the Genbank/EMBL gene database to identify any homology that exists between these sequences and known genes. Nine sequences were found to be homologous with known genes; the remaining thirteen did not reveal any significant homology and may represent novel genes.

The known genes identified included;

14 Iq JH:- This is a rearranged immunoglobulin switch mu fragment that is present in a number of genes. This sequence is thought to act as a translocation site for a variety of genes including the bcl2 and bcl3 protooncogenes. Such translocation of bcl2 and bcl3 has been linked with the onset of uncontrolled cell proliferation and is often found associated with human lymphoma development (Crossen *et al* 1993; Siete *et al* 1993). Differential display indicated that 14 Iq JH was upregulated in fibronectin modulated SMC's, but whether the gene identified plays a similar role in controlling cell proliferation to those genes previously described cannot be determined until the gene that carries this sequence has been further characterised. However, it is interesting to speculate that gene translocation maybe

responsible for initiation of an altered cell phenotype such as that observed in fibronectin modulated SMC's.

Canine SRP 72:- The Signal Recognition Particle (SRP) is a cytoplasmic ribonucleoprotein particle consisting of six polypeptide chains, it binds to the signal sequence on nascent polypeptides when they emerge from a translating ribosome. The SRP is responsible for targeting proteins to the endoplasmic reticulum. The SRP 72 polypeptide has been proposed to function specifically in targeting, but possibly participates also in the elongation arrest function of SRP (Lutke *et al* 1993). It is not clear what role upregulation of SRP 72 expression in plastic cultured SMC's could have on SMC phenotypic modulation, although it could be proposed that regulation of SRP 72 expression may ultimately control protein import into the endoplasmic reticulum. Alterations in protein import could have significant consequences regarding cell physiology which may be manifested as modulation of cell phenotype.

Human Ionising Radiation Resistance Protein:- This gene was indicated to be upregulated in fibronectin modulated SMC's. No information regarding the biological function of this gene has been published, therefore the significance of differential expression of this gene in relation to SMC phenotypic modulation cannot be assessed at the present time.

Iduronate-2-sulphatase:- This gene encodes a lysosomal sulphatase enzyme. Deficiency of this enzyme results in storage of the glycosaminoglycans heparan sulphate and dermatan sulfate, which leads to the lysosomal storage disorder (mucopolysaccharidosis) also known as Hunter's syndrome. Degradation of glycosaminoglycans such as heparan and dermatan sulphate provides a mechanism for the release of growth factors sequestered by such molecules. The activity of enzymes involved in this matrix degradation are therefore often required to support cell proliferation. The potentially decreased expression of this

particular enzyme in the plastic cultured SMC's may provide a possible explanation for the low proliferation rates of these cells.

P54:- This gene encodes a nuclear RNA binding protein that is highly homologous to the recently identified human splicing factor PSF (Dong *et al* 1993). Biochemical complementation, antibody inhibition, and immunodepletion experiments demonstrate that PSF is an essential pre-mRNA splicing factor required early in spliceosome formation (Patton *et al* 1993). P54 was initially identified as a gene upregulated in fibronectin modulated SMC's. It is possible that induction of spliceosome formation could result in alternative splicing of specific genes involved in modulating the phenotypic properties of SMC's cultured on fibronectin.

Pentaxin 3:- The pentaxin 3 gene encodes an acute phase protein whose plasma level is found to be considerably elevated during inflammatory responses (Alles *et al* 1994). Another member of the pentaxin family, serum amyloid A, also an acute phase protein, was found to be upregulated within atherosclerotic lesions (Meek *et al* 1993). Although the biological function of Pentaxin 3 remains to be elucidated it may be a promising marker for SMC phenotype. However, the potential upregulation of pentaxin 3 gene expression in our more quiescent plastic cultured SMC's as indicated by differential display conflicts with studies indicating that it is a marker for inflammatory disease.

Vinculin / Meta-Vinculin:- These are intracellular cytoskeletal proteins found in both smooth and non-muscle tissues. They have been shown to be localized at the termini of actin bundles often associated within focal adhesions (Feramisco *et al* 1982). It is likely that vinculin and meta-vinculin within focal adhesions are involved in transducing intracellular signals induced as a result of SMC adhesion to fibronectin. Differential display indicated that vinculin or meta-vinculin gene expression was upregulated in fibronectin modulated SMC cultures, the upregulation of either gene may therefore provide a mechanism by which fibronectin can induce phenotypic modulation of SMC's, presumably as a result of as yet

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unknown integrin derived signal transduction events. Vinculin and meta-vinculin have previously been demonstrated to be markers of SMC phenotype. Immunostaining studies suggest that the ratio of meta-vinculin : vinculin decreases during the development of atherosclerotic lesions. Meta-vinculin is an alternatively spliced form of vinculin, so it remains to be determined whether the gene upregulated or alternative splicing pattern observed in fibronectin modulated SMC's represents vinculin or meta-vinculin (Meyer *et al* 1994).

Phosphotyrosyl protein phosphatase:- The PTPase, red cell acid phosphatase / adipocyte acid phosphatase was potentially found to be upregulated in plastic cultured SMC's. The precise function of these specific PTPases has yet to be published although all PTPases identified to date share the ability to regulate tyrosine phosphorylation. Studies have previously shown that PTPases can inhibit both PDGF receptor and EGF receptor signal transduction as a result of dephosphorylation of these receptors (Way & Mooney 1994; Gunaratne *et al* 1994). The inhibition of growth factor receptor activity can promote cell differentiation (Pani *et al* 1995). The activity of PTPase's in plastic cultured SMC populations could therefore promote a more differentiated phenotype.

Rat cytochrome p450 hydroxylase/aldosterone synthase:- This enzyme belongs to a family of enzymes that catalyses the detoxification of potentially harmful metabolites, its major site of production is within the liver (Alberts *et al* 1989). The expression of this gene was suggested by differential display to be upregulated in fibronectin modulated SMC's. The significance of this in terms of SMC phenotypic modulation is unknown.

The thirteen novel genes identified should not be discounted from further analysis as they may also have the potential to play an important, but as yet unidentified role in SMC phenotypic modulation.

Because of the high number of false positives it is vital that differential expression of all genes identified by differential display is confirmed before studying them further and this was done by screening northern blots.

Another very important consideration is that differentially expressed bands cut out of the differential display gel may contain additional undetectable overlapping, or unresolved cDNA products. Such a scenario may result in reamplification and sequencing of cDNA that may not necessarily represent the differentially expressed product observed on the gel. To check for this possibility random primed labelled probes were generated using reamplified DNA from differentially expressed bands cut out of the differential display gel. This method should allow labelling of all products contained within a band. These probes were then used to screen northern blots containing the relevant plastic and fibronectin modulated SMC mRNA samples. Even after taking these precautions all the differential display products that were successfully labelled and produced a transcript have all failed to reveal any differential expression on a northern indicating that they represent false positives **(Fig's IX.6-IX.14)**. The persistence of false positives maybe due to the fact that the phenotypic differences between fibronectin modulated SMC's with SMC's cultured on plastic are not that great and those differences observed on the display gel were exaggerated and therefore misleading. Therefore the problem of preferentially selecting PCR artefacts has not been eliminated. Alternatively differentially expressed genes originally identified may be unique to the RNA samples used for the differential display amplification and do not represent true fibronectin modulated genes that would also be identified in equivalent RNA samples used for northerns.

However, one encouraging differential display product is B6.5 which was upregulated in fibronectin modulated SMC's and was found to be homologous to either vinculin or meta-vinculin. Vinculin is a 130 Kd cytoskeletal protein that has been shown to be localised at the termini of actin bundles often associated within focal adhesions. Meta-vinculin is an alternatively spliced form of vinculin that contains an additional exon inserted at amino acid position 915 and meta-vinculin encodes a 150 Kd protein. Because the differential display product B6.5 is homologous to the common 3' end of both genes it could not be determined whether it is vinculin or meta-vinculin that is apparently upregulated in fibronectin modulated

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SMC's. Antibodies are however, available that detect both vinculin and meta-vinculin, which allowed a western blot to be performed on protein samples extracted from the two SMC populations **(FIG.IX.16.)**. The intensity of the protein bands observed on the western blot indicate that probably both vinculin but to a greater degree the meta-vinculin proteins are upregulated in fibronectin modulated SMC's, confirming the pattern of expression indicated by differential display.

Unfortunately previous studies carried out by other laboratories investigating the usefulness of meta-vinculin expression as a marker for SMC phenotypic modulation proposed it as a marker for contractile SMC's. This somewhat contradicts the theory suggested in this thesis that its upregulation in fibronectin modulated SMC's is associated with the onset of synthetic phenotypic characteristics. These previous studies, however, used *in situ* hybridisation studies to examine the level of meta-vinculin protein in the intima versus the media of atherosclerotic vessels, and they observed very small quantitative differences between these areas (Meyer *et al* 1994). The results obtained from these studies suggest that the meta-vinculin protein is not a distinctive marker of SMC phenotype. It is possible however, that in culture ECM molecules such as fibronectin may upregulate meta-vinculin expression, but under *in vivo* conditions other additional factors govern the expression of meta-vinculin. Although the upregulation of meta-vinculin by fibronectin is interesting, its non-exclusive expression in either a synthetic or a contractile SMC phenotype limits its use as a phenotypic marker for studying atherosclerosis or restenosis.

Thus differential display analysis has not identified any differences in gene expression induced upon SMC adhesion to fibronectin that could prove useful in characterising the phenotypic shift towards a synthetic phenotype. This failure to identify clear differences in gene expression between fibronectin and plastic modulated SMC cultures has prevented the analysis of ECM modulated genes by *in situ* hybridisation on sections obtained from atherosclerotic and restenotic lesions. Such analysis would have allowed the evaluation of *in*

vitro models by determining whether any of our *in vitro* differentially expressed genes are also differentially expressed *in vivo* between SMC's localised in normal vascular tissue with SMC's from atherosclerotic and restenotic lesions.

One further simple optimisation step that could be applied to the differential display procedure is the incorporation of a time course of transition from one population into the other under study. For example differential display could be performed on RNA samples obtained at sequential time points following drug treatment or during cell transformation. The analysis of such a time course on a differential display gel would allow the observation of either a stepwise increase or decrease in the level of a product as the time course progresses. The chances that such a stepwise increase or decrease represents an artefact or false positive seems very slim. So the use of such a time course would have a significant effect at reducing the number of false positives obtained with the differential display procedure. Caution will still have to be taken when performing differential display on a time course of RNA samples, for example if the modulation of SMC phenotype was investigated by comparing cells on plastic with a time course of adhesion to fibronectin it is likely that at the later time points differences resulting from cell confluency rather than fibronectin modulation could be identified. Therefore the choice of samples for analysis is important.

The identification of novel genes by differential display provides a useful additional application of the technique. Although all of the novel genes analysed so far have proved to represent false positives and are constitutively expressed between SMC's cultured on plastic and fibronectin. The fact that some of these genes have produced highly expressed transcripts on northern blots indicates that they may still serve as markers for the highly proliferative synthetic phenotype exemplified by such SMC's in culture. Therefore two of the novel genes (B4.1 and B5.1) identified by differential display performed on plastic compared to fibronectin cultured SMC's were characterised further.

Ideally any really useful SMC phenotype marker would be SMC specific, therefore the expression patterns of these genes between various cell types associated with vascular tissue (lymphocytes, monocytes, fibroblasts, endothelial cells, and smooth muscle cells) were examined (FIG's.IX.17 & IX.18.).

The two novel differential display fragments B4.1 and B5.1 both gave very similar transcript patterns when they were used to probe northerns; two clear bands of around 2Kb and Ikb were observed.

Instant imager analysis allowed the accurate assessment of the level of these transcripts between each RNA sample measured in cpm. By reprobing identical northerns with a 28S rRNA probe values in cpm were also obtained for the 28S rRNA gene transcripts. This allowed the calculation of the ratio of the novel transcript levels to 28S rRNA level for each sample (FIG's. IX.21-.24.). The levels of B5.1 transcripts did not differ between mRNA samples from SMC's cultured on plastic or fibronectin, HUVECS, U937 cells and non-motile and motile Molt4 cells, but there was significantly less in the MRC-5 fibroblasts. Levels of the smaller transcript of B4.1 was increased in U937 cells and motile Molt4 compared with the other samples. So this could be interesting in relation to motility. The expression of these genes in relation to cell cycle and proliferation was also investigated by examining their levels in SMC cultures gradually entering quiescence as a result of serum starvation. These results demonstrate that the small 1.3kb B4.1 transcript, is cell cycle regulated as the level of this transcript gradually increased with time following serum starvation (FIG.IX.18.). A transient 1Kb transcript identified with the B5.1 probe appears at 6 hours following serum starvation and then disappears by 12 hours following serum starvation (FIG.IX.20.). It can be speculated that this transcript could express some cell cycle inhibition molecule such as a specific cyclin dependent kinase inhibitor or may even represent a stress protein. But the fact that differences in the expression levels of these novel gene transcripts are quite small and are not SMC specific indicates that these novel genes

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would not be useful SMC markers and accordingly no further work was carried out on them. This approach could, however, lead to the identification of novel genes that may well represent useful markers of SMC phenotype.

Although the identification of differentially expressed genes between fibronectin and plastic modulated SMC cultures was hampered by the limitations of the differential display procedure, the inclusion of the optimisation steps described in this thesis together with incorporation of a time course between comparative RNA populations under study would significantly improve the technique providing a relatively useful tool for the detection and characterization of altered gene expression in eukaryotic cells. Several problems remain to be addressed, not least, that the downstream characterisation of differentially expressed genes is labour intensive. Short cuts for the confirmation and cloning of full length products would be required if more than a few RNA species were to be further investigated. The question of how efficiently rare mRNA species are displayed relative to abundant ones remains to be fully addressed (Liang *et al* 1993). Finally the problems of false positives must be eliminated in order to reduce the labour of further screening.

It could be indicated from the results presented in this thesis that quite dramatic alterations in SMC growth morphology and behaviour as induced by ECM may arise through quantitative rather than stark qualitative changes in cell phenotype. This suggestion raises the question of whether the phenotypic alterations of SMC's observed *in vitro* in response to alternative ECM substrate adhesion arise through mechanisms operating at a non-genomic level.

Numerous studies have characterised cell transformation or alterations in cell phenotype that are brought about by mechanisms that do not involve alterations in gene expression. Cellular transformation by many oncogenic viruses is mediated by alterations in signal transduction pathways that control normal growth and proliferation. Common targets for many transforming viruses are pathways regulated by protein phosphorylation. Thus the

alteration of phosphatase activity and subsequent changes in phosphorylation levels is an important step in transformation by these viruses (Mumby and Walter 1991). Morphological transformation of cells by the v-Src tyrosine kinase is incompletely understood. However, it is independent of nuclear functions and probably involves phosphorylation of targets associated with the cytoskeleton and focal adhesions leading to alterations in cell shape and attachment that can be transduced into changes in cell proliferation and migration (Fincham *et al* 1995).

Both protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) have evolved to a level of structural diversity that allows them to regulate many cellular processes including proliferation, migration, and differentiation. The levels of tyrosine phosphorylation required for each of these processes are achieved through the co-ordinated action of PTKs and PTPs. An imbalance between these enzymes may impair normal cell behaviour, leading to alterations in cell phenotype or transformation (Sun and Tonks 1994).

For example the treatment of cells with mitogens or the PKC activator 12-O-tetradecanoyl-phorbal-13-acetate (TPA) can lead to the activation of the kinase Raf-1. Activated Raf-1 triggers a protein kinase cascade by direct phosphorylation of mitogen activated protein (MAP) kinase kinase, resulting in phosphorylation of ternary complex factor and *Jun* by MAP kinase. Such co-operation between Raf-1 and PKC α has been implicated in the transformation of NIH3T3 cells (Kolch *et al* 1993). Also Pharmacologic differentiation of the promyelocytic leukemia HL60 is associated with an increase in cellular tyrosine phosphatase activity. Modulation of CD45 tyrosine phosphatase activity may play a role in differentiation or in maintaining HL60 cells in a non proliferative state (Buzzi *et al* 1992).

In mitotic cells abnormal activation of the protein kinase network at multiple points can contribute to oncogenic transformation (Pelech 1995). It can be envisaged that alterations in the activation of protein kinases associated with cell cycle control such as the

cyclin dependent kinases (CDK) could yield significant consequences upon cell proliferation and phenotype. Similarly changes in the phosphorylation state of kinases associated with cell anchorage and cytoskeletal elements could result in alterations in the regulation of cell motility and phenotype.

Therefore the induction of phenotypic changes in SMC's induced by ECM adhesion may arise from alterations in the balance between phosphatase and kinase activity of various signalling pathways rather than from alterations in gene expression. This proposal would explain the lack of variation in the levels of protein markers between SMC populations cultured on different ECM substrates **(section VIII)** and the failure to identify large numbers of differentially expressed genes between fibronectin and plastic modulated SMC cultures by differential display analysis **(section IX)**. More success in elucidating the role ECM has on SMC phenotype may be achieved by investigating the modification of candidate tyrosine phosphatase and kinase activity as markers of ECM induced modulation of SMC phenotype.

It seems more likely that the differences observed between cultured SMC's and tissue samples are significant and involve differential gene expression by SMC's. Phenotypic analysis of protein samples isolated from vascular tissue and SMC's in culture indicated that SMC's in culture are clearly representative of the synthetic phenotype while SMC's within the tissue samples are clearly in the contractile phenotype **(section VIII)**. Therefore the comparison of SMC's isolated *in vitro* with SMC's present within normal tissue samples *in vivo* may provide a model for the identification of differences in gene expression that are associated with the phenotypic modulation of SMC's from a contractile to a synthetic phenotype taking place in early atherosclerotic and restenotic lesion development. However, the comparison of tissue samples with cultured SMC's is technically difficult as a consequence of the mixed cell population present within tissue samples. Maybe through advances in molecular technology such as further optimisation of the differential display procedure or other techniques for analyzing differential gene expression taken together with a

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cautious approach, the comparison of SMC gene expression patterns between *in vitro* and *in vivo* conditions could identify potential candidate genes for SMC phenotypic markers.

Section XI CONCLUSIONS

In conclusion, adhesion to ECM substrates *in vitro* appears to modulate human vascular SMC phenotype, resulting in distinct differences in growth morphology and in cellular response to mitogenic and chemotactic signals. Plasma fibronectin, cellular fibronectin, collagen types I, III, IV and vitronectin seemingly promote synthetic-like phenotypic behaviour, whereas SMC growth on uncoated tissue culture plastic, Matrigel, and possibly also laminin suppresses these synthetic-like characteristics. Phenotypic analysis of SMC populations cultured on different ECM substrates using antibodies specific for SMC phenotypic marker proteins indicated that ECM did not induce a distinct switch from a contractile to a synthetic phenotype. These results suggest that ECM may induce subtle alterations in phenotype slightly shifting the phenotype of SMC's towards either a more synthetic-like or contractile-like phenotype.

The differential display procedure has proven to be technically difficult to perform efficiently and may only be worth attempting on two populations where a time course for the transition of one population into the other can be incorporated into the procedure. Optimisation of the differential display technique as performed for this thesis has, however, provided significant improvements to the procedure.

Differences in the protein levels of meta-vinculin and the large isoform (210 Kd) of MLCK were confirmed to be regulated apon matrix adhesion, however, previous studies do not demonstrate that meta-vinculin is a suitable marker of SMC phenotype. It is not possible to examine the expression of the large MLCK isoform between SMC phenotypes *in vivo* as a probe or antibody specific for the 210 Kd isoform has not yet been developed.

One possible novel SMC phenotype marker identified during the course of these studies is a 30 Kd low molecular weight isoform of tropomyosin. This protein is present in cultured human and rat SMC's, but appears to be totally absent in tissue samples obtained from normal human and rat vessels but is present in balloon injured rat intimal tissue. These

observations provide strong evidence that this tropomyosin isoform is expressed exclusively

in synthetic SMC's.

Acknowledgments

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