MORPHOGENESIS OF HUMAN MAMMARY EPITHELIAL CELLS *IN VITRO*.

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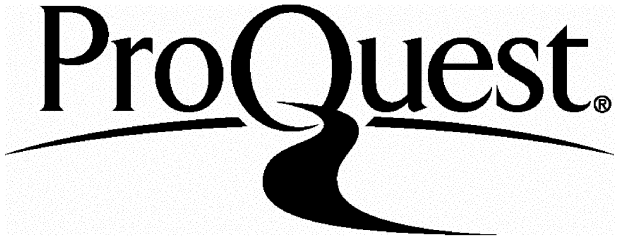

For my Grandfather,

Alexander Gibson

STATEMENT

All of the experimental work, results and ideas presented in this thesis, except where accordingly acknowledged, are entirely my own.

None of the work contained within this thesis has been submitted previously, either by myself or by any known persons, for examination within the University of London or any other awarding body.

ABSTRACT

MORPHOGENESIS OF HUMAN MAMMARY EPITHELIAL CELLS *IN VITRO*.

The human mammary gland *in vivo* is made up of branching epithelial ducts embedded in a supporting stroma. One of the earliest changes seen in the development of breast cancers is the disruption of normal tissue architecture, thereby leading to the loss of the organisation of the branching ducts. The objective of this study was to set up an *in vitro* system whereby the molecules involved in branching morphogenesis of a non tumorigenic human mammary epithelial cell line could be analysed. These molecules would be potential targets for alteration in tumorigenesis, and also have a potential role in mammary gland development *in vivo*. This thesis describes the *in vitro* system used, analysis of the roles of fibroblast secreted factors and integrins in the branching morphogenesis process, and the effect of overexpression of oncogenes on the ability of cells to form organised morphogenetic structures.

The *in vitro* system involves culturing a human mammary epithelial cell line (HB2) in 3-dimensions within a collagen type I (or fibrin) matrix. The cells grow to form compact, organised, spherical cysts, or can be induced to perform branching morphogenesis when cultured with conditioned medium from fibroblasts. We have found that there are at least two distinct soluble factors that can induce branching morphogenesis; hepatocyte growth factor (HGF), and an unidentified and potentially novel factor secreted by human foreskin fibroblasts (HFF). Preliminary characterisation of this factor has shown that it is a heat stable protein with a size of between 35 and 45 kDa.

In the analysis of the role of integrins in branching morphogenesis, we have analysed both the expression of integrins at the RNA level throughout the morphogenetic time course, and the effect of specific monoclonal antibodies (mAbs) to integrin subunits. Use of specific integrin mAbs that inhibit cell-matrix interaction, has shown that integrin mediated maintenance of cell-matrix interaction is vital both for the growth of HB2 cells and for maintaining cell-cell interactions in the branching structures. In both cases the integrins involved were found to differ depending on the matrix in

which the branching morphogenesis occurred, with the $\alpha_2\beta_1$ important in the collagen system and the $\alpha_v\beta_1$ in fibrin.

Both HGF and HFF conditioned medium are shown to stimulate the motility of HB2 cells on collagen, and it is hypothesised that the stimulation of cell motility is a necessary event in the branching morphogenesis process, and that the $\alpha_2\beta_1$ integrin has a specific role in branching morphogenesis by way of regulating this motility. Evidence for this is provided by showing that mAbs to the α_2 or β_1 integrin subunits that reduce HGF induced motility also inhibit branching morphogenesis induced by HGF.

Overexpression of the *v-Ha-ras* oncogene in HB2 cells also led to the disruption of cell-cell interactions in 3-dimensional morphogenetic structures. Overexpression of ras induced a reduced level of expression of the α_2 integrin subunit and a concurrent reduction in adhesion of the cells to collagen type I. Further evidence that the $\alpha_2\beta_1$ integrin-collagen interaction is involved in regulating cell-cell interaction in 3-dimensional structures was provided by showing that the HB2 ras colony phenotype could be significantly reverted with a mAb to the β_1 integrin subunit which increases the integrin mediated adhesion of cells to collagen.

ACKNOWLEDGEMENTS

The task of writing my thesis almost over, now comes the almost as challenging one of remembering to thank all the people that I should. My apologies in advance to anyone that I inadvertently miss!

I have been truly lucky, over the past four and a half years, to have been working in a lab with such a fantastic bunch of people, both past and present. I thank them all for making my time such a happy one, but in particular I must single out Joy, Caroline, Ros, Mike, Aurelia, Lucy and Brendan, without who's friendship and support the last few years would not have been what they were. I also extend huge thanks to my supervisor Joyce Taylor-Papadimitriou for all her help and support.

I have been fortunate, during my time at the ICRF, to reap the benefits of a huge amount of technical and scientific support, for which I must acknowledge in particular, Maureen Harrison and everyone in cell production; Chris Gilbert and Debbie Lyon for all their time and help with the time-lapse video-microscopy; Graham Clark in the equipment park; Derek, Clare and everyone else in the FACS lab, and last but certainly not least, the whole Photography department, who have spent countless hours on processing my films.

My thanks also to Fiona Watt for helpful discussion and guidance, as well as being a source for an abundance of antibodies; to all my other antibody sources; to Aurelia for help with the confocal microscopy; and to Feodor Berdichevsky for initiating the project with which I became involved, and to Dan Baeckstrom for continuing it.

As much fun as my time in the lab was, I must also pay tribute to people and friends outside the lab, without whose company and willingness to take me for beers, I would certainly not have preserved my sanity! My thanks and love then to Caroline, Neil, Ali, James, Peter, David and Ken - to name but a few! - and of course all my family.

It would be surprising, and I suppose unheard of, for someone to spend a period of four years in their life without some unhappiness, and while acknowledging the friendships gained and cemented during this time, I must pay my loving respects to Mark - a friend I tragically lost on the 24th April 1995, to a sudden and fatal asthma attack at the tender age of 30. I miss him.

I think that there can be no easy way to finish thanking people once you start; but I will bring this to a close by thanking the most important person last:- Thank you Charlie, you were, and still are, truly wonderful.

24 April 1997

One shuts one eye
Peers into oneself into every corner
Looks at oneself to see there are no spikes no thieves
No cuckoo's eggs

One shuts the other eye too
Crouches then jumps
Jumps high high high
To the top of oneself

Thence one drops by one's own weight
For days one drops deep deep deep
To the bottom of one's abyss

He who is not smashed to smithereens
He who remains whole and gets up whole
He plays

VASCO POPA

ABBREVIATIONS

EGF-R	Epidermal Growth Factor Receptor
ER	Oestrogen receptor
MDCK	Madin Derby Canine Kidney
HGF	Hepatocyte growth factor
rhHGF	recombinant human Hepatocyte growth factor
MuHGF	Murine hepatocyte growth factor
EGF	Epidermal growth factor
PDGF	Platelet derived growth factor
aFGF	acidic Fibroblast growth factor
bFGF	basic Fibroblast growth factor
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
FGF	Fibroblast growth factor
KGF	Keratinocyte growth factor/FGF7
NDF	Neu differentiation factor
CM	Conditioned medium
FCM	Fibroblast Conditioned Medium
HFFs	Human foreskin fibroblasts
HFF-CM	Human foreskin fibroblast conditioned medium
SH2	Src Homology-2
PI3-K	Phosphatidyl-inositol-3-kinase
Sos	Son of sevenless
PLC-γ	Phospholipase C-γ
MAP-K	Mitogen-activated protein-kinase
FAK	Focal Activation Kinase
ILK	Integrin-linked kinase
PKC	Protein kinase C
PMA	phorbol 12-myristate 13-acetate
IDC	Invasive Ductal Carcinoma
ILC	Invasive Lobular Carcinoma
vWF	von Willebrand Factor
FN	Fibronectin
VN	Vitronectin
FNG	Fibrinogen
ECM	Extracellular matrix
EHS	Engelbreth Holm Schwartz
MIDAS	Metal ion dependent adhesion site
Ab	Antibody
mAb(s)	monoclonal antibody(ies)
PEM	Polymorphic Epithelial Mucin
DTT	Dithiothreitol
uPA	urokinase plasminogen activator
WGL	Wheat germ lectin
Con-A	Concanavalin-A
TEMED	N, N, N', N'- tetramethylethylene diamine
ECL	Enhanced chemiluminescence
aa	amino acid
pmsf	phenylmethylsulphonylfluoride

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CHAPTER 1: GENERAL INTRODUCTION.

1.1. Structure of the mammary gland *in vivo*.

The mammary gland *in vivo* is made up of highly organised branching ducts and lobules, embedded in a fatty stromal matrix. The epithelial cells lining the ducts and lobular alveoli of the normal breast fall into two major phenotypically distinct categories (Figure 1.1); the basal or myoepithelial cells, which are firmly attached to the underlying basement membrane by specialised adhesion contacts, the hemidesmosomes, and the luminal epithelial cells, which sit on the basal cells, form junctions with them and each other, and line the lumen (Russo, J. and Russo, I., 1987). Although the contact with the basement membrane is less extensive than with the basal cells, projections from the luminal cells extend down to and interact with the basement membrane (Taylor-Papadimitriou, J. and Lane, E., 1987).

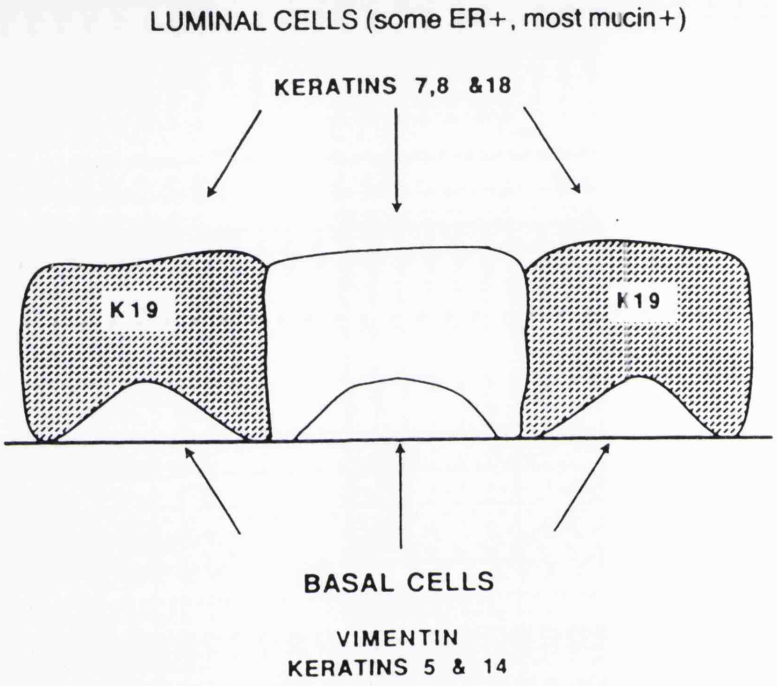
The two epithelial cell types can be distinguished by the profile of intermediate filaments which they express (Figure 1.1); with all the luminal cells expressing the simple epithelial keratins 7, 8, and 18, and the basal cells expressing keratins 5 and 14 and vimentin (Taylor-Papadimitriou, J. and Lane, E., 1987; Taylor-Papadimitriou, J., 1992). Subgroups of epithelial cells can also be identified, and of particular importance is the subgroup of luminal cells which express keratin 19 (Bartek, J. *et al.*, 1985b), since this profile of keratins is expressed by most primary and metastatic breast cancers (Bartek, J. *et al.*, 1985a). Other molecules which distinguish the two epithelial cell types are growth factor receptors; with Epidermal Growth Factor receptors (EGF-R) being more dominantly expressed in the basal cells, and oestrogen receptors (ER) expressed by only a fraction of luminal cells and not by basal cells (Petersen, O.W. *et al.*, 1987; Taylor-Papadimitriou, J., 1992). Basement membrane components such as laminin and collagen type IV are also expressed by the basal cells (Gusterson, B.A. *et al.*, 1982; Wetzels, R.H. *et al.*, 1989) and an epithelial specific mucin, MUC1, is expressed at the apical surface of luminal cells (Arklie, J. *et al.*, 1981).

Like other simple epithelia, the luminal epithelial cell layers of the mammary gland are highly polarised, with distinct basolateral and apical domains. The cell-cell junctions characteristic of epithelial cells are the tight junctions, (which physically separate the apical and basolateral

Figure 1.1.
Antigens expressed by basal and luminal epithelial cells of the normal adult human mammary gland.

The epithelial cells lining the ducts and lobular alveoli of the normal adult human mammary gland fall into two major categories which are phenotypically distinct; basal cells and luminal cells. The basal cells are firmly attached to the underlying basement membrane, while the luminal epithelial cells sit on the basal cells and line the lumen. Most breast cancers and cell lines developed from them correspond to the keratin 19+ luminal epithelial cell.

Key:
K19, keratin 19; ER, oestrogen receptor; mucin, polymorphic epithelial mucin (PEM)


LUMINAL CELLS (some ER+, most mucin+)
KERATINS 7,8 &18
K19
K19
BASAL CELLS
VIMENTIN
KERATINS 5 & 14

domains (Mandel, L.J. *et al.*, 1993), adherens junctions and desmosomes (Woods, D.F. and Bryant, P.J., 1993). These junctions act to maintain epithelial polarity, resulting in an impermeable protective cell barrier held in place by these junctions and by those made between the cells and the basement membrane. The epithelial junctions are complex, each containing a specific subset of proteins; for example, in the tight junctions molecules such as ZO-1 are found (Balda, M.S. *et al.*, 1993); in the adherens junctions, E-cadherin and associated molecules (for review see Birchmeier, W. and Behrens, J., 1994); and in the desmosomes, the desmosomal cadherins such as desmoglein and desmocollin (Troyanovsky, S.M. *et al.*, 1993). Members of the integrin family of molecules are often found in areas of cell-cell and cell-matrix contact (see later).

One of the first changes that occurs in breast cancer development is the loss of the organised ductal tissue architecture, which becomes progressively more pronounced as the cancer becomes more aggressive and invasive (Jensen, H.M., 1981; Millis, R., 1984), (Figure 1.2). During the loss of tissue architecture, the luminal cells proliferate to form multilayers instead of the single layer evident in the normal gland, and the epithelial polarity of the cells is lost. During this process, the expression of cell-cell and cell-matrix adhesion molecules important for maintaining polarity, such as the E-cadherin and some members of the integrin family are reduced (see later). These molecules are often distributed over the whole cell membrane surface, rather than in the appropriate basolateral domains. Conversely, the MUC-1 molecule is often up-regulated and overexpressed in breast cancers (Girling, A. *et al.*, 1989), and like the basolateral proteins, its normal (apical) distribution is lost.

1.2. The phenotype of the breast cancer cell.

From the earliest changes seen in the development of abnormal lesions, it is clear that the luminal rather than the basal epithelial cells form the proliferative component (Jensen, H.M., 1981; Millis, R., 1984). Moreover, it is clear from immunohistochemical studies that in most breast cancers (about 90%), the invasive breast cancer cell has a phenotype of the keratin 19 expressing luminal epithelial cell (Figure 1.1). This phenotype is also shown

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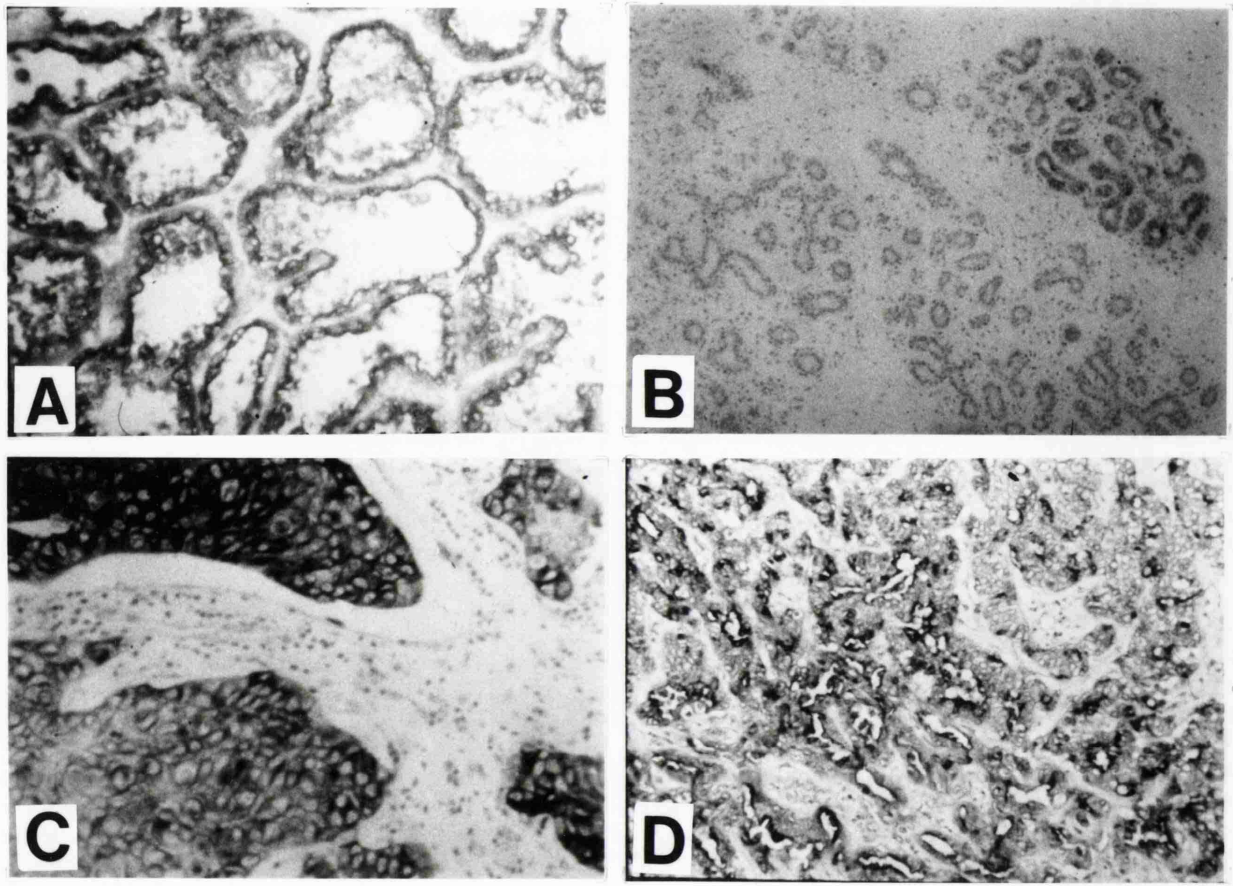
Figure 1.2.
Loss of organised tissue architecture in breast cancer.

Histological sections of A. lactating breast; B. Resting breast; C. a primary infiltrating ductal carcinoma; D. a breast cancer metastasis to a lymph node. The sections are stained with an antibody to the polymorphic epithelial mucin (PEM), which shows apical localisation in the normal gland. In the cancers, epithelial polarity is lost and PEM staining is found all around the cell and can be intracellular.


A
B
C
D

by the cancer cells in *in situ* tumours where the malignant cells are contained by a layer of apparently normal myoepithelial cells sitting on a basement membrane, and is maintained in many metastatic lesions and in cell lines derived from them (Bartek, J. *et al.*, 1985a). In benign lesions (particularly in fibroadenomas), myoepithelial cells and basement membranes are evident (Millis, R., 1984). However, expression of one or more basal markers (for example EGF-R, keratin 14, vimentin) can occur in a small proportion of breast cancers, and their expression is generally associated with a poor pateient prognosis (Domagala, W. *et al.*, 1990).

Although the phenotype of the breast cancer cell suggests that in most cases, it develops from the luminal lineage, the lack of information on the kinetic relationship between different phenotypes in the normal gland, does not allow unequivocal identification of the luminal cell as the initial target cell, which may have been a stem cell retaining the ability to develop into a differentiated luminal cell. However, for comparative studies between normal and malignant cells, either *in vivo* or *in vitro*, which might identify changes in phenotype and molecular mechanisms involved in malignant progression, the normal luminal epithelial cell must be the cell of choice.

1.3. Development of the mammary gland *in vivo*.

1.3.1. Developmental stages from the embryo to adult.

The mammary gland is an extremely interesting organ to study developmentally. This is because unlike most other organs, development occurs not only in the embryo, but also post-natally both at puberty and in the adult at pregnancy. The developmental stages of the human mammary gland are outlined below (Russo, J. and Russo, I., 1987).

In the foetus, the epidermal mammary ridge from which the mammary gland develops is formed during the 5th week (Dabelow, A., 1957). This mammary ridge thickens and parenchymal cells start to invade the underlying stroma during weeks 6 to 10. Between the 10th and 12th week epithelial buds sprout from the invading parenchyma and these buds become lobular in shape. Further branching into 15-25 epithelial strips or solid cords mark the "branching" stage, which occurs during

weeks 13 to 20. These solid cords then become canalised to form rudimentary ducts by lysis of the central epithelial cells during weeks 20 to 32.

From birth until the approach of puberty the mammary gland does little more than keep pace with the general growth of the body (Tanner, J., 1962). At puberty, the rudimentary ducts grow and divide to form a mammary tree with extensive ductal branching and terminal ductal lobular units (also called lobules).

Increased proliferation of the breast epithelium occurs during the luteal phase of each menstrual cycle (Anderson, T.J. *et al.*, 1982). Although this increase in proliferation is followed by an increase in apoptosis (Ferguson, D.J. and Anderson, T.J., 1981), mammary development induced by ovarian hormones during a menstrual cycle never fully returns to the starting point of the preceding cycle. Accordingly, each ovulatory cycle slightly fosters mammary development.

The early stages of pregnancy are characterised by further proliferation and branching of the distal elements of the ductal tree, giving rise to fully developed lobules. By the end of the first half of pregnancy, the definitive structure of the ductal tree is effectively settled. The second half of pregnancy is then characterised by the continuation and accentuation of the development of the secretory activity of the breast (Dabelow, A., 1957).

After lactation and weaning, involution occurs, involving the collapse of lobular structures and narrowing of the tubules (Geschickter, C., 1945). The connective tissue of the breast is also regenerated. Interestingly, despite the loss of a large number of mammary epithelial cells at involution, the breast is not completely returned to its pre-pregnant state and shows more glandular tissue than if pregnancy or pregnancy plus lactation had never occurred. Indeed this remains the case until menopausal involution sets in.

1.3.2. Role of epithelial-mesenchymal interactions in mammary gland development.

Epithelial-mesenchymal interactions have been shown to play an important role in both foetal and post-natal stages of mouse mammary gland development (for review see Cunha, G. and Hom, Y., 1996). In the mouse foetus, one of the first stages in mammary gland development is the formation of mammary epithelial buds from the ectoderm. Evidence suggests that the formation of these epithelial buds is induced by the mammary mesenchyme (Propper, A., 1972; Sakakura, T., 1987; Cunha, G. *et al.*, 1995). Moreover, once the epithelial buds have been induced, reciprocal interactions between the epithelia and mesenchyme occur, giving rise to regression of the epithelial bud in males, and the growth and rudimentary ductal branching through the stroma in females (Kratochwil, K., 1987). In postnatal development, mesenchymal-epithelial interactions have been shown to be responsible for rapid epithelial growth, and the distinctive pattern of ductal branching morphogenesis (Daniel, C. and Silberstein, G., 1987). Finally, the epithelial-mesenchymal interaction is maintained into adulthood where it may well continue to play an important role in growth and differentiation.

1.3.3. Branching morphogenesis in mammary gland development.

It is clear that the mesenchyme plays a vital role in the process of branching morphogenesis of epithelial cells in mammary gland development. An understanding of the molecular mechanisms involved in this mesenchymal-epithelial interaction is important, not only in the context of mammary gland development, but also in understanding both the basic and tumour biology of mammary cells, since proliferation and invasion of the stromal matrix occurs in normal morphogenesis and in tumorigenesis. In the one case, however, form is maintained and in the other, it is lost.

Interestingly, although not carried out in a mammary gland system, Montesano, R. *et al.*, 1991b, reproduced this mesenchymal induction of branching morphogenesis of epithelial cells *in vitro*. This was done by co-cultivation of MDCK cells (Madin Derby Canine Kidney epithelial

cells), in collagen type I (a major stromal component), with fibroblasts (the major cellular component of the mesenchyme), or fibroblast conditioned medium. In later studies, these investigators identified the molecule secreted by fibroblasts that could induce this branching morphogenesis as hepatocyte growth factor (HGF), and showed that in the MDCK kidney cell system, HGF was the only growth factor tested that could stimulate this effect (Montesano, R. *et al.*, 1991b).

In terms of mammary gland development, the known importance of mesenchymal-epithelial interactions in branching morphogenesis, and the fact that invagination of epithelial cells through a stromal matrix is a vital developmental process, made this result potentially very important. However, although HGF was an interesting candidate for a mesenchymal factor that could play a role in the induction of branching morphogenesis in the mammary gland, it was unknown whether HGF could stimulate this process in mammary epithelial cells.

1.4. Hepatocyte Growth Factor (HGF), and its cognate receptor c-met.

1.4.1. Characterisation and structure.

HGF was cloned in 1989 (Miyazawa, K. *et al.*, 1989; Nakamura, T. *et al.*, 1989), and in 1991 its cognate receptor was identified as the c-met transmembrane tyrosine kinase (Bottaro, D.P. *et al.*, 1991; Naldini, L. *et al.*, 1991). Biochemical characterisation of HGF, showed that it was a disulphide linked heterodimer, composed of a 69 kDa α subunit containing an N-terminus hairpin structure and four homologous kringle domains, and a 34 kDa β subunit, containing a serine protease-like motif. This motif gave HGF a structural homology to plasminogen, however HGF has no proteolytic activity due to the presence of two altered amino acid residues in the protease active triad site (Nakamura, T. *et al.*, 1989).

HGF is translated from a single mRNA as a single chain biologically inactive precursor called "preproHGF". HGF is secreted in this monomeric form, after which extracellular processing by a specific serine protease (Miyazawa, K. *et al.*, 1993; Mizuno, K. *et al.*, 1994), converts preproHGF to the biologically active mature form. Two naturally

occurring variants of HGF have been identified that are produced by alternate splicing; the first has a 5 amino acid deletion in the first kringle domain (Seki, T. *et al.*, 1990), and the second contains only the α chain N-terminal hairpin and the first two kringle domains (Chan, A.M. *et al.*, 1991; Miyazawa, K. *et al.*, 1991). HGF was found to be identical to an independently discovered protein called scatter factor (Gherardi, E. *et al.*, 1989; Weidner, K.M. *et al.*, 1991); so named because of its ability to "scatter" certain types of epithelial cells (Stoker, M. *et al.*, 1987), which involved a pronounced reduction in cell-cell association and the conversion of cells to a more motile and fibroblastic-like phenotype.

1.4.2. Biological properties of HGF.

HGF was originally partially purified from the serum of hepatectomised rats, was found to be a potent mitogen for mature hepatocytes in primary culture (Nakamura, T. *et al.*, 1984), and therefore thought to be an important mediator of liver regeneration. However, over the past 12 years HGF has been shown to be an extremely versatile molecule with diverse biological activities. These activities include the stimulation of mitogenesis of epithelial, endothelial, and carcinoma cells (Igawa, T. *et al.*, 1991; Rubin, J.S. *et al.*, 1991; Bussolino, F. *et al.*, 1992); motogenesis and cell separation of epithelial and carcinoma cells (Stoker, M. *et al.*, 1987; Weidner, K.M. *et al.*, 1990; Weidner, K.M. *et al.*, 1991); angiogenesis of endothelial cells (Bussolino, F. *et al.*, 1992; Grant, D.S. *et al.*, 1993), and growth inhibition of some tumour cells (Higashio, K. *et al.*, 1990; Tajima, H. *et al.*, 1991; Shiota, G. *et al.*, 1992), as well as the morphogenetic induction of MDCK epithelial cells to undergo branch formation described above. Interestingly, induction of these biological activities by HGF is dependent on the particular cell-type; for example, HGF does not stimulate mitogenesis in all types of epithelial cells, an interesting exception being MDCK cells (Montesano, R. *et al.*, 1991b).

The ability of HGF to control processes as diverse as mitogenesis, motogenesis, angiogenesis and morphogenesis, made HGF a prime candidate for having an important role in organogenesis and development (as described above). However, the ability to stimulate the same processes in carcinoma cells, also implicated HGF in the

uncontrolled growth, invasion and metastasis during tumorigenesis (see below).

1.4.3. Signal transduction from the HGF receptor, c-met.

After the identification of c-met as the cognate receptor for HGF, attention was focussed on the signalling induced through ligand interaction with the receptor.

The c-met receptor is a disulphide bonded heterodimer, composed of a 50 kDa α extracellular subunit, and a 145 kDa β transmembrane subunit, that contains the tyrosine kinase domain on the cytoplasmic side (Park, M. *et al.*, 1987). c-met is produced as a single chain precursor of 170 kDa, which then undergoes terminal glycosylation and proteolytic cleavage to produce the 190 kDa mature heterodimer (Giordano, S. *et al.*, 1989).

The c-met receptor undergoes autophosphorylation in response to HGF binding, which upregulates its kinase activity. This autophosphorylation of c-met enables the receptor to physically interact with and activate many cytoplasmic effectors of signal transduction that contain SH2 domains; such as PI3-k, Grb-2/Sos, pp60$^{c\text{-}src}$, and PLC-γ (Graziani, A. *et al.*, 1991; Graziani, A. *et al.*, 1993 Ponzetto, C. *et al.*, 1993; Ponzetto, C. *et al.*, 1994; Halaban, R. *et al.*, 1992). The interaction of such effector molecules with c-met occurs by the binding of their SH2 domains to a multifunctional docking site on c-met. This docking site contains two specifically phosphorylated tyrosine residues (tyrosine's 1349 and 1356), which have been shown to be vital for the biological function of c-met (Ponzetto, C. *et al.*, 1994). The interaction of HGF/c-met with the ras nucleotide exchanger Sos leads to activation of the ras proto-oncogene (Graziani, A. *et al.*, 1993). This pathway may well also lead to the observed induced phosphorylation of the mitogen activated protein kinase (MAP-K) (Ponzetto, C. *et al.*, 1994); a known downstream effector of ras that is involved in mitogenetic signal transduction pathways (Satoh, T. *et al.*, 1992).

The ability of HGF stimulated c-met to interact with and activate such a divergent number of signal transduction effector molecules, and the fast association and dissociation rates observed for these interactions

(Ponzetto, C. *et al.*, 1994), allows the activation of many different signal transduction pathways. This probably accounts for the ability of a single molecule (HGF) to induce such a wide range of different biological effects through a single cell surface receptor (Weidner, K.M. *et al.*, 1993). The actual mechanism by which single or multiple responses of cells to HGF is mediated is still unclear. However, it is postulated that if different biological responses involve interplay of different signal transduction pathways, this could conceivably be achieved by the competition of different effector molecules for a docking site such as that described above. Such competition might occur for example by differing affinities of SH2 effectors for c-met, or perhaps different local concentrations of effectors, or even different profiles of effector molecules in different cells (Ponzetto, C. *et al.*, 1994).

1.4.4. HGF stimulation of cell separation and motility.

The importance of the ras pathway in HGF stimulated cell separation and motility was shown by Hartmann, G. *et al.*, 1994. These investigators transfected MDCK cells with a dominant-negative *ras* construct under an inducible promoter, and showed that induction of expression of the dominant negative ras inhibited the scattering response of MDCK cells to HGF. The ras/MAP-K pathway is a common pathway by which mitogenesis is induced in cells (Satoh, T. *et al.*, 1992). HGF induced activation of ras and MAP-K make it likely that HGF uses this pathway to effect changes in gene expression in the nucleus and to induce proliferation. In contrast, the ras signal that leads to the stimulation of motility and scattering must finally reach the cell adhesion system and actin cytoskeleton.

Results suggest that the downstream signalling from ras to the actin cytoskeleton may involve the small GTP binding proteins, rho and rac. Which of these two molecules play a role in HGF induced motility seems to vary between cell types; with the activation of rho being shown to be essential for motility of keratinocytes (Takaishi, K. *et al.*, 1994), and the activation of ras and rac required for the HGF induced stimulation of spreading and motility in MDCK cells (Ridley, A.J. *et al.*, 1995). It should be noted that in both the keratinocyte and the MDCK system it was

found that although rho and ras/rac respectively were necessary, they were not sufficient for HGF induced motility.

As the "scattering" effect of HGF involves a cell dissociation as well as a stimulation of motility, roles for alterations in cell-cell and cell-matrix adhesion systems have also been examined. The E-cadherin cell-cell adhesion system has been postulated to play a role in HGF induced cell separation. This is because HGF has been shown to stimulate the increased phosphorylation of β-catenin and plakoglobin (Shibamoto, S. *et al.*, 1994), an event shown in the case of β-catenin (Kinch, M.S. *et al.*, 1995), to impair the interaction of β-catenin with E-cadherin, or the interaction of the cadherin-catenin complex with the cytoskeleton, thereby having a detrimental effect on cell-cell adhesion. The upstream kinases controlling β-catenin or plakoglobin phosphorylation are as yet unknown.

In terms of alterations in cell-matrix interactions, it has been shown that HGF stimulation of motility occurs as a two step process. The first step involves the induction of cell spreading and the formation of focal adhesions containing the β_1 integrin, cytoskeletal components, and phosphorylated pp125 focal adhesion kinase (FAK). The second step then involves disassembly of these focal adhesions (interestingly corresponding in time to the dephosphorylation of FAK), and the transformation of cells to a motile phenotype (Matsumoto, K. *et al.*, 1994). It therefore appears that HGF stimulates the motility of cells by regulating both the assembly and disassembly of focal adhesions. Furthermore, it seems likely that this control occurs by way of regulating the recruitment of integrins and cytoskeletal proteins to focal adhesions and probably involves the phosphorylation, dephosphorylation and function of FAK.

It has also been shown, by examining the naturally occurring variants of HGF described earlier, that different biological effects can be stimulated depending on which domains of the HGF molecule are present. Experiments showed that the HGF variant containing the 5 amino acid deletion in the first kringle domain could stimulate both mitogenic and motogenic responses in cells, but the variant containing only the N-terminal hairpin structure and the first two kringle domains could

stimulate motogenesis but not mitogenesis (Chan, B.M. *et al.*, 1991; Hartmann, G. *et al.*, 1992; Lokker, N.A. *et al.*, 1992). These results suggest that different domains of the HGF molecule may be responsible for the different biological activities, and moreover, as these variants are naturally occurring, they could also provide a mechanism for controlling a cells response to HGF.

1.5. Adhesion molecules in the normal mammary gland.

As described above, cell-cell and cell-matrix interactions play a particularly important role in maintaining the architecture and polarity of epithelial tissues. Studies in the mammary gland have tended to focus on two different types of molecule involved in these interactions; the first is the integrin family of molecules which are involved in both cell-cell and cell-matrix interactions, and the second is the E-cadherin molecule (and its associated catenins), which are components of the adherens junction and involved in cell-cell adhesion. Both these sets of molecules are thought to play an important role in normal tissue morphogenesis, and in the initial loss of tissue architecture and the subsequent invasion and metastasis seen in the development of breast cancer, their expression may be reduced. The evidence for the role of E-cadherin in these processes is described here in some detail, both because of its global relevance in the field and also because these roles make the E-cadherin adhesion system a potential focus for future investigations based on the results described in this thesis. However, the evidence for the roles of integrins in normal mammary gland and breast cancer development are described in more detail because these form the main focus of the thesis to follow.

1.5.1. Integrins.

Integrins are transmembrane glycoproteins important in the adhesion of cells to extracellular matrix components, and some are known to play a role in cell-cell adhesion, (Sriramarao, P. *et al.*, 1993; Symington, B.E. *et al.*, 1993; and for a comprehensive review see Hynes, R.O., 1992). However, it is now well established that integrins cannot merely be regarded as simple adhesion molecules, but are structurally dynamic and have the ability to transmit signals either into or out of cells; referred to

as outside-in and inside-out signalling respectively. The fact that integrins are structurally dynamic means that they can be expressed in different activation states (i.e. with different affinity for ligands). These induced changes in activation states have consequences both for cell adhesion and for signalling, and are mediated by conformational changes in the integrins, regulated from both the outside and inside of cells. "Outside-in" signalling, refers to the ability of integrins, on the binding of ligand, or a similar extracellular cue, to act as classical signal transduction units and trigger events inside the cell such as clustering into focal contacts, the phosphorylation of intracellular proteins, and altering the transcription of certain genes (see below).

Integrins consist of α and β heterodimers, of which 16 α and 8 β subunits have to this date been cloned (for review see Garratt, A. and Humphries, M., 1995). Individual β subunits can heterodimerise with different α subunits and vice-versa (although the latter case is less frequent), forming a large number of different integrin family members; examples of which are shown in Figure 1.3. Further diversity is achieved by the fact that individual integrins often bind more than one ligand (Figure 1.3), and individual ligands are often recognised by more than one integrin. Integrins have been found to be expressed in all cell types examined, and usually more than one type of integrin is expressed on a particular cell. The ligands bound by a particular integrin also vary between cell types; for example, endothelial cells and platelets both express the $\alpha_2\beta_1$ integrin but although endothelial cells bind both collagen and laminin via the $\alpha_2\beta_1$, platelets can only bind to collagen type I (Kirchhofer, D. *et al.*, 1990). Integrins can be conveniently subgrouped depending on the β subunit in the heterodimer, and in the cells of the mammary gland the most abundantly expressed integrins are members of the β_1 family, plus the $\alpha_6\beta_4$.

1.5.2. E-cadherin and associated proteins.

Cadherins are a large and expanding family of molecules that mediate cell-cell adhesion. For "classic" cadherins (the only ones discussed here), adhesion is mediated by a homophilic and Ca^{2+} dependent interaction between cadherin molecules on the surface of both adherent cells. Adhesion is mediated by the interaction of the extracellular domains of

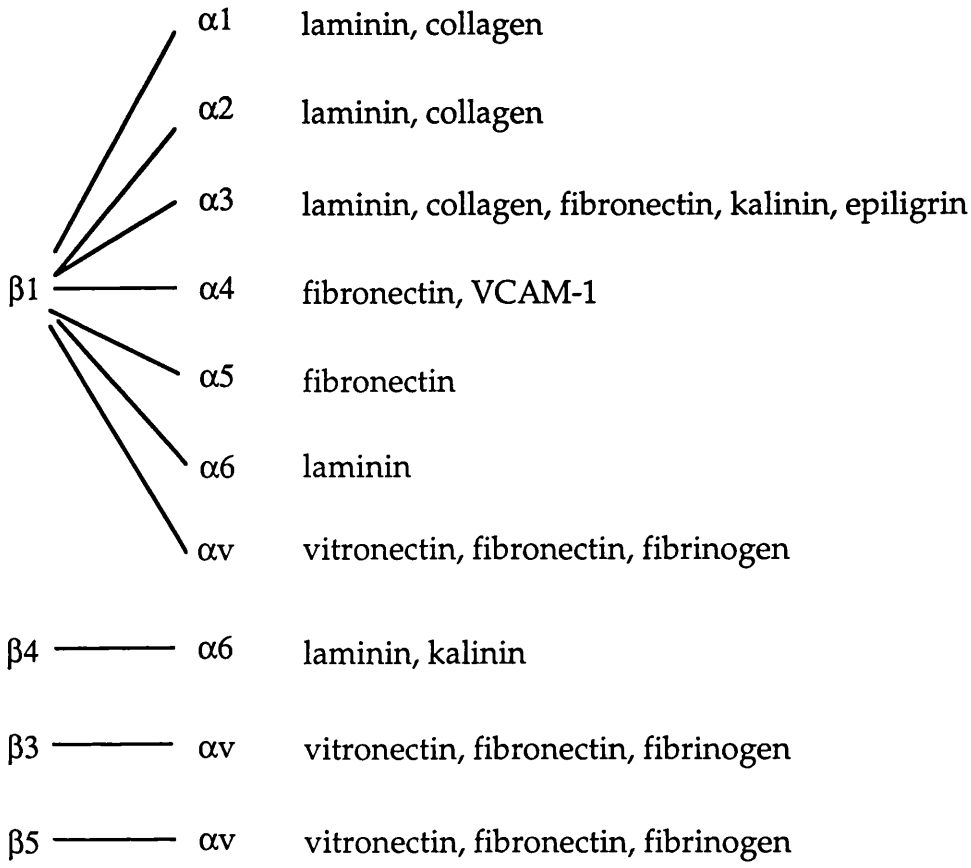


Figure 1.3.
Integrin - ligand binding specificities.

Integrin α-β heterodimers are shown with their ligands. The list of integrins and their respective ligands is selective, and is designed to show the major integrins discussed in this thesis.

the cadherin molecules, while the intracellular domain is associated with the catenins (α, β, and γ). The catenins couple the cadherin to the actin cytoskeleton network and are known to be vital for the efficient functioning of cadherins (for review see Kemler, R., 1993).

1.6. Integrin and E-cadherin expression in the mammary gland *in vivo*.

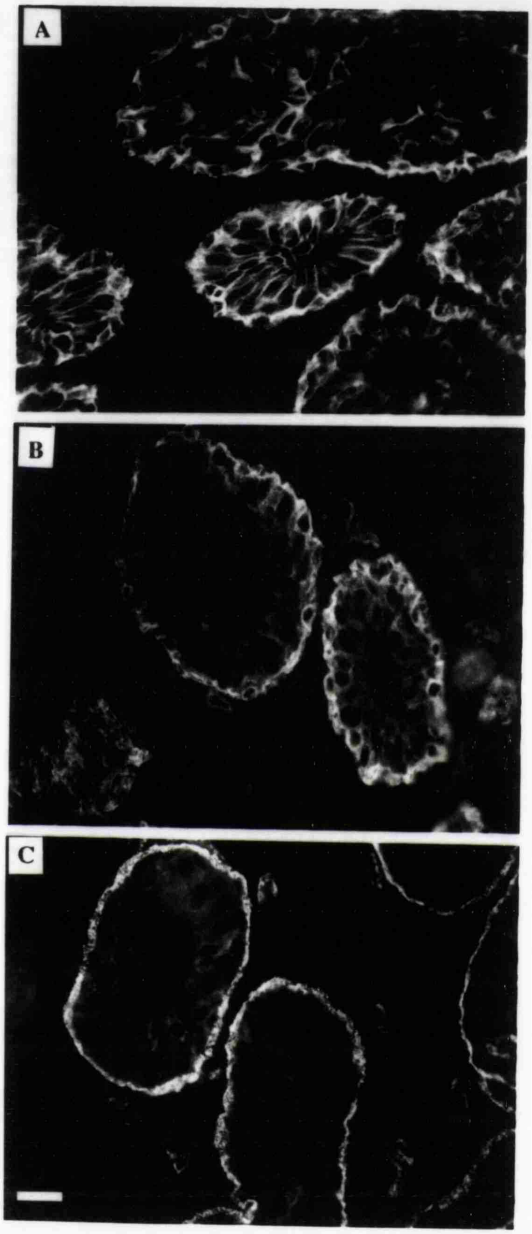
1.6.1. Integrins.

Integrin expression in the normal mammary gland has been fairly well documented and is very organised and regular. Three members of the integrin family are found to be abundantly expressed in the resting and lactating mammary gland, namely: $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ (Figure 1.4) (Koukoulis, G.K. *et al.*, 1991; Berdichevsky, F. *et al.*, 1994b). The α_v integrin subunit has also been shown to be moderately expressed in the basal cells and weakly expressed in the luminal cells of the adult mammary gland (Koukoulis, G.K. *et al.*, 1991). A study by Anbazhagan, R. *et al.*, 1995, has examined the expression profile of integrins in the infant breast and showed that the profile of expression is much the same as in the adult. Interestingly, no $\alpha_v\beta_3$ expression was detected in the infant breast and no β_3 subunit was detected in the adult mammary gland (Anbazhagan, R. *et al.*, 1995; Koukoulis, G.K. *et al.*, 1991). Therefore, it seems that the partner for α_v in the mammary gland might be either the β_1, β_5, β_6 or β_8 integrin subunit. Although the expression of the β_5, β_6 and β_8 integrin subunits in the mammary gland have not as yet been examined, the data presented in this thesis supports the presence of the $\alpha_v\beta_1$ integrin in the mammary gland *in vivo*.

Being a component of hemidesmosomes (Sonnenberg, A. *et al.*, 1991) the $\alpha_6\beta_4$ complex is found to be localised exclusively at the contact points of myoepithelial cells with the basement membrane (Figure 1.4 panel C). In contrast, the $\alpha_2\beta_1$ integrin is found evenly distributed along the basolateral cell surface in both cell layers as well as in regions of cell-basement membrane contact (Figure 1.4 panel A). Monoclonal antibodies to the $\alpha_3\beta_1$ integrin show strong staining of the basolateral

Figure 1.4.
Integrin expression in the normal resting adult mammary gland.

Indirect immunofluorescence staining of tissue sections of a normal resting adult mammary gland with antibodies to the A, α_2; B, α_3; and C, β_4 integrin subunits. Antibodies used: α_2, HAS4; α_3, J143; β_4, 3E1.


A
B
C

surface of myoepithelial cells (with a stronger staining in the cell-basement membrane contacts), and weaker staining of the basolateral surfaces of luminal cells (Figure 1.4 panel B) (Berdichevsky, F. *et al.*, 1994b).

It can be seen from the expression patterns described above that the integrins are in the correct place to be involved in cell-cell and cell-matrix adhesions in the mammary gland *in vivo*, the disruption of which by either a reduction in expression, or a change in organised position, could help convert the cells to a potentially more malignant phenotype. It is also clear that although the $\alpha_2\beta_1$ integrin is expressed strongly in both luminal and myoepithelial layers and is the most abundant integrin expressed in the luminal cells, in general a higher level of integrin expression tends to be found in the myoepithelial rather than the luminal cells.

1.6.2. Cadherins.

Of the classic cadherins both E-cadherin and P-cadherin are expressed in the normal mammary gland. E-cadherin is found abundantly on the cell-cell contact surfaces of both luminal and myoepithelial cells, whereas the P-cadherin is found in the cell-cell junctions of the myoepithelial cell layer only (Rasbridge, S.A. *et al.*, 1993). P-cadherin will be discussed no further here, as to date this molecule has not been a major focus of investigation in the mammary gland/breast cancer field. Moreover, it is also is expressed in the myoepithelial rather than the luminal epithelial cells, thereby making it less likely to play a significant role in breast cancer development.

1.7. Role of integrins and E-cadherin in breast cancer development.

A role for these molecules in breast cancer development has been implicated both in studies examining expression levels in breast cancers *in vivo*, and also in studies using breast cancer cell lines *in vitro*.

1.7.1. In vivo studies on expression.

(i) Integrins.

The expression of integrins in breast carcinomas has been documented using immunohistochemical methods and by *in situ* hybridisation. Some of the data is potentially conflicting. However, in general, integrin levels are reduced in breast cancers (Zutter, M.M. *et al.*, 1990; Pignatelli, M. *et al.*, 1991; Jones, J.L. *et al.*, 1992; Natali, P.G. *et al.*, 1992; Zutter, M.M. *et al.*, 1993; Berdichevsky, F. *et al.*, 1994a; Glukhova, M. *et al.*, 1995), and are also expressed in a more disorganised manner. In interpreting the results, it is important to remember that the majority of breast cancers show the phenotype of the luminal epithelial cells, and that with the exception of the $\alpha_2\beta_1$, integrin expression in these cells is normally low compared to the myoepithelial cells. Any studies looking at changes in the levels of integrin expression in breast cancers should therefore be considered with the cellular phenotype of the tumour in mind. For example, expression of the α_3 integrin subunit has been reported to be reduced in invasive carcinomas (Pignatelli, M. *et al.*, 1991; Glukhova, M. *et al.*, 1995). However, this data may simply reflect the higher expression of $\alpha_3\beta_1$ in the myoepithelial compartment, which is not represented in the majority of tumours because they are of the luminal phenotype.

Levels of the $\alpha_2\beta_1$ integrin have been found to be reduced in breast carcinomas (Jones, J.L. *et al.*, 1992), and several studies have shown that an increased loss in expression correlates with increasing tumour grade, i.e. expression is more reduced as the tumour becomes less differentiated and the normal tissue structure more disrupted (Pignatelli, M. *et al.*, 1991; Zutter, M.M. *et al.*, 1993). Once again it is very important to consider the phenotype of cells in the tumour when interpreting these results as is shown in the study by Berdichevsky, F. *et al.*, 1994a. These investigators subdivided breast tumours for analysis into those with a completely luminal phenotype (group L), and those with basal elements (group B), and found that while most of the high grade ductal carcinomas in group L showed a reduced expression of $\alpha_2\beta_1$, the same category of tumours in group B expressed high levels.

The expression of the α_6 integrin subunit in normal breast is mainly associated with the β_4 subunit (Berdichevsky, F. *et al.*, 1994b), and as such

is found in the hemidesmosomes which bind the myoepithelial cells to the basement membrane. Like laminin, collagen IV and keratin 14, the expression of the α_6 or β_4 integrin subunits can therefore be regarded as markers for basal cells. This point is also illustrated in the study by Berdichevsky, F. *et al.*, 1994a, where group B tumours were found to express the β_4 integrin more frequently than group L (82% vs. 22%), and all group B tumours expressed the α_6 subunit. In general, markers of basal phenotype such as laminin, collagen IV, keratin 14, vimentin, and the EGF-R are only expressed by a small subset of breast tumours, but are predictive of a poor patient prognosis (Domagala, W. *et al.*, 1990). Assuming that the α_6 integrin is another marker for the basal phenotype may provide an explaination for recent results (Friedrichs, K. *et al.*, 1995), suggesting that high levels of expression of the α_6 integrin in breast cancer patients was associated with shorter survival.

Taken together, these results show that while reduced $\alpha_2\beta_1$ integrin expression is frequent in undifferentiated tumours with the luminal phenotype, integrin expression can also be regarded as a marker for basal elements in a tumour.

(ii) E-cadherin and associated proteins

E-cadherin expression is often reported to be reduced or even absent in breast carcinomas *in vivo*. The expression pattern seems to depend on the grade, the differentiation state, and the type of tumour:- Invasive ductal carcinomas (IDC) are usually positive for E-cadherin, but there is a good inverse correlation between the level of E-cadherin expression and grade (Gamallo, C. *et al.*, 1993; Moll, R. *et al.*, 1993; Oka, H. *et al.*, 1993; Rasbridge, S.A. *et al.*, 1993), with the higher grade and less differentiated tumours expressing the E-cadherin in a patchy and heterogeneous manner compared to more differentiated tumours. On the other hand, in invasive lobular carcinomas (ILC), and lobular carcinoma *in situ*, (thought to be a precursor of ILC), E-cadherin expression is lost in a large proportion of cases (Moll, R. *et al.*, 1993; Rasbridge, S.A. *et al.*, 1993), and in the cases where expression is detectable, it is usually very low and abnormally positioned (Rasbridge, S.A. *et al.*, 1993). Mutation of the E-cadherin gene has been reported in ~50% of invasive lobular breast carcinoma's (Berx, G. *et al.*, 1995), and recently it has also been shown that hyper-methylation of the E-cadherin promoter region may be a

mechanism of E-cadherin silencing in human carcinomas (Yoshiura, K. *et al.*, 1995).

Good expression of E-cadherin in some carcinomas does not of course mean that the molecule is functionally active. For example, certain mutations in the E-cadherin gene or changes in the E-cadherin associated cytoplasmic proteins, the catenins, could weaken the adhesive properties of the molecule. Data on the expression of the catenins in breast carcinomas *in vivo* is as yet limited. However, expression of the α-catenin has been shown to be reduced in some breast cancers (Takayama, T. *et al.*, 1994), showing not only a significant inverse correlation between α-catenin expression and tumour grade, but also a significant correlation with a reduced expression of E-cadherin.

1.7.2. In vitro studies on α_2 integrin expression and function in cell lines, and the suggested importance of the $\alpha_2\beta_1$ integrin in mammary gland morphogenesis.

As described above there is a correlation in breast cancers *in vivo* between down regulation in α_2 integrin expression and loss of normal tissue morphology. This correlation was shown by Berdichevsky, F. *et al.*, 1994a, to be reflected in an *in vitro* system, where cell lines were cultured in 3-dimensions within collagen type I, (an extracellular matrix/stromal component). The authors examined α_2 integrin expression in a panel of non-tumorigenic human mammary epithelial cell lines (Bartek, J. *et al.*, 1991); cell lines developed from benign lesions or primary tumours (Shearer, M. *et al.*, 1992), and established primary and metastatic breast cancer cell lines. They showed that (as observed *in vivo*), a high level of α_2 integrin expression was maintained by the normal cell lines and those derived from benign lesions, whereas a reduced expression was associated with breast cancer cell lines of the luminal phenotype. Moreover, when these cell lines were cultured in 3-dimensions in collagen type I, the cell lines developed from normal and benign components (with normal levels of α_2 integrin expression), formed organised, compact cyst like structures. On the other hand, the breast cancer cell lines (with reduced α_2 integrin expression), had lost this ability and either did not grow at all within the matrix, grew as single cells, or grew in chaotic, disorganised aggregates.

The loss in ability of breast cancer cell lines to form organised 3-dimensional structures *in vitro* was also observed in a study by Petersen, O.W. *et al.*, 1992, whereby normal and malignant human mammary epithelial cells were cultured in the reconstituted basement membrane product Matrigel, rather than collagen type I. Furthermore, in the matrigel system, the normal but not the malignant cells underwent growth arrest after 3-dimensional structures were formed. Both the collagen and matrigel assays therefore suggest that signals from extracellular matrix components are important for controlling normal cell growth and maintaining normal cell tissue architecture and organisation, and that tumour cells may no longer receive or respond to these signals. Obvious candidates for mediators of these signals are integrins, and the observed correlation between normal levels of expression of the α_2 integrin, on a luminal epithelial cell background, and the ability of cells to form organised morphogenetic architecture both *in vivo* and *in vitro*, strongly implicates the $\alpha_2\beta_1$ integrin as playing a major role in morphogenetic processes. Also, the fact that these cell lines *in vitro* reflect integrin expression *in vivo* so accurately, suggests that they provide good *in vitro* models in which to study the functional roles of integrins (and in particular $\alpha_2\beta_1$) in normal mammary epithelia and mammary tumours.

Interestingly, and again reflecting the situation *in vivo* where the disruption of tissue architecture is one of the first changes that occurs in the development of breast cancer, the loss of the morphogenetic ability of mammary tumour cells to form compact 3-dimensional structures *in vitro*, and the concurrent reduction in α_2 integrin expression, appears to be an early event in malignant progression (Shearer, M. *et al.*, 1992). Thus cell lines derived from benign breast lesions formed smooth compact organised structures, like the cell lines derived from normal milk epithelia; whereas cell lines derived from primary breast tumours had lost the ability to form such structures, even though they were not sufficiently transformed to form colonies in soft agar or tumours in nude mice. This shows that *in vitro*, the loss in ability of tumour cells to form organised structures occurs before the cells acquire the property of anchorage independent growth. Indeed *in vivo*, anchorage

independence might well be predicted to be a property of a more aggressive later stage carcinoma.

From the studies by Meredith, J.J. *et al.*, 1993, and Frisch, S.M. and Francis, H., 1994, it is apparent that extracellular matrix acts as a survival factor for normal epithelial cells by inhibiting apoptosis, and that integrins are important for this effect. In other words, integrins, through their interaction with the extracellular matrix, play an important role in the growth of normal epithelial cells. It is therefore possible that a mechanism by which cancer cells become anchorage independent is that they acquire the ability to bypass the integrin mediated signals required for cell growth/survival.

The proposed role for integrins in the processes of normal mammary gland morphogenesis, which requires anchorage dependent growth, may involve their function as cell-cell/cell-matrix adhesion molecules, and/or their function as transducers of signals from the extracellular environment. In analysing such potential functions of the integrins it is relevant to understand (i) what is known of integrin structure, (ii) the general mechanisms which regulate integrin-ligand interaction, (iii) the control of integrin mediated adhesion, and (iv) integrin mediated signal transduction.

1.8. Integrin structure.

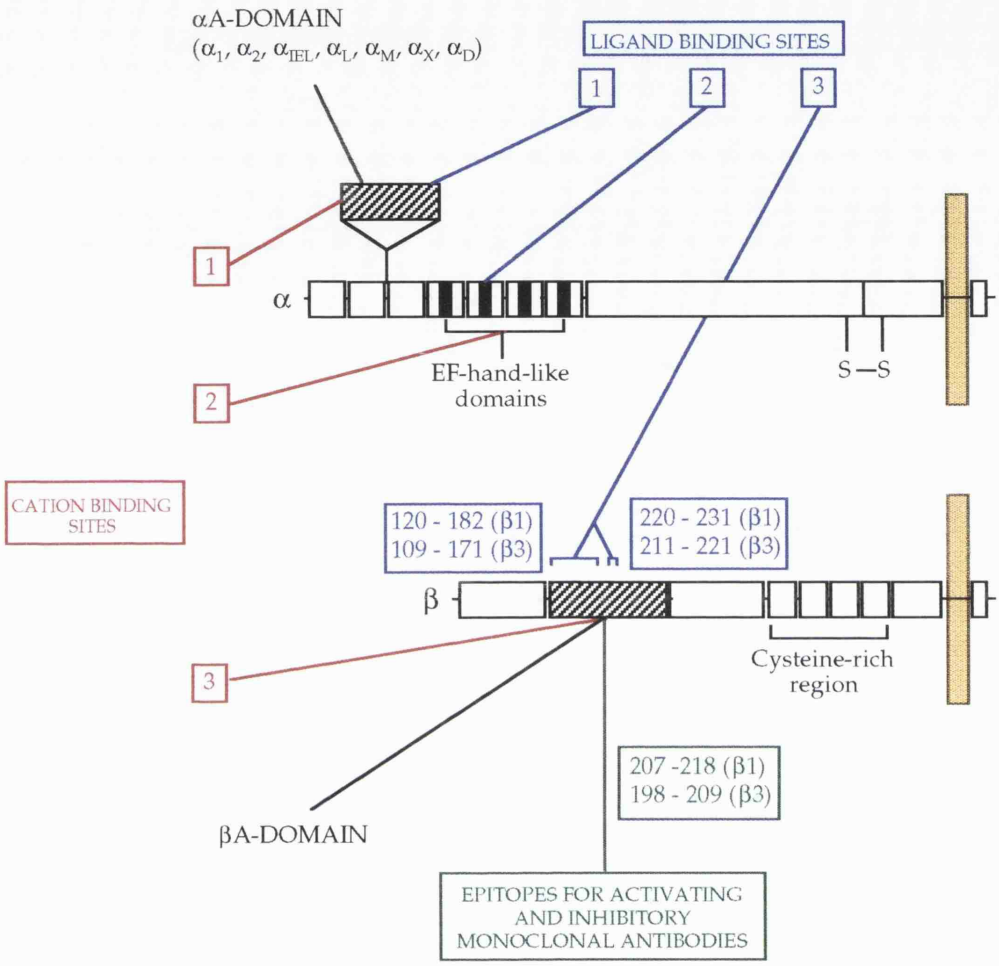
Several notable features of integrin structure have been identified (Figure 1.5). All integrin subunits have large extracellular domains, a single membrane spanning region, and a small cytoplasmic domain (with the exception of the β_4 subunit that has a large intracellular domain, Tamura, R.N. *et al.*, 1990). The heterodimers are non disulphide bonded, although there are intrachain disulphide bonds present. α subunits have a seven-fold tandem repeat at the N-terminus. The last 3 or 4 of these repeats (depending on the α subunit), show sequence homology to divalent cation binding structures called EF hands, found in divalent cation binding proteins such as calmodulin (Tuckwell, D.S. *et al.*, 1992). Some α subunits contain an extra domain of about 200 aa inserted between repeats 2 and 3. This domain is called an "I"-domain ("insert" domain) or the A-domain because of its sequence

Figure 1.5.
Active sites in integrins.

A variety of approaches have identified three main regions of integrins (labelled 1, 2, 3), as active sites for ligand binding. Interestingly, the same three regions are thought to interact with divalent cations which also effect ligand binding.

The boxed ranges of numbers indicate amino acid residues of the β_1 or β_3 integrin subunits identified as being involved in ligand binding, or identified as regions where epitopes for various anti integrin antibodies are localised.

(Adapted from Figure 1 in review by Garratt and Humphries 1995)


αA-DOMAIN
(α1, α2, αIEL, αL, αM, αX, αD)
LIGAND BINDING SITES
1
2
3
1
α
2
EF-hand-like
domains
S—S
CATION BINDING
SITES
120 - 182 (β1)
109 - 171 (β3)
220 - 231 (β1)
211 - 221 (β3)
β
3
Cysteine-rich
region
207 -218 (β1)
198 - 209 (β3)
βA-DOMAIN
EPITOPES FOR ACTIVATING
AND INHIBITORY
MONOCLONAL ANTIBODIES

homology to the A-domain in von Willibrand Factor (vWF). The main structural feature of β subunits is a cysteine rich region that consists of four homologous EGF like repeats (Calvete, J.J. *et al.*, 1989).

1.9. Integrin-ligand interaction.

1.9.1. Ligand active sites.

Integrin binding sites in extracellular ligands have been identified as short (3-5 aa) peptides containing an acidic residue of either aspartate or glutamate. The first integrin binding site identified, which is present in several potential integrin ligands, was the RGD site (Arg-Gly-Asp) (Pierschbacher, M.D. and Ruoslahti, E., 1984). However, presence of the motif does not automatically guarantee integrin binding, and only four ligands containing this motif have been unequivocally shown to bind to integrins; fibronectin (FN), vitronectin (VN), vWF, and fibrinogen (FNG), (Pierschbacher, M.D. and Ruoslahti, E., 1984; Humphries, M.J., 1990; Beacham, D.A. *et al.*, 1992). Other ligand binding sites so far identified are LDV (Leu-Asp-Val), present in FN and some members of the Ig superfamily of adhesion molecules (Wayner, E.A. *et al.*, 1989; Komoriya, A. *et al.*, 1991), QAGDV (Gln-Ala-Gly-Asp-Val) in FNG (Kloczewiak, M. *et al.*, 1984), and also DGEA (Asp-Gly-Glu-Ala) in collagen type I (Staatz, W.D. *et al.*, 1991). Staatz, W.D. *et al.*, 1991 demonstrated the presence of the DGEA active site in collagen type I that is recognised by the $\alpha_2\beta_1$ integrin, by showing that DGEA peptides could effectively inhibit $\alpha_2\beta_1$-mediated adhesion of platelets to collagen type I, but not effect $\alpha_5\beta_1$ mediated adhesion to FN, or $\alpha_6\beta_1$-mediated adhesion to laminin. Moreover, in T47D breast adenocarcinoma cells, which are known to use $\alpha_2\beta_1$ as a collagen type I and laminin receptor, DGEA peptides were shown to inhibit adhesion to both collagen and laminin.

It is still unclear how the specificity of integrin-ligand interaction is determined; whether it is within the short peptide motif itself, or whether integrins make multiple contacts with different regions of the ECM ligand and the combination of contacts gives the specificity. The proposal that a combination of contacts may be required for maximum binding is supported by the presence of a synergy site in FN that co-operates with the RGD site in integrin binding (Aota, S. *et al.*, 1994;

Obara, M. *et al.*, 1988), although it is unknown whether such synergy sites are found in other ECM components.

1.9.2. Integrin active sites.

3 distinct regions of integrin subunits have been found to participate in ligand binding (Figure 1.5), although as yet the relative contributions of these three sites is unknown. The first is the A-domain/I-domain (found in some α subunits), the second are the EF hand like domains, a conserved repeat found in all α subunits, and the third is the putative A-domain (containing two identified ligand binding regions) found in β subunits. Each of these regions also corresponds to the location of cation binding sites composed of a consensus DxSxS sequence (Figure 1.5). This is interesting given the requirement for cations in integrin mediated adhesion to ECM ligands.

The αA-domain region was first thought to be involved in ligand binding due to its homology with the vWF A domain, which was known to mediate protein-protein interactions with collagen. The role for αA-domains (where present), in ligand binding has been directly shown for the α_L, α_M, and α_2 integrins, by the ability of recombinant A-domains to bind to ECM components (Diamond, M.S. *et al.*, 1993; Landis, R.C. *et al.*, 1994; Zhou, L. *et al.*, 1994; Kamata, T. and Takada, Y., 1994b; Tuckwell, D. *et al.*, 1995), and is further supported by the mapping of epitopes of Abs that block integrin-ECM adhesion to this region (Kamata, T. *et al.*, 1994aa). The crystal structure of the A domain in α_M has recently been determined (Lee, J.O. *et al.*, 1995), part of which was termed the MIDAS region (metal ion dependent adhesion site), which contained a metal binding pocket composed of a DxSxS sequence and two distal amino acids.

The EF hand-like cation binding domains of the α subunit and two small regions of the βA-domain (Figure 1.5), were found to be important in integrin-ligand interaction by chemical cross linking of acidic ECM recognition peptides and also by mutagenesis studies (Charo, I.F. *et al.*, 1991; D'Souza, S.E. *et al.*, 1988; Smith, J.W. and Cheresh, D.A., 1988; D'Souza, S.E. *et al.*, 1990; Smith, J.W. and Cheresh, D.A., 1990; Charo, I.F. *et al.*, 1991; D'Souza, S.E. *et al.*, 1991). The βA-domain is so called because

of its sequence and predicted structural homology to the αA-domain, containing a MIDAS cation binding motif of the consensus sequence DxSxS, immediately followed by a ligand binding region which has been shown to bind RGD (Pasqualini, R. *et al.*, 1995).

1.9.3. Role of divalent cations in integrin-ligand interaction.

Divalent cations are known to be essential for integrin interaction with ligand. Initial theories advocated a direct role in binding, by virtue of the cation being co-ordinated between integrin and ligand by the acidic residue in the ECM binding motif, in addition to the residues in the integrin DxSxS metal binding pocket (Edwards, J.G. *et al.*, 1988). However, the current theory is that cations are not directly involved in ligand binding (Kamata, T. and Takada, Y., 1994b), but mediate the formation of a ternary complex containing the cation, integrin and ligand, and are then displaced when binding actually occurs (D'Souza, S.E. *et al.*, 1994).

Divalent cations are also thought to induce changes of integrin conformation, and thereby regulate binding competence (Dransfield, I. and Hogg, N., 1989; Dransfield, I. *et al.*, 1992). This is also important for their role in integrin-ligand interaction. Different divalent cations have different effects on ligand binding ability. In general Mg^{2+} activates ligand binding and Mn^{2+} further activates it, but Ca^{2+} inhibits ligand binding (Dransfield, I. *et al.*, 1992; Masumoto, A. and Hemler, M.E., 1993). It is thought that these different effects may be mediated by the presence of multiple functional cation binding sites on integrins, and differing affinities of cations for these sites (Smith, J.W. *et al.*, 1994; Mould, A.P. *et al.*, 1995).

1.10. Mechanisms of integrin activation/ Control of integrin mediated adhesion.

The ability of integrins to bind ligand can be altered without changing the number of integrins expressed on the surface of cells. This ability is known to be vital for processes such as the inflammatory response, but may also play a role in other biological processes such as development, cell proliferation, differentiation, motility, and even tumour

progression. Integrin activation and increased adhesion usually occurs when a conformational change is induced in the extracellular domain of the integrin giving it a higher affinity for ligand (Kouns, W.C. *et al.*, 1990; O'Toole, T.E. *et al.*, 1990; Sims, P.J. *et al.*, 1991). These conformational changes can either be induced by extracellular stimuli, or by inside-out signalling from intracellular stimuli.

1.10.1. Extracellular Stimuli.

Extracellular stimuli which activate integrins can be divalent cations such as Mg^{2+} or Mn^{2+} as described above, or so called "activating" Abs. These Abs (for example the β_1 antibodies TS2/16 or 8A2, Arroyo, A.G. *et al.*, 1992; Kovach, N.L. *et al.*, 1992; Wayner, E.A. and Kovach, N.L., 1992; Wiel, v.d. *et al.*, 1992; Arroyo, A.G. *et al.*, 1993) recognise epitopes that are expressed on integrins in the resting state, and mediate a direct change in conformation of the integrin to an activated state with an increased affinity for ligand. Interestingly, in the β_1 integrin subunit it has been shown that the epitopes for such activating Abs lie in the βA-domain, at residues 207-218, between the two putative ligand binding sites (Takada, Y. and Puzon, W., 1993) (Figure 1.5). The fact that domains of integrins involved in ligand binding, cation binding and activation, are all localised to the same region (the βA-domain), is interesting in terms of the interdependence of these processes in the regulation of integrin adhesive function. Many inhibitory Abs of the β_1 integrin also map to this region (Takada, Y. and Puzon, W., 1993), presumably thereby enabling them to interfere with the integrin-ligand interaction. The protein sequence predicts this region to contain a β-bend, which is thought to allow the polypeptide chain to reverse sharply in orientation and thus potentially mediate the conformational change (Takada, Y. and Puzon, W., 1993). Further studies into the mechanism of conformational changes have been enabled by the discovery of other "reporter" Abs that either recognise neo-epitopes exposed after the conformational change associated with activation, or epitopes exposed only after ligand binding.

To explain the regulation of integrin activation it is hypothesised that integrins are constantly changing states between inactive and active conformations (i.e. in a state of conformational equilibrium); and that addition of an activator such as an activating Ab or divalent cation,

pushes the equilibrium towards the active state, and adhesion thereby increases. It must be borne in mind though that the resting state of integrins (i.e. the "inactive" state), will vary a lot between different cell types; for example on circulating platelets, the $\alpha_{IIb}\beta_3$ integrin shows very little adhesion to fibrinogen until integrin function is activated (Phillips, D.R. *et al.*, 1991; Savage, B. and Ruggeri, Z.M., 1991; Savage, B. *et al.*, 1992), whereas the β_1 integrins on epithelial cells that mediate interaction to ECM ligands have a high level of basal adhesion which can be further activated by treatment with Abs or divalent cations.

1.10.2. Intracellular stimuli and inside-out signalling.

For inside-out signalling, intracellular molecules must interact with regions of the integrin cytoplasmic domains that enable the transduction of signals from the intracellular to the extracellular domains, and thereby regulate the integrin binding affinity. Analysis of various integrin subunits has identified sites on both α and β cytoplasmic domains that are important for the regulation of ligand binding affinity. On the β_3 and β_2 subunits, a site at the C-terminus has been identified (Hibbs, M.L. *et al.*, 1991; O'Toole, T.E. *et al.*, 1994), and a site essential for ligand binding has also been identified in the β_1 subunit cytoplasmic domain (O'Toole, T.E. *et al.*, 1995). Truncations of some α subunits have shown that it is sequences C-terminal of a conserved GFFKR motif that are important in mediating adhesion (Shaw, L.M. and Mercurio, A.M., 1993; Kawaguchi, S. and Hemler, M.E., 1993; Filardo, E.J. and Cheresh, D.A., 1994), and that the GFFKR motif itself may be responsible for holding the integrins in an inactive state (O'Toole, T.E. *et al.*, 1994).

The current model for the regulation of the binding affinity of integrins by inside-out signalling, predicts the presence of cytoplasmic co-factors, whose synthesis, mobilisation, modification (e.g. by phosphorylation) and degradation are controlled in a cell specific way. Binding of these co-factors to specific regions in the cytoplasmic domains of integrin molecules is predicted to induce an energy dependent conformational change, which is then transferred to the extracellular domain and leads to the opening or closing of the ligand binding pocket (O'Toole, T.E. *et al.*, 1994).

1.10.3. Increasing integrin avidity.

Regulation of integrin avidity is another mechanism by which adhesiveness can be changed. This is thought to be mainly associated with events that involve integrin-cytoskeletal interactions; including organisation of the cytoskeleton, clustering integrins at specialised adhesion sites, and integrin-mediated cell spreading. These events generally occur after the initial ligand-receptor interaction has taken place, and are therefore sometimes referred to as "post receptor occupancy events" (Danilov, Y.N. and Juliano, R.L., 1989; Sanchez, M.P. *et al.*, 1993; Faull, R.J. *et al.*, 1994). The stimulation of integrin-mediated adhesion by phorbol esters (such as PMA) via the activation of the protein kinase C (PKC) pathway, is an example of a process that is now thought to occur through the alteration of integrin avidity rather than affinity (Danilov, Y.N. and Juliano, R.L., 1989; Faull, R.J. *et al.*, 1994).

1.11. Integrins and signal transduction.

Integrins are bone-fide mediators of signals from the extracellular domain to the inside of cells. This is shown by the fact that integrin activation, either by mAbs or ligand, leads to the tyrosine and serine phosphorylation of proteins (Guan, J.L. *et al.*, 1991; Kornberg, L.J. *et al.*, 1991; Kapron, B.C. *et al.*, 1993), an increase in intracellular pH (Schwartz, M.A. *et al.*, 1991), Ca^{2+} influx (Schwartz, M.A., 1993), a change in phosphoinositide turnover (McNamee, H.P. *et al.*, 1993), and changes in gene expression (Werb, Z. *et al.*, 1989). Understanding the mechanism of integrin signal transduction is an extremely important and expanding area of current research. Some important points are outlined below, but for more detailed reviews on integrin mediated signal transduction see Clark, E.A. and Brugge, J.S., 1995; Schwartz, M.A. *et al.*, 1995.

Immunofluoresence studies indicate that on complexing with ligand, integrins are redistributed to form clusters. This clustering is thought to serve two main purposes. The first is to increase the avidity/strength of the mechanical interaction between cell and ligand. The second is to sequester the cytoplasmic domains of integrins into focal adhesions, which form the sites of interaction between ECM, multiple cytoskeletal proteins, and signalling molecules. Such focal adhesions are thought to

mediate downstream signal transduction in addition to maintaining cell shape and structure (Sastry, S.K. and Horwitz, A.F., 1993; Geiger, B. *et al.*, 1995).

The cytoplasmic domains of integrins are capable of modulating activation of integrins (see above), and of directing integrins to focal adhesions (mediated by the β subunit (LaFlamme, S.E. *et al.*, 1992; Reszka, A.A. *et al.*, 1992). However, the absence of any obvious catalytic domains in integrin cytoplasmic tails, suggested that in order to mediate the changes in phosphorylation of proteins observed on integrin ligation, integrins would have to recruit tyrosine and serine kinases. Two such kinases have been identified; the first being FAK (a tyrosine kinase Zachary, I. and Rozengurt, E., 1992), and the second being the recently discovered "integrin linked kinase" (ILK), which is a novel 59 kDa serine/threonine kinase (Hannigan, G.E. *et al.*, 1996). Little is known as yet about the role of ILK in integrin signalling, but the association of FAK with integrin cytoplasmic tails is thought to be one of the first events that occurs in integrin signalling after integrin clustering (Miyamoto, S. *et al.*, 1995). Induced autophosphorylation of FAK is then thought to enable the recruitment of other cytoplasmic effectors of signal transduction (such as pp60src and Grb-2), via interactions of the SH2 domains of the effector molecules with the phosphorylated residue in FAK and the phosphorylated residues in other molecules of the complex as it is built up (Schlaepfer, D.D. *et al.*, 1994; Clark, E.A. and Brugge, J.S., 1995; Richardson, A. and Parsons, J.T., 1995). The recruitment of a significant number of such effector proteins to the focal contact (by complex protein-protein and protein-phosphoprotein interactions) is then proposed to lead to the activation of downstream signal transduction pathways. For example, the MAP-K pathway via ras is activated (Kapron, B.C. *et al.*, 1993; Schlaepfer, D.D. *et al.*, 1994; Chen, Q. *et al.*, 1994), thereby providing a link from integrins to the nucleus and hence a potential to control gene expression. Also, other pathways dependant on protein phosphorylation, such as the activation of PLC-γ and hence PKC (known to be involved in the modulation of cytoplasmic pH) and Ca^{2+} mobilisation pathways are postulated to be activated (for review see Richardson, A. and Parsons, J.T., 1995).

1.12. Effect of oncogenes on the expression of epithelial specific molecules and integrins.

If changes in E-cadherin and the $\alpha_2\beta_1$ integrin are important for loss of tissue morphology and malignant progression of breast cancers, it is important to know how the loss of function, or down regulation of these molecules is induced. Oncogenes are obvious candidates, particularly with the observation that transformation of epithelial cells with oncogenes *in vitro* often leads to conversion of cells to an undifferentiated fibroblastic/mesenchymal phenotype.

Proto-oncogenes form normal components of signal transduction pathways, from cell surface receptors (e.g. c-erbB2 and EGF-R), through intracellular kinases (e.g. c-src, ras), to transcription factors such as c-fos and c-jun. It is therefore possible to speculate that constitutively active or overexpressed oncogenes at any point in signal transduction pathways contribute to tumour progression by ultimately inducing constitutive activity of c-fos (or some other transcription factor), that in turn alters expression of epithelial and mesenchymal specific proteins, leading to an undifferentiated and more aggressive cell phenotype.

Overexpression of representative proto-oncogenes from each point of the signal transduction pathway described above, have been shown to induce epithelial-mesenchymal transition in *in vitro* studies involving MDCK or mammary epithelial cells. For example, expression of *c-erb*B2 (D'Souza, B. *et al.*, 1993), *bcl*-2 (Lu, P.J. *et al.*, 1995), *v-src* (Behrens, J. *et al.*, 1993), *c-fos* (Reichmann, E. *et al.*, 1992) and activated *ras* oncogenes (Kinch, M.S. *et al.*, 1995), can all lead to epithelial-mesenchymal conversion.

Such converted epithelial cells show much reduced intercellular contacts, implying that cell adhesion systems have been affected, and also suggesting that oncogene expression may indeed be upstream regulators of adhesion molecules. For this reason, most investigations into the effects of oncogene overexpression focus on studying changes in the level of expression and function of epithelial specific molecules involved in cell-cell adhesion (such as E-cadherin and ZO-1), but not the integrins. However, changes in the levels of expression of integrins in

the epithelial-mesenchymal transition have been examined in a mammary system, where the oncogenes *bcl-2* and *c-erbB2* have been overexpressed in normal human mammary epithelial cells (D'Souza, B. *et al.*, 1993; D'souza, B. and Taylor, P.J., 1994; Lu, P.J. *et al.*, 1995). In both cases the levels of the α_2 integrin (but interestingly not the α_3 subunit), were found to be markedly reduced, as well as the level of E-cadherin.

Interestingly, with the *bcl-2* transfectants, the highest expressors, and hence the cells where epithelial-mesenchymal conversion was most pronounced, also showed high level expression of vimentin. Vimentin is not only a mesenchymal marker but has been shown *in vitro*, (Sommers, C.L. *et al.*, 1991), and *in vivo*, (Domagala, W. *et al.*, 1990), to be a marker of more aggressive tumours. The distribution of ZO-1 (a tight junction component) was also found to be irregular in the *bcl-2* transfectants, showing that the polarity of the cells and the junctional complexes had been disrupted.

In the activated ras induced epithelial-mesenchymal conversion (Kinch, M.S. *et al.*, 1995), the level of the $\alpha_2\beta_1$ integrin was not reported, but unlike the situation with c-erbB2 or bcl-2, the E-cadherin expression level was unchanged. However, E-cadherin expression was less organised, and on further analysis it was shown that the associations between E-cadherin, β-catenin and the cytoskeleton were disrupted. This loss of association was thought to be due to phosphorylation of β-catenin induced by transformation. In this case the epithelial-mesenchymal conversion could be explained by loss of E-cadherin function rather than expression. This interpretation was further supported by the observation that tyrosine kinase inhibitors could re-establish the association of β-catenin with E-cadherin and revert the cells to the epithelial phenotype.

Other evidence to support the idea that an increase in phosphorylation of proteins, and in particular the catenins, could play a central role in epithelial-mesenchymal conversion is provided by the action of growth factors such as hepatocyte growth factor and epidermal growth factor, which promote epithelial-mesenchymal conversion and "scatter" certain epithelial cells. These growth factors stimulate phosphorylation of many proteins which have interestingly been shown to include β-catenin and plakoglobin (Shibamoto, S. *et al.*, 1994).

These studies show that there are at least two possible mechanisms by which oncogenes can act to affect cell adhesion molecules and other epithelial markers downstream. The first is to alter the expression of these molecules at the RNA and protein level, as in the studies with c-erbB2 and bcl-2, where E-cadherin and $\alpha_2\beta_1$ are reduced and vimentin is increased (D'Souza, B. *et al.*, 1993; D'souza, B. and Taylor-Papadimitriou, J., 1994; Lu, P.J. *et al.*, 1995). The second mechanism is to alter the function of adhesion molecules, by inducing, for example, post translational modification of proteins as seen in the ras studies (Kinch, M.S. *et al.*, 1995), where phosphorylation of β-catenin led to its dissociation from E-cadherin and loss of E-cadherin function.

1.13. Tumour progression - Invasion and metastasis.

For the initial stages of both invasion and metastasis, tumour cells must clearly become more motile. Individual tumour cells or a group must become able to detach from the primary tumour, and invade through the basement membrane (if still present), and the underlying stroma (probably also requiring the secretion of proteases). This apparent requirement for an increase in motility, production of proteases and a reduction in adhesion of a subset of tumour cells to the primary tumour, has suggested roles for motility factors such as HGF. Loss of adhesion molecules such as integrins and E-cadherin may also be implicated in invasion and metastasis.

1.13.1. Role of E-cadherin in invasion and metastasis.

E-cadherin has not as yet been shown to play a direct role in the control of cell motility or protease secretion. However, it is thought that a reduction in the expression/function of E-cadherin or the associated catenins, which play such a vital role in cell-cell adhesion, is a common mechanism by which tumour cells acquire a more invasive and metastatic phenotype.

The facts that E-cadherin expression correlates positively with degree of differentiation in invasive ductal breast carcinomas, that a complete loss or reduction in E-cadherin expression is often seen in invasive lobular

carcinomas (discussed above), and that undifferentiated tumours with reduced expression of E-cadherin and α-catenin are more likely to metastasise (Oka, H. *et al.*, 1993; Takayama, T. *et al.*, 1994), support the theory that normal expression of E-cadherin is important not only to maintain differentiation but also to prevent invasion and metastasis. Moreover, these *in vivo* observations have been supported by *in vitro* studies which show that the expression level of E-cadherin can be an indicator of the invasive capacity of breast cancer cells, and that the invasive capacity of certain breast cancer cells can be reduced by expressing the E-cadherin gene (Frixen, U.H. *et al.*, 1991).

1.13.2. Role of integrins in invasion and metastasis.

Changes in levels or function of integrins are thought to play a role in the processes of invasion and metastasis because of their proposed function in cell motility and adhesion. By far the most common change observed in breast carcinomas is the down regulation of integrin subunits that are normally expressed at high levels (see earlier); indeed down-regulation of the α_2 integrin has been shown to be associated with loss of tumour differentiation (see earlier) and increased metastatic disease (Arihiro, K. *et al.*, 1993). It is quite conceivable that this reduction in integrin expression makes the cells of the tumour less adhesive to each other, less tightly anchored to the ECM, and therefore more prone to separation from the primary tumour mass and invasion.

While it is likely that down regulation of cartain integrin function is important for the early stages of invasion, at later stages of metastasis, where cells need to re-attach to other cells and matrices, in order to extravasate blood or lymph and re-establish the tumour at the new site, increased adhesion to various ligands and therefore increased expression of other integrins may be required. This would explain data whereby experimental metastases can be reduced by treatment of carcinoma cells with anti-integrin antibodies. For example, in a study by Newton, S.A. *et al.*, 1995, experimental metastasis were significantly reduced by pre-treatment of MDA-MB-231 cells with anti-α_5 or β_1 but not α_2 integrin Abs, before being tail vein injected into athymic nude mice.

The role of integrins in motility on ECM components is related to their ability to modulate adhesion to substrate and, therefore, provide a means of traction by controlling anchorage. Indeed, when motility of epithelial/carcinoma cells is stimulated by a motility factor such as HGF, integrins are found to be re-localised to focal contacts, along with FAK and cytoskeletal components (Matsumoto, K. *et al.*, 1994). Moreover, the fact that epithelial cell motility is inhibited on ECM by blocking Abs to certain integrin subunits (Berdichevsky, F. *et al.*, 1992; Chen, J.D. *et al.*, 1993) strongly implicates a functional role for integrins in the motility process. It is clear that a balanced, intermediate level of adhesion must be attained for maximum cell movement, and that either too great or too little adhesion to substrate would inhibit this process, as shown to be the case in smooth muscle cells (DiMilla, P.A. *et al.*, 1993).

Interestingly, in a recent study (Tremble, P. *et al.*, 1995), it was shown that engagement of integrins with ligand could transduce signals that lead to the controlled stimulation of transcription of proteolytic enzymes such as collagenase, stromelysin-1 and the 92 kDa gelatinase via specific elements in the promoter regions of these protease genes. This finding not only has important implications for the role of integrins in controlled protease secretion in developmental processes, but could also mean that changes in integrin function, such as those that occur in breast cancer, could lead to the loss of control of protease production, and hence aid the processes of invasion and metastasis.

1.13.3. Role of the motility factor, HGF in invasion and metastasis.

The stimulation of motility and cell separation ("scattering") of normal epithelial cells by HGF is well documented, and has been discussed earlier in this introduction. A similar stimulation of motility and cell separation in tumour cells, could therefore stimulate their invasion into, and dissemination through the stroma. Indeed, it has been shown in a number of *in vitro* systems, that HGF can stimulate the invasion of tumour cells into collagen (a major stromal component), or basement membrane-like matrices, (Nakayama, Y. *et al.*, 1993;Weidner, K.M. *et al.*, 1990). Moreover, a role for HGF in breast cancer *in vivo* is suggested by the fact that a significant proportion of breast cancer patients have increased levels of HGF present in their serum, or in tissue extracts, and

that a high level of expression of HGF is an even more significant factor in predicting a shorter relapse free and overall survival, than is lymph node status (Yamashita, J. *et al.*, 1994). High levels of HGF also show a significant association with invasive and metastatic disease (Taniguchi, T. *et al.*, 1994; Taniguchi, T. *et al.*, 1995).

HGF has been shown to stimulate the secretion of proteases such as urokinase plasminogen activator (uPa) and induce the increased expression of the uPa receptor by MDCK epithelial cells (Pepper, M.S. *et al.*, 1992). If a similar stimulation occurred in breast carcinoma cells *in vivo* (as it does in human sarcoma and renal cancer cell lines*in vitro*, where it is also associated with increased invasiveness, (Nakayama, Y. *et al.*, 1993; Jeffers, M. *et al.*, 1996), then this could play a vital role in tumour cell invasion, by stimulating focal degradation of ECM. Indeed, in breast cancer patients, the presence of high levels of uPa in tumours is used as an indicator of poor prognosis in node negative patients (Janicke, F. *et al.*, 1993).

When considering a potential role for HGF in stimulating the invasion of tumour cells, it is relevant to consider the cellular source of HGF. In the normal situation HGF is not secreted by epithelial cells, but only by mesenchymal cells such as fibroblasts or adipocytes, both of which are found in the breast and have been shown to produce HGF both *in vivo* and *in vitro*, (Rahimi, N. *et al.*, 1994; Sasaki, M. *et al.*, 1994; Tuck, A.B. *et al.*, 1996). It is therefore possible that production of HGF could occur either from mesenchymal cells within the breast, or alternatively, changes could occur within the carcinoma cells themselves to allow HGF secretion and action by an autocrine mechanism. In a recent study (Tuck, A.B. *et al.*, 1996) HGF mRNA was detected not only in the stromal mesenchymal cells of the breast, but additionally in both benign and malignant epithelium. Also, in examples of IDC the leading edge of the tumour often had strong expression of both c-met and HGF.

The question as to the source of the HGF in breast cancers can be addressed *in vitro* by analysis of HGF secreted by breast cancer cell lines. Secretion of HGF by breast carcinoma cells *in vitro* is not common, and the first example was found only recently where a murine mammary carcinoma cell line called Sp1 was found to secrete HGF and stimulate

autocrine invasion into basement membrane (Rahimi, N. *et al.*, 1996). In general therefore, it is thought possible that either paracrine or autocrine mechanisms could mediate the effect of HGF on carcinoma cell invasion, although autocrine stimulation is more likely to be developed later in tumorigenesis because it requires secretion of HGF by epithelial carcinoma cells which is not a normal event.

It should be noted that although HGF is a good candidate to play an important role in mammary gland development, it could only play this role by having its secretion tightly controlled, and ultimately turned off once development is complete. In order for carcinoma cells to harness HGF for invasion and metastasis, HGF production would have to be re-stimulated in an uncontrolled way. Although this undoubtedly occurs, factors responsible for this induction have not yet been identified but are an important consideration, possibly in terms of therapy.

1.14. Aims of the thesis project.

It is known that branching morphogenesis of epithelial cells is an important part of the development of the mammary gland *in vivo*, and that the underlying mesenchyme plays a vital role in this process. Moreover, in an *in vitro* morphogenesis system developed to study branching morphogenesis in the MDCK kidney cell line, a fibroblast secreted molecule known as HGF was identified as playing an important role in branching morphogenesis. Hence, HGF is a good candidate for a mesenchymal factor involved in branching morphogenesis in the mammary gland. An initial aim of this project was to set up an *in vitro* system, whereby non-tumorigenic human mammary epithelial cells could be cultured within an extracellular matrix component such as collagen type I, and branching morphogenesis could be stimulated by fibroblast factors. This system could then be used to establish whether HGF could stimulate branching morphogenesis in a mammary system, and if any other fibroblast secreted factors with a similar role could be identified.

Loss of tissue architecture is one of the first changes that occurs in the development of breast cancers *in vivo*. Changes in the $\alpha_2\beta_1$ integrin have been implicated as having an important role in the loss of tissue

architecture, and, therefore, in the process of normal morphogenesis. The second aim of this project was to use the *in vitro* morphogenesis system developed, to establish whether or not the $\alpha_2\beta_1$ integrin (and indeed other integrins) played a role in morphogenesis of mammary epithelial cells. It was also hoped that developing an *in vitro* system whereby roles for motility factors such as HGF, and adhesion molecules such as integrins in normal cell morphogenesis could be analysed, would also give an insight as to the potential role of both these types of molecules in the processes of invasion and metastasis.

Finally, a functional role for integrins has been implicated in the anchorage dependent growth of normal cells. It is thought possible that bypassing integrin mediated growth signals may be a means by which tumour cells can become capable of anchorage independent growth. The overexpression of oncogenes is known to transform cells into being anchorage independent, and has also been shown to reduce the expression of the $\alpha_2\beta_1$ integrin. It is proposed that the roles of integrins in cell growth and the effect of oncogenes on the processes of growth and morphogenesis could also be examined in such an *in vitro* system.

CHAPTER 2: MATERIALS AND METHODS.

MATERIALS.

2.1. Chemicals and Solvents.

All chemicals used were of analytical grade (or tissue culture grade if more appropriate), and were obtained from either BDH or Sigma Chemicals Ltd, except where otherwise stated.

2.2. Radiochemicals.

All radiochemicals were obtained from Amersham International.

2.3. Enzymes.

Restriction endonucleases were obtained from New England Biolabs, Boehringer Mannheim or Promega.

2.4. Cell Culture flasks and plates.

These were obtained from Nunc or Falcon.

2.5. Cell Culture Media

E4 (DMEM), 2x E4, E4 minus phenol red, E4 minus Ca^{2+} and Mg^{2+}, and RPMI were made up and supplied through the ICRF media service.

Fetal calf serum	Gibco Life Sciences

2.6. Miscellaneous

Fuji Medical X-ray film	Fuji, Japan.
Other film	Kodak
Filtration units (0.2 and 0.45 μm)	Nalgene
Geneticin (G418)	Gibco-BRL
Jetsorb Kit	Genomed, (UK agent - ams Biotechnology)

ECL Kit	Amersham
Hybond-N+ and C	Amersham
Citifluor	Chem. Labs, Canterbury
Agarose	FMC Bioproducts
Sephadex beads	Pharmacia
Protein A-Sepharose	Pharmacia

2.7. Buffers and solutions.

All solutions were prepared using sterile de-ionised water and stored at room temperature unless otherwise stated. Solutions were usually sterilised by autoclaving, or by filtering through a 0.22µm filter unit as appropriate.

2.7.1. Cell Culture

Trypsin/Versene mix for detaching cells:	Trypsin (0.25% in Tris Saline) and Versene (EDTA, 0.2g/l in PBSA) stocks produced by the Media Production Unit at the ICRF. 4 mls of the Trypsin stock were mixed with 16 mls of the Versene stock to give the trypsin/versene mix with which cells are detached. This gives a final trypsin concentration of 0.05% and an EDTA/versene concentration of 0.16g/l.
Collagenase buffer:	130 mM NaCl 10 mM $CaCl_2$ 20 mM Hepes pH 7.2
TEGPED:	0.04% Trypsin 0.1% pancreatin 0.004% Versene 0.25% EGTA
Heat inactivated BSA:	BSA was dissolved in PBSABC and heated at 56°C for 30 minutes.

2.7.2. Protein methods

2x SDS PAGE sample buffer :	4% SDS 20% glycerol 0.125 M Tris-HCl pH 6.8 0.2% Bromophenol blue +/- 0.2M dithiothreitol (DTT)
10x SDS PAGE Running buffer:	0.25 M Tris 1.92 M Glycine 1% SDS
Western blotting transfer buffer:	25 mM Tris 190 mM glycine 20% methanol
Buffer P:	20 mM Tris (pH 7.5) 5 mM $MgCl_2$

2.7.3. DNA methods.

ψa medium:	5g/l Bacto Yeast Extract 20g/l Bacto Tryptone 5g/l $MgSO_4$ 0.75g/l KCl Adjust to pH 7.6 with KOH 14g/l Bacto Agar (produced by Media Production Unit at the ICRF).
ψb medium:	ψa medium without the agar

Tfb1 buffer:	30 mM potassium acetate
	100 mM $RbCl_2$
	10 mM $CaCl_2$
	50 mM $MnCl_2$
	15% glycerol
	Adjust to pH 5.8 with 0.2M acetic acid
	sterilise by filtration
Tfb2 buffer:	10 mM MOPS
	75 mM $CaCl_2$
	10 mM $RbCl_2$
	15% glycerol
	Adjust to pH 6.5 with KOH
	sterilise by filtration
L-Broth:	10g/l Bacto Tryptone
	5g/l Bacto yeast extract
	10g/l NaCl
	(produced by Media Production Unit at the ICRF)
L-Agar:	L-Broth with 15g/l Bacto Agar
DNA preparation solution A:	50 mM glucose
	25 mM Tris (pH 8.0)
	10 mM EDTA
	5 mg/ml lysozyme
DNA preparation solution B:	5M KOAc
	Glacial acetic acid (115 ml/l)
10x TBE:	0.89M Trizma base
	0.89M Boric acid
	10mM EDTA pH 8.0

DNA sample buffer (5x): 25%(w/v) Ficoll
0.5% (w/v) SDS
50mM EDTA
0.025% (w/v) Bromophenol Blue
0.025% (w/v) Xylene cyanol

OLB buffer:

Solution O: 1.2M Tris-HCl pH 8.0
0.125M $MgCl_2$
(store at 4°C)

Solution A: 1 ml of solution O
18µl β-mercaptoethanol
5µl each of dATP, dTTP, dGTP
(each triphosphate dissolved in TE pH 7.0 at a concentration of 0.1M)
Aliquot and store at -20°C

Solution B: 2M Hepes pH 6.6
(pH with 4M NaOH)

Solution C: Hexadeoxyribonucleotides evenly suspended in TE at 90 OD units/ml
(obtained from Pharmacia)

OLB is made by mixing solutions A:B:C in a ratio of 100:250:150.
stored in aliquots at -20°C

TE: 10mM Trizma base
1mM EDTA
(adjusted to the appropriate pH)

2.7.4. RNA methods

20x SSC: 3M NaCl
0.3M Tri-Sodium Citrate

Depc treated water: Add 1µl Depc (diethyl pyrocarbonate)/ml of distilled water, then autoclave

LSGD buffer for RNA extraction:
4M guanidine thiocyanate
25 mM sodium citrate
1% β-mercaptoethanol - (added just before use)

10X MOPS:
0.2M MOPS (3-[N-Morpholino] propane-sulfonic acid)
50 mM sodium acetate
800 mls Depc water
adjust to pH 7.0 with NaOH
add 10 mM EDTA pH 8
make up to 1 litre with Depc water, filter through 0.22µm filter to sterilise and store in the dark at room temperature

RNA sample buffer:
1 mM EDTA (pH 8)
0.25% bromophenol blue
50% glycerol
aliquot and store at -20°C

Deionised Formamide: Formamide was stirred with AG 501-X8 mixed bed resin (Biorad) for 1 hour and then filtered twice through Whatmam N1 paper and stored in aliquots at -20°C.

Methylene blue staining solution:
0.03% methylene blue
0.3M sodium acetate (pH 5.2)

1M sodium phosphate buffer (pH 7.2):
68.4 mls 1M Na_2HPO_4
31.6 mls 1M NaH_2PO_4

Northern blot prehybridisation buffer:
0.2M sodium phosphate buffer (pH 7.2)
1mM EDTA
7% SDS
45% formamide
made up to appropriate volume with Depc water and stored in aliquots at -20°C
250µg/ml salmon sperm DNA (added just before use)

2.7.5. Other buffers and solutions.

PBSA:
137mM NaCl
3.4mM KCl
20mM Na_2HPO_4
1.8mM KH_2PO4
pH 7.2

PBSB:
36mM $CaCl_2$

PBSC:
20mM $MgCl_2$

All these forms of PBS produced by Media Production Unit at the ICRF. To obtain complete PBS 400mls of PBSA is mixed with 10 mls each of PBSB and PBSC.

2.8. Antibodies.

Integrin Antibodies.

All integrin Abs used were purified mouse monoclonals unless otherwise stated.

Name	Integrin subunit specificity	Source of Antibody	Functionality [1] (if applicable to this thesis)
TS2/7	alpha 1	Dr. M. Hemler Dana-Faber Cancer Institute, Boston, MA	n/a. [3]

HAS 3	alpha 2	Dr. F. Watt ICRF, London (Tenchini, M.L. *et al.*, 1993)	Non blocking
HAS 4	alpha 2	Dr. F. Watt ICRF, London (Tenchini, M.L. *et al.*, 1993)	Non blocking
HAS 6	alpha 2	Dr. F. Watt ICRF, London (Tenchini, M.L. *et al.*, 1993)	Non blocking (except of adhesion of keratinocytes to collagen IV)
P1E6	alpha 2 (ascites fluid initially, purified available later)	Chemicon International Inc.	Blocking
5E8	alpha 2	Dr. Bankert Roswell Park Cancer Institute, Buffalo, NY (Zylstra, S. *et al.*, 1986)	Blocking
6F1	alpha 2	Dr. Coller Mount Sinai Medical Centre, New York (Coller, B.S. *et al.*, 1989)	Blocking
AB1936	alpha 2 (rabbit polyclonal)	Chemicon International Inc.	n/a [2]
P1B5	alpha 3 (ascites fluid)	Chemicon International Inc.	Blocking
DH4-B4	alpha 3 (rabbit polyclonal)	Dr. F. Watt ICRF, London	n/a. [2]
J143	alpha 3 (ascites fluid)	Dr. A. Albino Sloan-Kettering Institute, New York, (Fradet, Y. *et al.*, 1984)	n/a. [3]

B5G10	alpha 4	Dr. M. Hemler Dana-Faber Cancer Institute, Boston, MA	n/a. [3]
mAb16	alpha 5 [4]	Dr. K. Yamada NIH, Bethesda, MD	n/a. [3]
GoH3	alpha 6 [4] (tissue culture supernatent)	Dr. A. Sonnenberg University of Amsterdam, Amsterdam	Blocking
L230	alpha v	ATCC	Blocking
MAB1980	alpha v	Chemicon International Inc.	n/a. [5]
MAR 5	beta 1 (ascites fluid)	Dr. M. Colnaghi	Non blocking
P5D2	beta 1	ATCC	Blocking
mAb13	beta 1 [4]	Dr. K. Yamada (Akiyama, S.K. *et al.*, 1989)	Blocking
TS2/16	beta 1	ICRF hybridoma unit	Activating
8A2	beta 1	Dr. N. Kovach University of Washington, Seattle, WA	Activating
3E1	beta 4	Chemicon International Inc.	n/a. [3]

n/a = not applicable.

1. The "functionality" of the integrin Abs refers to their documented ability to affect adhesion of cells to appropriate ECM components:- "Non blocking" meaning no effect, "blocking" meaning an inhibition of adhesion is induced, and "activating" meaning an increase in adhesion is induced.
2. Used for Western blotting
3. Used for immunoprecipitation
4. Derived from rat
5. Used for FACS analysis

Other Antibodies

(mouse monoclonal unless otherwise stated)

HECD-1, anti human E-cadherin, from R&D Systems Europe.
HMFG-1 and HMFG-2, anti polymorphic epithelial mucin antibodies developed in the laboratory of Dr. Joyce Taylor-Papadimitriou.
W632, anti human MHC Class I, supplied by Dr Julia Bodmer, ICRF.
Polyclonal Ab to rhHGF, a gift from the Matsubishi Kasei Corporation (Yokohama, Japan).
C-28 rabbit polyclonal Ab to 140 kDa β subunit of c-met from Santa Cruz Biotechnology Inc.
Anti phosphotyrosine Ab from Upstate Biotechnology Inc, (Cat No 05-321).

Secondary anti immunoglobulin Abs, FITC or peroxidase conjugated, from DAKO.

2.9. DNA probes for Northern blots.

α_2 integrin. Full length α_2 cDNA (~ 5.4kb) cloned into Sal I and Xba I sites of pF neo vector (constructed in the laboratory of Dr. R. Germain). Provided by Dr M. Hemler, Dana-Faber Cancer Institute, Boston, MA.
α_3 integrin. (Clone 3.38S). 800 bp EcoRI fragment in pGEM-4 (Promega) vector. Provided by Dr M. Hemler.
α_v integrin. 2.0 kb Sac I fragment. Dr J. Loftus, Scripp's Institute, CA.
MUC1. "Clone 7" - 420 bp fragment from the laboratory of Dr J. Taylor-Papadimitriou, ICRF.
18S RNA. 200 bp Pst I fragment in p100D9 vector.

2.10. Growth factors.

Recombinant human hepatocyte growth factor (rhHGF) was a gift from the Mitsubishi Kasei Corporation, Yokohama, Japan.
Keratinocyte growth factor (KGF) from Promega.
Acidic fibroblast growth factor (aFGF) from AMS Biotechnology.
Basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) from Amersham.
Transforming growth factor β and transforming growth factor α from Calbiochem.
Epidermal growth factor (EGF) from Sigma.
Neu differentiation factor (NDF), a gift from Professor W. Birchmeier

2.11. Cell lines and standard growth medium.

Epithelial cell lines:

MTSV1-7	Immortalised non tumorigenic mammary epithelial cell line (human). Luminal epithelial cells from human milk were immortalised by SV40 large T Ag transfection. Grown in E4 + 10% FCS + 5 µg/ml hydrocortisone, + 10 µg/ml insulin. (Bartek, J. *et al.*, 1991)
HB2	Subclone of MTSV1-7. Grown in same medium. (Berdichevsky, F. *et al.*, 1994b).
ce1	MTSV1-7 transfected with and overexpressing the *c-erbB2* oncogene, co-transfected with a plasmid conferring neomycin resistance. Also a control cell line, MTSV1-7 neo, transfected with the neomycin resistance plasmid only. Grown in the same medium as MTSV1-7 supplemented with 500 µg/ml G418 (D'Souza, B. *et al.*, 1993).
HB2 ras	HB2 transfected with and overexpressing the *v-Ha-ras* oncogene, co-transfected with a plasmid conferring neomycin resistance. Also a control cell line, HB2 neo, transfected with the neomycin resistance plasmid only. Grown in the same medium as MTSV1-7 supplemented with 500 µg/ml G418 (Ye, J.P. *et al.*, 1996).
MDCK	Madin-Derby Canine Kidney, grown in E4 + 10% FCS.

Fibroblast cell lines:

MRC-5	human embryonic lung, grown in E4 + 10% FCS.
Swiss 3T3K	murine, grown in E4 + 10% FCS.
HFF	human foreskin fibroblasts, grown in E4 + 10% FCS.

All cell-lines were obtained from cell production at the ICRF, or were generated in Dr Joyce Taylor Papadimitriou's laboratory; apart from HB2 ras and HB2 neo which were generously donated by Dr Paula Pitha Rowe's laboratory.

METHODS.

2.12. Cell Culture methods.

2.12.1. Growth and maintenance of cells.

The cell lines used in this study and their culture media are described above. The cells were maintained in sterile plastic tissue culture dishes or flasks at 37°C in a humidified atmosphere containing 10% carbon dioxide. Monolayers were passaged by trypsinization, which was carried out by rinsing the monolayer twice in versene solution and then incubating with trypsin/versene solution in the incubator at 37°C. After 2-5 minutes cells could be dislodged from the plastic by gentle tapping and were then resuspended in prewarmed complete medium to inactivate the trypsin. The cells that had been resuspended in complete medium could then be reseeded into new flasks. For cell lines which were more stubborn to remove, or for experiments where exposure of cells to trypsin needed to be kept to a minimum, cells were first placed in the incubator at 37°C with versene for 10 minutes (or until the cells started to round up), before adding the trypsin/versene mix which then removed the cells almost immediately.

2.12.2. Storage and recovery of cells.

It was important that cell lines were not maintained for too long in culture. Therefore stocks of early passage cell lines were stored in liquid nitrogen to be recovered when required. Cells were prepared for frozen storage by pelleting at 1300 rpm, resuspending at the desired concentration in "freezing" medium (containing 70% standard growth medium, 20% FCS, and 10% DMSO), and aliquoting into prelabelled cryovials (Nunc, UK). Vials were wrapped in tissue paper to prevent rapid freezing and placed at -70°C overnight before transfer to liquid nitrogen for long-term storage.

Cells were recovered from liquid nitrogen by rapid thawing at 37°C in a water bath. The cells were then washed once in standard growth medium to remove the DMSO and the pellet resuspended in an appropriate volume of medium to seed the cells in tissue culture flasks.

2.12.3. Preparation of cells in a single cell suspension for cell culture experiments.

Some experiments described below require the cells to be at a certain concentration in terms of the number of cells/ml. A single cell suspension was obtained by passing trypsinised cells that had been resuspended in growth medium through a 23G needle several times, after which the cell number/ml was counted using a heamocytometer. The cells could then be diluted in more growth medium to the required concentration for the particular experiment.

2.12.4. Preparation of conditioned medium from cells.

Conditioned medium was usually prepared by the Cell Production unit at the ICRF. Cells were grown to confluency in standard growth medium, then washed twice with PBSA and cultured for 72 hours in serum free medium. The conditioned medium was then harvested, filtered through a 0.2µm filter, and stored at 4°C. For the purposes of protein purification and characterisation the conditioned medium was used in its serum free state (unless stated otherwise). However when used for cell culture experiments it was supplemented with 10% FCS, and if required for the particular cell line, hydrocortisone and insulin to the appropriate concentration. Unless stated otherwise the conditioned medium was used undiluted.

2.12.5. Collagen gel morphogenesis assay.

A preparation of solubilised bovine dermal collagen type I was purchased as Vitrogen 100 from Imperial Laboratories Ltd. Vitrogen 100 is supplied as a solution of solubilised native collagen at a concentration of approximately 3 mg/ml in 0.012N HCl . Vitrogen 100 is 99.9% pure collagen of which 95-98% is collagen type I with the remainder being collagen type III. In order to use the Vitrogen 100 for cell culture assays the solution must be neutralised. This is done by mixing 8 volumes of Vitrogen 100 with 1 volume of sterile 10x PBSA and 1 volume of sterile 0.1M NaOH. If necessary, the pH is then adjusted to 7.4 +/- 0.2 units by the addition of further 0.1M NaOH or 0.1M HCl .

Cells were embedded and grown in collagen gels as follows. Cells were harvested, made into a single cell suspension and counted as described in section 2.12.3. The cells were then diluted to a concentration of $1x10^5$/ml and mixed with neutralised Vitrogen 100 at a ratio of 1:10 to give a final cell concentration of $1x10^4$/ml. This mixture was then plated into 24 well tissue culture plates at a volume of 500 µl/well and placed at 37°C to induce polymerisation of the collagen. After gelation occurred the cells within the gels were cultured either in standard growth medium, in fibroblast conditioned medium (FCM), or in standard medium supplemented with a specified concentration of growth factor, (usually rhHGF which was routinely used at a concentration of 10 ng/ml unless otherwise stated). This was done by adding an equal volume of the appropriate medium on top of the polymerised matrix. Cells within gels were refed with the appropriate fresh medium every 3-4 days.

Morphogenesis was completed after 6-8 days in culture, at which point photographs were taken and/or quantitation of the morphogenetic phenotypes of the colonies that had formed was carried out (see section 2.12.9. below). All morphogenesis assays were set up in duplicate.

2.12.6. Morphogenesis assays using antibodies to rhHGF.

For these experiments the FCM (or the standard medium supplemented with purified rhHGF), was incubated with various concentrations of the polyclonal Ab to rhHGF, or a control Ab HMFG-1, for 4 hours at 4°C before being used to feed cells that had been embedded in collagen gels. Medium used to refeed gels was treated in exactly the same way.

2.12.7. Fibrin gel morphogenesis assay.

Bovine fibrinogen (Calbiochem) was dissolved in serum free E4 (supplemented with hydrocortisone and insulin at the appropriate concentrations if required) to a final concentration of 2.5 mg/ml. This solution was then filtered through a 0.45µm filter. Cells were harvested in exactly the same way as for the collagen gel morphogenesis assay, except that after the trypsin had been inactivated with complete medium containing FCS, the cells were washed twice with serum free medium before they were made into a single cell suspension and counted. This was

done in order to remove the FCS which would initiate premature clotting of fibrinogen if present. (It should be noted that for experiments where morphogenesis in fibrin and collagen was to be compared all the cells were taken through the fibrin gel preparation procedure).

Cells were diluted to $1x10^5$/ml in serum free E4 and mixed at a ratio of 1:10 with the fibrinogen solution to give a final concentration of $1x10^4$ cells/ml. Clotting of the fibrinogen to form fibrin was then initiated by adding a 1/10 volume of human plasma thrombin (made up in serum free E4) to a final concentration of 2.5 U/ml. This cell and fibrinogen mixture was then quickly plated into 24 well plates (500 µl/well) before clotting occurred. Clotting is initiated almost immediately at room temperature, therefore throughout this manipulation the fibrinogen and thrombin stocks were kept on ice, and the cell/fibrinogen mixture was plated into 24 well plates that were also on ice. The plates were placed at 37°C to complete the clotting process and then cells within the gels were fed with appropriate medium, as for the collagen gel assays. As in the case with collagen gels, morphogenesis was completed after 6-8 days in culture and photographs taken using a CONTAX 135 mm camera, connected to a Zeiss IM35 inverted microscope.

2.12.8. Morphogenesis assays using antibodies to integrin subunits.

For these experiments, cells were harvested and prepared for embedding in collagen or fibrin gels as described above. At this point cells were washed twice with E4 and incubated with the appropriate concentrations of integrin antibodies for 15 minutes at room temperature with occasional mixing, or left with no Ab treatment as a control. The cell suspensions (still containing Ab) were then mixed 1:10 with neutralised collagen or fibrinogen and plated as described above. After gelation, cells within gels were fed with the appropriate growth medium unsupplemented with Abs, and also when refed during the assay no further Ab was added. Concentrations of Abs used varied between experiments and are therefore given in the text and figure legends for individual experiments.

2.12.9. Definition of the morphogenetic phenotypes of colonies and photography and quantitation of morphogenesis experiments.

Definition of a "branching colony": Morphogenetic structure must have at least 5 processes extending away from the central cell body; and one of these processes must be at least the length of the cell body diameter.

Definition of a "cyst": Morphogenetic structure which has less than five processes extending away from the central cell body; or a structure which has at least 5 processes extending away from the central cell body but none of these processes fits the criteria defined for a "branching colony" of being at least the length of the cell body diameter.

Definition of a "Dissociated colony": Morphogenetic structure must be composed of at least 5 single cells or small cell clusters, that are completely separated from each other in 3-dimensional space.

Photography

Photographs were taken using a CONTAX 135 mm camera, connected to a Zeiss IM35 inverted microscope. Photographs were taken at either low or high power magnifications of 6.5x and 25.5x respectively.

Quantitation

Quantitation of the morphogenetic phenotype of colonies was carried out between days 6 and 8 of the morphogenesis assay. Morphogenetic colonies (at least 30 colonies in each of the duplicate wells for each condition) were scored into "branching", "cyst" and "dissociated" phenotypes according to the definitions above. The number of colonies of a particular phenotype were then converted into a percentage of total colonies, and the mean of the duplicate wells and the associated standard error of the mean (s.e.m) was calculated. It is this mean value and s.e.m. that is plotted on the graphs presented in this thesis.

2.12.10. Matrigel morphogenesis assay.

Basement Membrane Matrigel™ was purchased from Collaborative Research Incorporated. Matrigel™ is prepared from the Engelbreth-Holm-Swarm (EHS) mouse tumour and is a solubilised tissue basement

membrane which contains laminin, collagen type IV, heparin sulphate proteoglycan, entactin and also naturally occurring growth factors found in the EHS tumour.

The Matrigel was diluted 1:1 in E4 and 100µl plated into the central well of organ culture dishes. Dishes were placed at 37°C for 30 minutes to allow gelation to occur. $5x10^3$ cells in a volume of 200µl standard growth medium were then seeded on top of the matrix and allowed to adhere before adding an extra 500µl of standard growth medium. Dishes were refed with growth medium every 3-4 days of the assay and the morphogenesis was generally completed after 6-8 days in culture. Photographs were taken at either low or high power magnifications of 6.5x and 25.5x respectively.

2.12.11. Growth curves of cells growing in collagen gels.

Cells were set up in collagen gels in duplicate as for the morphogenesis assay described in section 2.12.5. above, the only differences being that the cell density used was 5×10^4/ml and that the cultures were set up in 35 mm dishes with a 2 ml volume of collagen rather than in 24 well plates with a volume of 500 µl. After polymerisation of the collagen, cells within gels were fed with various conditions and allowed to grow for seven days and form morphogenetic structures as normal.

In order to count the total number of cells making up the morphogenetic structures, the structures had to be removed from the collagen gels and made into a single cell suspension. This was done by washing the gels for two x 5 minutes with PBSA and then digesting the collagen gel with an equal volume of 5 mg/ml collagenase dissolved in collagenase buffer.

Digestion of the gel takes 30-40 minutes at 37°C and is aided initially by using a scalpel to cut around the periphery of the collagen gel and to cut the gel into quadrants, and at later times by gently pippetting the dispersed gels up and down to solubilise the remaining collagen. After the collagen matrix was completely solubilised, the cells were spun out by centrifugation at 200g for 10 minutes. The cells were extracted as individual colonies which were dispersed by incubation with TEGPED solution at 37°C for up to 45 minutes and syringing through a 23G needle. The number of cells in duplicate cultures were then counted using a hemocytometer.

2.12.12. Adhesion assays.

96 well plates were coated with either 100µl of neutralised Vitrogen 100 collagen type I or 100µl of 0.1% BSA in PBSA (to measure non specific adhesion), and placed at 37°C for 2 hours in order for polymerisation of the collagen to occur. Adhesion assays were carried out on triplicate sets of wells. The wells were blocked for at least 1 hour at 37°C with 100 µl of 0.1% heat inactivated BSA in PBSA, and then washed twice with PBSA before cells were plated. Throughout the adhesion assay, "washes" of wells were carried out by flooding the plate with the appropriate wash solution and then flicking it away.

Cells were loaded with the fluorescent dye BCECF-AM [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester, Molecular Probes, Inc]. This cell labelling technique depends on the BCECF-AM dye being taken up by cells and being hydrolysed by intracellular esterases to release BCECF free acid which is retained by the cells. For this reason, unless otherwise stated, the cell washes and cell labelling were carried out with serum free and phenol red free E4 due to the risk of these containing endogenous esterases.

Cells were harvested with trypsin from monolayer cultures that had been seeded for two days, washed twice, and resuspended in a single cell suspension to give a labelling concentration of $5x10^6$ cells/ml. The BCECF-AM dye was added at a concentration of 1/500 (3.2µM final concentration) and incubated with the cells for 30 minutes at 37°C with gentle mixing. After loading the cells were washed twice and diluted to an appropriate concentration (in phenol red free E4 + 10% FCS) for plating in the adhesion assay. At this point if the effect of integrin Abs on adhesion was to be analysed, the cells were incubated with the appropriate Ab for 15 minutes at room temperature with occasional mixing. The concentrations of Abs used varied between experiments and are given in the appropriate figure legends.

100µl of each cell suspension was then plated onto the matrix and BSA coated wells in triplicate. The number of cells plated per well varied slightly between experiments (from 1.0×10^4 - 2.5×10^4) and is noted in the

appropriate figure legends. The plating was carried out on ice and as quickly as possible to minimise possible cell adhesion to matrix before all conditions had been plated. The cells were then placed at 37°C for 10 to 40 minutes (depending on the particular experiment) to allow adhesion to occur, after which the non adherent cells were removed from the wells by washing three times with phenol red free E4. At this point, to be able to quantitate the number of cells bound, triplicate wells containing 100µl of the original labelled cells were plated to obtain a fluorescent value for the total number of cells plated at the start of the assay. The fluorescence of individual wells was then measured at a wavelength range of 485-538 nm on a Titertek Fluoroskan II plate reader (Labsystems), and the number of cells bound / mm^2 was calculated as follows.

$$\text{Cells bound /mm}^2 = \frac{BF}{TF} \times \frac{\text{No. of cells plated/well}}{\text{area of well/mm}^2}$$

Where BF = bound fluorescence after washing, and is a measure of the specific cell binding to collagen. This was obtained by taking the average fluorescence reading from wells of cells adherent to collagen and subtracting the average fluorescence reading from wells of cells adherent to BSA for each cell line or cell treatment.
TF = Total Fluorescence, and was the average reading from the triplicate wells containing 100µl of the initial labelled cell suspension.
The area of the wells used in these experiments was 38.5 mm^2.

2.12.13. Scatter assay.

35 mm dishes were coated with 1 ml of polymerised collagen type I or fibrin (prepared as for the morphogenesis assays), or left uncoated. $5x10^4$ cells in a single cell suspension in standard growth medium were plated per well in a volume of 0.5 ml, and the cells allowed to adhere for 30 minutes. The cells were then fed with an additional 1.5 mls of either standard medium, HFF-CM, or a specified concentration of HGF and cultured for 24-48 hours after which time the scattered response could be seen. The morphology of cells was photographed at 24-48 hours using a CONTAX 135 mm camera, connected to a Zeiss IM35 inverted microscope. Photographs were taken at either low or high power magnifications of 6.5x and 25.5x respectively.

2.12.14. Time-lapse videomicroscopy to measure cell motility.

Cells were harvested and made into a single cell suspension of $1x10^5$ cells/ml as described above. If the experiment involved the use of Abs, then at this point cells were incubated with the appropriate Ab for 15 minutes at room temperature with occasional mixing, in the same way as described in section 2.12.8 for the morphogenesis assay. The cells were then plated onto polymerised collagen gels in 35 mm dishes and stimulated (or not) with HFF-CM and rhHGF in the same way as for the scatter assay. These dishes were then set up for time-lapse videomicroscopy, carried out using Olympus IMT 2 microscopes enclosed within environmental chambers. High resolution monochrome CCD cameras (Sony M370CE) were attached to each microscope. Images were recorded using broadcast quality video recorders (Sony Betacam PVW-2800) driven externally by animation controllers (BAC 900 from EOS Electronics AV Ltd. EOS House, Weston Sq. Barry, S. Wales).

A field of well dispersed cells containing 30 to 40 cells was chosen for each condition. Images were recorded every 1 minute for a 24 hour period. The recordings were downloaded onto a VHS videorecorder (Mitsubishi B-82) linked to a computer and the cells that remained within the observed field for the duration of the film were tracked using Cell Motility software (EOS Electronics). A printout of the track of each cell was obtained together with the distance moved and motility. The mean distance and motility for each cell condition was calculated.

2.13. Cell biology and biochemical methods

2.13.1. Fluorescence analysed cell sorting (FACS) analysis.

Monolayer cells were trypsinized, washed in PBS and a minimum of 5×10^5 cells were resuspended in 100µl of the appropriate specific antibody diluted in growth medium, (or in 100µl of growth medium alone as a negative control), in the well of a solid 96 well microtitre plate (Linbro). After 30 min incubation on ice, cells were washed 3 times in cold PBS. Cells were then resuspended in 100µl of fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse, or rabbit anti-rat immunoglobulins as appropriate diluted 1:40 in growth medium and incubated on ice in the dark for 30 min. After

washing as above, cells were resuspended in 300 µl PBS and 5000 cells were analysed by a Becton-Dickinson FACScan flow cytometer.

2.13.2. Immunofluorescence staining and confocal microscopy of cultured cells.

Cells were grown on 9 mm diameter, acid washed and sterilised glass coverslips in the wells of a 24 well tissue culture plate and were processed according to the antibody used. All cells were briefly washed with complete PBS and then fixed either with methanol/acetone (1:1, precooled to -20°C; used when cells were stained with HMFG-2) for 10 minutes, or with 3% formaldehyde in complete PBS for 10 minutes at room temperature (used when cells were stained with HECD-1). After brief washing with PBSA, cells were blocked with 20% FCS in PBSA for 30 minutes. Again cells were briefly washed with PBSA and incubated with the appropriate Ab (HMFG-2 tissue culture supernatent was used undiluted and HECD-1 was used at a concentration of 10µg/ml diluted in 5% FCS in PBSA), for 1 hour at room temperature. Cells were washed 3-4 times in copious amounts of PBSA prior to incubation with the second antibody (FITC conjugated rabbit anti mouse Ig (DAKO) diluted 1:40 in 5% FCS/PBSA) for 30 min. The cells were then washed 3-4 times in PBS and the coverslips mounted in Citifluor (glycerol/PBS). Confocal analysis was carried out on a Nikon Optiphot Microscope with Biorad software, which made it possible to view horizontal (Z-series) and vertical (XZ-series) sections through the cells.

2.13.3. Extraction of whole cell protein lysates from monolayer cultures.

Cells growing in monolayer cultures in 100 mm dishes or 80 cm^2 flasks (ideally at 70-90% confluence), were cooled on ice. The cells were washed twice with cold complete PBS and a small volume (0.8 - 1.0 ml), of the appropriate prechilled lysis buffer was added. The cells were then incubated on ice for 15 minutes, making sure that lysis buffer was completely covering the cells at all times. The lysate and cell debris were scraped into a microfuge tube and the insoluble membrane and nuclear protein removed by centrifugation at 13 000 rpm for 10 minutes at 4°C. The supernatent containing soluble protein was taken for immediate use in experiments or alternatively stored at -70°C after quick freezing in a dry ice and methanol bath.

The particular lysis buffers used varied depending on the purpose of the particular experiment. The basic mixture consisted of a non ionic detergent in a Tris buffer, and contained salt and also a combination of protease inhibitors. If the E-cadherin protein was to be analysed then Ca^{2+} ions were included in the lysis buffer to minimise trypsin induced cleavage of the E-cadherin protein. On the other hand, for experiments where the ability of HGF and HFF-CM to induce phosphorylation of the HGF receptor (c-met) was studied then phosphatase inhibitors such as vanadate were included.

The exact recipes for particular lysis buffers used in this thesis are given below, and denoted by a letter which is used to distinguish them hereafter.

Lysis buffer A.

1% NP40
50 mM Tris-HCl pH 7.5
150 mM NaCl
5 mM $MgCl_2$
2 mM PMSF
10 µg/ml Leupeptin

Lysis buffer B (used for E-cadherin studies).

1% Triton X100
50 mM Hepes pH 7.5
100 mM NaCl
1 mM $CaCl_2$
2 mM PMSF
10 µg/ml Leupeptin
10 µg/ml Aprotinin
10 µg/ml Trypsin Inhibitor

Lysis buffer C (used for phosphorylation studies)

1% Triton X100
50 mM Tris pH 7.5
100 mM NaCl
1 mM sodium vanadate
2 mM p-nitrophenyl phosphate (Sigma 104)
20 mM sodium fluoride
10 µg/ml Leupeptin
10 µg/ml Aprotinin
10 µg/ml Trypsin Inhibitor
10 µg/ml Pepstatin
10 µM Benzamidine

2.13.4. Extraction of lysates from cells stimulated with HGF or HFF-CM.

In experiments where the ability of rhHGF and HFF-CM to induce phosphorylation of the HGF receptor (c-met) was studied, monolayers of cells were subjected to serum starvation overnight and then stimulated for 15 minutes with the appropriate factor in serum free medium before lysates were made. Serum starvation was carried out by washing cell monolayers several times with serum free E4 and then incubating the cells in serum free E4 overnight. Starvation was carried out in order to distinguish phosphorylation induced by HGF or HFF-CM from that induced by any serum factors.

2.13.5. Bio-Rad (Bradford) microassay to estimate protein concentration.

To establish a standard curve, a serial dilution (1.42 - 0.05 mg/ml) of BSA was carried out in distilled water, and 5µl aliquots of each dilution plated in duplicate in 96 well microtitre plates, with 5µl of distilled water plated as a reagent blank control. Several dilutions of unknown samples (using dilutions of lysis buffer alone as a reagent blank control), were also carried out in distilled water, and 5µl of these samples were plated in duplicate in the microtitre plate. The Bio-rad detection reagent was diluted 1/5 in distilled water and filtered through a Whatman 2v filter paper. 200 µl of this was then added to each of the assay wells and the colour reaction allowed to fully develop. The absorbances of samples were measured at an optical

density of 595 nm on a Titertek Multiskan MC plate reader. The concentrations of unknown protein samples were then calculated from the BSA standard curve (obtained by plotting OD 595 readings against the protein concentrations of BSA standards).

2.13.6. Cell surface labelling with ^{125}Iodine.

Cells growing in monolayers on tissue culture plastic at 70-90% confluence were treated with versene until cells started to round up, and then removed completely by brief trypsinization. The cells were then washed twice with complete PBS and resuspended in complete PBS containing 50 mM glucose as a single cell suspension of 1 x 10^7 cells/ml, before labelling with $Na^{125}I$ using the lactoperoxidase -glucose oxidase method (Hubbard, A. and Cohn, Z., 1972). After labelling for 20 minutes at room temperature with $Na^{125}I$ at 1 mCi/ml, the cells were washed three times in cold E4 to remove unincorporated radiolabel. Labelled cell surface proteins were then extracted by resuspending the final cell pellet in 1 ml of cold lysis buffer A, and lysing on ice for 15 minutes, after which the nuclear proteins were removed by centrifugation at 13 000 rpm for 10 minutes.

2.13.7. Immunoprecipitation.

(i) Of integrins

The amount of incorporated radiolabel in iodinated cell protein extracts, made as described above, was measured using TCA precipitation (Harlow, E. and Lane, D.P., 1988). Samples containing equal numbers of TCA-precipitable counts (usually 500 000 cpm) were then used for immunoprecipitation of integrin proteins as described in Adams and Watt 1990. Lysates were incubated on ice with an excess of the appropriate integrin Ab for 1-2.5 hours. Rabbit anti mouse Ig or rabbit anti rat Ig antibodies (DAKO) were then added in excess and incubated on ice for 1 hour. 50 µl of a 10% protein-A sepharose bead suspension was then added, and the mixture tumbled end-over-end for 1 hour at 4°C. [The suspension of protein A sepharose beads was made by swelling 100 mg of beads in 1 ml PBSA for 15 minutes on ice, washing the beads three times in PBSA and resuspending in 1 ml of lysis buffer A]. The beads and the associated immune complexes were washed 4 times in lysis buffer A, the second wash

being a high salt wash containing 500 mM NaCl rather than 150 mM. The washed beads were then resuspended in 50µl of 2x SDS-PAGE sample buffer without DTT reducing agent, and the proteins eluted from the beads and denatured by boiling for 5 minutes. After this the samples were loaded and resolved on 7.5% SDS polyacrylamide gels, (see below).

(ii) Of the HGF receptor c-met

Lysates containing 500 µg of protein were precleared with 100µl of 10% protein-A sepharose beads by tumbling for 1 hour at 4°C. The beads were spun out and the supernatent then incubated with 2 µg of a rabbit polyclonal Ab to c-met (C-28 Santa Cruz Biotechnology Inc.) by tumbling for 2 hours at 4°C. 50 µl of protein-A sepharose were then added and the lysates tumbled at 4°C for 1 hour. The sepharose beads were then washed 4 times in lysis buffer C and resuspended in 50µl of 2x SDS PAGE sample buffer containing DTT. The proteins were eluted from the beads as described above and resolved on a 7.5 % SDS polyacrylamide gel before Western blotting and detection with an anti phosphotyrosine Ab (see below).

2.13.8. SDS polyacrylamide gel electrophoresis of proteins

Proteins were fractionated on SDS polyacrylamide gels according to Laemmli, U.K., 1970, using apparatus from Genetic Research Instrumentation Ltd.

The running gel mixture was poured between two glass plates separated by 1.5 mm spacers and overlaid with isopropanol to aid polymerisation and eliminate meniscus. Running gels containing 7.5% acrylamide were prepared from a stock solution of 30% acrylamide and 0.8% N, N' -bis-methylene acrylamide (Millipore), and also contained a final concentration of 0.375M Tris-HCl (pH 8.8) and 0.1% SDS. The gels were polymerised by the addition of 0.06% TEMED and 0.03% ammonium persulphate. After polymerisation, the isopropanol was removed and the stacking gel poured on top of the running gel and allowed to polymerise around a well former. The stacking gel consisted of 5% acrylamide, 0.125M Tris-HCl (pH 6.8), and 0.1% SDS and was polymerised in the same way as the running gel. Protein samples were added to an equal volume of 2x SDS-protein sample buffer

(+/- the reducing agent DTT as appropriate), and boiled for 5 minutes before loading. Rainbow molecular weight protein markers or ^{14}C methylated markers (Amersham International) were also run on gels to allow determination of molecular weight.

Gels were electrophoresed in 1x SDS-PAGE running buffer at 100V through the stacking gels and 150V through the running gel, until the desired separation was achieved. After separation of radiolabelled proteins, gels were fixed in 20% methanol/10% acetic acid, dried, and exposed to film; or after separation of unlabelled proteins, gels were subjected to Western blotting (see below).

2.13.9. Western Blotting

Western blotting was performed as described by Harlow, E. and Lane, D.P., 1988 using Biorad apparatus. Hybond-C extra nitro-cellulose membrane and 4 sheets of 3MM filter paper were cut to the same size as the gel. The membrane was soaked for 2 minutes in distilled water then 5 min in transfer buffer. A 'transfer sandwich' was assembled, taking care that all components were immersed in transfer buffer and air bubbles were excluded. The sandwich was assembled in the order of a support pad, 2 sheets of the 3MM filter paper, the gel, the membrane, another 2 sheets of 3MM, and another support pad, and the completed sandwich placed in the transfer tank with the membrane closest to the positive electrode. Transfer was carried out at 30V overnight in transfer buffer at 4°C, after which the membrane was probed with specific antibodies and signals detected by the enhanced chemiluminescence technique (ECL), as outlined below.

2.13.10. Probing of Western blots and ECL detection of proteins.

All washes were carried out in buffers made up with PBSA unless the E-cadherin protein was being detected in which case complete PBS was used. All steps were carried out at room temperature. After transfer, the membrane was washed briefly in PBS and blocked for 2 hours in PBS/5% skimmed milk/0.1% Tween. All subsequent washes and Ab incubations were carried out in PBS/1% skimmed milk/0.1% Tween unless stated otherwise. After blocking, the membrane was washed briefly and incubated with an appropriate concentration (noted in individual figure legends) of

primary Ab for 2 hours. The membrane was then washed 4 times (1 x 15 minutes and 3 x 5 minutes), and the appropriate secondary Ab (horse radish peroxidase conjugated rabbit anti mouse or swine anti rabbit Ig, DAKO) at a concentration of 1/2000, was incubated with the membrane for 1 hour. 4 washes were carried out as before with two additional 5 minute PBSA washes included at the end. The ECL reaction was then carried out according to the manufacturers instructions and any signal detected by exposing the membrane to film for a few seconds.

2.14. Protein Purification Methods

2.14.1. Concentration of Fibroblast Conditioned Medium

Before being used for factor purification, conditioned medium was concentrated in a 50ml stirred cell concentrator (Amicon) using a 10 000Da MWt low protein binding membrane (Amicon) according to the manufacturers instructions. For quantities larger than 50 ml, repeated loadings of conditioned medium were made.

For smaller quantities (<15ml), spin columns were used with a low protein binding membrane (10 000Da MWt cut-off) according to the instructions supplied by the manufacturer (Millipore).

2.14.2. Column Chromatography of Human Foreskin Fibroblast Conditioned Medium

The following methods were used:

(i) Heparin-Agarose Affinity Chromatography

Heparin-agarose chromatography matrix (Heparin Affi-gel) was purchased from Bio-Rad Laboratories. The matrix was packed into a Bio-Rad gravity column and used according to manufacturers instructions.

(ii) Lectin Affinity Chromatography

Wheat germ lectin (WGL) sepharose and Concanavalin A (ConA) sepharose affinity chromatography matrices were purchased from Pharmacia Ltd.

Matrices were equilibrated and run according to the instructions supplied by the manufacturer. Proteins bound to the wheat germ lectin column were eluted with 0.3M N-acetyl glucosamine and 0.3M α-D-methyl mannoside was used to elute the proteins bound to the Concanavalin A column.

(iii) Anion-Exchange Chromatography

The weak anion exchange matrix, DEAE-sepharose and the strong anion exchange matrix, Q-sepharose were purchased from Pharmacia Ltd. Each column was used according to the manufacturers instructions and eluted using a stepped-gradient of increasing salt concentrations.

(iv) Cation-Exchange Chromatography

The strong cation exchange matrix, SP-sepharose (Pharmacia Ltd), was used in the same way as the anion exchange matrices described above.

(v) Superdex 75 Size Exlusion Chromatography

A pre-packed Superdex 75 column (Pharmacia Ltd) was used in conjunction with the Pharmacia SMART^{TM} system. The system was run at 50µl/min, collecting 50µl sample volumes. Samples were collected from 12 minutes after loading. Size exclusion markers (MWt range 6 500 - 67 000Da) were used to calibrate the column (Sigma).

2.15. Molecular Biology Methods

The plasmids containing DNA fragments to be used as probes for Northern blots (described in section 2.9 above), were usually obtained in small quantities. The plasmids were therefore transformed into competent bacteria and large scale plasmid preparations made, the DNA of which was then digested with the appropriate restriction endonucleases and the required inserts purified to be used as probes (see below).

2.15.1. Bacterial transformation

i) Bacterial strains

The *Escherichia coli* XL-1 Blue (Stratagene, USA) bacterial strain was used throughout to propagate and amplify plasmid DNA. All plasmids described herein contained the β-lactamase gene which allowed the selection of bacterial transformants on L-Agar containing ampicillin at a concentration of 50 to 100 μg/ml and 40μg/ml tetracycline.

ii) Preparation of competent bacteria

Bacteria were streaked out on ψa plates and incubated inverted at 37°C overnight. A single colony was picked and inoculated into 5 ml of ψb broth and incubated overnight at 37°C with shaking. The culture was subcultured 1: 20 into 100 ml of prewarmed ψb broth and grown at 37°C until an OD_{550} of approximately 0.48 was reached. Cells were chilled on wet ice for five minutes then recovered by centrifugation in prechilled corex tubes at 6 000 r.p.m. at 4°C for 5 minutes. The resultant cell pellet was resuspended in 0.40 volumes of Tfb1 buffer and left on wet ice for 5 minutes. Cells were recovered again, as before, and the resultant cell pellet was resuspended in 0.04 volumes of Tfb2 buffer. The resuspended cells were left on wet ice for 15 minutes then aliquotted into freezing vials (200 μl of cells per vial), and snap frozen on dry ice. For long term storage, cells were stored under liquid nitrogen.

iii) Transformation of competent bacteria

Competent cells were thawed on wet ice for approximately 15 minutes. For each transformation, a minimum of 40 μl of cell suspension were added to 10-20 ng of each DNA sample, and the tubes were placed on wet ice for approximately 30 minutes, mixing gently every 10 minutes. After incubation on ice, the cells were heat shocked at 42°C for 90 seconds and returned to ice. 160 μls of L-Broth were added to the cell-DNA mixture and the tubes incubated at 37°C for 30 to 45 minutes. Transformed cells were then spread onto pre-dried L-Agar plates containing 100 μg/ml ampicillin and 40μg/ml tetracycline and incubated overnight at 37°C.

2.15.2. Preparation of plasmid DNA

Large scale LiCl plasmid preparation: A single colony was picked from a freshly plated culture and inoculated into 5 ml L-Broth containing 100 µg/ml ampicillin and 40µg/ml tetracycline. After incubation for greater than 5 hours, the culture was subcultured into 400 ml of L-Broth supplemented with 100 µg/ml ampicillin and 40µg/ml tetracycline. Cultures were incubated overnight at 37°C with shaking.

Bacterial cells were harvested by centrifugation at 7 000 r.p.m. for 10 minutes. The cells were resuspenden in 20 mls of DNA preparation solution A (see Materials section) and incubated at room temperature for 10 minutes. 40 mls of freshly made 0.2M NaOH/1% SDS were added and the cell suspension mixed well and placed on ice for 5 minutes. 20 mls of DNA preparation solution B (see Materials section) were then added and the solution mixed and left on ice for 15 minutes. Cell debris was pelleted by centrifugation for 10 minutes at 7 000 r.p.m. and the supernatant transferred into a sterile beaker by filtering through sterile gauze. 0.6 volumes of ice-cold propan-2-ol were added to the supernatent which was then mixed well and centrifuged for 10 minutes at 7 000 r.p.m.

The resultant nucleic acid pellet was washed with 70% ethanol, resuspended in 4 ml TE pH 8.0 and transferred to a corex tube. An equal volume of ice-cold 5 M LiCl was added to each tube. Tubes were covered with parafilm, inverted to mix and spun at 10 000 r.p.m. for 5 mins at 4°C. After centrifugation, the supernatant was poured into a corex tube and DNA was precipitated by the addition of 2.5 volumes of 100% ethanol and then incubating on dry ice for 30 minutes. DNA was pelleted by centrifugation at 10 000 r.p.m. for 10 minutes. The pellet was resuspended in 70% ethanol, centrifuged as in the previous step and the pellet allowed to dry for approximately 30 minutes before dissolving in 1 ml of TE pH 8.0 containing 40 µg/ml pre-boiled RNase A. This mixture was then incubated at 37°C for 15 minutes before an equal volume of 13% polyethylene glycol (PEG) 6000 in 1.6 M NaCl was added and the tubes mixed and incubated at 4°C for 1 hour. DNA was pelleted by centrifugation at 10 000 rpm for 5 minutes, dissolved in 400 µl TE pH 8.0 and transferred to a 1.5 ml microcentrifuge tube.

Each DNA sample was extracted three times with an equal volume of Tris-buffered phenol: chloroform: iso-amyl alcohol (25: 24: 1). DNA was precipitated from the final aqueous phase by the addition of 0.1 volumes of 3 M NaOAc pH 5.5, 2.5 volumes of ice-cold 100% ethanol and incubating on dry ice for 30 minutes. The DNA was pelleted by centrifugation in the bench-top microcentrifuge for 30 minutes at room temperature. The resultant DNA pellet was then washed with 70% ethanol, dried and dissolved in 300 µl of sterile distilled water. The concentration, purity and integrity of the plasmid DNA was determined, and the DNA aliquotted and stored at -20°C.

2.15.3. Restriction digests of plasmid DNA

Restriction endonuclease digestion of plasmid DNA was typically carried out in a total volume of 30 µl. 10x buffers for the respective restriction endonucleases were supplied by the manufacturer, and used at a final working concentration of 1x. For digests in which more than one restriction endonuclease was utilised simultaneously the optimal buffer was determined according to the manufacturer's instructions. If it was determined that both enzymes would not digest completely together, the two respective digests were carried out sequentially. Plasmid DNA (typically 1-5 µg) was digested for a minimum of one hour at the optimal temperature. All restriction endonuclease digests were typically carried out with a 3-5 fold excess of enzyme.

2.15.4. Agarose gel electrophoresis of DNA.

Agarose gels were prepared by dissolving agarose at 0.8-3.0% (w/v) in 1x TBE buffer in a microwave oven. The solution was allowed to cool, after which ethidium bromide was added to a final concentration of 0.1 µg/ml, and the gel was poured into a gel mould containing a well former. Gels were allowed to set at room temperature. DNA samples were prepared by the addition of DNA sample buffer to 1/5th final volume, and loaded into the wells of the gel submerged in 1x TBE buffer. Electrophoresis was carried out in 1x TBE buffer prepared from 10x TBE stock solution. Gels were electrophoresed at 5 to 7 V/cm at room temperature until the desired range of separation of the DNA fragments was achieved. DNA was visualised by illumination over a long wave u.v. light box, and photographed with

polaroid film. The sizes of fragments were estimated by comparison of their mobility relative to molecular weight markers of known size. Molecular weight markers utilised were as follows:
a) HindIII restriction endonuclease digestion of bacteriophage λ DNA.
b) HaeIII restriction endonuclease digestion of bacteriophage ϕX174 DNA.

2.15.5. Isolation of DNA fragments from gels.

i) Isolation of DNA fragments from low melting point agarose

Low melting point (LMP) agarose gel purification was utilised for the preparation of the α_3 integrin DNA probe described in this thesis. Low melting point (LMP) agarose gels were prepared in 1x TBE as for standard agarose gels, except that LMP gels were allowed to set at 4°C. 4-6μg of plasmid DNA was digested with the appropriate restriction enzymes as described in section 2.15.3. The digested DNA samples were then loaded onto the gel and electrophoresed at 4°C in 1x TBE buffer at 5 V/cm. After suitable separation, fragments to be isolated from LMP gels were cut out of the gel as a thin gel slice. This was carried out over long wave u.v. illumination. Excess agarose was trimmed from around the DNA within the gel slice, and the slice was transferred to a sterile 1.5 ml microcentrifuge tube that had been preweighed. The tube containing the gel slice was then reweighed and the weight of the gel slice was calculated. Sterile distilled water was added to the tube at the ratio of 3 ml water to 1 gram of gel. The tube was subsequently boiled for 10 minutes after which time the probe was aliquotted into separate tubes, the concentration of DNA estimated by agarose gel electrophoresis, and the tubes stored at -20°C.

ii). Isolation of DNA fragments from TBE-agarose gels using the Jetsorb kit.

This method was utilised for the preparation of the α_2 integrin, MUC1, 18S and α_v integrin DNA probes described in this thesis. Restriction digests of the appropriate plasmid with the appropriate enzymes was carried out as normal and the digested DNA separated by electrophoresis on a standard TBE-agarose gel. The insert to be purified was then cut from the gel in the same way as described for the LMP agarose technique and the DNA fragment isolated from the gel slice according to the manufacturer's instructions.

2.15.6. Labelling DNA fragments by random priming

This method was used for the preparation of all labelled DNA probes described in this thesis and follows the method of Feinberg, A.P. and Vogelstein, B., 1984. DNA fragments were isolated in low melting point agarose or by use of the Jetsorb kit as described above. Approximately 50 ng of DNA in a volume of 32µl was heated at 100°C for 5 minutes, centrifuged briefly and placed on ice for 5 minutes. 10µl of OLB buffer and 2 µl BSA (10 mg/ml solution) were added, followed by 2.5 units (1µl) of Klenow DNA polymerase and 50 µCi [α^{32}P]dCTP (5 µl). Labelling was allowed to progress for at least 4 hours at room temperature.

2.15.7. Purification of radiolabelled DNA probe to remove unlabelled fragments.

A 1ml sephadex G-50 column was made as follows: Glass wool was use to plug the bottom of a 1 ml disposable syringe. Sephadex G-50 that had been equillibrated in TE (pH 8) was then packed in the syringe by several rounds of filling the syringe with sephadex solution and subsequent low speed centrifugation. The column was washed twice with TE pH 8 and the labelled DNA probe was then loaded onto and spun through the column. The flow through containing the labelled DNA fragments (the unlabelled fragments being retained in the column), was used to probe Northern blots as described below.

2.15.8. Isolation of total RNA from monolayer cell cultures

This method was used to extract RNA from monolayers of cells growing on tissue culture plastic or on top of collagen type I or fibrin gels. Cells were grown in 10 cm tissue culture plates and in experiments where ECM components were used the plates were coated with 10 mls of polymerised collagen type I (Vitrogen 100) or fibrin (prepared as described above for the morphogenesis assays), before the cells were seeded.

In all RNA manipulation, glassware, plasticware and electrophoresis tanks were treated so as they were free of RNases (Sambrook, J. *et al.*, 1989). Glassware was prepared by autoclaving, and gel trays, electrophoresis

tanks and plasticware were prepared by treating with 3% hydrogen peroxide. In the preparation of solutions, Depc treated water was used wherever possible.

Total RNA was prepared by the guanidine isothiocyanate extraction method as described (Chomczynski, P. and Sacchi, N., 1987). Cells growing as a monolayer were washed twice with ice-cold PBSA. A total of 10 mls of LSGD buffer (see Materials section) containing 4 M GTC was then added in sequential volumes of 4 mls, 3 mls and 3 mls to lyse the cells, and the lysed cell solution was transferred into 50 ml polystyrene Falcon tubes on ice. Approximately 1/10 volume (1 ml) of 2M sodium acetate (pH 4.1) was added and mixed, followed by an equal volume (11 mls) of water saturated phenol (Rathburn) and 2 mls chloroform: isoamyl alcohol (mixed in a 49:1 ratio). The samples were well mixed by vortexing and left on ice for 15 mins to allow phase separation. The samples were then centrifuged at 5 000 rpm for 20 mins at 4°C and the top aqueous phase transferred to a fresh tube. An equal volume (or slight excess) of iso-propanol was then added and the RNA precipitated overnight at -20°C. The RNA was pelletted by centrifugation at 5 000 rpm for 20 mins at 4°C and resuspended in 1 ml LSGD and an equal volume of isopropanol. The samples were placed at -20°C overnight and the RNA pelleted by centrifugation as described above. The RNA pellet was washed with 75% ethanol and air dried for about 20 minutes after which the pellet was dissolved in 30µl Depc water and transferred to an RNase free microfuge tube to be stored at -20°C.

A small amount of the RNA sample was loaded on a 1.3% (w/v) ethidium bromide containing agarose-TBE gel and was electrophoresed in 1xTBE at 5V/cm at room temperature. This technique was used to assess the quality of the RNA and also to approximately estimate the concentration.

2.15.9. Isolation of total RNA from cells growing within collagen gels.

Cells were set up in collagen gels in duplicate as for the morphogenesis assay described in section 2.12.5. above, the only differences being that cells were plated in 6 mls of collagen in 6 cm tissue culture plates and a higher cell density was used, which differed depending on the time point at which the RNA was to be extracted. For Day 1 and Day 3 extractions 0.7×10^6 cells/ml were plated and for Day 7 extractions 1×10^5 cells/ml were plated.

After polymerisation of the collagen, cells within gels were fed and allowed to grow and form morphogenetic structures as normal until the time point for extraction.

Cells were extracted from the collagen by the collagenase method as described in section 2.12.11; except that after the cells were spun out of the solubilised collagen by centrifugation, the pellet was chilled and lysed in 2 mls of LGSD buffer. Total RNA was then extracted as described in section 2.15.8.

2.15.10. Agarose gel electrophoresis of RNA.

RNA was fractionated by electrophoresis through a 1.3% (w/v) 1xMOPS-agarose gel containing 6.7% (v/v) formaldehyde. 1.3g of agarose was dissolved in 72 mls of Depc water and 10 mls of 10x MOPS buffer by heating in a microwave oven. The mixture was then allowed to cool to 60°C after which 18 mls of 37% v/v formaldehyde was added. The gel was cast in a gel-tray and tank that had been previously soaked in hydrogen peroxide as described above.

To 6 µl of RNA samples in Depc water (containing approximately equal amounts of RNA as estimated from TBE-agarose gel electrophoresis as described above, and preferably containing 10-20µg), 2.5 µl 10x MOPS, 4 µl formaldehyde (37% v/v solution) and 12.5 µl deionised formamide were added. Each sample was incubated at 65°C for 15 minutes then chilled on wet ice. After the addition of 2.5 µl RNA sample buffer, each sample was loaded onto the MOPS-agarose gel and electrophoresed at 5 V/cm for 2-3 hours in 1x MOPS buffer.

2.15.11. Northern blotting

At the completion of electrophoresis, the gel was transferred to a wash box and washed briefly several times in Depc water. The gel was then washed for 30 minutes with 0.05M NaOH and in 20x SSC for 45 minutes. RNA was transferred overnight to Hybond N+ nylon membranes by capillary elution in the presence of 20x SSC as described (Sambrook, J. *et al.*, 1989). After transfer, the RNA was immobilised onto the membrane by u.v. cross linking and baking at 80°C for 1 hour in a vacuum oven. The quality of the RNA

and efficiency of transfer were assessed by staining an extra lane of the baked membrane (run especially for this purpose) in methylene blue staining solution for approximately 1 minute, followed by destaining in water for 1-2 minutes (Wilkinson, M. *et al.*, 1990).

The Northern blot was washed briefly in 2x SSC and prehybridised for 1-2 hours at 42°C in prehybridisation buffer in hydrogen peroxide treated plastic sandwich boxes. The prehybridisation buffer was prepared by heating it to 65-70°C and adding salmon sperm DNA (that had been heated for 5 mins at 100°C and cooled on ice), to a final concentration of 250µg/ml. After prehybridisation the appropriate radiolabelled DNA probe (prepared and purified as described in sections 2.15.6 and 2.15.7), was denatured by heating to 100°C for 5 minutes, then chilled on ice and added to the prehybridised blot. Hybridisation was then allowed to proceed overnight at 42°C.

After overnight hybridisation, membranes were washed at 65°C. The wash protocol was as follows: Wash twice for 15 minutes each in 2x SSC, 0.1% SDS. Wash twice for 15 minutes each in 1x SSC, 0.1% SDS. If background radioactivity is still high carry out two further high stringency washes for 15 minutes each in 0.5x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS respectively. The blot was then wrapped in cling film and exposed to film at -70°C to detect the signal. Signals were then quantitated using an LKB Ultroscan XL Laser Densitometer. If required the Northern blots were stripped for reprobing by washing in boiling 0.5% SDS.

CHAPTER 3: ESTABLISHMENT OF AN *IN VITRO* SYSTEM FOR STUDYING THE MOLECULES INVOLVED IN BRANCHING MORPHOGENESIS OF HUMAN MAMMARY EPITHELIAL CELLS.

INTRODUCTION.

Loss of tissue architecture, and loss of organisation of the branching ducts of the mammary gland is one of the first changes that occurs in breast cancer development. This reflects the loss of a differentiated function of the normal epithelial cell, i.e. the ability to form an ordered 3-dimensional structure. The development of an *in vitro* system to identify molecules which play a crucial role in organised branching morphogenesis of normal human mammary epithelial cells, may therefore identify candidate molecules that become dysfunctional in breast cancer development.

In the development of any *in vitro* system, in order to make comparisons between normal and malignant cells at the molecular level, it is very important to define the phenotype of the malignant cell in terms of the normal cell lineage, and to work with normal cells which show the phenotype corresponding to that of the malignant cell. The cell phenotype in the mammary gland, from which the vast majority (~ 90%) of breast cancers develop, corresponds to the keratin 19 positive, luminal epithelial cell, (Bartek, J. *et al.*, 1985a). It was, therefore, important to analyse normal morphogenesis using this cell type.

Cell-cell and cell-matrix interactions are pivotal in maintaining tissue architecture, such that changes in the level or function of molecules involved in these interactions would need investigation. The integrin family of molecules are known to play an important role in mediating cell-matrix and cell-cell interactions (Hynes, R.O., 1992; Sriramarao, P. *et al.*, 1993; Symington, B.E. *et al.*, 1993). In breast cancers, certain integrin family members (and in particular the $\alpha_2\beta_1$ integrin), had been reported to show a reduction/loss in expression, which correlated with increasing grade of tumour, i.e. increased disruption of tissue architecture (Zutter, M.M. *et al.*, 1990; Zutter, M.M. *et al.*, 1993). These properties made integrins good candidates for molecules involved in organised branching morphogenesis.

To study the role of integrins in branching morphogenesis of human mammary epithelial cells, an appropriate *in vitro* model system was required. In the induction of branching morphogenesis of epithelial organs *in vivo*, a role for the fibroblast rich, underlying mesenchyme had been implicated, (Nogawa, H. and Mizuno, T., 1981; Sakakura, T., 1987; Saxen, L. and Sariola, H., 1987). Montesano, R. *et al.*, 1991b, had reproduced this mesenchymal induction of branching morphogenesis *in vitro*, by showing that a kidney epithelial cell line (MDCK) could be induced by fibroblast produced factors to form branching structures in collagen type I matrices, as opposed to the spherical cysts that developed in standard growth medium. Following this example, we therefore attempted to develop a human mammary epithelial cell line, of the correct normal cell phenotype, which underwent branching morphogenesis under these conditions.

RESULTS

3.1. Derivation of the cell line and development of the *in vitro* branching morphogenesis system.

The human mammary epithelial cell line, MTSV1-7, which shows the luminal epithelial phenotype of interest, was originally developed from normal breast epithelial cells, cultured from human milk. (Bartek, J. *et al.*, 1991). Due to the short *in vitro* life span of primary breast epithelial cells, the MTSV1-7 line was produced by immortalisation of cells with SV40 large TAg. This immortalisation gave rise to a cell line with an extensive *in vitro* life span, which was important for any relatively long term *in vitro* assays. More importantly, the immortalisation did not confer tumorigenicity to the cells, as assessed by their inability to form tumours in nude mice and to grow in soft agar. Also, the immortalisation did not alter the original luminal epithelial phenotype of the cells (Bartek, J. *et al.*, 1991).

The MTSV1-7 cell line formed compact three-dimensional structures when grown in collagen type I gels in standard growth medium. However, the structures formed were found to be heterogeneous in nature, with some spherical cysts, some colonies that were performing branching morphogenesis, and other colonies in between these two

extreme phenotypes. In order to use this collagen gel system to induce branching morphogenesis with fibroblast factors and investigate the role of integrins, it would be advantageous to have a cell line showing a more homogeneous, spherical cyst phenotype in standard growth medium, which could be induced to branch by exogenously added fibroblast factors. Accordingly the HB2 cell line was developed.

When the MTSV1-7 cell line was grown on matrigel (a laminin rich basement membrane preparation from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells), branching networks and complex structures were formed in a significant proportion of colonies (Figure 3.1.1.A.). To try and develop a cell line with a more homogeneous phenotype, and a reduced propensity to branch in the absence of external stimuli, two subclones of the MTSV1-7 cell line were picked from those cultured on matrigel. These subclones were picked because they did not form branching networks on matrigel, but remained as spherical balls (Figure 3.1.1.B).

One subclone, MTSV1-7HB2 (referred to hereafter as HB2), when cultured in collagen type I gels, in standard growth medium, formed compact, spherical cysts in a very homogeneous manner, (Figure 3.1.2). The homogeneous formation of cyst structures, was precisely the behaviour required of a human mammary epithelial cell line to attempt to induce branching morphogenesis with fibroblast factors. Indeed, when the HB2 cell line was cocultivated in collagen gels with various types of fibroblast cells, [primary human breast fibroblasts, (Chang, S.E. *et al.*, 1982); human foreskin fibroblasts, HFFs; human embryonic lung fibroblasts, MRC-5; and 3 stable mouse fibroblast lines, Balb/c 3T3, Swiss 3T3, Swiss 3T3-L1], branching morphogenesis was seen in up to 90% of colonies (data not shown, Berdichevsky, F. *et al.*, 1994b).

3.2. Integrin expression in HB2 cells.

In order for the HB2 cell line to be used in an *in vitro* system for studying the role of integrins in branching morphogenesis of human mammary epithelial cells, it was important that the integrins expressed by the HB2 cells were comparable to those expressed by the mammary gland *in vivo*.

Figure 3.1.1.
Growth of MTSV1-7 cells and the subclone HB2 on matrigel.

Either MTSV1-7 cells (panel A) or HB2 cells (panel B) were plated on a Matrigel™ basement membrane matrix and cultured in standard growth medium. The morphogenetic phenotypes of colonies were photographed at high power magnification after 7 days of growth.

Scale bar denotes 100 μm

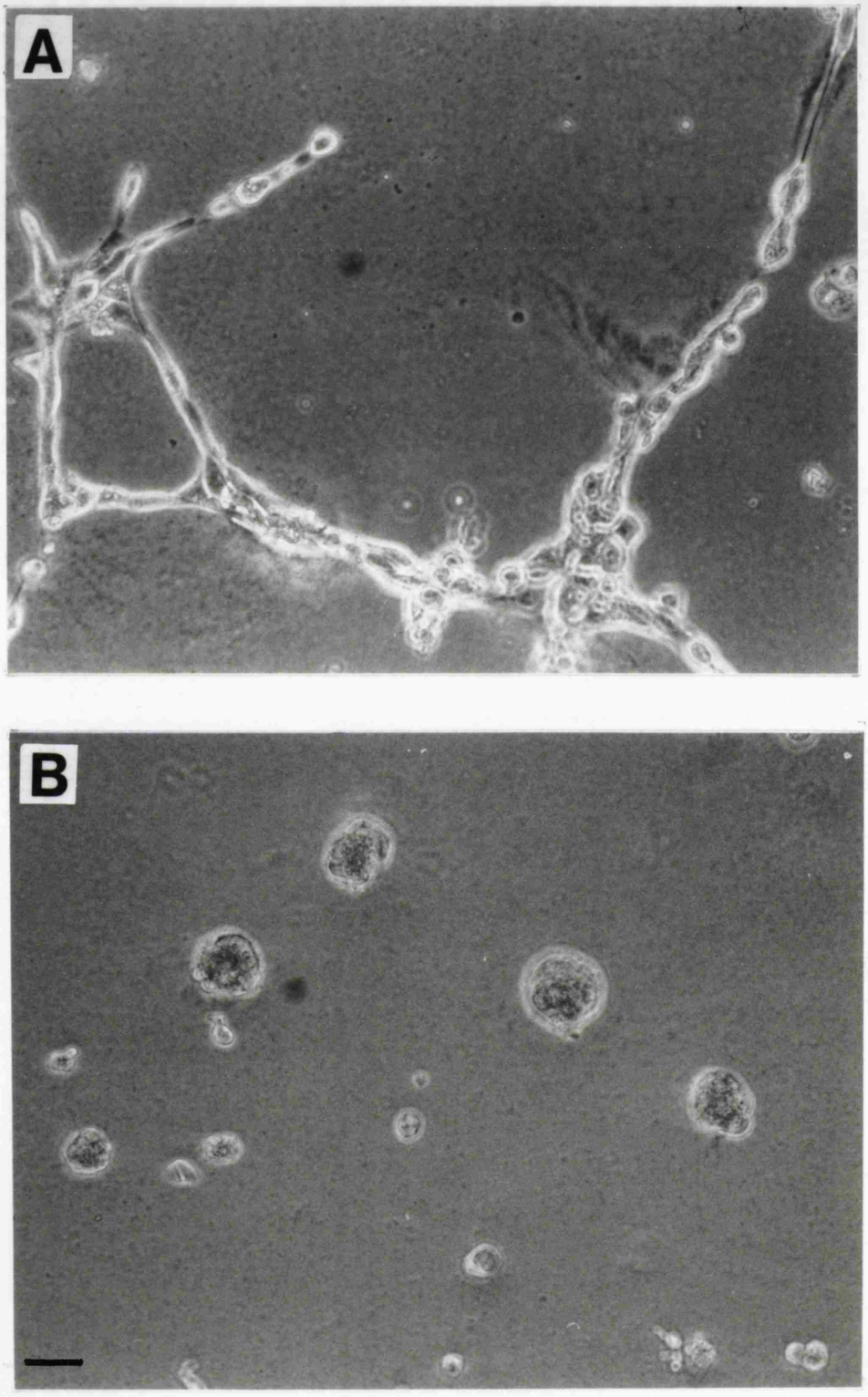
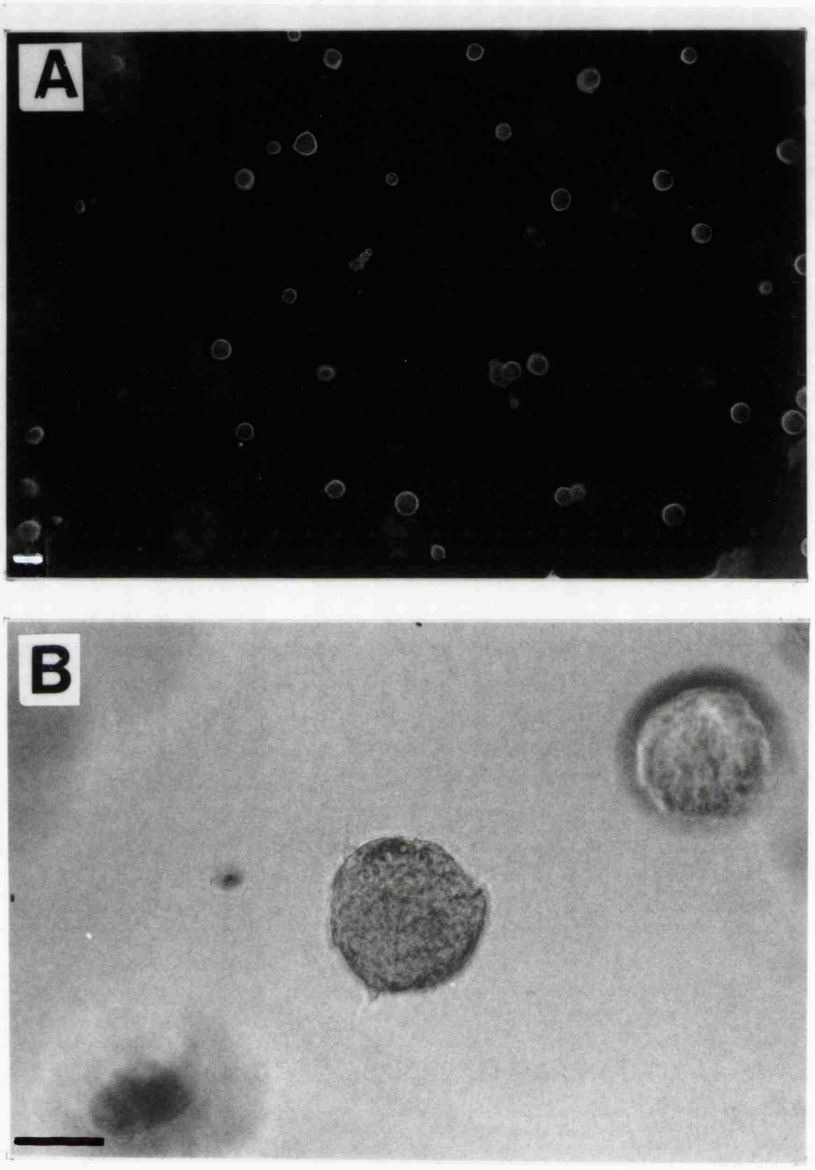

A
B

Figure 3.1.2.
The HB2 subclone of MTSV1-7 forms compact spherical cyst structures when cultured within a collagen type I matrix.

HB2 cells were embedded in collagen gels and cultured in standard growth medium. The morphogenetic phenotypes of colonies formed were photographed after 7 days of growth at both low power (panel A), and high power magnification (Panel B).

Scale bar on photographs taken at low power denotes 200 μm
Scale bar on photographs taken at high power denotes 100 μm


A
B

The major integrins expressed by the mammary gland *in vivo*, are the $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ integrins (Berdichevsky, F. *et al.*, 1994b; Koukoulis, G.K. *et al.*, 1991). The α_v integrin subunit has also been shown to be moderately expressed in the basal cells and weakly expressed in the luminal epithelial cells of the adult mammary gland (Koukoulis, G.K. *et al.*, 1991). When analysed by immunoprecipitation (Figure 3.2.1.A.), by flow cytometry, (Figure 3.2.1.B.), or by immunoflourescence, (data not shown), the profile of integrin expression detected on the surface of HB2 cells showed the α_2, α_3, and β_1 subunits to be expressed at high levels, the α_v subunit at an intermediate level (by flow cytometry only), and the α_6 and β_4 subunits detectable at lower levels (β_4 subunit shown by immunoprecipitation only). This profile of integrin expression accurately reflects that seen *in vivo*, suggesting that the HB2 cell line is an appropriate one to use in our *in vitro* studies.

3.3. Other epithelial specific molecules expressed by HB2 cells.

It was shown that the HB2 subclone had retained the luminal epithelial cell phenotype of the parental MTSV1-7 cell line, in that it expressed the simple epithelial keratins 8, 18, and 19, and also the polymorphic epithelial mucin (PEM), but not keratin 14, collagen type IV or laminin (data not shown). It was also shown that the HB2 cells had the ability to form a highly polarised epithelial monolayer when grown on glass coverslips, with the PEM expressed at the apical surface, and the cell adhesion molecule, E-Cadherin, expressed at the lateral surface in regions of cell-cell contact (Figure 3.3.1).

The ability to form a tight polarised monolayer expressing lateral markers like E-Cadherin (and other junction specific molecules such as ZO-1) in cell-cell contacts, and markers such as PEM on the apical surface, is typical of normal epithelial cells, and is seen in the normal mammary gland *in vivo*. It has been shown in breast cancer cell lines *in vitro* (Aurelia Rughetti -personal communication); in normal mammary epithelial cell lines transfected with proto-oncogenes such as *bcl-2*, *c-fos* and *c-jun* (Reichmann, E. *et al.*, 1992; Lu, P.J. *et al.*, 1995; Fialka, I. *et al.*, 1996); and also in breast carcinomas *in vivo*, (Jensen, H.M., 1981; Millis, R., 1984), that this tightly organised epithelial polarity is lost, with

Figure 3.2.1.A.
Integrins expressed by HB2 cells as detected by immunoprecipitation.

HB2 cells were surface labelled with $Na^{125}Iodine$ using the lactoperoxidase - glucose oxidase method, and integrins immunoprecipitated from samples of whole cell lysates using specific antibodies to integrin subunits. Immunoprecipitated integrins were eluted in 2x SDS PAGE sample buffer without DTT and were resolved by SDS-PAGE on a 7.5% gel under non-reducing conditions. The antibodies used were: TS2/7-α_1 (lane 1); HAS4-α_2 (lane 2); J143-α_3 (lane 3); B5G10-α_4 (lane 4); mAb16-α_5 (lane 5); GoH3-α_6 (lane 6); MAR5-β_1 (lane 7); 3E1-β_4 (lane 8).

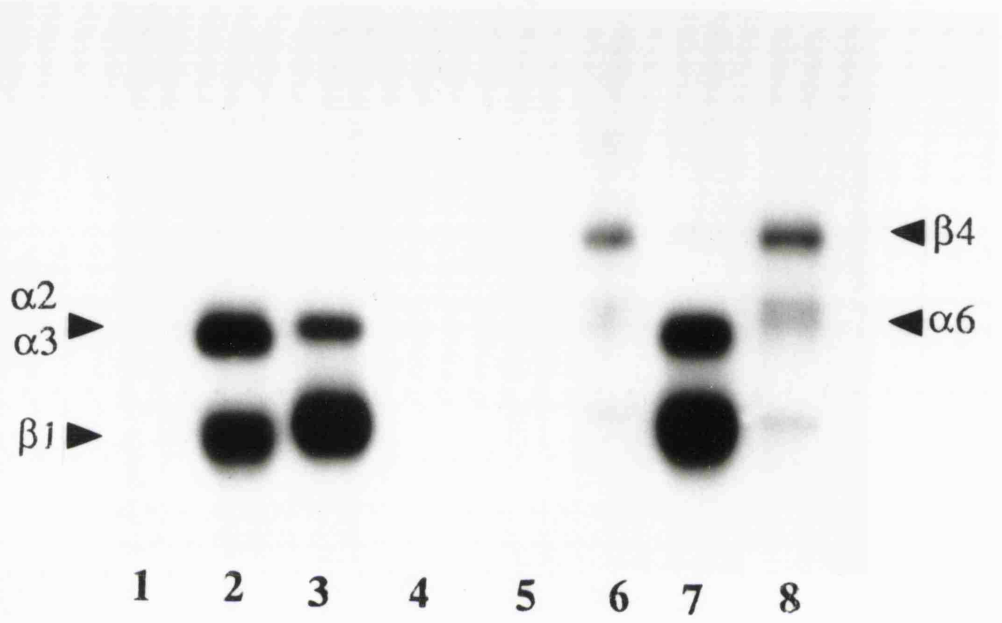
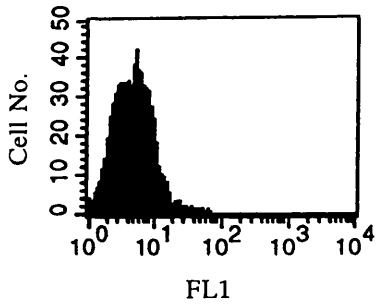
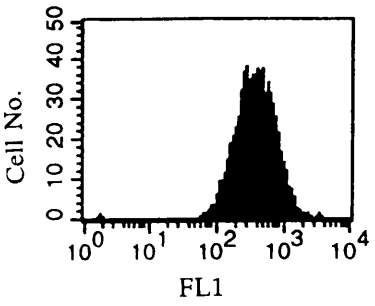

α2
α3
β1
β4
α6
1 2 3 4 5 6 7 8

Figure 3.2.1.B.
Integrins expressed by HB2 cells as detected by FACS analysis.

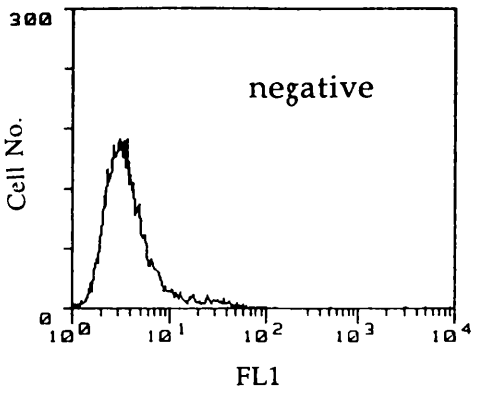
The expression of α_2, α_3, α_v, α_6 and β_1 integrin subunits on HB2 cells is shown by FACS analysis using specific integrin antibodies. The antibodies used were: HAS4-α_2; P1B5-α_3; MAB 1980-α_v; MAR5-β_1; GoH3-α_6. Cells incubated with second antibody alone [i.e. FITC conjugated rabbit anti-mouse Ig (left hand side) or rabbit anti-rat Ig (right hand side)] are used as a negative control.

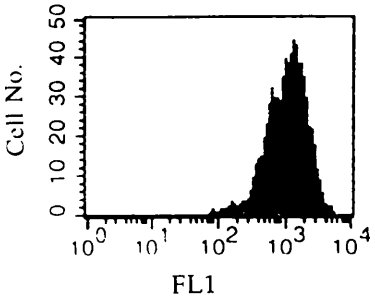


negative

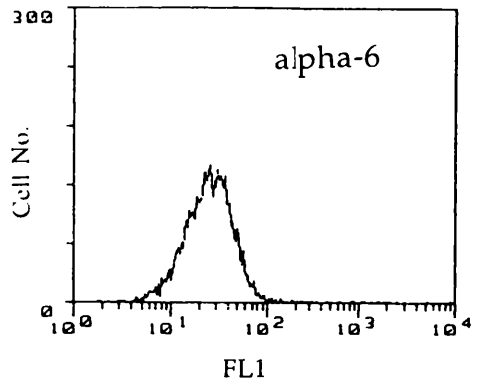


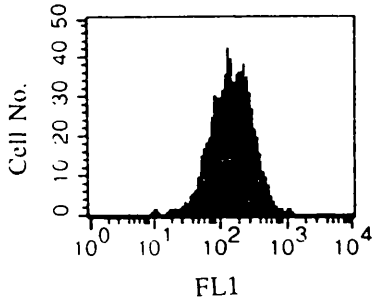
alpha-2



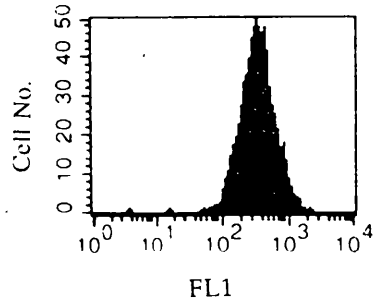


alpha-3





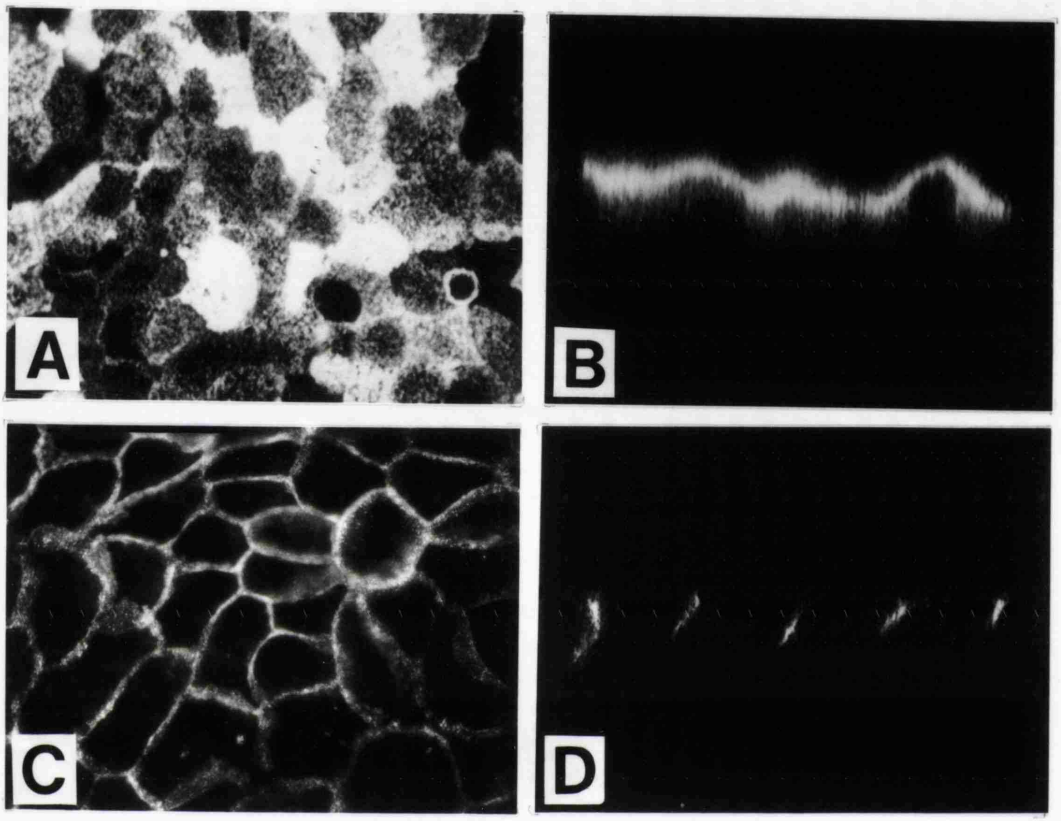
alpha-v



beta-1

Figure 3.3.1.
HB2 cells in monolayer culture form a well organised, polarised epithelial monolayer.

HB2 cells were grown to confluence on glass coverslips and stained by immunoflourescence techniques using Abs against the apical specific PEM (panels A and B), and the cell-cell adhesion molecule E-cadherin (panels C and D). Analysis by confocal microscopy was carried out to view horizontal (panels A and C) and vertical (panels B and D) sections of the cells. Abs used were HMFG-2 against PEM, and HECD-1 against E-cadherin.


A
B
C
D

the basolateral and apical specific molecules becoming distributed over the whole cell surface.

3.4. The induction of branching morphogenesis of HB2 cells by cocultivation with fibroblasts, is due to the secretion of a soluble factor(s).

To establish whether the induction of branching morphogenesis of HB2 cells by fibroblasts was due to the secretion of a soluble factor rather than by some other mechanism, conditioned medium (CM) was taken from several of the fibroblast lines listed above, and used to stimulate HB2 cells embedded in collagen gels. Figure 3.4.1 shows that fibroblast conditioned medium (FCM), from MRC-5, Swiss 3T3, and HFF cells could induce the branching morphogenesis of HB2 cells in a dose dependent manner. Both the ability of CM to induce branching morphogenesis without the necessity of cocultivation with the fibroblast cells themselves, and the dose dependence of the branching morphogenesis response, show that the induction of branching morphogenesis is indeed due to a soluble, secreted factor or factors. The induction of branching morphogenesis of HB2 cells by HFF-CM is shown pictorially in Figure 3.5.2. panels E/F.

3.5. Recombinant human Hepatocyte Growth Factor can induce branching morphogenesis of HB2 cells in collagen gels.

Montesano, R. *et al.*, 1991b, had shown that a protein known as hepatocyte growth factor (HGF) could induce branching morphogenesis of MDCK cells. It was, therefore, important to test this growth factor in the HB2 *in vitro* system. Figure 3.5.1. shows that recombinant human HGF (rhHGF) induces branching morphogenesis of HB2 cells in a dose dependent manner, and is effective at concentrations as low as 1-2 ng/ml. A maximal response of HB2 cells to rhHGF is seen at a concentration of 10ng/ml, after which the response plateau's out. The percentage of colonies induced to perform branching morphogenesis at a rhHGF concentration of 10ng/ml (the concentration routinely used), varies between experiments and between batches of rhHGF, but can be up to 100%. The branching morphogenesis induced by rhHGF is shown pictorially in Figure 3.5.2. panels C/D.

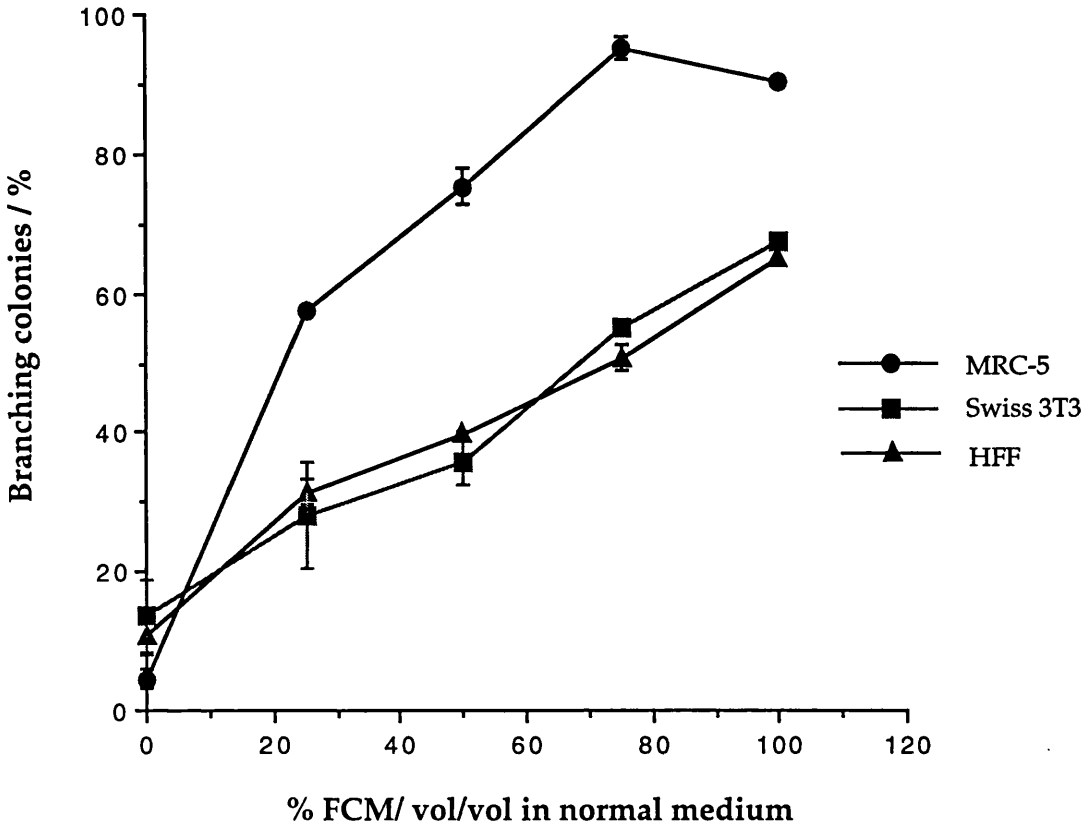


Figure 3.4.1.

Induction of branching morphogenesis of HB2 cells in a dose dependent manner with FCM from different types of fibroblasts.

HB2 cells were embedded in collagen gels and cultured with different amounts of conditioned medium from MRC-5, HFF, or Swiss 3T3 fibroblast cells. The percentage of colonies showing branching morphogenesis was determined after 6 days in culture.

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies branching was expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

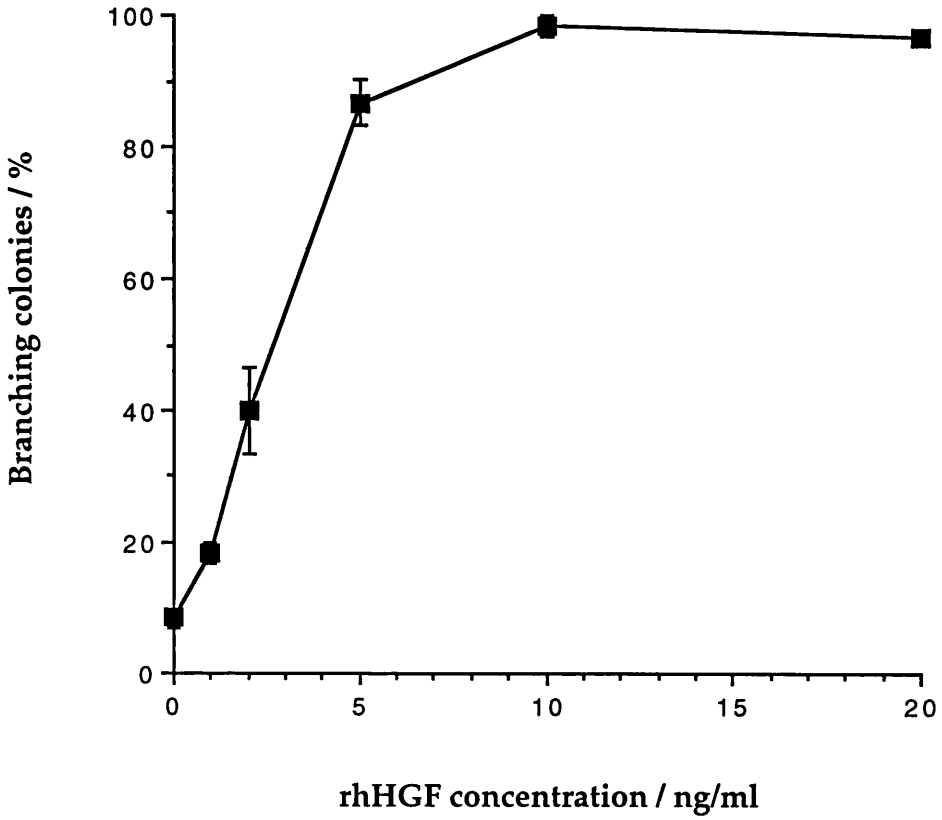


Figure 3.5.1.
Induction of branching morphogenesis of HB2 cells in a dose dependent manner with rhHGF.

HB2 cells were embedded in collagen gels and cultured with different concentrations of rhHGF. The percentage of colonies showing branching morphogenesis was determined after 7 days in culture.

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies branching was expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

Factor	Concentration/ ng/ml
Basic fibroblast growth factor (bFGF)	1 - 30 ng/ml
Acidic fibroblast growth factors (aFGF)	1 - 30 ng/ml
Platelet derived growth factor (PDGF)	1 - 20 ng/ml
Mouse hepatocyte growth factor (MuHGF)	1 - 100 ng/ml
Transforming growth factor β (TGF β)	0.1 - 20 ng/ml
Epidermal growth factor (EGF)	10 - 100 ng/ml
Keratinocyte growth factor (KGF)/FGF 7	1 - 50 ng/ml
FGF 3	1 - 50 ng/ml
EGF + bFGF (10 ng/ml)	50 ng/ml
KGF/FGF 7 + EGF (10 ng/ml)	1 - 50 ng/ml
KGF/FGF 7 + aFGF (10 ng/ml)	1 - 50 ng/ml

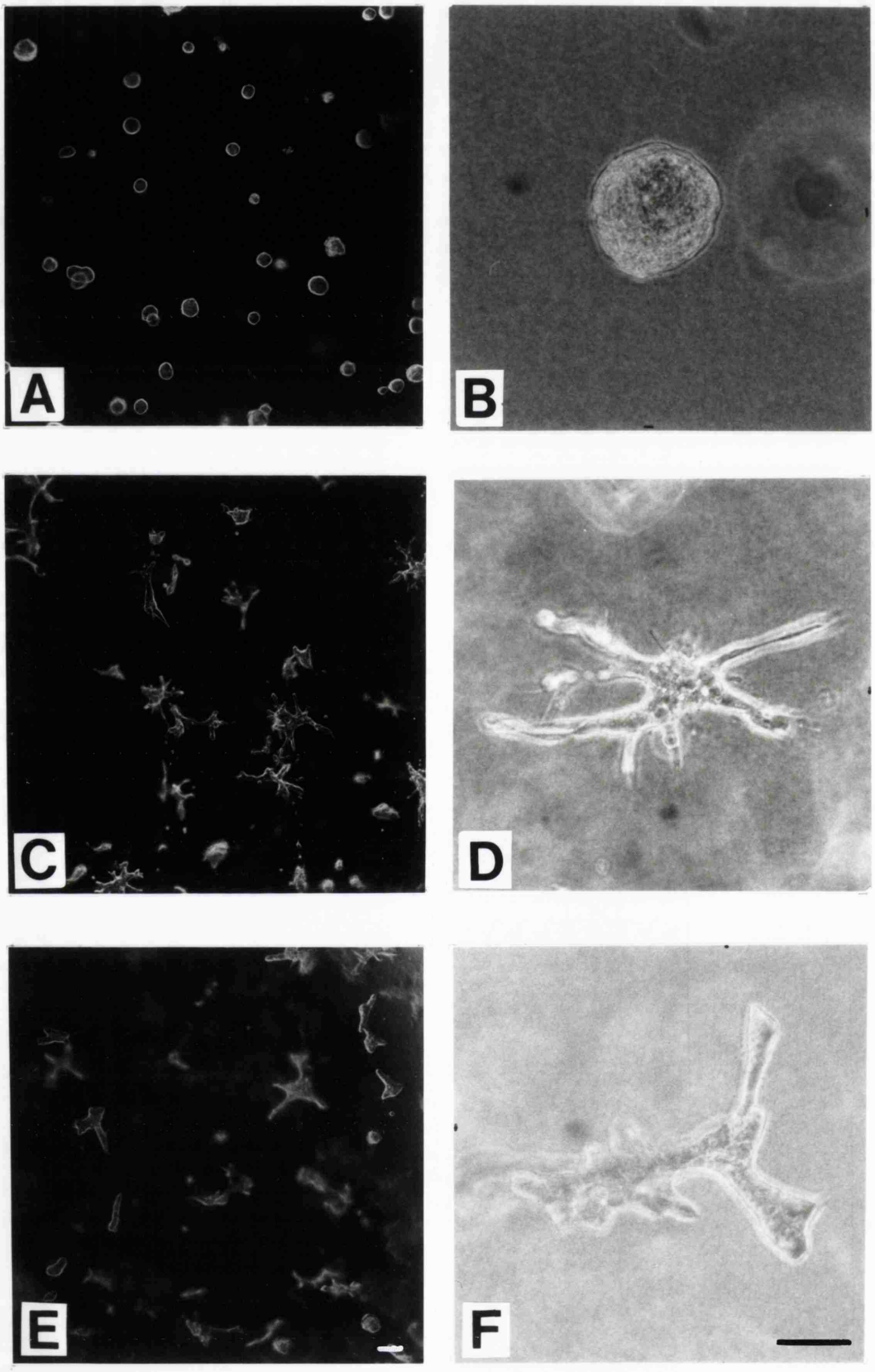
Table 3.5.1.
Growth factors tested which were unable to induce branching morphogenesis of HB2 cells.

HB2 cells were embedded in collagen gels and cultured in the presence of standard growth medium supplemented with the various growth factors listed in the table above, at the concentrations, and combinations indicated. None of the growth factors, or combinations of growth factors tested were shown to induce branching morphogenesis of HB2 cells.

Figure 3.5.2.
Induction of branching morphogenesis of HB2 cells in collagen gels by rhHGF and Human Foreskin Fibroblast Conditioned Medium, (HFF-CM).

HB2 cells were embedded in collagen gels and cultured in the presence of standard growth medium alone (panels A/B), growth medium supplemented with 10 ng/ml rhHGF (panels C/D), or conditioned medium from human foreskin fibroblasts (panels E/F). The morphogenetic phenotype of colonies were photographed after 7 days of growth at both low power (panels A/C/E) and high power (panel B/D/F) magnification.

Scale bar on photographs taken at low power denotes 200 μm
Scale bar on photographs taken at high power denotes 100 μm


A
B
C
D
E
F

Many other known growth factors have been tested, at a range of concentrations, and in various combinations, (Table 3.5.1), however none have been shown to have the ability to induce branching morphogenesis in this system.

3.5.1. HB2 cells express the HGF cell surface receptor, c-met.

The cognate cell surface receptor for HGF is c-met; a disulphide bonded heterodimeric transmembrane tyrosine kinase, made up of a 140 kDa β subunit (which contains the tyrosine kinase domain), and a 50 kDa α subunit. The presence of c-met on HB2 cells was demonstrated by Western blotting of whole cell lysates, and probing with a specific Ab to the 140 kDa β subunit of the c-met receptor under reducing conditions. Figure 3.5.3. shows the level of expression of c-met, and the fact that the expression level is not affected by rhHGF stimulation of HB2 cells. The 170 kDa protein that can be seen is the non disulphide bonded single chain c-met precursor protein.

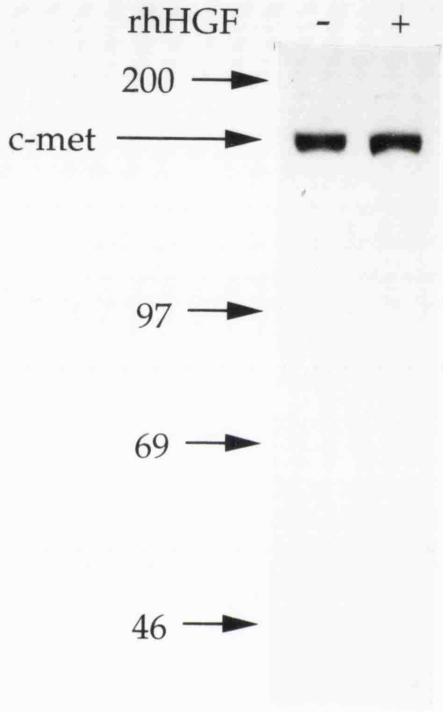
3.6. The stimulation of branching morphogenesis by human foreskin fibroblast conditioned medium (HFF-CM).

The demonstration that rhHGF could induce branching morphogenesis of HB2 cells, and was the only growth factor tested that had been shown to have this effect, meant that HGF was a prime candidate to be the soluble factor mediating the induction of branching morphogenesis by the MRC-5, Swiss 3T3, and HFF conditioned medium.

It was well documented that MRC-5 human embryonic lung fibroblasts secreted HGF (Stoker, M. *et al.*, 1987). Indeed Montesano, R. *et al.*, 1991b, had shown that in the MDCK system, the branching morphogenesis induced by MRC-5 fibroblasts, could be attributed solely to their secretion of HGF, (Montesano, R. *et al.*, 1991b). Swiss 3T3 cells are also documented to endogenously express HGF mRNA (Rong, S. *et al.*, 1992). However, in the HB2 (human) system, a purified recombinant murine HGF was found not to stimulate branching morphogenesis (Table 3.5.1.), thereby making it unlikely that HGF was the functional factor secreted by 3T3 cells.

Figure 3.5.3.
HB2 cells express the HGF cell surface receptor, c-met.

HB2 cells were grown on tissue culture plastic and starved overnight in serum free medium. Cells were then stimulated (+), or not (-) with 10 ng/ml rhHGF and proteins extracted in lysis buffer C. 50 μg of total protein was resolved by SDS-PAGE on a 7.5 % acrylamide gel under reducing conditions, and Western blotted onto a nitro-cellulose membrane. The membrane was then probed with a specific Ab (C-28) to the 140 kDa β subunit of the c-met heterodimer at a concentration of 0.05 μg/ml and was detected by ECL. The positions of migration of standard molecular weight markers are also shown.


rhHGF
-
+
200
c-met
97
69
46

Since the experiments by Montesano, R. *et al.*, 1991a had suggested that HFFs did not secrete HGF, it was of interest to investigate further the factor produced by human foreskin fibroblasts which induces branching morphogenesis.

3.6.1. The induction of branching morphogenesis of HB2 cells by HFF-CM is not due to the presence of HGF in the conditioned medium.

To confirm that the soluble factor in HFF-CM that was inducing branching morphogenesis of HB2 cells was not HGF, a functional blocking polyclonal antibody to rhHGF was obtained. This blocking Ab was used to try to inhibit the induction of branching morphogenesis by HFF-CM (Figure 3.6.1), after the antibody's functionality had been demonstrated by blocking branching morphogenesis induced by rhHGF (data not shown).

It can be seen from Figure 3.6.1. that although the blocking Ab to rhHGF inhibited MRC-5 induced branching morphogenesis of HB2 cells in a dose dependent manner, it had no effect on the branching morphogenesis induced by the HFF-CM. A non specific Ab, HMFG-1, directed against a transmembrane, non secreted, epithelial specific mucin, used at the same concentrations as the rhHGF Ab, had no effect on branching morphogenesis induced with either type of FCM (Figure 3.6.1.).

This experiment strongly indicated that a soluble factor other than HGF was responsible for the morphogenetic activity associated with the HFF-CM.

3.7. Further evidence that the soluble factor inducing branching morphogenesis in HFF-CM is not HGF.

3.7.1. HGF but not HFF-CM is sensitive to boiling temperatures.

HGF is known to be a heat labile protein (Stoker, M. *et al.*, 1987; Nakamura, T. *et al.*, 1989). The effect of boiling on the morphogenetic activity of CM from MRC-5, 3T3, and HFF fibroblasts, and standard growth media containing 10ng/ml of rhHGF was assayed. Results from a

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies branching was expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

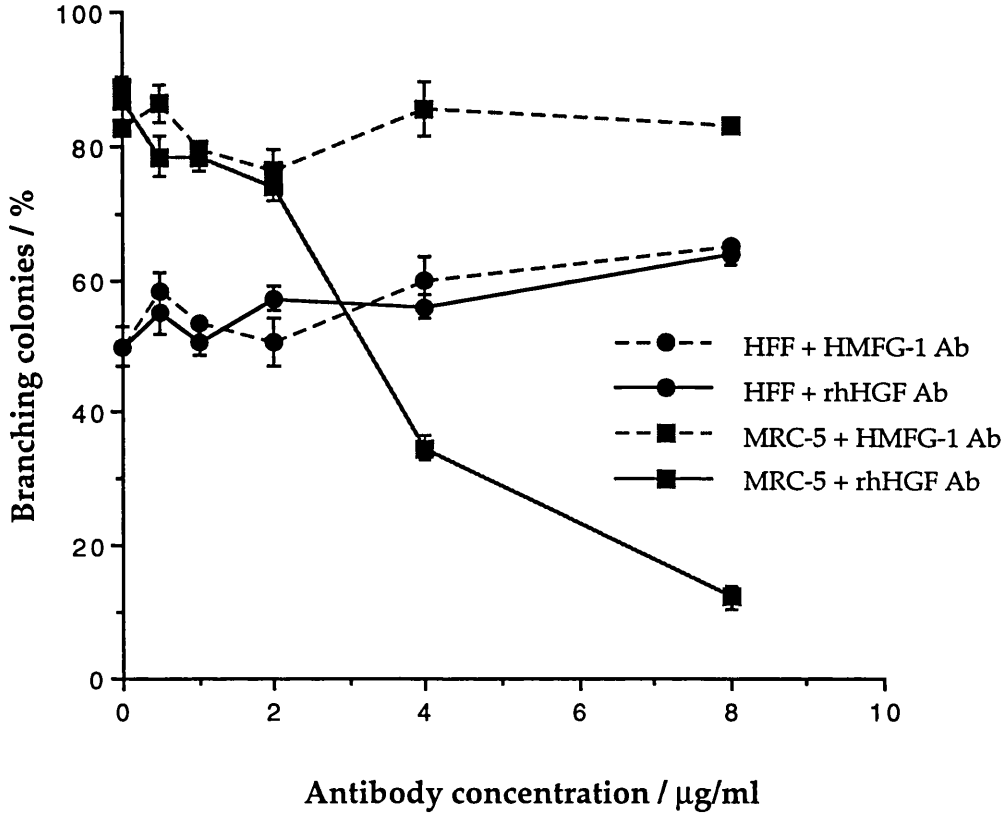


Figure 3.6.1.
Effect of a blocking antibody to rhHGF on branching morphogenesis of HB2 cells induced by MRC-5 and HFF conditioned medium.

HB2 cells were embedded in collagen gels, and cultured with MRC-5 or HFF conditioned medium that had been pre-incubated with various concentrations of Ab at 4°C for 4 hours. The Ab used to pre-incubate the conditioned media was either a blocking Ab to rhHGF, or a non-specific Ab to an epithelial mucin, (HMFG-1). The percentage of colonies showing branching morphogenesis was determined after 6 days in culture.

The histogram shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies branching was expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

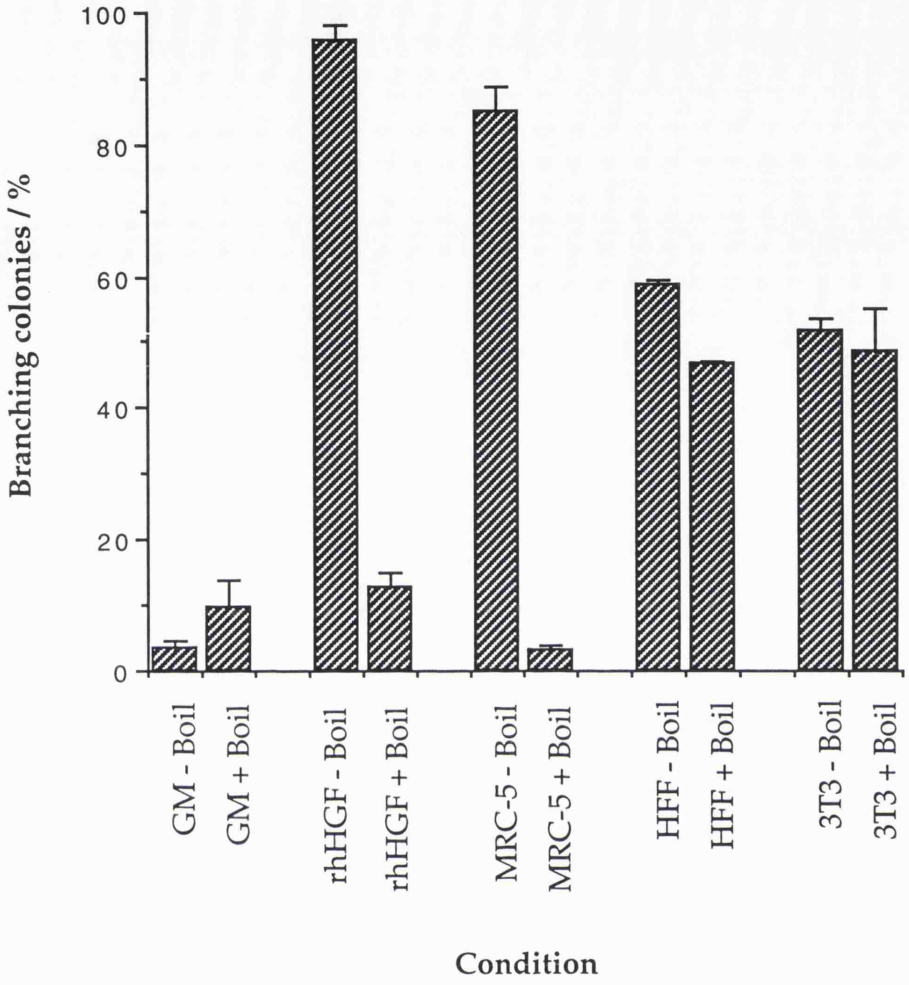


Figure 3.7.1.
Effect of heat treatment on the ability of fibroblast factors to induce branching morphogenesis of HB2 cells.

HB2 cells were embedded in collagen gels and cultured in the presence of growth media alone (GM), growth media supplemented with 10ng/ml rhHGF, or conditioned medium from different types of fibroblasts (MRC-5, HFF, or Swiss 3T3). In addition, each of the solutions were heat treated by placing in a boiling waterbath for 3 minutes (+ Boil), before being used to stimulate morphogenesis of HB2 cells. The percentage of colonies showing branching morphogenesis was determined after 7 days in culture.

typical experiment are shown quantitatively in Figure 3.7.1. It can be seen that, as expected, boiling leads to the abrogation of the branching morphogenetic activity of rhHGF containing media, and therefore MRC-5 FCM. However, boiling does not lead to the abrogation of the branching activity of HFF-CM or Swiss 3T3 FCM.

3.7.2. rhHGF but not HFF-CM can induce "scattering" of MDCK cells on plastic.

HGF was originally identified as a protein called "scatter factor" (Stoker, M. *et al.*, 1987), so named because of the observation that it induced a "scattered" morphology in many different types of epithelial cells. Epithelial cells that have been induced to scatter by HGF no longer form compact characteristic epithelial islets with tight cell-cell junctions, but look more fibroblastic and motile with fewer cell-cell contacts.

Further evidence that the morphogenetic factor in HFF-CM was not HGF, came from experiments showing that although MDCK cells were induced to "scatter" on plastic by rhHGF at concentrations of 5-50 ng/ml (Figure 3.7.2. panel B), HFF-CM had no such effect (Figure 3.7.2. panel C). Also, we have shown (as have Montesano, R. *et al.*, 1991a; Montesano, R. *et al.*, 1991b), that although rhHGF could induce branching morphogenesis of MDCK cells in collagen gels, HFF-CM could not (data not shown).

3.7.3. rhHGF but not HFF-CM can induce branching morphogenesis of HB2 cells in fibrin gels.

rhHGF can induce branching morphogenesis of HB2 cells when grown in fibrin, as well as collagen gels (Figure 3.7.3. panels C/D). However, when HFF-CM was used to try and induce branching morphogenesis in fibrin, no effect was seen, and spherical cysts were formed (data not shown), as seen in growth medium alone (Figure 3.7.3 panels A/B).

Figure 3.7.2.
rhHGF but not HFF-CM induces scattering of MDCK cells.

$5x10^4$ MDCK cells were plated in standard medium onto 35 mm tissue culture dishes. The cells were allowed to attach and then stimulated with standard growth medium (panel A), 50 ng/ml rhHGF (panel B), or HFF-CM (panel C). Photographs were taken at high power magnification after 48 hours of growth.

Scale bar denotes 100 μm

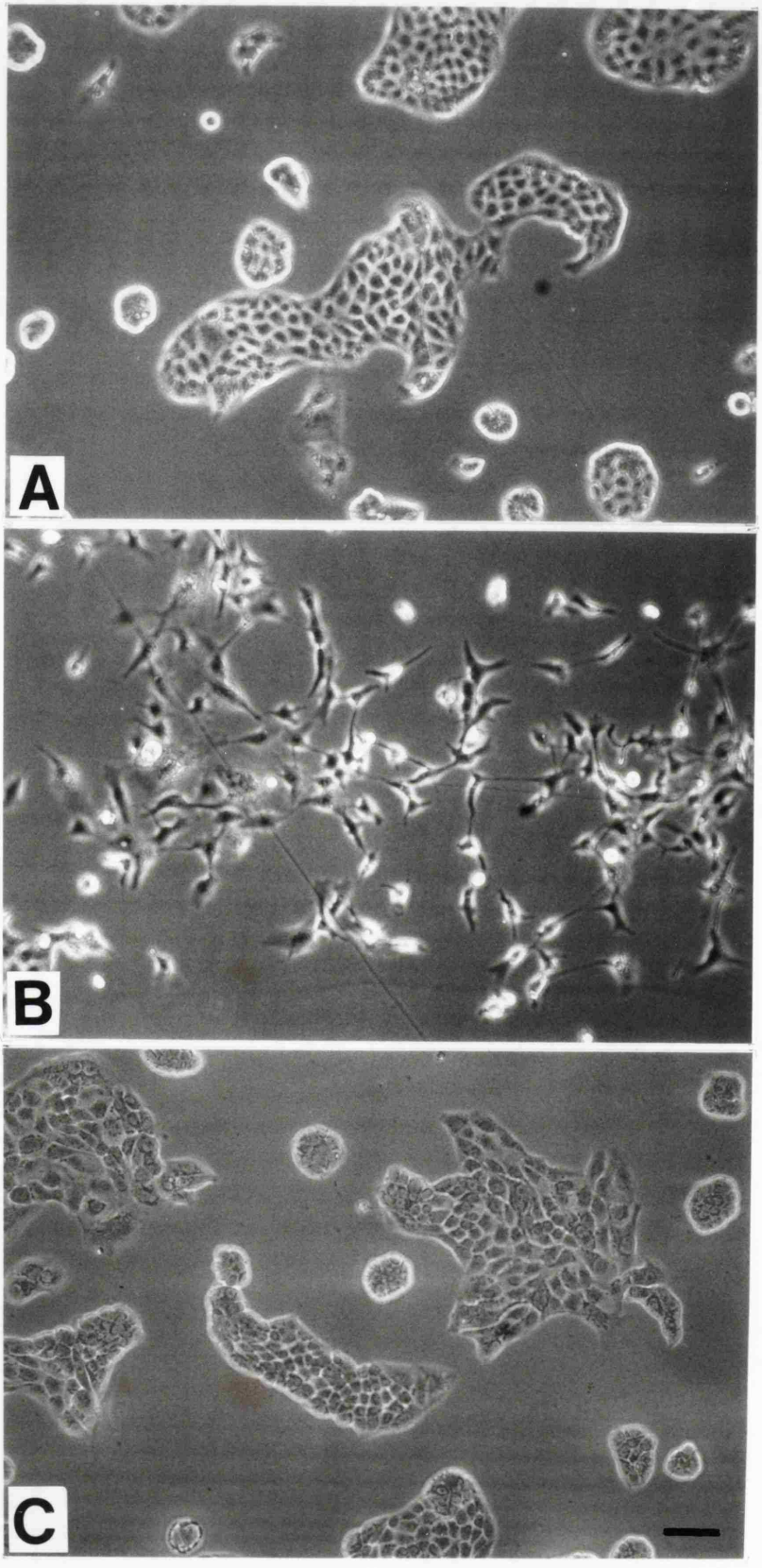
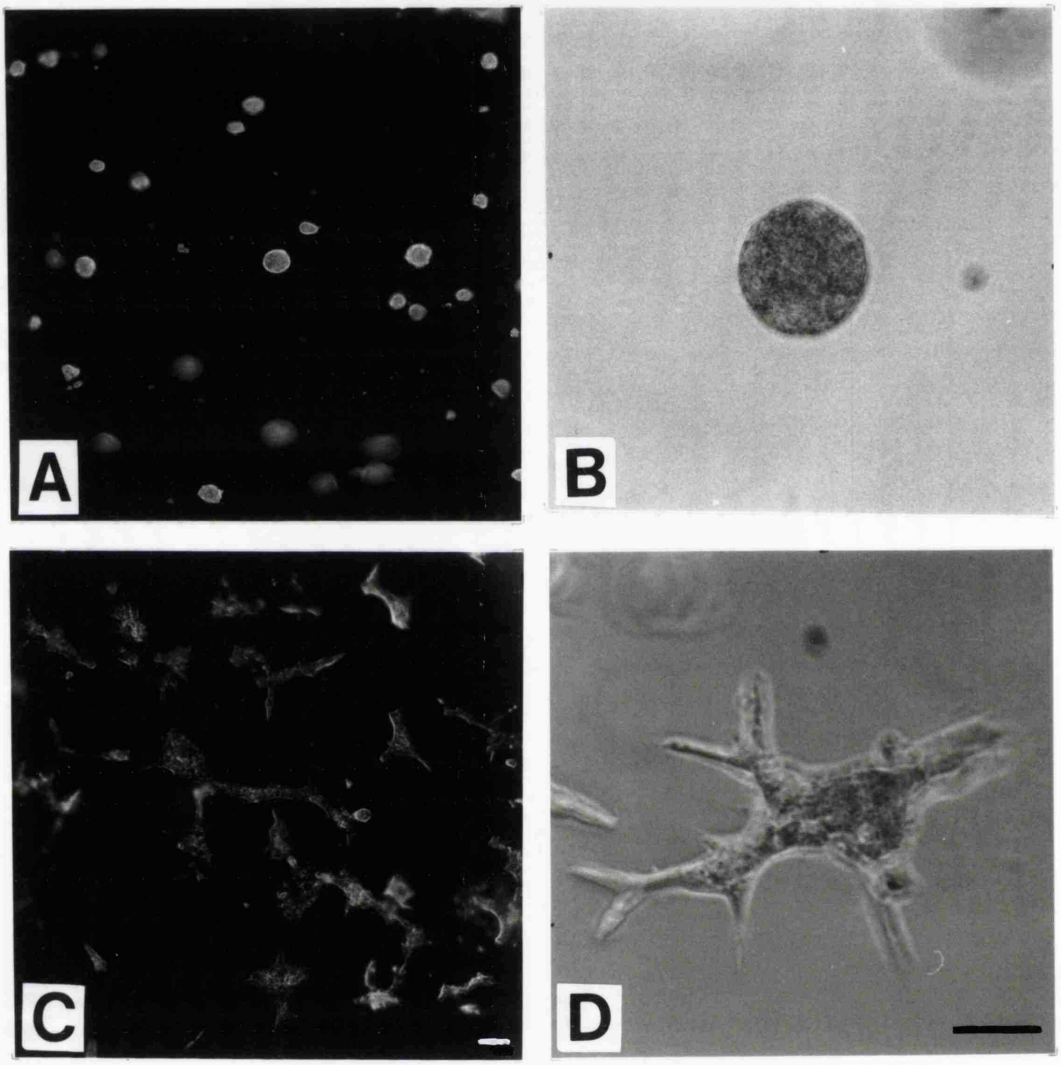

A
B
C

Figure 3.7.3.
rhHGF induces branching morphogenesis of HB2 cells in fibrin gels.

HB2 cells were embedded in fibrin gels and cultured in the presence of standard growth medium alone (A/B), or growth medium supplemented with 10 ng/ml rhHGF (C/D). The morphogenetic phenotypes of colonies were photographed after 7 days of growth, at both low power (panels A and C), and high power (panels B and D) magnification.

Scale bar on photographs taken at low power denotes 200 μm
Scale bar on photographs taken at high power denotes 100 μm


A
B
C
D

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies branching was expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

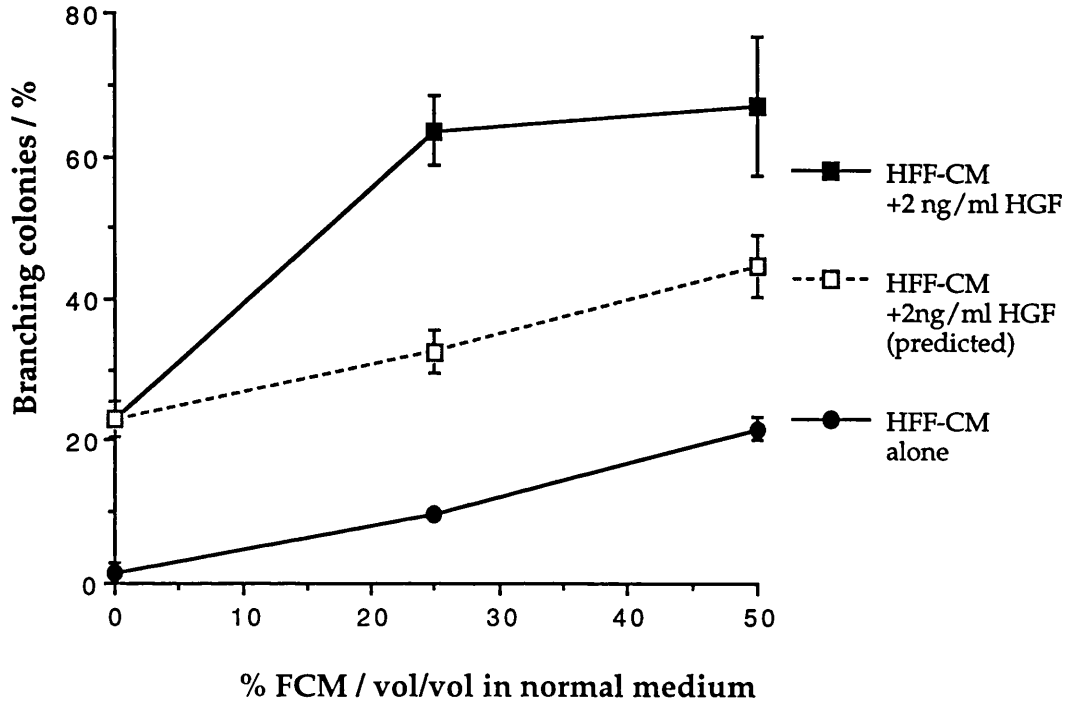


Figure 3.7.4.

Synergistic action of HFF-CM and rhHGF on the induction of branching morphogenesis of HB2 cells in collagen gels.

HB2 cells were embedded in collagen gels and cultured with 0, 25, or 50% concentrations of HFF-CM alone, or these same concentrations supplemented with 2ng/ml rhHGF. The percentage of colonies showing branching morphogenesis was determined after 7 days in culture.
By determining the percentage of colonies showing branching morphogenesis with different concentrations of HFF-CM alone (shown by the unbroken circled line —●—), and also with 2ng/ml rhHGF alone, a curve was plotted showing the predicted percentage of colonies branching if the individual effects of rhHGF and HFF-CM on branching morphogensis were additive, (--□--). The unbroken squared line (—■—) shows the actual percentage of colonies branching when the two factors were present in combination.

3.7.4. The effects of rhHGF and HFF-CM on stimulating branching morphogenesis are synergistic and not additive.

If the morphogenetic factor in HFF-CM was HGF, then it would be expected that small, sub-optimal doses of each, used together would have an additive effect on branching morphogenesis. This, however, is not the case, as can be seen from the data shown in Figure 3.7.4, where the predicted percentage of colonies branching if rhHGF and HFF-CM effects were additive are shown (as the squared symbol dotted line) compared to the actual percentage branching (shown as the squared symbol solid line). A synergistic effect has also been seen with 0.5ng/ml rhHGF and sub-optimal concentrations of HFF-CM (data not shown).

The observation was made that the structures formed by rhHGF and HFF-CM in combination, tended to be more complex, with more, and longer branches, than those induced by either factor alone.

The concentrations of rhHGF and HFF used for these experiments were carefully chosen. They lay within the linear parts of the individual dose response curves for the induction of branching morphogenesis, and were not near the parts of the curves where the branching morphogenesis response started to plateau out (see Figures 3.4.1. and 3.5.1. for HFF-CM and rhHGF dose response curves respectively).

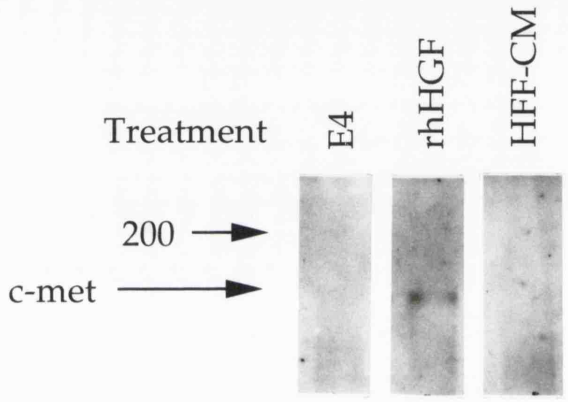
3.7.5. The soluble factor present in HFF-CM is not an HGF receptor agonist.

Having shown that the factor with morphogenetic activity in HFF-CM was not HGF, it was relevant to ask whether it stimulated branching morphogenesis through the HGF cell surface receptor, c-met. c-met is a transmembrane tyrosine kinase receptor, and binding of HGF induces phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor, which then stimulates further downstream signalling.

To test whether a ligand for c-met is the functional factor in HFF-CM, the effect on c-met phosphorylation of incubation of HB2 cells with HFF-CM was compared to the effect of rhHGF. It was shown that rhHGF but

Figure 3.7.5.
The soluble factor present in HFF-CM is not an HGF receptor agonist.

HB2 cells were grown on tissue culture plastic and starved overnight in serum free medium. Cells were then stimulated with E4 containing 10 ng/ml rhHGF, serum-free HFF-CM, or unsupplemented E4, and proteins extracted in lysis buffer C. The c-met protein was immunoprecipitated from 500 μg of total cell lysate using 2 μg of a specific mAb (C-28) to the 140 kDa β subunit of the c-met heterodimer. The precipitated proteins were eluted in 2x SDS PAGE sample buffer containing DTT, resolved by SDS-PAGE on a 7.5 % gel under reducing conditions and Western blotted onto a nitro-cellulose membrane. The membrane was then probed with a specific Ab to phosphotyrosine and was detected by ECL. The position of the 140 kDa subunit of the c-met heterodimer is indicated, as is the position of the 200 kDa standard molecular weight marker myosin.


Treatment
E4
rhHGF
HFF-CM
200
c-met

not HFF-CM induced autophosphorylation of the c-met receptor in HB2 cells (Figure 3.7.5). This was done by immunoprecipitating c-met from whole cell lysates of treated HB2 cells, and Western blotting the immunoprecipitates with anti-phosphotyrosine Abs (Figure 3.7.5) and also by sequentially probing Western blots of whole cell lysates with anti-c-met receptor, and anti-phosphotyrosine antibodies (data not shown). From the experiments described above, it was concluded that there were at least two factors, secreted by fibroblasts, that could induce branching morphogenesis of HB2 cells in collagen gels; the first being HGF, and the second, an unknown, potentially novel factor, secreted by HFFs.

3.8. Mechanism of the induction of branching morphogenesis of HB2 cells by fibroblast secreted factors.

3.8.1. rhHGF or HFF-CM do not significantly increase the proliferation of HB2 cells growing in collagen gels.

HGF has been reported to have a mitogenic effect on certain epithelial and endothelial cell types, (Igawa, T. *et al.*, 1991; Rubin, J.S. *et al.*, 1991; Bussolino, F. *et al.*, 1992), but not on others, such as MDCK epithelial cells (Montesano, R. *et al.*, 1991a). It was, therefore, important to know whether the branching morphogenesis of HB2 cells induced by rhHGF, and indeed that induced by the unknown factor in HFF-CM, was due to a morphogenetic effect, or simply the product of a mitogenic response. To do this, HB2 cells were cultured in collagen gels as normal, and stimulated with either rhHGF, or epidermal growth factor (EGF). EGF is a known mitogen for mammary epithelial cells, and although shown to alter the morphology of HB2 colonies in collagen gels, did not induce branching morphogenesis (Table 3.5.1).

It had been shown (Figure 3.5.1), that rhHGF could induce branching morphogenesis of HB2 cells from a concentration as low as 1ng/ml, and that a maximal branching response was seen at 10ng/ml, which was also the concentration used routinely in experimental assays. It was therefore decided to use concentrations of rhHGF of 2.5ng/ml, and 10ng/ml (both of which induced a good branching response), and a concentration of EGF of 30ng/ml, which altered the morphology of the HB2 cells in collagen gels, but did not induce branching morphogenesis. At the end

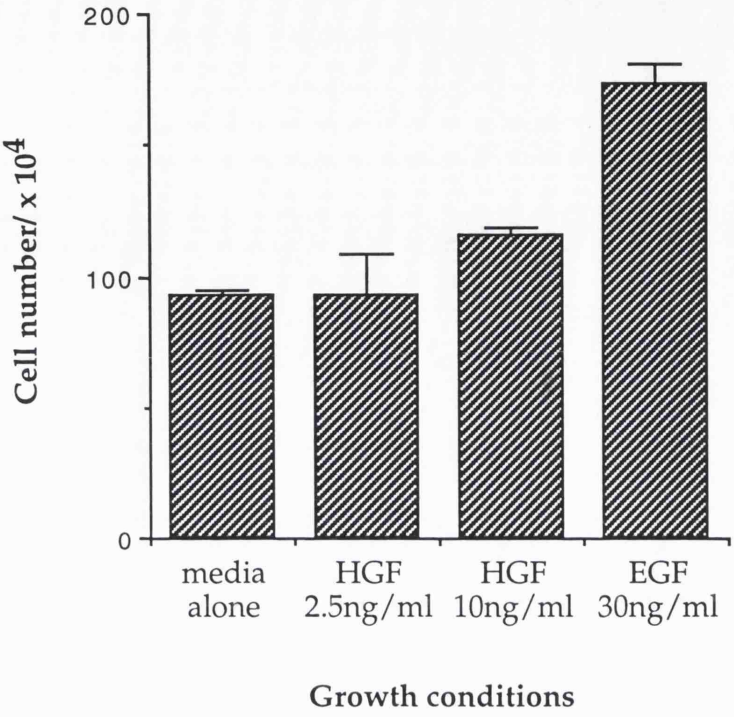


Figure 3.8.1.
Effect of rhHGF on HB2 cell proliferation during branching morphogenesis.

$10x10^4$ HB2 cells were embedded in collagen gels and cultured in normal growth media, in media supplemented with rhHGF at a concentration of 2.5 or 10ng/ml, or in media supplemented with EGF at 30ng/ml. After 7 days of culture, colonies were removed from collagen by collagenase treatment, made into a single cell suspension, and counted.

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the total number of cells were counted and the mean plotted, together with the standard error showing the deviation about the mean.

of the morphogenetic time course the total number of cells in the various conditions were counted, and compared to the total number where cells had been cultured with standard growth medium alone, and therefore had not been induced to form branching structures. The results are shown in Figure 3.8.1, where it can be seen that although EGF stimulated the growth of HB2 cells by a factor of roughly two-fold, rhHGF at either concentration, had no significant effect on proliferation, compared to media alone.

This result shows that at a concentration of rhHGF required to induce a maximal branching response in HB2 cells, no increase in the proliferation of HB2 cells was seen, and therefore, increased proliferation did not play a role in the branching morphogenesis mechanism.

In separate experiments (data not shown), it was also shown that HFF-CM did not significantly increase proliferation of HB2 cells in collagen gels.

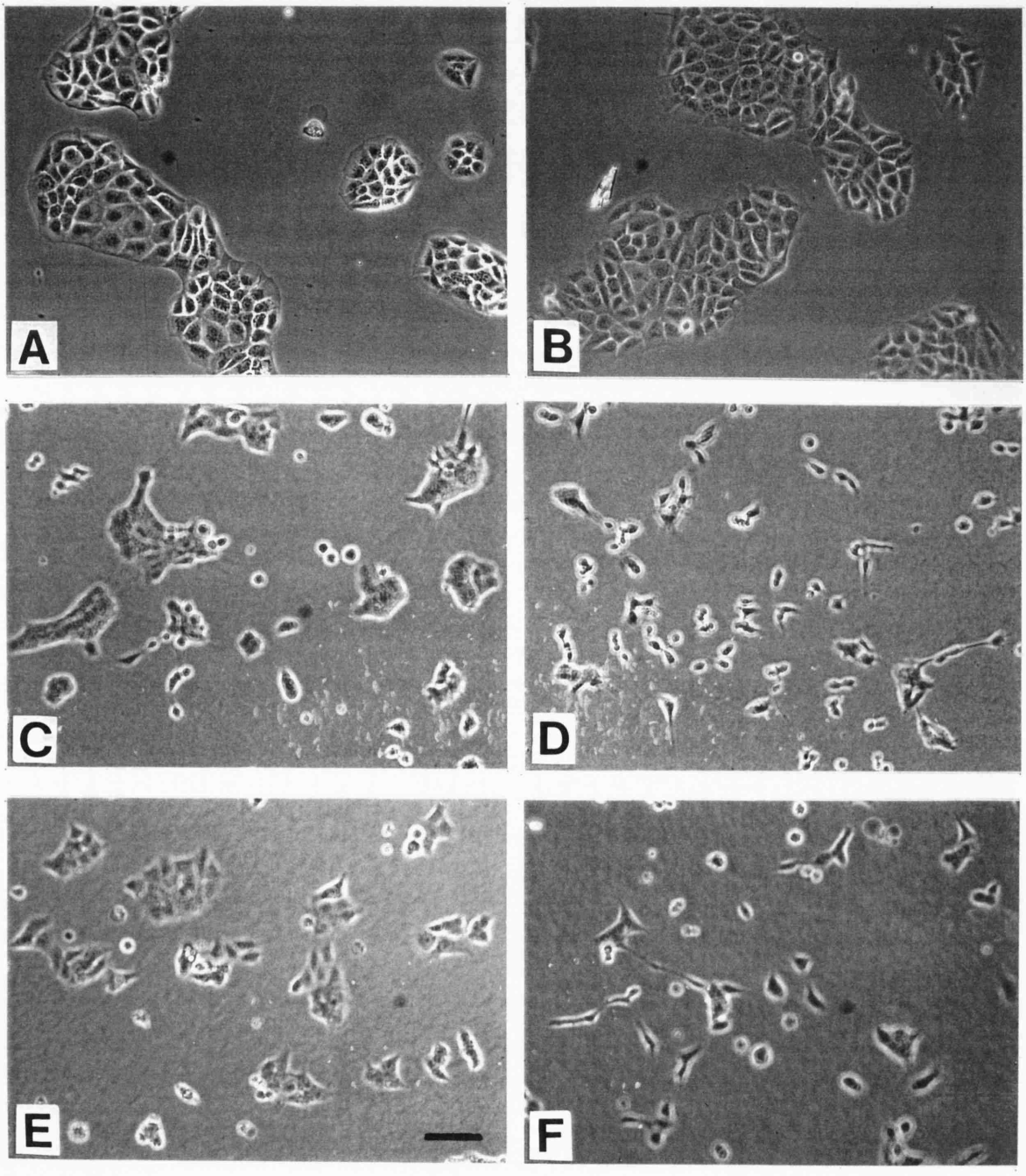
3.8.2. rhHGF induces a "scattered" morphology of HB2 cells on collagen and fibrin matrices.

Unlike the effect induced in MDCK cells, rhHGF did not induce scattering of HB2 cells on tissue culture plastic. However, it was observed that HB2 cells could be induced to scatter on both collagen and fibrin matrices by rhHGF (Figure 3.8.2). HFF-CM was also found to induce scattering of HB2 cells on collagen gels, but not on fibrin or plastic (data not shown), although the scattering response was not as marked as that seen with rhHGF (only clearly seen when a 3-fold concentrated sample of HFF-CM was used).

These results, showing that HFF-CM was less potent than HGF at inducing scattering of HB2 cells on collagen, correlate with the observation that HFF-CM is not as potent as HGF at inducing branching morphogenesis of HB2 cells. Moreover, the fact that rhHGF induced branching morphogenesis of HB2 cells when cultured within both fibrin and collagen matrices, while HFF-CM induced branching morphogenesis of HB2 cells only when cultured in collagen, suggests that reproduction of the 2-dimensional scattering response in a 3-

Figure 3.8.2.
Induction of "scattering" of HB2 cells on collagen and fibrin matrices by rhHGF.

$5x10^4$ HB2 cells were plated in standard medium onto 35 mm tissue culture dishes that were left uncoated (A/B), coated with a polymerised collagen matrix (C/D), or coated with a fibrin matrix (E/F). The cells were allowed to attach and then stimulated with standard medium (left hand side), or 10 ng/ml HGF (right hand side). Photographs were taken after 48 hours of growth.
Scale bar denotes 100 μm


A
B
C
D
E
F

dimensional form, might play an important part in the branching morphogenesis process.

3.8.3. rhHGF and HFF-CM stimulate the motility of HB2 cells on collagen gels.

The scattered response induced by rhHGF is often interpreted to represent a stimulation of motility of epithelial cells. This was verified by using time-lapse microscopy. HB2 cells were plated on collagen gels, and either given no stimulation, stimulated with various concentrations of rhHGF, or a 3-fold concentrated sample of HFF-CM. The movement of 36 or 48 cells was monitored for 24 hours, and the average speed of cells calculated for the various conditions (see Materials and Methods). The results are shown in Table 3.8.3. where it can be seen that both rhHGF and HFF-CM significantly increase the motility of HB2 cells on collagen gels, compared to the motility seen in media alone, and that the rhHGF stimulation occurs in a dose dependent manner.

From these experiments in a 2-dimensional system, showing the induction of a scattered phenotype and a corresponding stimulation of motility of HB2 cells on matrix components with rhHGF (and HFF-CM). It seemed likely that the stimulation of cell separation and motility, when transferred into a 3-dimensional system, might play an important part in the mechanism of branching morphogenesis.

This *in vitro* morphogenesis system, wherein a non-tumorigenic human mammary epithelial cell line, HB2, is cultured in 3-dimensions in collagen type I and/or fibrin matrices, has been used:
1). To biochemically characterise the unknown factor in HFF-CM that can stimulate branching morphogenesis, (Chapter 4).
2). To investigate the role of integrins and integrin mediated cell-ECM contact, in the branching morphogenesis process and in epithelial cell growth, (Chapter 5), and
3). To assess the effect of overexpression of the *v-Ha-ras* oncogene on the organised morphogenesis of human mammary epithelial cells, (Chapter 6).

The mean speed of cells in medium alone is 12.94 μm/hr. The calculated 95% confidence interval using a two-tailed t test of significance lies between 11.22 μm/hr and 14.66 μm/hr. Experimental means lying outside this range deviate sufficiently from the control to be deemed significantly different.

Treatment	rhHGF concentration/ ng/ml	Average speed [1]/ µm/hour	Overall average speed [2]/ µm/hour	s.e. overall average speed [3]/ µm/hour
media alone	0	11.55 15.34 14.31 10.54	12.94	1.96
HGF	20	19.36	19.36	n/a
	15	17.83 18.93	18.38	0.55
	10	16.09 12.25	14.17	1.92
HFF-CM[4]	n/a	21.76	21.76	n/a

Table 3.8.3.
Stimulation of motility of HB2 cells on collagen by rhHGF and HFF-CM.

$5x10^4$ HB2 cells were plated in standard growth medium onto 35 mm tissue culture dishes coated with 1 ml of polymerised collagen. The cells were allowed to attach and then stimulated with standard growth medium alone, standard medium containing rhHGF at the concentration indicated, or HFF-CM. The motility of individual cells was monitored by time-lapse videomicroscopy as described in Materials and Methods.

Footnotes:

1. This column lists the average speed of 36 or 48 cells calculated for each condition in individual experiments.
2. This column shows the overall average speed of cells for a particular condition, calculated from all the experiments done.
3. This column shows the standard error of the overall average speeds given in 2.
4. The HFF-CM had been concentrated in an Amicon stirred cell, and was used in the motility assay at a final concentration that was three times that of the harvested medium.

n/a = not applicable.

DISCUSSION AND CONCLUSIONS.

This chapter describes the establishment of an *in vitro* system, whereby organised branching morphogenesis of a non-tumorigenic human mammary epithelial cell line could be induced by fibroblast secreted factors. This system provides a basis for investigating the molecules involved in the organised morphogenesis of human mammary epithelial cells, including the roles of integrins and the effect of oncogenes, which will be discussed in Chapters 5 and 6 respectively.

From the results described in this chapter, it was concluded that there were at least two distinct soluble factors, secreted by fibroblasts, that could induce branching morphogenesis of human mammary epithelial cells; the first being HGF, and the second being an as yet unidentified, and potentially novel factor, secreted by HFFs. These results were the first describing the induction of branching morphogenesis in a mammary epithelial cell line by HGF and HFF-CM.

It was also concluded that the mechanism of induction of branching morphogenesis of HB2 cells by HGF or HFF-CM, was not due to the stimulation of increased proliferation, but was likely to involve the stimulation of increased motility and cell separation by HGF or HFF-CM. However, it should be stressed that the stimulation of motility, although it seems probable that it is necessary, is unlikely to be sufficient for the induction of branching morphogenesis in a 3-dimensional system.

The choice of collagen type I and fibrin matrices for the *in vitro* system.

(i) Collagen type I.

Collagen type I was chosen as a matrix component in this *in vitro* system for several reasons:
1) Using a matrix that consisted of one matrix component rather than several (for example like Matrigel), would make the system inherently more simple, and hence easier to analyse at the molecular level, which was the aim of the project. Matrigel is also known to bind growth factors, further complicating the interpretation of experiments.

2) Being a major component of the stroma, collagen type I is a relevant matrix in terms of the morphological development of the human mammary gland as seen in the embryo, at puberty, and at pregnancy. In the embryonic stages of human mammary gland development, epithelial cells have to invade the stroma of the breast to form a primitive epithelial duct with poorly developed branching side arms (Dabelow, A., 1957; Russo, J. and Russo, I., 1987). At puberty and at pregnancy these epithelial ducts undergo more extensive branching morphogenesis, meaning the cells again have to invade the stroma, to form the ductal tree characteristic of the adult and pregnant mammary gland respectively (Russo, J. and Russo, I., 1987).

3) A stromal matrix is also invaded by breast cancer cells during tumour progression (after the cells have invaginated through, or separated from, the basement membrane); thereby making it a relevant matrix for investigating the processes of tumour cell invasion.

(ii) Fibrin.

Although fibrin/fibrinogen as a matrix, is not as relevant for mammary gland development, or breast cancer progression as collagen type I, it provides a useful comparison to the collagen gel system when trying to analyse the mechanism of branching morphogenesis and the molecules involved.

The results described above have shown a role for the fibroblast secreted factor HGF in branching morphogenesis of HB2 cells, both in the fibrin and collagen matrix systems. The other molecules hypothesised to play a role in branching morphogenesis by virtue of their mediating interactions with the extracellular matrix (ECM), and also by mediating transduction of signals from the ECM which could be important for the formation of organised structures, are the integrins. Different integrins have been shown to act as receptors for fibrin/fibrinogen ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_M\beta_2$, $\alpha_{IIb}\beta_3$), and for native collagen type I ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$). The HB2 cells have been shown to express the α_2, α_3, α_v, and β_1 integrin subunits (Figures 3.2.1. A and B), thereby giving the cells the potential to interact with both matrices via integrin receptors. This meant that comparison of the fibrin gel system with the collagen system, would

prove to be especially useful in analysing the role of integrins, and more specifically integrin mediated interaction with ECM, in branching morphogenesis of HB2 cells, (see Chapter 5).

Role of matrix in regulating cell motility.

The results described in this chapter show that although HGF can induce scattering of HB2 cells on both collagen type I and fibrin matrices, it cannot induce scattering on tissue culture plastic. This is an interesting observation considering that growing cells on tissue culture plastic in the presence of serum, is the equivalent of growing cells on a VN matrix coated surface (Edwards, J.G. *et al.*, 1987; Underwood, P.A. and Bennett, F.A., 1989). These results imply a requirement for a specific integrin-ligand interaction in the motility response.

Possible explanations for the lack of a scattering response of HB2 cells to HGF on VN (serum coated tissue culture plastic) have not been analysed. However, it may be that the HB2 cells lack the major integrin receptor required for migration on VN, whereas they do express the integrins required for migration on fibrin and collagen. The expression of the β_3 integrin subunit on HB2 cells has not been analysed. However, the majority of breast epithelial cell lines examined do not express this subunit (John Marshall - personal communication) and neither does the mammary gland *in vivo*. As the $\alpha_v\beta_3$ is documented to be an important integrin involved in cell migration on VN (Leavesley, D.I. *et al.*, 1992; Aznavoorian, S. *et al.*, 1996), then lack of the β_3 subunit in HB2 cells (as is likely), might conceivably explain the lack of HGF induced motility on VN.

It is also known that HB2 cells, when cultured on tissue culture plastic, lay down a cell deposited matrix composed mainly of epiligrin (Berdichevsky, F. *et al.*, 1994b). The $\alpha_3\beta_1$ integrin is a known epiligrin receptor (Carter, W.G. *et al.*, 1991). However it seems that, at least in the case of HB2 cells, the epiligrin-$\alpha_3\beta_1$ integrin interaction does not allow the HGF induced motility and cell separation seen on the fibrin and collagen matrices.

Induction of branching morphogenesis by Swiss 3T3 CM.

Although the results described in this chapter are primarily concerned with the stimulation of branching morphogenesis by HGF, and the unknown factor present in HFF-CM; the factor in Swiss 3T3 CM that induces branching morphogenesis may also be of interest. It has been shown that Swiss 3T3 cells express HGF mRNA (Rong, S. *et al.*, 1992); however, HB2 cells were not able to branch in response to a recombinant murine HGF tested. This apparent species specificity in the ligand-receptor interaction of HGF had also been observed for the induction of tumorigenicity by HGF (Rong, S. *et al.*, 1992).

Since murine HGF does not induce branching morphogenesis of HB2 cells, it would appear that the functional factor in Swiss 3T3 CM is not murine HGF. This is further supported by the observation that the factor in Swiss 3T3 CM that induces branching morphogenesis is heat resistant, as is the factor in HFF-CM. It is possible, given its heat resistant property, and the evidence that it is not HGF, that the factor in Swiss 3T3 CM is the same factor, or at least the murine analogue, of the factor in HFF-CM. We have however focused our efforts on characterising the factor(s) in HFF-CM which induce branching morphogenesis (see Chapter 4), and have not analysed further the Swiss 3T3-CM.

CHAPTER 4: BIOCHEMICAL CHARACTERISATION OF THE UNKNOWN FACTOR PRESENT IN HUMAN FORESKIN FIBROBLAST CONDITIONED MEDIUM THAT INDUCES BRANCHING MORPHOGENESIS OF HUMAN MAMMARY EPITHELIAL CELLS.

INTRODUCTION.

In the previous chapter it was shown that a non-tumorigenic human mammary epithelial cell line, HB2, could be induced to perform branching morphogenesis in collagen gels, by culturing in CM from MRC-5, Swiss 3T3, and HFF cells (Figure 3.4.1.). The induction of branching morphogenesis occurred in a dose dependent manner (Figure 3.4.1.), and rhHGF was the only growth factor tested that could replicate this branching morphogenesis (Figure 3.5.2. and Table 3.5.1).

HGF was shown to be the factor in MRC-5 CM that induced branching morphogenesis. However the soluble factor secreted by HFFs was shown to be different to HGF, and potentially novel. With the soluble factor secreted by HFFs being potentially novel, it was important to biochemically characterise it, with the long term view of possibly trying to purify it.

In all experiments where the unknown factor was being characterised, it was necessary to quantitate the activity of a treated sample, or a column fraction, in the biological branching morphogenesis assay. The usual time course of this assay is 6-7 days, to allow structures to grow to an appropriate size to get good photographs, and to become as intricate as well developed as possible. However, by 3-4 days in culture, colonies have put out processes and formed small branching colonies. These Day 4 colonies can be counted and were used to assess activity in biochemical fractions.

In each experiment, the activity has been measured by calculating the percentage of colonies showing branching morphogenesis, in different fractions, or under different conditions. The main difference in the quantitation of branching colonies over this shorter time course, is in the definition of a branching colony, which has been made less stringent,

because the colonies have had less time to develop (particularly in terms of the length of the branches formed).

Two definitions of a branching colony have been used in these experiments, (see individual figure legends). The first is less stringent, and includes every colony with one or more protrusions, although if only one protrusion is seen, it must be at least the length of the cell body diameter. The second is more stringent, in that the colony must have at least three protrusions, although only one of these must be at least the length of the cell body diameter. Both definitions easily distinguish an active sample from an inactive one; the only problem with the first definition being that because it is less stringent, there is sometimes quite a high background level of branching in cells that have been grown with normal growth medium alone, or an inactive HFF sample. In experiments where this background is exceptionally high, the results have also been shown with the background branching subtracted, to show more clearly where the actual activity is seen.

RESULTS

4.1. Basic properties of the unknown factor present in HFF-CM.

Initially, some simple experiments were carried out to establish that the morphogenetic activity in HFF-CM was indeed due to a protein, and to establish more completely the sensitivity of this protein to temperature, and also to reducing agents and changes in pH.

4.1.1. The morphogenetic activity of HFF-CM is attributable to a protein.

Figure 4.1.1 shows the trypsin-sensitive nature of the morphogenetic activity, and that this activity is not sensitive to treatment with agents that will digest nucleic acids. Due to the fact that these treatments had an optimum temperature of 37°C, HFF-CM treated at 37°C but in the absence of any additives, was used as the positive control for activity. As can be seen from Figure 4.1.1. this heat treatment of HFF-CM did not affect its activity.

The histogram shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies branching was expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

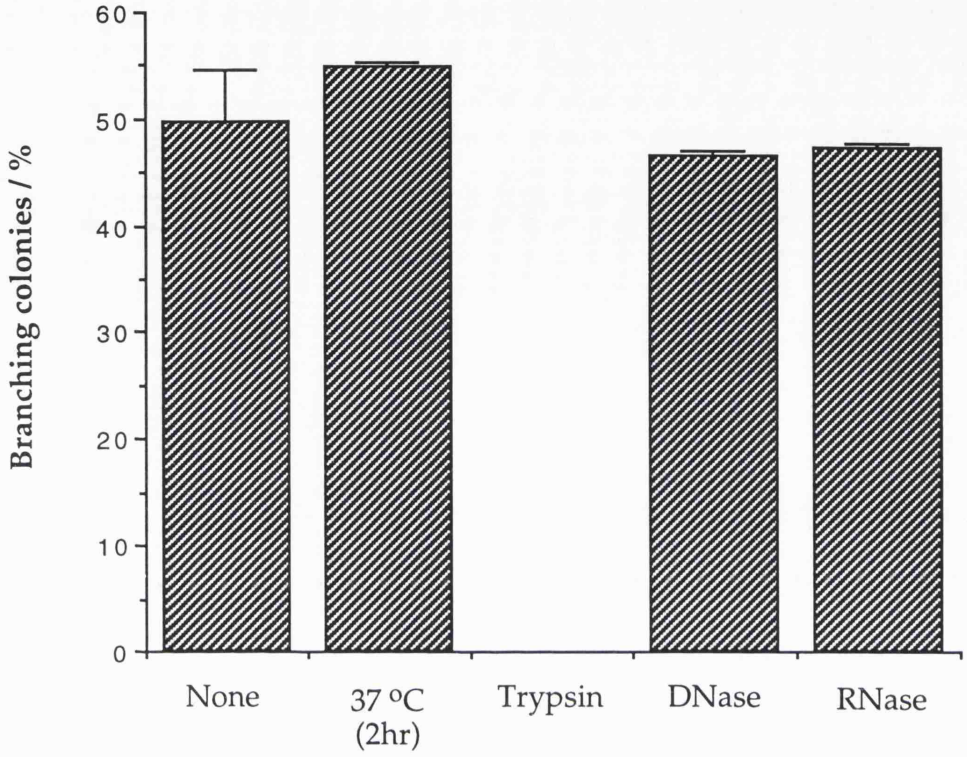


Figure 4.1.1.
Effect of trypsin, DNase and RNase on the ability of HFF-CM to induce branching morphogenesis of HB2 cells in collagen gels.

HFF-CM was pre-treated at 37°C for 2 hours with Trypsin (0.1mg/ml), DNase (30U/ml), RNase (40µg/ml), or left in the absence of any treatment. Each medium was then assayed for its ability to stimulate branching morphogenesis of HB2 cells in collagen gels. As a control for the heat treatment, HFF-CM that had not been heat treated was assayed for comparison. The percentage of colonies showing branching morphogenesis was determined after 4 days, with a branching colony defined as one with at least 3 protrusions from the central cell body, with one being at least the length of the cell body diameter.

4.1.2. Sensitivity of the morphogenetic activity of HFF-CM to heat treatment.

The effect on HFF-CM activity, of exposure to a boiling temperature for a short time, had already been examined (Figure 3.7.1). To examine more closely the temperature sensitivity of the protein, treatment was carried out over a range of temperatures, and exposure to each temperature was carried out for 30 minutes. The longer exposure time than 3 minutes, and the lower temperatures than boiling, were chosen mainly with the long term view of potentially using the heat resistance of this protein as a purification step. For the large volumes of conditioned medium that would inevitably be involved in a purification, heating at a lower temperature than boiling would be more manageable, and also, the time taken for this large volume to reach the required temperature would be faster and therefore the treatment more controlled.

It can be seen from Figure 4.1.2. that with a 30 minute heat treatment the activity is stable up to a temperature of 60°C, but above this temperature the activity starts to decrease quite quickly.

4.1.3. Sensitivity of the morphogenetic activity of HFF-CM to reducing agents.

Table 4.1.3. shows that the HFF-CM morphogenetic activity is not sensitive to the reducing agent dithiothreitol (DTT). This suggests that the activity is not dependent on any intrachain disulphide bonds, and that the active form of the protein is not a disulphide bonded homo- or heterodimer. The active form of HGF is a disulphide bonded heterodimer, produced by proteolytic cleavage and processing of a single precursor protein, (Naka, D. *et al.*, 1992; Naldini, L. *et al.*, 1992). rhHGF was treated with DTT as a control to show that the reducing agent was working. Table 4.1.3. shows that rhHGF activity is sensitive to DTT at the same concentration and conditions used to treat the HFF sample.

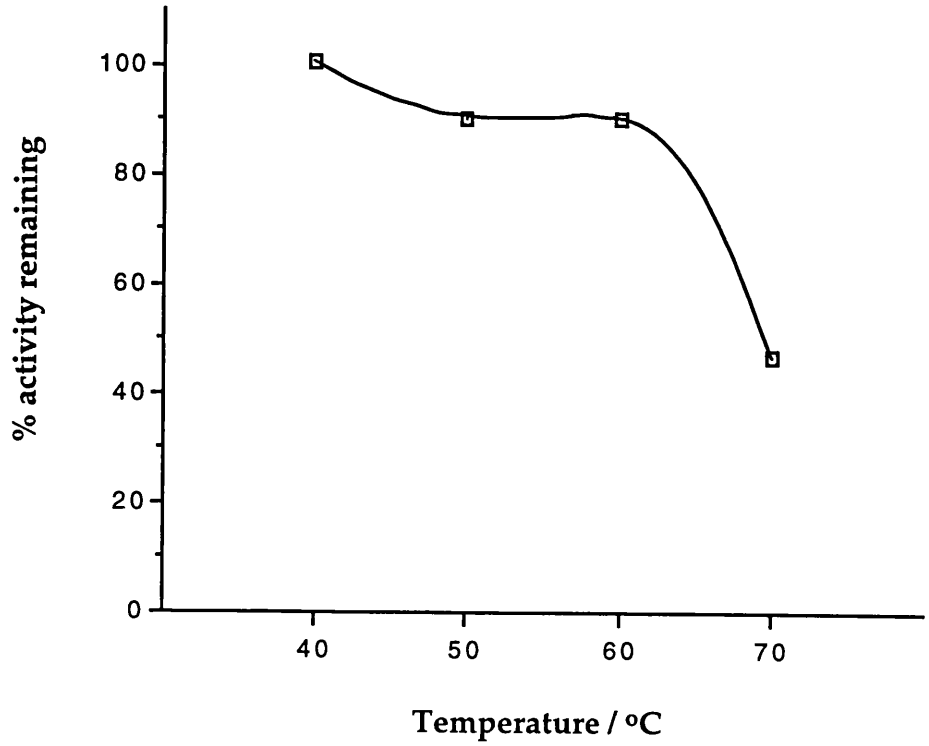


Figure 4.1.2.
Effect of variations in temperature on ability of HFF-CM to induce branching morphogenesis of HB2 cells in collagen gels.

HFF-CM was heated at temperatures of 40, 50, 60, and 70°C for 30 minutes. The heated media was then clarified at 13 000 r.p.m. for 15 minutes to remove precipitated protein and then assayed for its ability to induce branching morphogenesis of HB2 cells in collagen gels. Non-heat treated HFF-CM was also assayed and the "% activity" remaining in the heat treated samples was calculated by making the non-heat treated activity 100%. The proportion of colonies showing branching morphogenesis was determined after 4 days, with a branching colony defined as one with at least 1 protrusion extending from the central cell body, although, if only one protrusion was seen, it had to be at least the length of the cell body diameter.

The graph shows the results of a typical experiment consisting of duplicate samples for each condition.

Growth medium and treatment	Proportion of colonies branching	% of colonies branching
HGF + 0mM DTT	38/45	84.4
HGF + 10mM DTT	1/51	2.0
HFF + 0mM DTT	22/54	40.7
HFF + 10mM DTT	17/45	37.8

Table 4.1.3.
Effect of the reducing agent dithiothreitol (DTT) on the ability of HFF-CM and rhHGF to induce branching morphogenesis of HB2 cells in collagen gels.

HFF-CM was concentrated 100-fold in an Amicon stirred cell concentrator. A 25µl volume of concentrated HFF-CM, or 25µl of E4 containing 10ng rhHGF, was treated, or not, with 10mM DTT. After 1 hour on ice the samples were clarified at 13 000 r.p.m. for 15 minutes to remove precipitated protein. The samples were then assayed (the HFF-CM at a dilution of 1/50, and the rhHGF at a concentration of 10 ng/ml) for the ability to induce branching morphogenesis of HB2 cells in collagen gels. The proportion of colonies showing branching morphogenesis was determined after 4 days, with a branching colony defined as one with at least 3 protrusions from the central cell body, with one being at least the length of the cell body diameter.

4.1.4. Sensitivity of the morphogenetic activity of HFF-CM to pH changes.

The solubility of a protein varies with pH depending on the charge characteristics of the protein in question. Most proteins become less soluble, and eventually precipitate, as the pH of their buffer becomes closer to the isoelectric point (i.e.p.) of the particular protein.

Varying the pH of the buffer in which the HFF protein is dissolved, and measuring the pH at which the activity is lost, should not only give us some idea as to the i.e.p. of the protein, but also give us some indication of whether it would be possible to fractionate the activity on an ion exchange column.

This experiment was done with the HFF protein by altering the pH of the HFF-CM in a range between 4.1 and 8.8 pH units with standard laboratory buffers (Table 4.1.4.).

As described in the Table legend, the HFF-CM used in this experiment, was a sample that had been concentrated 100-fold. This was vital because the biological assay to quantitate the activity involves the growth of cells, and must therefore be carried out at a neutral pH. Having such a concentrated sample meant that the pH alteration could be carried out in a small volume, precipitated protein removed by centrifugation, and the activity tested by diluting the sample 1/50 in growth medium of pH 7.0. This large dilution overrode the buffering effects of the buffer used to alter the pH, and allowed the cells to grow at the required neutral pH. This procedure, whereby the pH of the HFF-CM was altered to precipitate protein and then returned to a pH of 7.0 for the biological activity to be assayed, meant that removing the precipitated protein by clarification after the pH treatment was vital, to eliminate the possibility of the protein renaturing again during the biological assay.

The results of this experiment are shown in Table 4.1.4. and it can be seen that the protein in question becomes gradually less soluble as the pH becomes more acidic, with a significant loss in biological activity

Buffer	pH	Proportion of colonies branching	% of colonies branching
50mM NaOAc	4.1	12/48	25.0
50mM NaOAc	5.2	28/59	47.5
20mM NaPi	6.5	29/61	47.5
20mM Tris	6.8	32/60	53.3
20mM NaPi	7.2	36/65	55.4
20mM Tris	7.5	40/68	58.8
20mM Hepes	7.9	27/45	60.0
20mM Tris	8.8	41/77	53.2
HFF (non buffered)	8.0	38/57	66.7

Table 4.1.4.
Effect of variations in pH on ability of HFF-CM to induce branching morphogenesis of HB2 cells in collagen gels.

HFF-CM was concentrated 100-fold in an Amicon stirred cell concentrator. The pH of 25µl volumes of concentrated HFF-CM were altered with various buffers, clarified at 13 000 r.p.m. for 15 minutes to remove precipitated protein and then assayed (at a dilution of 1/50) for the ability to induce branching morphogenesis of HB2 cells in collagen gels. Non-buffered concentrated HFF-CM was also assayed as a control. The proportion of colonies showing branching morphogenesis was determined after 4 days, with a branching colony defined as one with at least 3 protrusions from the central cell body, with one being at least the length of the cell body diameter.

occurring between 5.2. and 4.1. pH units. This result suggests that the i.e.p. of the protein with morphogenetic activity in HFF-CM is acidic.

4.2. The soluble factor in HFF-CM that induces branching morphogenesis is unlikely to be a glycoprotein.

Lectin columns have proved to be invaluable tools in the purification of many glycoproteins, due to their affinity for commonly occurring sugars. The binding reaction of a lectin with its specific sugar residue is analogous to the interaction of an antibody with its Ag. HFF-CM was passed sequentially down both a wheat germ lectin (WGL) sepharose, and a Concanavalin A (Con A) sepharose column, but the morphogenetic activity failed to bind to either (Table 4.2.1). WGL binds specifically to N-acetyl-glucosaminyl residues that are found on both O- and N- linked sugars, and Con A binds specifically to molecules that contain α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related residues.

The fact that the activity in HFF-CM does not bind to either of these lectin columns, strongly suggests that the common sugar moieties that both WGL and Con A are specific for, are not found on this protein, and that the protein is not likely to be glycosylated.

4.3. The soluble factor in HFF-CM that induces branching morphogenesis is not a heparin binding protein.

The protein with morphogenetic activity in HFF-CM does not bind to heparin agarose (Table 4.3.1). This was tested because HGF (Weidner, K.M. *et al.*, 1990) and other known growth factors, such as members of the FGF family (for example KGF/FGF 7), have been shown to bind heparin (Panos, R.J. *et al.*, 1993).

4.4. Ion exchange chromatography of HFF-CM.

4.4.1. Anion exchange chromatography.

The acidic i.e.p. of the protein with morphogenetic activity in HFF-CM, suggests that it may bind to an anion exchange column. In order to assess

Table 4.2.1.
Attempted fractionation of HFF-CM morphogenetic activity on sequential lectin - sepharose columns.

50 mls of HFF-CM was loaded onto a 2 ml Wheat germ lectin (WGL) sepharose column (column 1), after which the flow through was taken and loaded onto a 2 ml concanavalin A (Con A) sepharose column (column 2). Both columns were equilibrated and run in Buffer P containing 100 mM NaCl. Elution from both columns was carried out by competition, with 3 x 1ml washes of 0.3M N-acetyl glucosamine for the WGL column and 0.3M α-D methyl mannoside for the Con A column. All column fractions - (loads, eluates, and flow throughs) - were assayed for the ability to induce branching morphogenesis of HB2 cells in collagen gels. The loads and flow throughs were assayed neat, and the eluates at a dilution of 1/10. The proportion of colonies showing branching morphogenesis was determined after 4 days, with a branching colony defined as one with at least 1 protrusion extending from the central cell body, although, if only one protrusion was seen, it had to be at least the length of the cell body diameter.

Column 1: Wheat germ lectin Sepharose.

Column fraction	Proportion of colonies branching	% of colonies branching	Presence of activity
Load - HFF-CM	26/44	59.1	Yes
Elution 1	2/39	5.1	No
Elution 2	1/35	2.8	No
Elution 3	1/30	3.3	No
Flow through/ Load Column 2	21/35	60.0	Yes

Column 2: Concanavalin A Sepharose.

Column fraction	Proportion of colonies branching	% of colonies branching	Presence of activity
Load/ Flow through Column 1	21/35	60.0	Yes
Elution 1	2/41	4.9	No
Elution 2	1/31	3.2	No
Elution 3	1/31	3.2	No
Flow through	20/38	52.6	Yes

Column: Heparin Agarose.

Column fraction	Proportion of colonies branching	% of colonies branching	Presence of activity
Load - HFF-CM	25/40	62.5	Yes
Elution 1	6/35	17.1	No
Elution 2	5/35	14.3	No
Flow through	15/33	45.5	Yes

Table 4.3.1.
Attempted fractionation of HFF-CM morphogenetic activity on a heparin-agarose column.

20 mls of HFF-CM was loaded onto a 2 ml heparin-agarose column. The column was equilibrated and run in Buffer P containing 100 mM NaCl and elution was carried out with 2 x 1ml of Buffer P containing 2M NaCl. All column fractions - (load, eluates, and flow through) - were assayed for the ability to induce branching morphogenesis of HB2 cells in collagen gels. The loads and flow throughs were assayed neat, and the eluates (after desalting through a G25 spin column) at a dilution of 1/5. The proportion of colonies showing branching morphogenesis was determined after 4 days, with a branching colony defined as one with at least 1 protrusion extending from the central cell body, although, if only one protrusion was seen, it had to be at least the length of the cell body diameter.

this, HFF-CM was passed down both a weak (DEAE-sepharose), and a strong (Q-sepharose) anion exchange column, Table 4.4.1.

From the final columns of Tables 4.4.1.A and B, it can be seen that when HFF-CM is passed down the DEAE-sepharose weak anion exchange column, none of the activity binds, and all the activity is found in the flow-through of the column (Table 4.4.1.A). However, when HFF-CM is passed down the Q-sepharose strong anion exchange column, although the majority of the activity is again in the flow through, there is a small but significant amount of activity that has bound and elutes in the 100 mM salt fraction (Table 4.4.1.B). These results imply that the unknown protein binds weakly to an anion exchange matrix.

4.4.2. Cation exchange chromatography.

The active flow through from the DEAE-sepharose anion exchange column described in the previous section, as well as original HFF-CM, was subsequently passed down a SP-sepharose strong cation exchange column. In both cases, all the activity was found to be in the flow through (data not shown), showing that the morphogenetically active protein did not bind to a cation exchange column.

4.5. Size determination of the soluble factor that induces branching morphogenesis in HFF-CM.

It was also important, as part of the characterisation, to get some idea of the size of the protein in question. In preliminary experiments using Millipore molecular weight (M.W.) cut off spin columns, it was seen that activity was still retained in the concentrate of a 30 kDa cut off spin column. This implied that the protein was more than 30 kDa, although the molecular weight cut offs are known not to be very accurate.

To size the morphogenetically active protein more accurately, HFF-CM was run on a size exclusion column. To accurately determine the size of the protein by this method, it is necessary to load a small volume of HFF-CM, of the order of 100 µl. Therefore, a large volume of HFF-CM was concentrated using a 50 ml Amicon stirred cell concentrator, followed by a 15 ml Millipore spin column, both fitted with M.W. cut-off

Table 4.4.1.
Fractionation of HFF-CM activity on A. a weak anion-exchange column, and B. a strong anion-exchange column.

20 mls of HFF-CM was loaded onto a 2 ml DEAE-Sepharose weak anion-exchange column, (A), or a 2 ml Q-Sepharose strong anion-exchange column, (B). Both columns were equilibrated and run in Buffer P containing 50mM NaCl. Elution from both columns was carried out in 2ml volumes of a salt gradient. All column fractions - (loads, eluates, and flow throughs) - were assayed for the ability to induce branching morphogenesis of HB2 cells in collagen gels. The loads and flow throughs were assayed neat, and the eluates (after desalting through G25 spin columns) at a dilution of 1/3. The proportion of colonies showing branching morphogenesis was determined after 4 days, with a branching colony defined as one with at least 1 protrusion extending from the central cell body, although, if only 1 protrusion was seen, it had to be at least the length of the cell body diameter.

A.

Column 1: DEAE Sepharose.

Column fraction	Proportion of colonies branching	% of colonies branching	% colonies branching, above media alone background
Load - HFF-CM	39/51	76.5	47.9
Flow through	34/46	73.9	45.3
100 mM	6/38	15.8	None
200 mM	8/40	20.0	None
400 mM	11/42	26.2	None
600 mM	6/35	17.1	None
1M	6/38	15.8	None
2M	2/45	4.4	None
medium alone	14/49	28.6	n/a*

B

Column 2: Q-Sepharose.

Column fraction	Proportion of colonies branching	% of colonies branching	% colonies branching, above "media alone" background
Load - HFF-CM	39/51	76.5	47.9
Flow through	28/43	65.1	36.5
100 mM	15/36	41.7	13.1
200 mM	14/40	35.0	6.4
400 mM	15/50	30.0	1.4
600 mM	10/40	25.0	None
1M	16/52	30.8	None
2M	3/35	8.6	None
medium alone	14/49	28.6	n/a*

* n/a = not applicable.

membranes of 10 kDa. (The appropriate experiments were done to make sure that a significant amount of biological activity was retained in both these methods of protein concentration).

Once the sample had been concentrated (typically by 600 - 1000x), a maximum volume of 100µl was loaded onto a Pharmacia Superdex 75 size exclusion column and run using the Pharmacia SMART™ system. The Superdex 75 column allows good separation of proteins between M.W. of 3 000 and 70 000 Da, which was appropriate based on pilot experiments.

A set of M.W. protein standards were run before the samples were loaded, to allow a standard curve to be plotted and hence the M.W. range of the active fraction to be calculated. The columns were run at a flow rate of 50µl/minute, and 50µl fractions were collected. Fractions containing proteins within an appropriate size range, calculated from the M.W. standards, were tested in the branching morphogenesis assay for biological activity. Figures 4.5.1.A. and B. show the results of biological assays, on fractions from two separate runs of different batches of HFF-CM (batches 6 and 7). Two different batches of HFF-CM were used to try and control for any spurious activity that might be detected, which should, by its very nature, not be present in the same fraction in different batches of HFF-CM.

Figures 4.5.1.A. and B show that the main morphogenetic activity of HFF-CM batch 6 (HFF-CM-6) is in fraction 12 (4.5.1.A), and in HFF-CM batch 7 (HFF-CM-7) is in fractions 11 and 12 (4.5.1.B). In a subsequent run, using a Tris based buffer with a higher salt concentration (500 mM NaCl), with yet another batch of HFF-CM (batch 8), activity was again eluted in fraction 12, showing that it was in this fraction, and corresponding molecular weight range, that the highest concentration of the protein was consistently found.

The same run programme was used for all the size exclusion columns, and in this programme fraction 12 corresponds to an elution volume of 1150-1200 µl. The M.W. standard curve used to calibrate the column on which HFF-CM batches 6 and 7 were run is shown in Figure 4.5.2. By using the curve equation shown, the M.W. range corresponding to the

Table 4.5.1.A.
Fractionation of HFF-CM - 6 activity on a Superdex 75 size exclusion column.

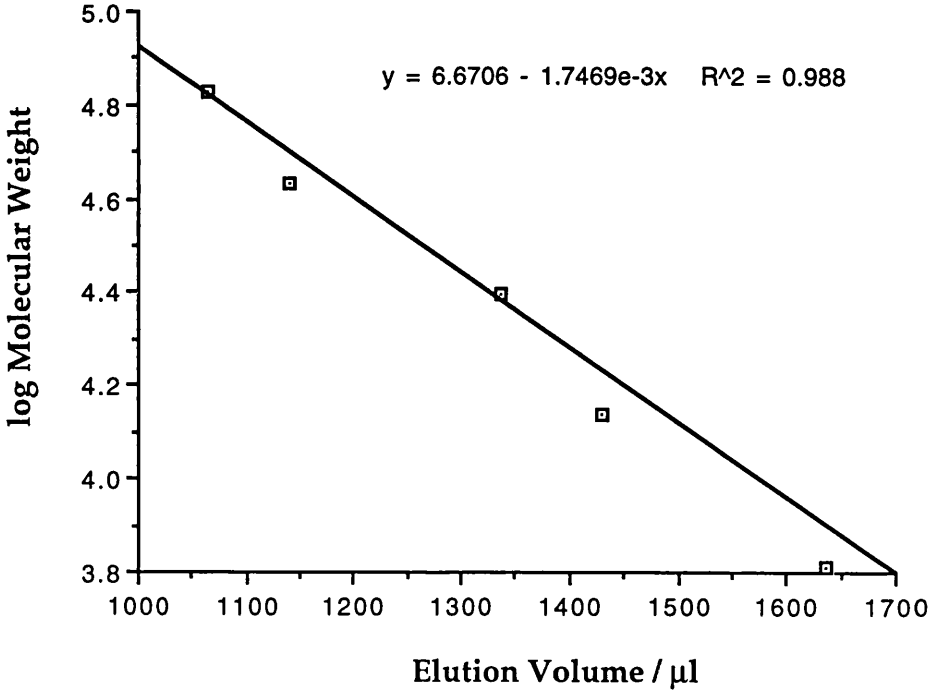
150 mls of HFF-CM - batch 6 were concentrated to 250µl in a 50ml Amicon stirred cell concentrator, followed by a 2ml Millipore spin column, both using 10kDa cut-off membranes. 100µl of this concentrate was loaded onto a Superdex 75 size exclusion column run using the Pharmacia SMART™ system. The column was equilibrated and run in Buffer P containing 200mM NaCl at a flow rate of 50µl/minute, and 50µl fractions were collected. Column fractions 9-23 were assayed for the ability to induce branching morphogenesis of HB2 cells in collagen gels. The load was tested at a dilution of 1/50 and the fractions at a dilution of 1/40. The proportion of colonies showing branching morphogenesis was determined after 4 days, with a branching colony defined as one with at least 3 protrusions from the central cell body, with one being at least the length of the cell body diameter.

Column fraction	Proportion of colonies branching	% of colonies branching
Load	17/64	27.0
9	2/65	3.1
10	3/72	4.2
11	4/74	5.4
12	11/82	13.4
13	5/88	5.7
14	5/71	7.0
15	6/75	8.0
16	9/106	8.5
17	3/84	3.6
18	1/69	1.4
19	2/59	3.4
20	4/68	5.9
21	2/63	3.2
22	3/62	4.8
23	4/64	6.3

Table 4.5.1.B.
Fractionation of HFF-CM - 7 activity on a Superdex 75 size exclusion column.

200 mls of HFF-CM - batch 7 were concentrated to 170µl in a 50ml Amicon stirred cell concentrator, followed by a 2ml Millipore spin column, both using 10kDa cut-off membranes. 70µl of this concentrate was loaded onto a Superdex 75 size exclusion column run using the Pharmacia SMART™ system. The column was equilibrated and run as described in Figure 4.5.1.A. Column fractions 9-23 were assayed for the ability to induce branching morphogenesis of HB2 cells in collagen gels. The load was tested at a dilution of 1/200 and the fractions at a dilution of 1/40. The proportion of colonies showing branching morphogenesis was determined after 4 days, with a branching colony defined as one with at least 3 protrusions from the central cell body, with one being at least the length of the cell body diameter.

Column fraction	Proportion of colonies branching	% of colonies branching
Load	33/70	47.1
9	6/77	7.8
10	5/69	7.2
11	15/90	16.7
12	16/85	18.8
13	7/78	9.0
14	7/78	9.0
15	7/69	10.1
16	7/78	9.0
17	7/84	8.3
18	8/80	10.0
19	4/74	5.4
20	1/52	1.9
21	2/71	2.8
22	1/69	1.4
23	1/83	1.2



Standard proteins used for callibration	Molecular Weight/Da
Albumin	67 000
Ovalbumin	43 000
Chymotrypsin A	25 000
RNAase	13 700
Aprotinin	6 500

Figure 4.5.2.

Molecular Weight standard curve and proteins used for callibration of the Sephadex 75 column used to fractionate HFF-CM -6 and 7.

fraction 12 elution volume is 37.5 - 45.9 kDa. The M.W. standards used to calibrate the column that the HFF-CM batch 8 was run on gave a M.W. range for fraction 12 of 36.0 - 44.2 kDa. Putting the results of these runs together gives a size range for the unknown protein of 36 - 46 kDa, that appears to be very consistent both between batches of HFF-CM, and between different experiments.

DISCUSSION AND CONCLUSIONS.

The unidentified, and potentially novel factor, secreted by HFFs that induces branching morphogenesis of HB2 human mammary epithelial cells has been partially characterised biochemically. It is a 36 - 46 kDa, heat stable protein, with an active form that is unlikely to be glycosylated, or to contain inter- or intrachain disulphide bonds required for function. The protein probably has an acidic i.e.p. and binds weakly to an anion, but not a cation exchange column. The unknown factor has also been shown not to bind heparin.

Heregulin as a candidate molecule for the unknown factor secreted by HFFs that induces branching morphogenesis of HB2 cells.

A recent paper by Yang, Y. *et al.*, 1995, showed a sequential requirement for both HGF, and the protein Neuregulin, for the induction of branching morphogenesis and differentiation of the mouse mammary gland. This paper showed that in organ cultures of mouse mammary glands, HGF promoted branching morphogenesis of the ductal trees, but inhibited the production of secretory proteins; whereas neuregulin (also called neu differentiation factor, NDF), stimulated lobular alveolar budding and the production of milk proteins.

HGF and NDF are also expressed in the mesenchymal cells of the mouse mammary gland during appropriate stages in development, as are their receptors; c-met (for HGF), and c-erbB3 and c-erbB4 (for NDF) (Yang, Y. *et al.*, 1995). It should also be noted that NDF is known to use c-erbB2/c-erbB3, and c-erbB2/c-erbB4 heterodimers as receptors as well as c-erbB3 or c-erbB4 homodimers (Plowman, G.D. *et al.*, 1993; Carraway, K.3. *et al.*, 1994; Sliwkowski, M.X. *et al.*, 1994). c-erbB2 is also known to be expressed in the mammary gland *in vivo* (Slamon, D.J. *et al.*, 1987), therefore, any

combination of NDF receptors could be involved in NDF function in the mammary gland. The described morphogenetic effect of NDF in a mouse mammary gland system, meant that heregulin (the human homologue of NDF), was a good candidate for the unknown factor produced by HFFs that induced branching morphogenesis of the human mammary epithelial cell line, HB2.

Heregulin is known to exist in a number of differentially spliced forms (Ben-Baruch, N. and Yarden, Y., 1994), however it was initially purified as a 45 kDa protein (Holmes, W.E. *et al.*, 1992); which is within the molecular weight range determined in the characterisation of the unknown factor. Heregulin/NDF biological activity has also been shown to be resistant to heat treatment (Yarden, Y. and Peles, E., 1991); which is another property of the morphogenetic activity of HFF-CM (Figure 3.7.1. and 4.1.2).

Although the molecular weight and heat resistant properties of heregulin were consistent with the discovered properties of the unknown factor secreted by HFFs, three known biochemical properties of heregulin/NDF, made it extremely unlikely that it was the unidentified factor in HFF-CM. These properties were:
1) Its affinity for heparin: Binding to heparin-sepharose was a key step in the purification of both heregulin and NDF (Holmes, W.E. *et al.*, 1992; Peles, E. *et al.*, 1992).
2) The heregulin/NDF biological activity is sensitive to reducing agents (Peles, E. *et al.*, 1992). Treatment with reducing agent only slightly reduces the molecular weight of NDF on SDS-PAGE, implying that it is a single chain protein, and not a dimer. However, the loss of biological activity of heregulin/NDF with reducing agent treatment, makes it likely that intra-chain disulphide bonding is important for function.
3) All mesenchymally derived isoforms of heregulin/NDF have been shown to be glycosylated with mainly O-linked sugars, which results in an affinity for WGL (Yarden, Y. and Peles, E., 1991; Peles, E. *et al.*, 1992). The exception to this is the β3 subtype of heregulin/NDF, which is not a secreted form (Ben-Baruch, N. and Yarden, Y., 1994).

In this chapter it has been shown that the unknown factor in HFF-CM does not bind heparin (Table 4.3.1), or WGL (Table 4.2.1), and that its

biological activity is insensitive to the reducing agent DTT (Table 4.1.3). Thus, although heregulin/NDF was a good candidate for the unknown factor by virtue of its size, its heat resistance and its morphogenetic effect in an organ culture system; biochemical evidence suggested that the unknown factor was not any of the splice variants of heregulin/NDF identified so far.

Recent experiments carried out by Dan Baeckstrom have confirmed that heregulin is not the unknown factor secreted by HFFs that induces branching morphogenesis of HB2 cells. Heregulin has been shown to be a very potent inducer of branching morphogenesis of HB2 cells. However, whereas the heregulin induced branching of HB2 cells could be blocked by an antibody to erbB3 (a heregulin cell surface receptor), the branching induced by HFF-CM could not be blocked in this way.

CHAPTER 5: ROLE OF INTEGRINS IN *IN VITRO* MORPHOGENESIS.

INTRODUCTION

Several different lines of evidence both *in vivo*, and using collagen gel assays *in vitro*, suggest a role for integrins in mammary cell morphogenesis. Firstly, *in vivo* studies have shown that in breast cancers, levels of expression of the α_3, α_6, and in particular the α_2 integrin are reduced compared to those seen in the normal mammary gland (Zutter, M.M. *et al.*, 1990; Pignatelli, M. *et al.*, 1991; Jones, J.L. *et al.*, 1992; Natali, P.G. *et al.*, 1992; Zutter, M.M. *et al.*, 1993; Berdichevsky, F. *et al.*, 1994b; Glukhova, M. *et al.*, 1995; Zutter, M.M. *et al.*, 1995). Importantly, an increased reduction in expression of the α_2 integrin was shown to be correlated with increasing grade of carcinomas, and therefore with increased disruption of tissue architecture and morphology (Zutter, M.M. *et al.*, 1990; Pignatelli, M. *et al.*, 1991; Zutter, M.M. *et al.*, 1993). Further evidence for a role for the $\alpha_2\beta_1$ integrin in morphogenesis was obtained from studies by Berdichevsky, F. *et al.*, 1994a, and D'Souza, B. *et al.*, 1993, which showed that α_2 integrin expression was reduced on a number of breast cancer cell lines *in vitro*, and on a non-tumorigenic human mammary epithelial cell line transfected with the *c-erbB2* oncogene. In both cases, this reduced α_2 integrin expression correlated with a loss in ability of cells to form organised structures in collagen gels.

Experiments described in Chapter 3 of this thesis also indirectly suggested a role for integrins in morphogenesis, and more specifically in the process of branching morphogenesis. Data was presented which suggested that the stimulation of motility/scattering of HB2 cells by HGF was an important part of the branching morphogenesis mechanism. It was also shown that rhHGF could stimulate scattering (an indication of motility) on both collagen and fibrin matrices but not on plastic. This requirement for particular ECM components for HGF to induce motility therefore suggested that integrins might be involved in the motility response, and hence in the process of branching morphogenesis.

The evidence outlined above strongly suggested a role for integrins in cell morphogenesis. However this evidence only suggested a role for

integrins indirectly, hence it was important that experiments to directly analyse the potential role of integrins in morphogenesis should be carried out.

One way to analyse the potential role of integrins in morphogenesis is to analyse the expression levels of integrins during morphogenesis, and to see whether or not these levels are regulated either in response to ECM components, or in response to fibroblast factors that stimulate branching. This analysis has been carried out at the RNA level in the HB2 *in vitro* morphogenesis system, and the results are described in Part I of this chapter.

Alternatively, a way to directly examine the role of integrins in morphogenesis, is to assess the effect of specific monoclonal antibodies (mAbs) to integrin subunits on the morphogenetic process. With this approach in mind, a comparison of the effects on morphogenesis, of mAbs that are known to inhibit function, activate function, and those that are reported to have no functional effect on integrins, has been carried out in the same *in vitro* system. The results of experiments using various integrin mAbs as investigative tools will be described in Part II of this chapter.

As described in Chapter 3, branching morphogenesis of HB2 cells can be induced with either HFF-CM, or with purified rhHGF. It was shown that the soluble factor in HFF-CM was distinct from HGF, although it is, as yet unidentified and unpurified. Most of the investigations into the role of integrins in branching morphogenesis, described in both parts of this chapter, were carried out using rhHGF, rather than HFF-CM. This was done because of the availability of a purified recombinant HGF protein. It was hoped that using a known and purified growth factor, (that had already been the subject of much research as to its mechanisms of action, and downstream signal transduction pathways), would make it easier to analyse any effects seen in the *in vitro* system at the molecular level. Where the experiments have also been carried out with HFF-CM, these have been noted in the appropriate results sections.

RESULTS PART I. ANALYSIS OF INTEGRIN EXPRESSION DURING MORPHOGENESIS.

5.1. Integrin expression by HB2 cells is down regulated at the RNA level when cells are grown on top of fibrin or collagen type I matrices.

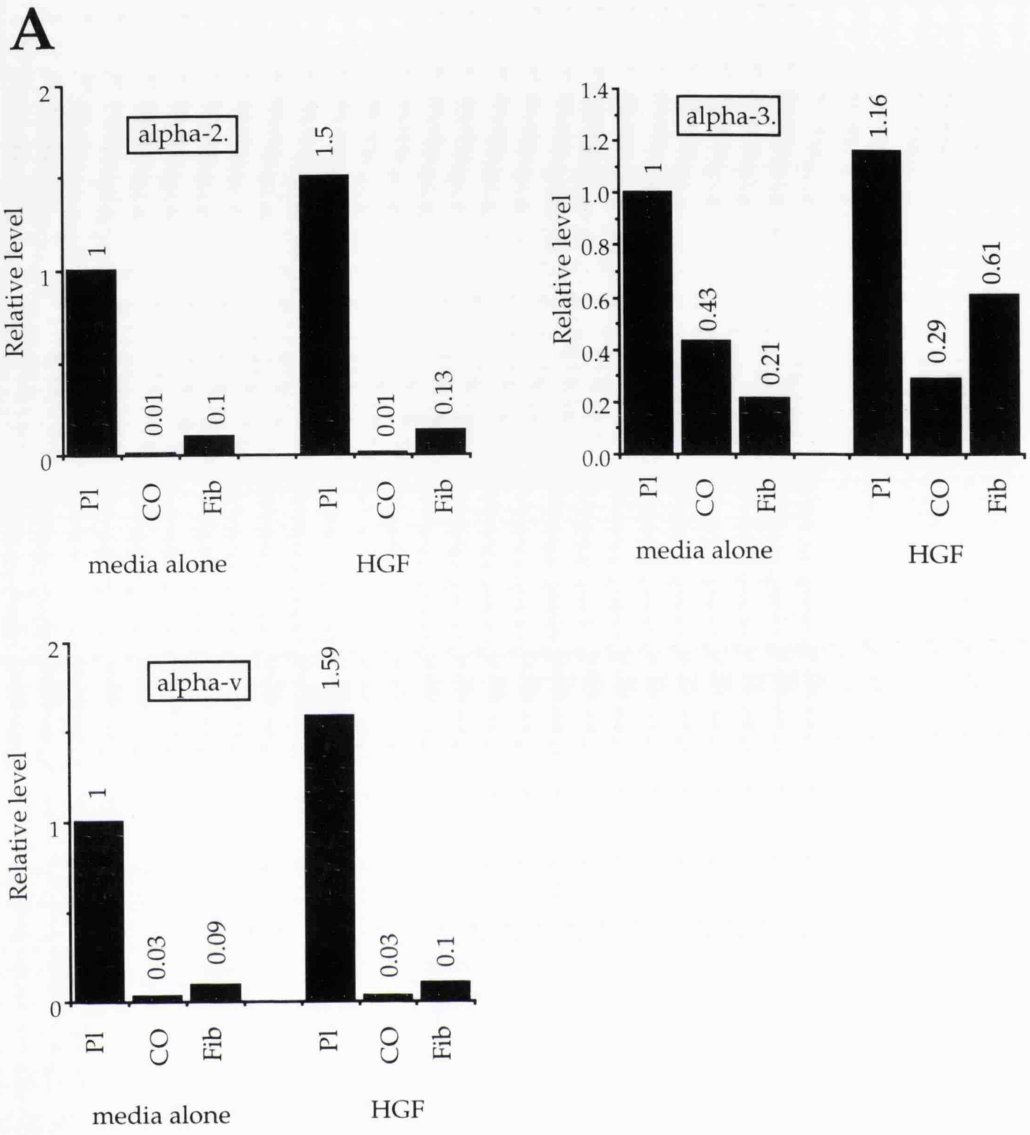
As outlined above, it had been shown that HGF could only induce the motility response in HB2 cells if they were in contact with an ECM component such as collagen or fibrin, and from this it was proposed that specific integrins may be involved in the motility process. In order to analyse the potential role of integrins in HGF induced motility, it was important to know if contact of HB2 cells with ECM components altered the expression levels of integrins. This was done by plating an equal number of HB2 cells on either tissue culture plastic, polymerised collagen type I, or polymerised fibrin gels, and analysing the levels of integrins expressed after 24 hours. The integrin subunits analysed were the α_2, α_3, and α_v subunits, and the results are shown both pictorially and graphically in Figure 5.1.1. It can be seen that a marked down regulation of each of these integrin subunits occurred when HB2 cells were cultured on ECM components compared to plastic. On both matrices the down regulation was most extensive for the α_2 and α_v subunits with a less marked but significant decrease in expression of the α_3 subunit.

It could be proposed that a general mechanism of increasing cell motility on ECM components (for example when cells are cultured with HGF), might involve the loosening of integrin mediated contact of cells with the matrix. This could potentially occur by cells, in the presence of HGF, being induced to down-regulate integrin expression even further. In the experiment shown in Figure 5.1.1. it can be seen that in terms of the absolute levels of integrins expressed in cells growing on ECM components, there is no difference between levels of the α_2 and α_v subunits in the presence and absence of HGF. However, on collagen the absolute levels of the α_3 integrin in the presence of HGF, are lower than in its absence. This has been observed previously in this system, in experiments carried out by Feodor Berdichevsky (Berdichevsky, F. *et al.*, 1994b), and it is possible (and indeed suggested by Berdichevsky, F. *et al.*, 1994b), that the α_3 integrin plays a specific role in the regulation of

Figure 5.1.1.
Integrin expression by HB2 cells is down regulated at the RNA level when cells are grown on top of fibrin or collagen type I matrices.

$4x10^6$ HB2 cells were seeded onto tissue culture plastic (Pl), or on the top of polymerised collagen type I (CO) or fibrin (Fib) gels, in either standard growth medium alone or standard medium supplemented with 20 ng/ml rhHGF. After 18 hours, RNA was extracted from the monolayers of cells by the GTC extraction method (see Materials and Methods). The RNA was fractionated by electrophoresis and transferred to Hybond N+ nylon membranes which were subsequently hybridised with radiolabelled DNA probes to detect the α_2, α_3, and α_v integrins. A probe to detect 18S RNA was used as a control for loading.

After exposure of the hybridised membranes to film, the signals were quantitated by densitometric scanning, and the results plotted graphically (panel A) after being normalised for any variations in the amount of RNA loaded using the 18S RNA signals. The graphs were plotted by making the level of individual transcripts from cells growing on plastic in standard medium 1.0 unit, and comparing other signals thereto. The autoradiographs of signals were also photographed and are shown in panel B.


A
alpha-2.
Relative level
2
1
0
1
0.01
0.1
1.5
0.01
0.13
Pl
CO
Fib
Pl
CO
Fib
media alone
HGF
alpha-3.
Relative level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
0.43
0.21
1.16
0.29
0.61
Pl
CO
Fib
Pl
CO
Fib
media alone
HGF
alpha-v
Relative level
2
1
0
1
0.03
0.09
1.59
0.03
0.1
Pl
CO
Fib
Pl
CO
Fib
media alone
HGF
B
alpha-2
alpha-3
alpha-v
18S
Pl
CO
Fib
Pl
CO
Fib
media alone
HGF

motility and the process of branching morphogenesis within collagen gels. Alternatively, it is conceivable that a general ECM induced down-regulation of all integrins, leads to the priming of cells for motility, by loosening their contact with the matrix. In this case, HGF would play no role in modulating the down regulation of expression, but would simply provide the motility stimulus that the cells could respond to, if they had been primed. This second model might also explain why HB2 cells were not induced to scatter on plastic in the presence of HGF, because when grown on plastic the integrin levels remained high and the cells would, therefore, not be primed for motility.

5.2. Expression of integrins at the RNA level during branching or cyst morphogenesis of HB2 cells growing within collagen gels.

From the experiments described above, it seemed that a down regulation of integrin expression was associated with the interaction of cells with certain ECM components, and that this effect (possibly in addition to a further HGF mediated effect on expression of the α_3 integrin), might conceivably play a role in priming the cells for HGF-induced motility in a 2-dimensional system. If these observed effects were indeed important for motility, then it would be interesting to investigate whether a similar ECM induced down-regulation of integrin expression occurred when the cells were cultured in the 3-dimensional system, and if so whether HGF could modulate the down regulation in this case.

The expression of integrins in HB2 cells over a morphogenetic time course in 3-dimensions, in the presence or absence of rhHGF, was investigated in the collagen gel system. RNA was extracted from HB2 cells at different stages in morphogenesis, at the Day 1, Day 3 and Day 7 time-points. In the morphogenesis assays, HB2 cells are embedded in collagen (or fibrin) as single cells and at Day 1 (18-24 hours) spherical single cells are still present with no obvious proliferation having yet occurred. At this point no phenotypic differences are apparent between cells that have been stimulated or not with HGF. By Day 3 cells have proliferated and formed small clusters. At this point, cells in the presence of HGF have started to put out processes, look motile, and branch; a clear difference being seen between these colonies and the spherical non-spiking cysts seen in the absence of HGF. Over the rest of

the morphogenetic time course, the branching or cyst structures seen at Day 3 grow larger as cells proliferate, and by Day 7 fully developed branching or cyst-like structures are present.

The levels of expression of the α_2, α_3, and α_v integrin subunits over a morphogenetic time-course in collagen are shown graphically in Figure 5.2.1.A, and pictorially in Figure 5.2.1.B. The level of integrin expression at Day 1 on plastic is also shown for comparison. It can be seen that for cells growing in a 3-dimensional situation, there is a similar collagen induced down-regulation of integrins as seen in 2-dimensions. However in 3-dimensions the down-regulation is delayed, and occurs between the Day 1 and Day 3 time points, as opposed to within the first 24 hours. In fact it can be seen that there is a significant increase in integrin expression at the Day 1 time point, (as compared to the level on plastic), before the down regulation takes place.

These profiles of integrin expression in cells undergoing morphogenesis in collagen gels are reproducible between experiments. Moreover, the distinctive pattern of integrin expression seen does not seem to reflect general, non-specific changes in the expression of molecules at the RNA level. This was shown by analysis of the levels of the apically expressed mucin MUC-1 over the same time course, (Figure 5.2.1.A and B). In contrast to the up, then down regulation seen with the integrin subunits, MUC-1 was expressed at a lower or equivalent level in collagen than the level seen on plastic at Day 1, and then showed a general increase in expression throughout the time-course.

5.3. HGF increases the level of integrin expression in HB2 cells growing both on plastic and initially within collagen gels.

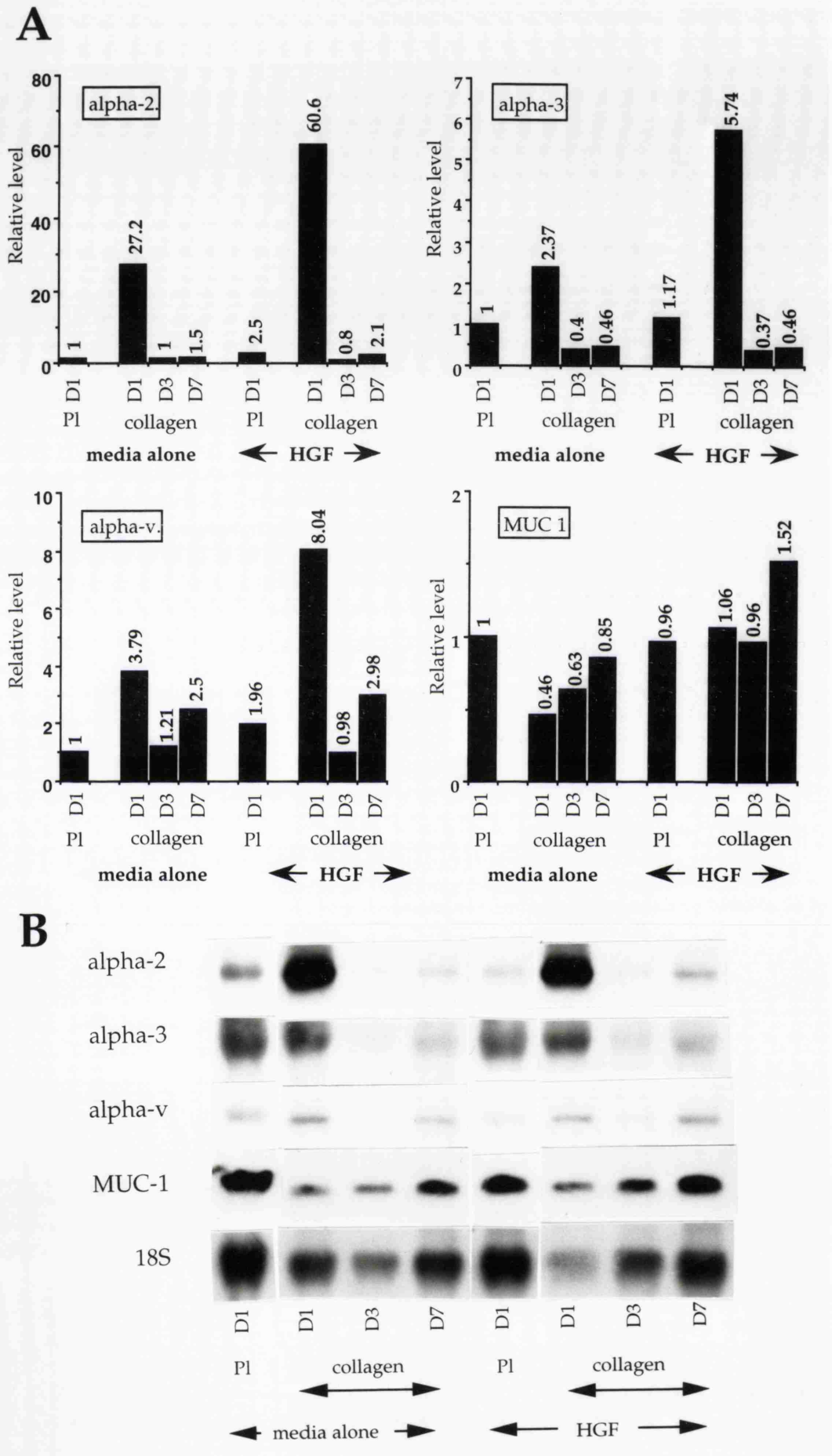
Looking at the levels of integrin expression during the morphogenetic time course when cells are growing within collagen gels (Figure 5.2.1.A and B), it can be seen that the only major difference between cells growing in the presence and absence of HGF is the significant stimulation of expression at Day 1 in the presence of HGF. This stimulation is consistently seen in different experiments but is not thought to play a role in the induction of morphogenetic programmes by HGF. This is because a stimulation in expression of integrins by HGF is

Figure 5.2.1.
Expression of integrins at the RNA level during branching or cyst morphogenesis of HB2 cells growing within collagen gels.

$4x10^6$ HB2 cells were either seeded onto tissue culture plastic, or a particular density of cells were embedded in collagen gels in order that RNA could be extracted at various morphogenetic time points (see Materials and Methods). Cells were then cultured in either standard growth medium alone or standard medium supplemented with 20 ng/ml rhHGF. RNA was extracted from the cells growing on plastic after 18 hours (day 1, D1), and from cells growing within the collagen at days 1, 3 and 7 (D1, D3 and D7). RNA was extracted from monolayers of cells by the GTC extraction method, which was also used to extract RNA from the cells growing within collagen gels, after they had been removed from the collagen by collagenase treatment (see Materials and Methods).

The RNA was fractionated by electrophoresis and transferred to Hybond N+ nylon membranes which were subsequently hybridised with radiolabelled DNA probes to detect the α_2, α_3, and α_v integrins and also the MUC 1 gene (encoding the polymorphc epithelial mucin, PEM). A probe to detect 18S RNA was used as a control for loading.

After exposure of the hybridised membranes to film, the signals were quantitated by densitometric scanning and the results plotted graphically (panel A), after being normalised for any variations in the amount of RNA loaded using the 18S RNA signals. The graphs were plotted by making the level of individual transcripts from cells growing on plastic in standard medium alone 1.0 unit, and comparing other signals thereto. The autoradiographs of signals were also photographed and are shown in panel B.


A
alpha-2
alpha-3
alpha-v
MUC 1
Relative level
1
27.2
1
1.5
2.5
60.6
0.8
2.1
1
2.37
0.4
0.46
1.17
5.74
0.37
0.46
1
3.79
1.21
2.5
1.96
8.04
0.98
2.98
1
0.46
0.63
0.85
0.96
1.06
0.96
1.52
D1
Pl
collagen
media alone
HGF
B
alpha-2
alpha-3
alpha-v
MUC-1
18S

also seen in cells growing on plastic (Figures 5.1.1.A and B, 5.2.1.A and B, and other data not shown), which show no morphogenetic response to HGF. Moreover when compared, the relative increases in expression induced by HGF on plastic and in collagen are similar within the same experiment, further supporting the idea that the stimulation of integrin expression by HGF is a general phenomenon and has no functional relevance to morphogenetic processes. The only exception to this occurs in the case of the α_3 integrin subunit, where although an HGF induced increase in expression in collagen of 2-3 fold is always seen, the up-regulation on plastic is not always so high. It was also noted (and can be seen in Figure 5.2.1.A), that HGF stimulated the expression of all integrins in collagen at Day 1 by approximately 2-3 fold, i.e. that no differential up-regulation was detected between different integrin subunits.

5.4. The down regulation of integrin expression during morphogenesis is collagen induced.

In order to show that the down-regulation of expression between Day 1 and Day 3 was collagen induced, (as was the case with the down-regulation in the 2-dimensional system), the expression levels of integrins at Day 1 and Day 3 in collagen were compared to those of cells grown on plastic. These experiments (data not shown) showed that although there was a tendency for a small down-regulation of integrin expression to occur in cells growing on plastic between Day 1 and Day 3 (in particular with the α_2 subunit); the down-regulation that occurred in cells growing within collagen during the same time period was substantially greater.

5.5. A lower absolute level of expression of integrins in the presence of HGF cannot be responsible for any increased motility of cells during branching morphogenesis.

It had been proposed from earlier experiments carried out by growing HB2 cells on polymerised collagen (Berdichevsky, F. *et al.*, 1994b), and from the results shown in Figure 5.1.1., that a lower level of expression of the α_3 integrin subunit in the presence of HGF might be involved in the motility response of cells to HGF. However, in the case of cells

growing within collagen gels, when morphogenesis begins following down-regulation i.e. after Day 3, no significant difference in the absolute levels of any of the integrin subunits analysed, in the presence versus the absence of HGF was observed (see Figure 5.2.1.A and B). This means that a lower absolute level of one or more integrins cannot account for the induction of motility and branching morphogenesis in the presence of HGF. However, the expression profiles in the 3-dimensional system do support the second hypothesis, that the collagen induced down regulation of integrins may generally prime the cells for motility, allowing them to migrate if such a stimulus is provided.

It is interesting to note in the time-course experiments, that because of the stimulation of integrin expression at Day 1 by HGF, the relative collagen-induced down-regulation to reach the same absolute levels by Day 3, has to be much greater in the presence of HGF. It has also been noted that the down regulation of integrin expression between Day 1 and Day 3 by collagen, is induced to different degrees for different integrins. In each experiment the trend is the same with the α_2 integrin being down regulated more than the α_3, which is in turn, down-regulated more than the α_v. For example, in the experiment shown in Figure 5.2.1. in the absence of HGF, the α_2 integrin was down regulated 27.2x; the α_3, 5.9x; and the α_v, 3.1x. In the presence of HGF the α_2 was down regulated 75.8x; the α_3, 15.5x; and the α_v, 8.2x. This shows that in the presence as opposed to the absence of HGF, the same trend in the regulation of integrin expression applies, but illustrates the fact that the level of down-regulation has to be greater because of the induced increase in expression at Day 1.

DISCUSSION AND CONCLUSIONS. PART I.

From the results presented in this part of chapter 5, we conclude that both collagen and fibrin induce a down-regulation of integrin expression, compared to the level on plastic, and that this down-regulation may play a role in priming cells for increased motility which can be stimulated by HGF. The collagen induced down-regulation of integrins was also shown to occur during morphogenesis of HB2 cells in 3-dimensions, although in this situation, the down-regulation was delayed and occurred between the Day 1 and Day 3 time points.

Moreover, this collagen induced down-regulation appeared to have some degree of specificity; as the apical mucin MUC-1 showed a general increase in level throughout the time course, and also for the integrins themselves, down-regulation appeared to be induced by differing amounts for different integrins.

In the 3-dimensional situation, it was shown that a differential down-regulation of integrins (in terms of absolute levels of expression), did not occur in the presence compared to the absence of HGF and could not, therefore, form part of the mechanism by which motility and branching morphogenesis was induced.

HGF was shown to stimulate integrin expression both on plastic and at the Day 1 time point in 3-D culture within collagen. However, the fact that the relative level of stimulation was similar on both plastic and within collagen showed that it was unlikely that this phenomenon played any role in the HGF induction of morphogenetic processes.

Model for the induction of branching morphogenesis by HGF and the potential role for integrins in this process.

In early experiments carried out in this *in vitro* system by Feodor Berdichevsky, a specific role for the α_3 integrin in the branching morphogenesis process was proposed. Evidence for this came from experiments which showed that treatment of HB2 cells with a mAb to the α_3 subunit (P1B5), could actually stimulate branching morphogenesis of a significant proportion of colonies within collagen gels in the absence of HGF. It was then shown that in HB2 cells growing on collagen gels, the level of the α_3 integrin was lower in the presence of HGF than in its absence, (a result also repeated in these studies, see Figure 5.1.1). Given these results it was therefore proposed that a change in function or an increased down-regulation of the α_3 integrin might play a role in the branching morphogenesis process, (Berdichevsky, F. *et al.*, 1994b).

The experiments carried out here have taken this analysis a step further, by actually analysing the levels of expression of integrins during the morphogenesis process occurring in 3-dimensions within collagen,

rather than the expression in a 2-dimensional situation. These results have excluded the possibility that a differential down-regulation of the α_3 integrin (and indeed the α_2 and α_v integrins), to a lower absolute level in the presence of HGF plays a role in branching morphogenesis, although it is still possible that the alteration of α_3 integrin function is involved.

However, the general, and to some degree specific, down-regulation of all the integrins analysed, leads us to propose that such a general down-regulation of integrins, induced by the collagen (or fibrin) matrix, could lead to the priming of cells for motility by loosening their contact with the matrix. Then, in the absence of a motility stimulus, further proliferation would give rise to spherical cysts, but in the presence of a motility stimulus such as HGF branching morphogenesis would occur. In support of this model, it should be noted that the time-point of down-regulation, although different in both the 2-dimensional and 3-dimensional systems, corresponds with the time at which motility is observed, i.e. by 24 hours and by 3 days respectively. It is probable that such a profound down regulation of integrins is induced by collagen for additional reasons than simply to prime the cells for branching morphogenesis, if indeed such a stimulus occurs. For example, it might also be necessary for cells to loosen their contact with the matrix for a more fundamental property such as cell division. To determine if the collagen induced down regulation of integrins is an important part of the morphogenesis process and/or cell proliferation, it would be interesting to express various integrin subunits in HB2 cells under the control of heterologous promoters (to prevent down-regulation being induced), and observing the effect this had on cell proliferation and the ability to undergo branching morphogenesis.

RESULTS PART II. ANALYSIS OF THE FUNCTIONAL ROLE OF INTEGRINS IN *IN VITRO* MORPHOGENESIS.

This functional analysis was carried out by assessing the effect of specific monoclonal antibodies to various integrin subunits on morphogenesis. An antibody that inhibits integrin function is referred to as a "blocking" Ab, and is defined by its ability to reduce integrin mediated adhesion of cells to an appropriate ECM component. Conversely, an Ab that

"activates" integrin function, increases the integrin mediated adhesion to an appropriate ECM component. Abs that are reported to have "no functional effect", are usually defined as such, simply because they have not been shown to have any effect on cell adhesion to ECM components. In this thesis, Abs of this type are referred to as "non blocking" Abs. Epitopes of "non blocking" α_2 Abs usually map outside the "A domain", which is a region of the α_2 integrin subunit (and indeed other integrin α subunits), known to be important in the integrin-ECM interaction, and is where all epitopes of α_2 integrin blocking Abs so far mapped have been located (Kamata, T. *et al.*, 1994a). Similarly, non blocking Abs to the β_1 subunit have been shown to map outside the putative "β-A domain" that is important for ligand binding; whereas activating and inhibitory Abs have been shown to map within this region (Takada, Y. and Puzon, W., 1993).

5.6. Effect of blocking mAbs to integrins on rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

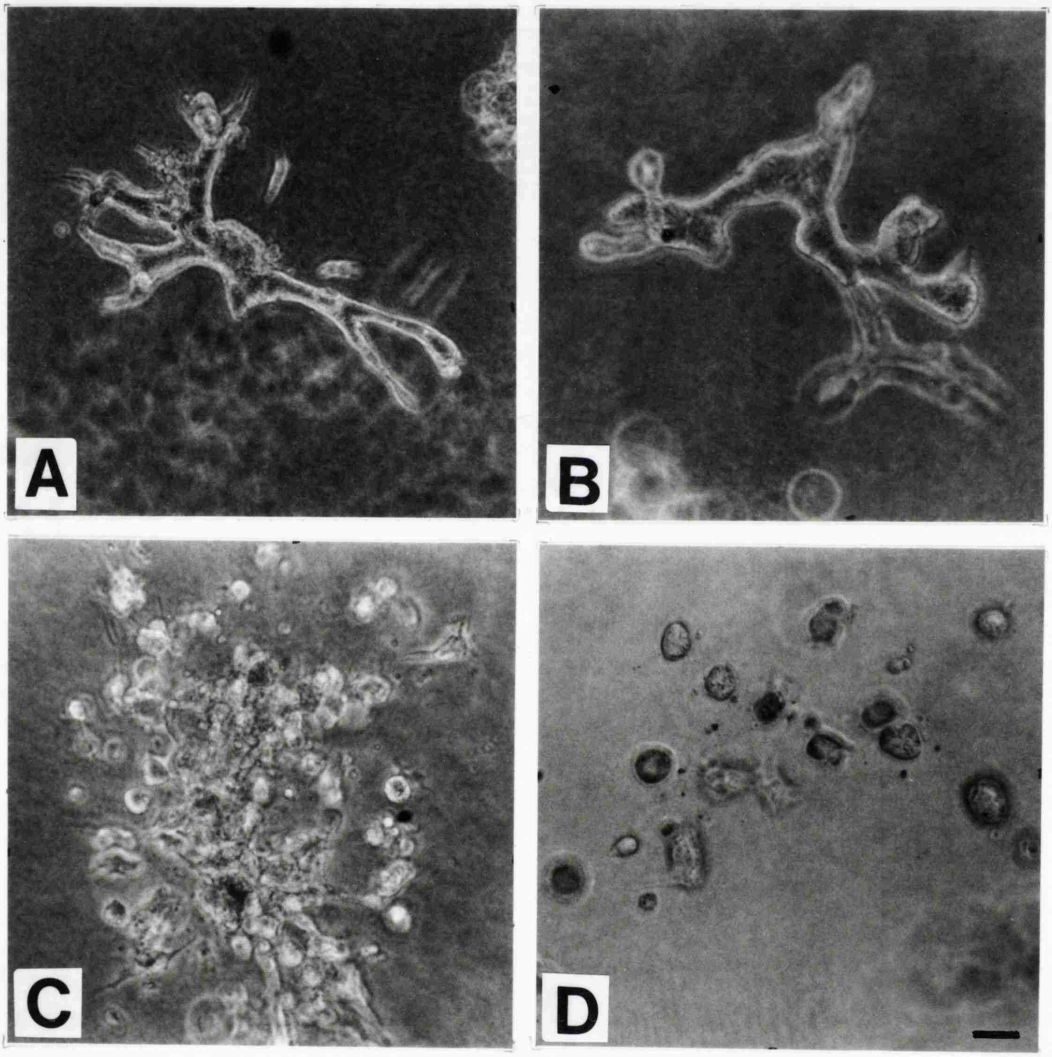
The major integrins expressed by the mammary gland *in vivo*, and the HB2 cells *in vitro*, are the $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$ integrins, (Chapter 3; Koukoulis, G.K. *et al.*, 1991; Berdichevsky, F. *et al.*, 1994b). The first approach, therefore, was to look at the effect on branching morphogenesis, of blocking antibodies to each of these integrins, to identify which, if any, had a functional role in the morphogenetic process.

Blocking Abs to the α_2 and β_1 integrin subunits (P1E6 and mAb13 respectively), reduced the formation of compact, organised branching structures in the presence of rhHGF, instead giving rise to the formation of non-compact "dissociated" structures, in which the cells making up the colonies showed reduced cell-cell contacts [compare Figure 5.6.1. panel C. (P1E6 treated), and 5.6.1. panel D (mAb13 treated), with 5.6.1. panel A. (no Ab treatment)]. In contrast, blocking Abs to either the α_3 (P1B5, Figure 5.6.1. panel B.), or the α_6 integrin subunit (GoH3, data not shown), had no visible effect on the branching morphogenesis induced by rhHGF.

Figure 5.6.1.
Effect of blocking antibodies to α_2, α_3, and β_1 integrin subunits on rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

HB2 cells were pre-treated with the anti-integrin mAbs: α_2 (P1E6, panel C); α_3 (P1B5, panel B); and β_1 (mAb13, panel D); or given no treatment (panel A), before embedding in collagen gels and culturing in the presence of 10ng/ml rhHGF. The concentration of mAbs used were: P1E6, 1/500 ascites; P1B5, 1/100 ascites; mAb13, 2µg/ml. The morphogenetic phenotypes of colonies were photographed after 8 days of growth at a magnification of 51x.

Scale bar denotes 100 µm


A
B
C
D

The results of a typical experiment are shown quantitatively in Figure 5.6.2. The morphogenetic phenotypes of the colonies formed were classified into three groups; branching colonies, spherical cyst colonies, and dissociated colonies (as defined in Materials and Methods). The proportion of colonies in each morphogenetic group was expressed as a percentage of the total number counted, and the mean percentage and the standard error of this mean (for duplicate plates) was plotted. (This same method of quantitation has been used in all experiments examining morphogenetic phenotype).

It can be seen from Figure 5.6.2. that in the absence of any Ab, or in the presence of the α_3 integrin blocking Ab P1B5, the majority of the colonies formed were of the branching phenotype; whereas, in the presence of the α_2 or β_1 blocking Abs (P1E6 and mAb13 respectively), the majority of the colonies formed were of the dissociated phenotype. This dissociated phenotype, induced in the presence of blocking α_2 or β_1 integrin Abs, was also seen when cells were cultured in the presence of HFF-CM (Berdichevsky, F. *et al.*, 1994b). However, dissociation was not seen when the cells were cultured with media alone, i.e. in the absence of fibroblast secreted factors.

5.7. Effect of other blocking Abs to α_2 and β_1 integrins on rhHGF induced branching morphogenesis in collagen gels.

The results described above imply that the function of the $\alpha_2\beta_1$ integrin is important in the branching morphogenesis of HB2 cells in collagen gels, and that a major role for this integrin is to maintain cell-cell interactions in the branching morphogenesis process.

In order to confirm this role, the effects of other blocking Abs to the α_2 or β_1 integrin subunits on branching morphogenesis were investigated, to see if a similar effect was seen. Two further blocking Abs to the α_2 integrin (6F1 and 5E8), and one further blocking Ab to the β_1 integrin (P5D2), were tested. Figure 5.7.1. shows the quantitative results of a typical experiment, and it can again be seen that when blocking Abs to either the α_2 or β_1 integrin were used (P1E6, 6F1, 5E8 - α_2 ; P5D2 - β_1), the predominant phenotype of colonies formed was the dissociated phenotype. However, a blocking Ab to the α_v integrin subunit (L230), an

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies in each morphogenetic group were expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

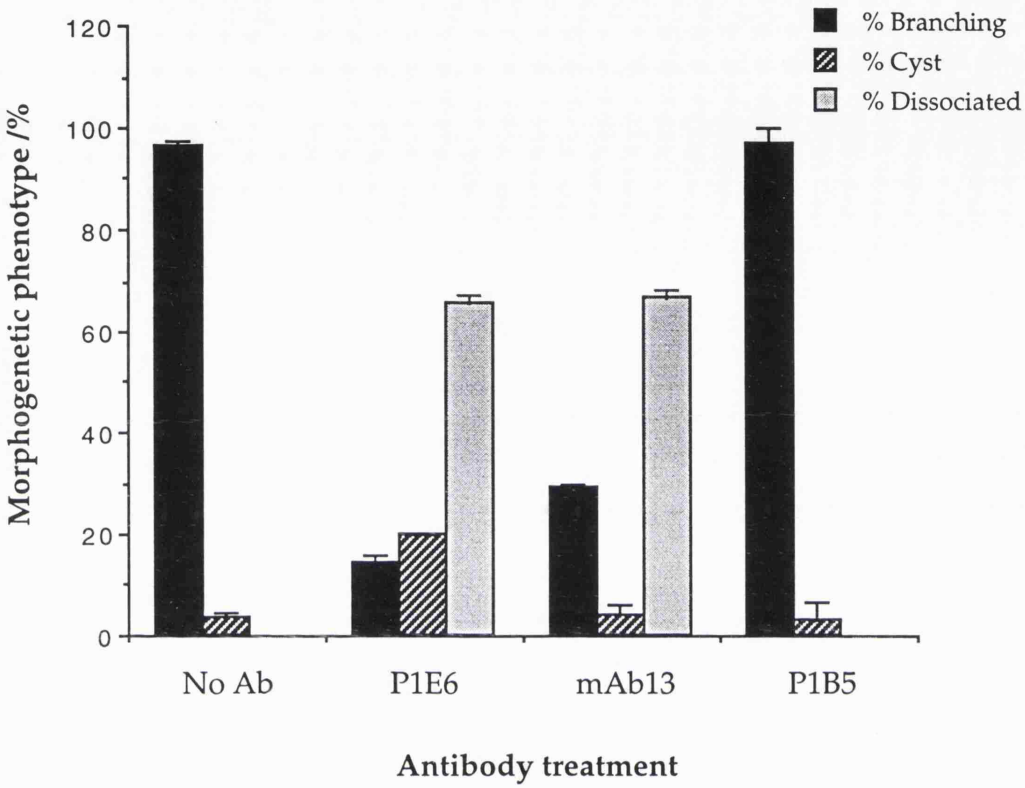


Figure 5.6.2.
Effect of blocking antibodies to α2, α3, and β1 integrin subunits on rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

Cells were pre-treated with the anti-integrin mAbs: α2, P1E6; α3, P1B5; and β1, mAb13, or given no treatment, before embedding in collagen gels and culturing in the presence of 10ng/ml rhHGF. The concentrations of mAbs used were: P1E6, 1/500 ascites; P1B5, 1/100 ascites; mAb13, 2μg/ml. The morphogenetic phenotypes of colonies were counted after 8 days of growth.

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies in each morphogenetic group were expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

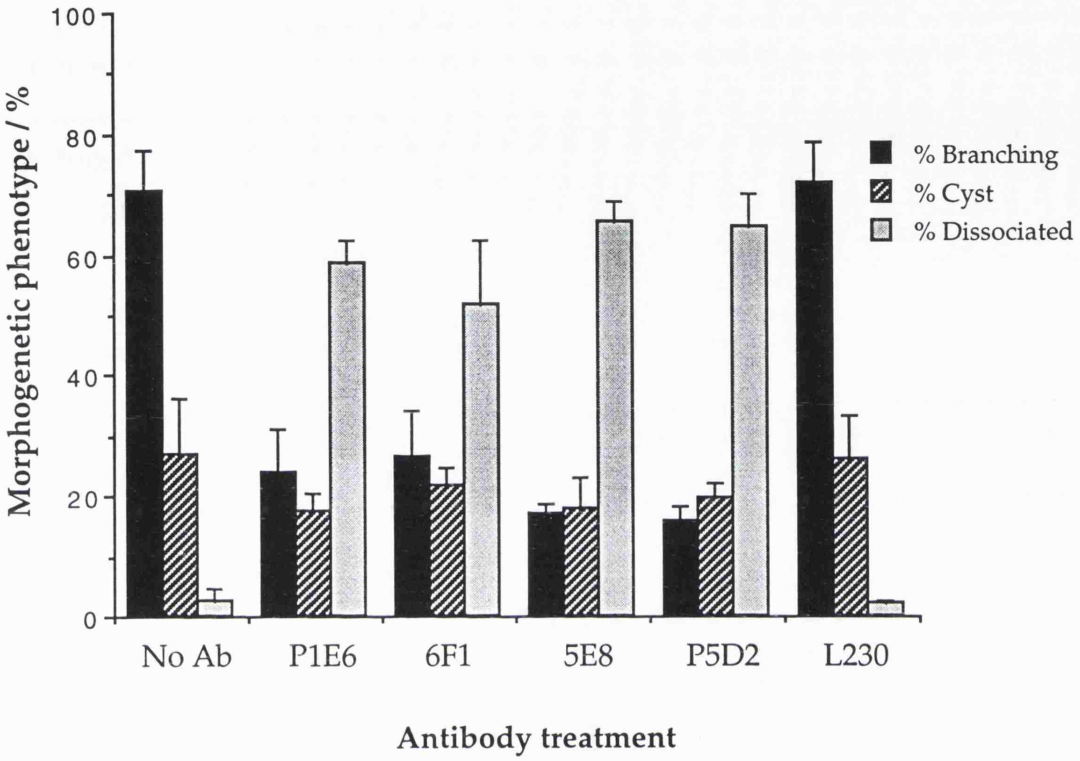


Figure 5.7.1.
Effect of blocking antibodies to α2, αv, and β1 integrin subunits on rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

Cells were pre-treated with the anti-integrin mAbs: α2 - P1E6, 6F1, or 5E8; αv, L230; and β1, P5D2, or given no treatment, before embedding in collagen gels and culturing in the presence of 10ng/ml rhHGF. The concentrations of mAb used were: P1E6, 1/5000 ascites; 6F1, 5μg/ml; 5E8, 2μg/ml; P5D2, 3.6μg/ml; L230, 1/250. The morphogenetic phenotypes of colonies were counted after 7 days of growth.

integrin that is not thought to be directly involved in the adhesion of cells to polymerised collagen, had no phenotypic effect on branching morphogenesis. Pictorial examples of the dissociated phenotype formed with the α_2 blocking Ab 6F1, and the β_1 blocking Ab P5D2, are shown in Figure 5.7.2. (6F1 treated cells in 5.7.2. panels E/F, and P5D2 treated cells in panels G/H), with examples of α_2 blocking Ab P1E6 treated cells for comparison (Figure 5.7.2. panels C/D). The dissociated phenotypes induced by the α_2 blocking Ab 5E8, were similar to those seen in Figure 5.7.2. panels C-H (data not shown).

The experiments described in Figures 5.7.1. and 5.7.2. have been repeated several times, and although the exact percentage of colonies in each morphogenetic category, for each condition, does vary between experiments, the overall result is consistently reproducible. As can be seen from the error bars on the figures, the variations in morphogenetic phenotype, within duplicates in the same experiment, are usually relatively small.

5.8. Effect of α_2 and β_1 integrin blocking Abs on adhesion of HB2 cells to collagen gels.

Although the blocking antibodies which gave the dissociated phenotype in our morphogenesis assay, were, by definition, documented to block adhesion of cells to ECM. It was important to confirm that they were blocking adhesion in our particular experimental system, using the HB2 cell line and polymerised collagen type I gels. Figure 5.8.1. shows that the α_2 blocking Abs P1E6 (Figure 5.8.1.A/B/C), 5E8, and 6F1 (Figure 5.8.1.A), and the β_1 blocking Ab P5D2 (Figure 5.8.1.B) reduced the adhesion of HB2 cells to polymerised collagen as expected. On the other hand, a blocking Ab to the α_v integrin subunit (L230) had no significant effect on the adhesion of HB2 cells to polymerised collagen (Figure 5.8.1.B).

Figure 5.8.1.C. shows that an α_3 blocking Ab, P1B5, unlike the α_2 blocking Abs, does not significantly affect the adhesion of HB2 cells to polymerised collagen. This correlates well with P1B5 having no visible effect on branching morphogenesis, (Figures 5.6.1. panel B and 5.6.2). This result suggests that the $\alpha_2\beta_1$ is the major integrin receptor by which the HB2 cells adhere to polymerised collagen type I.

Figure 5.7.2.
Effect of other blocking antibodies to α_2 and β_1 integrin subunits on rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

HB2 cells were pre-treated with the anti-integrin mAbs: α_2 (P1E6, panels C/D; 6F1, panels E/F); and β_1 (P5D2, panels G/H); or given no treatment, (panels A/B), before embedding in collagen gels and culturing in the presence of 10ng/ml rhHGF. The concentration of mAbs used were: P1E6, 1/500 ascites; 6F1, 5 μg/ml; P5D2, 3.6 μg/ml. The morphogenetic phenotypes of colonies were photographed at both low power (panels A/C/E/G) and high power (panels B/D/F/H) magnification after 7 days of growth.
Scale bar on photographs taken at low power denotes 200 μm
Scale bar on photographs taken at high power denotes 100 μm

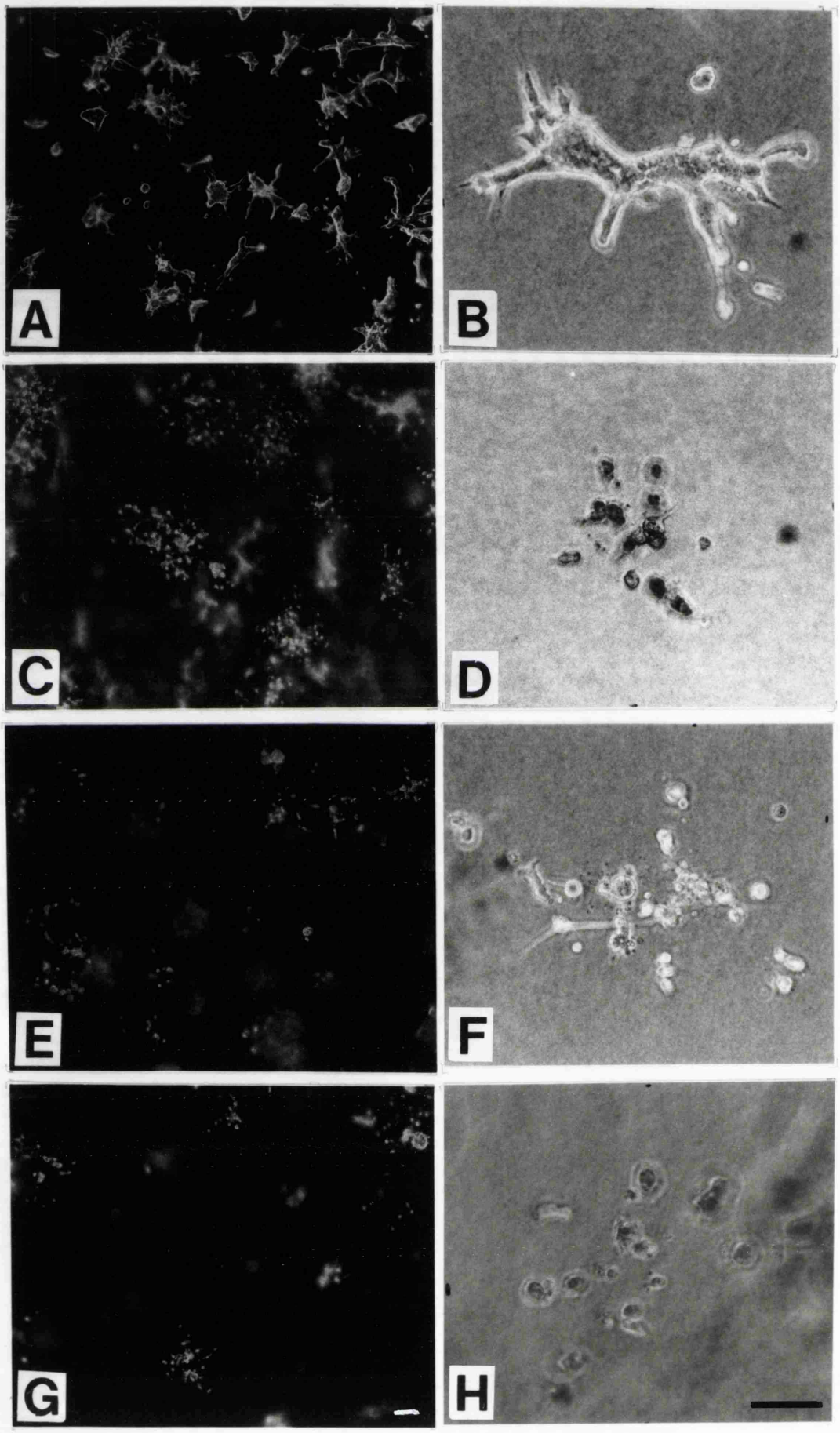

A
B
C
D
E
F
G
H

Figure 5.8.1.
A. Effect of blocking antibodies to the α_2 integrin subunit on adhesion of HB2 cells to collagen.

$1.4x10^4$ HB2 cells were pre-treated with the anti-α_2 integrin mAbs P1E6, 5E8, or 6F1 at a concentration of 5 μg/ml, or given no treatment, before cells were plated onto polymerised collagen in triplicate and allowed to adhere for 15 minutes. After the non adherent cells were removed by washing, the number of cells bound/mm^2 was calculated as described in Materials and Methods.

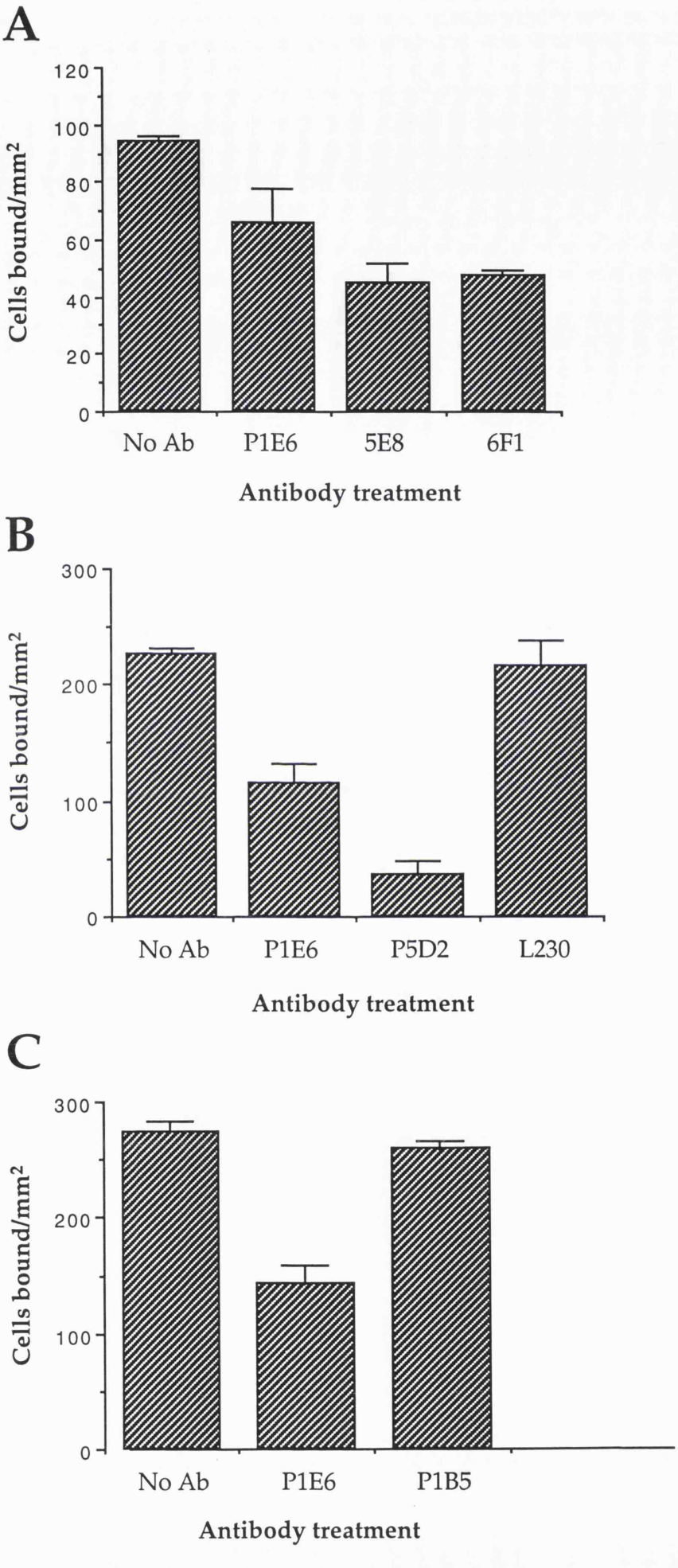
B. Effect of blocking antibodies to the α_2, α_v, and β_1 integrin subunits on adhesion of HB2 cells to collagen.

$1.7x10^4$ HB2 cells were pre-treated with the anti-integrin mAbs: α_2, P1E6; β_1, P5D2; and α_v, L230, or given no treatment, before cells were plated onto polymerised collagen in triplicate and allowed to adhere for 30 minutes. After the non-adherent cells were removed by washing, the number of cells bound/mm^2 was calculated as described in Materials and Methods. The concentrations of mAbs used were: P1E6, 5 μg/ml; P5D2, 4.5 μg/ml; L230, 1/50.

C. Effect of blocking antibodies to the α_2 and α_3 integrin subunits on adhesion of HB2 cells to collagen.

$1.8x10^4$ HB2 cells were pre-treated with the anti-integrin mAbs: α_2, P1E6; and α_3, P1B5, or given no treatment, before cells were plated onto polymerised collagen in triplicate and allowed to adhere for 20 minutes. After the non-adherent cells were removed by washing, the number of cells bound/mm^2 was calculated as described in Materials and Methods. The concentrations of mAbs used were: P1E6, 1/200 ascites; P1B5, 1/200 ascites.

The graphs show the results of typical experiments. For each set of triplicates the mean number of cells bound/mm^2 was plotted, together with the standard error showing the deviation about the mean.


A
120
100
80
60
40
20
0
Cells bound/mm²
No Ab
P1E6
5E8
6F1
Antibody treatment
B
300
200
100
0
Cells bound/mm²
No Ab
P1E6
P5D2
L230
Antibody treatment
C
300
200
100
0
Cells bound/mm²
No Ab
P1E6
P1B5
Antibody treatment

The quantitative effects of these Abs on adhesion, are subject to variation between experiments, but the overall result is consistently reproducible. The other β_1 blocking Ab used in our morphogenesis assays, mAb13, has also been shown to reduce the adhesion of HB2 cells to polymerised collagen type I. (Berdichevsky, F. *et al.*, 1994b).

These experiments suggested that a reduction in the $\alpha_2\beta_1$ mediated cell-collagen interaction, led, indirectly, to the loss of cell-cell interactions in the 3-dimensional morphogenesis assay. However, at this stage it could not be ruled out that the blocking Abs weren't directly inhibiting an integrin mediated cell-cell interaction at the cell-cell interface: The $\alpha_2\beta_1$ integrin had been reported to directly mediate cell-cell adhesion of keratinocytes via an interaction with the $\alpha_3\beta_1$ integrin (Symington, B.E. *et al.*, 1993), and in the mammary gland *in vivo* is also expressed at cell-cell junctions, i.e. in the correct position to mediate cell-cell interactions (Berdichevsky, F. *et al.*, 1994b).

5.9. The individual integrins involved in the maintenance of cell-cell interactions in rhHGF induced branching morphogenesis, are different depending on the matrix in which the HB2 cells are cultured.

The integrins which are involved in adhesion of cells to matrix differ according to the matrix involved. Consequently, if the inhibition of cell-matrix interaction is indirectly responsible for the loss of cell-cell interaction, then the integrins involved in mediating the effect would be matrix dependent. Conversely, if the $\alpha_2\beta_1$ integrin is directly involved in cell-cell interactions, these interactions should be dependent on the function of this integrin even in non-collagen matrices.

To test these alternatives, we examined the effect of various integrin antibodies on rhHGF induced branching morphogenesis of HB2 cells in fibrin gels. The $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been shown to be direct receptors for fibrin/fibrinogen, but not for native polymerised collagen type I (see later discussion), and we have shown that the HB2 cells express the α_v integrin subunit (Figure 3.2.1.B). Thus, it can be deduced that if the effect of inhibition of integrin function on cell-cell adhesion is indirect, i.e. operating through inhibition of interaction of the integrin with the matrix, then α_v integrins and not $\alpha_2\beta_1$ should be

important for morphogenesis in fibrin gels. This was the case as is illustrated in Figure 5.9.1.A. which shows that the α_2 blocking Ab P1E6 had no significant effect on rhHGF induced branching morphogenesis in fibrin, thus providing confirmation that the $\alpha_2\beta_1$ integrin plays no functional role in branching morphogenesis in the fibrin system. In the experiment illustrated in Figure 5.9.1.A. the α_2 integrin Ab P1E6 was tested at a concentration which gives maximum dissociation in collagen (1/500 ascites). However no effects on morphogenesis were seen at a range of concentrations going up to 20μg/ml purified Ab.

In contrast to the results with blocking antibodies to α_2, a blocking Ab to the α_v integrin (L230) did have an effect on branching morphogenesis in fibrin gels. At an Ab concentration of 1/500 a dissociated phenotype was observed in 9.8 - 35.7% of colonies in different experiments. The degree of dissociation was a factor of 1.8 - 4.5 times more than the basal levels of dissociated colonies seen with rhHGF alone. The β_1 blocking antibody, P5D2, also led to the formation of dissociated colonies in fibrin, at a frequency of between 13.0 - 30.2% of colonies in different experiments, at an Ab concentration of 3.6μg/ml. This dissociation was a factor of 3.8 - 8.2 times more than the basal levels of dissociated colonies seen with rhHGF alone. The dissociative effects of the α_v and β_1 blocking Abs in fibrin, were smaller than those seen with the α_2 and β_1 integrin blocking Abs in collagen, but consistently seen in all experiments. Quantitation of the morphogenetic phenotypes formed in a typical experiment is shown in Figure 5.9.1.B.

Pictorial examples of branching and dissociated colonies, induced in collagen compared to fibrin gels in the presence of α_2, α_v, and β_1, blocking Abs are shown in Figure 5.9.2.

5.10. A high concentration of blocking integrin Abs leads to growth inhibition of HB2 cells in collagen or fibrin matrices.

To see the maximal dissociated phenotype in collagen or fibrin matrices with the appropriate blocking integrin Abs, a concentration of between 2 and 5μg/ml purified Ab (or 1/500 dilution of the anti-α_2 Ab P1E6, or the anti-α_v Ab L230), was used to pre-incubate the cells before embedding in

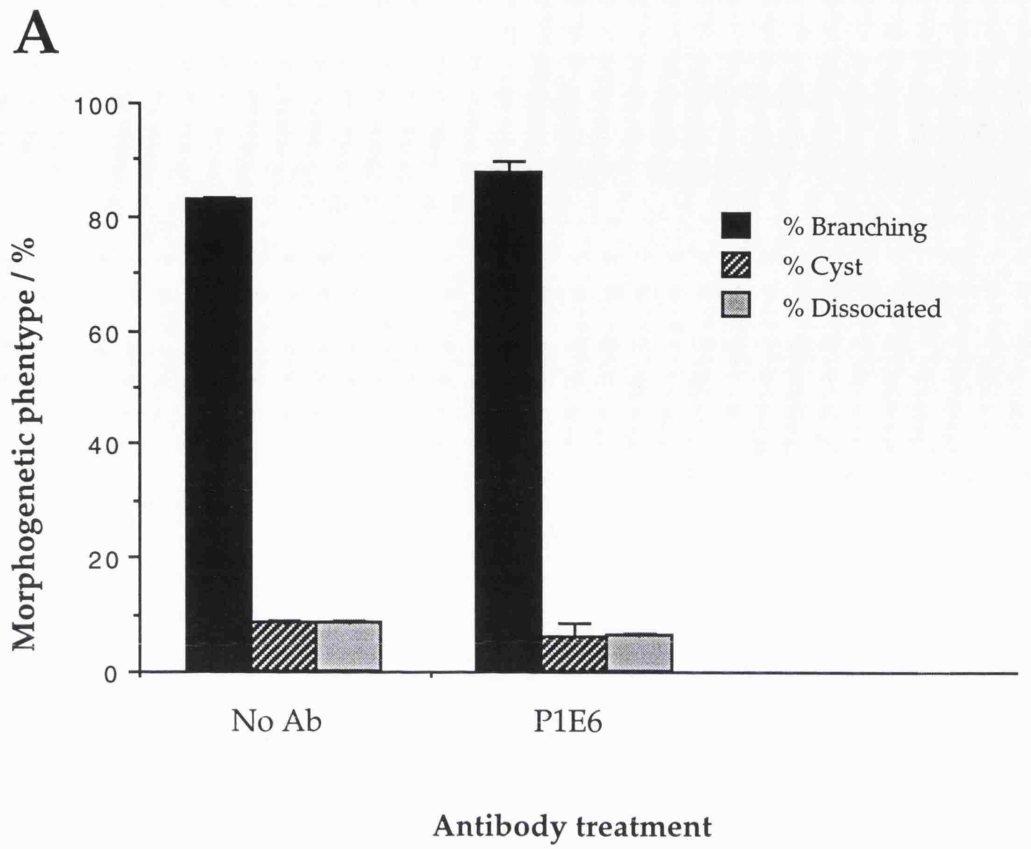
Figure 5.9.1.
A. Effect of a blocking antibody to the α_2 integrin subunit on rhHGF induced branching morphogenesis of HB2 cells in fibrin gels.

HB2 cells were pre-treated with the anti-α_2 integrin mAb P1E6 at a concentration of 1/500 ascites, or given no treatment, before embedding in fibrin gels and culturing in the presence of 10ng/ml rhHGF. The morphogenetic phenotypes of colonies were counted after 7 days of growth.

B. Effect of blocking antibodies to the α_v and β_1 integrin subunits on rhHGF induced branching morphogenesis of HB2 cells in fibrin gels.

HB2 cells were pre-treated with the anti-α_v (L230) or anti-β_1 (P5D2) integrin mAbs, or given no treatment, before embedding in fibrin gels and culturing in the presence of 10ng/ml rhHGF. The concentrations of mAbs used were: L230, 1/500; P5D2, 3.6 μg/ml. The morphogenetic phenotypes of colonies were counted after 7 days of growth.

The graphs show the results of typical experiments consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies in each morphogenetic group were expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.


A
100
80
60
40
20
0
Morphogenetic phentype / %
% Branching
% Cyst
% Dissociated
No Ab
P1E6
Antibody treatment

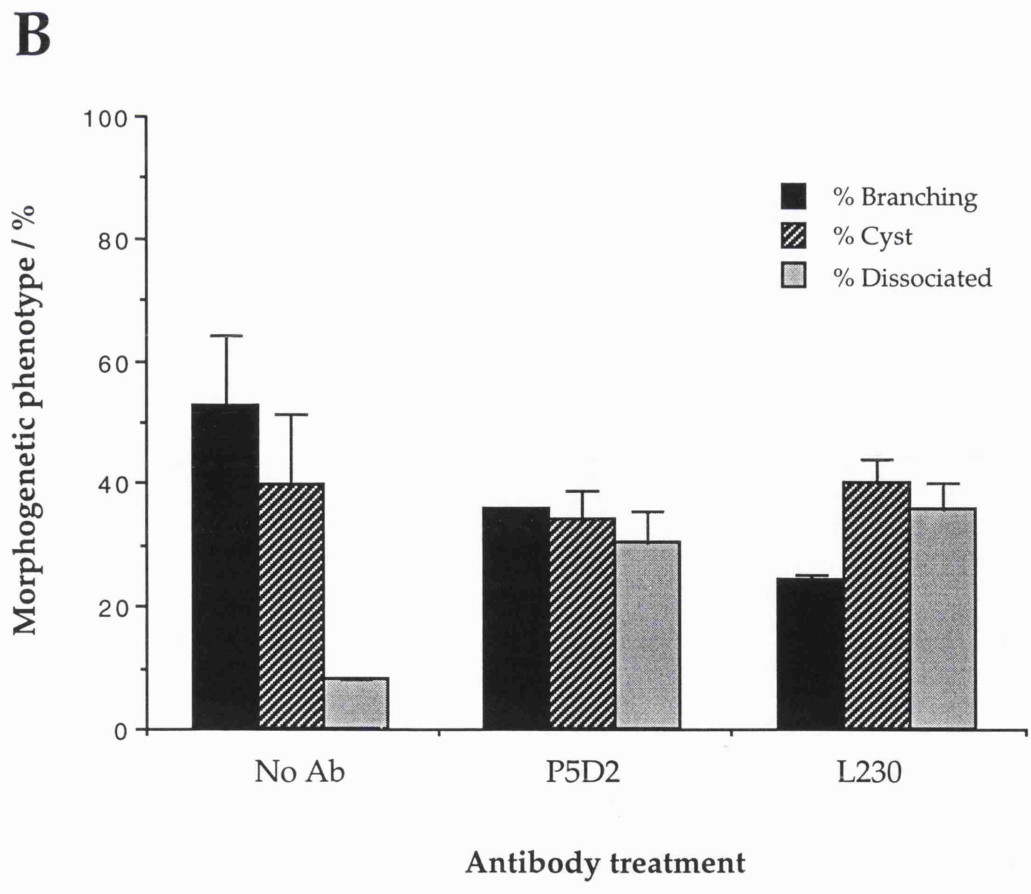
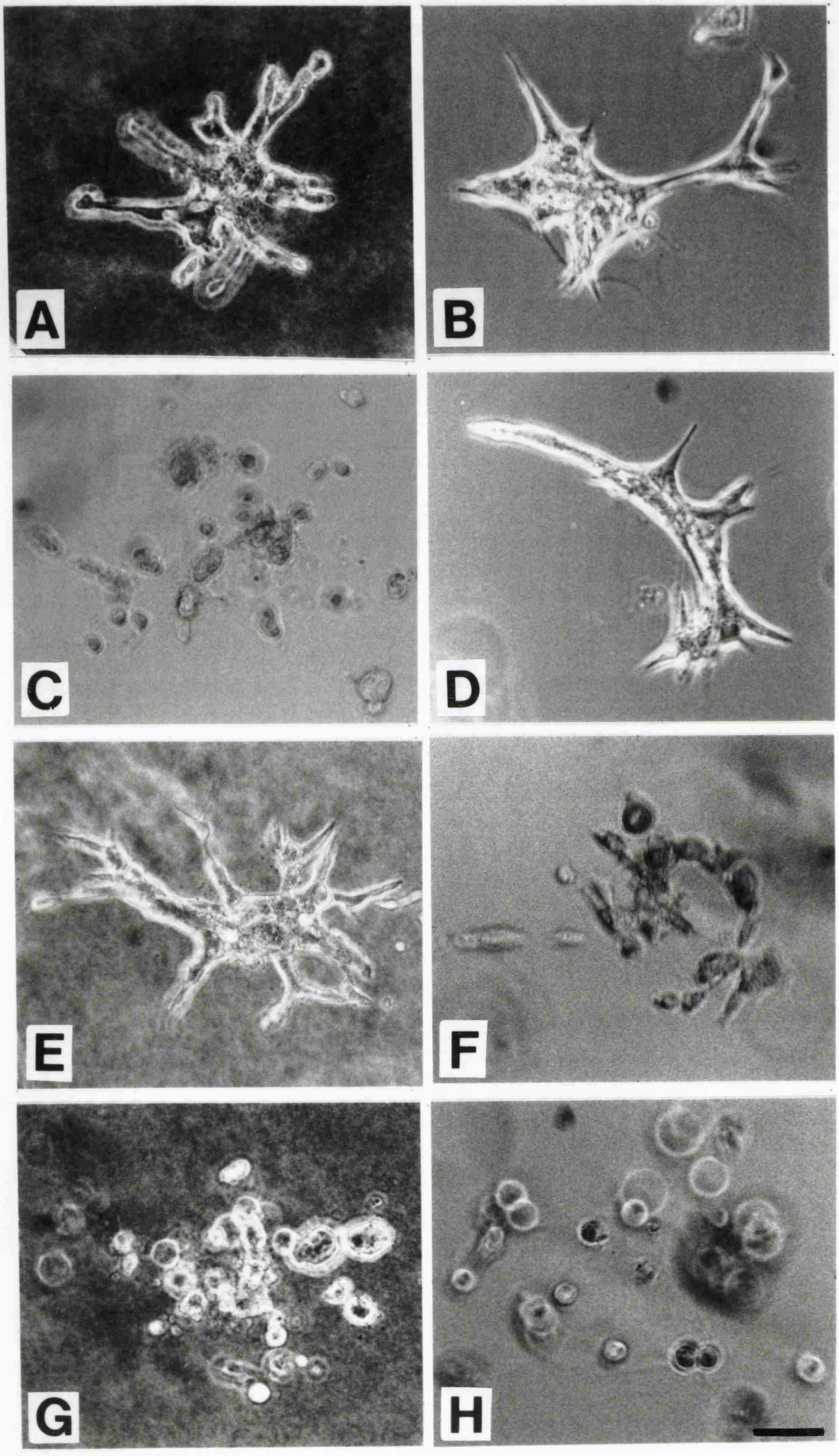

B
100
80
60
40
20
0
Morphogenetic phenotype / %
% Branching
% Cyst
% Dissociated
No Ab
P5D2
L230
Antibody treatment

Figure 5.9.2.
Effect of blocking antibodies to α_2, α_v, and β_1 integrin subunits on rhHGF induced branching morphogenesis of HB2 cells in collagen and fibrin gels.

HB2 cells were pre-treated with the anti-integrin mAbs: α_2, (P1E6, panels C/D); α_v, (L230, panels E/F); and β_1, (P5D2, panels G/H); or given no treatment, (panels A/B), before embedding in collagen gels (left hand side); or fibrin gels (right hand side), and culturing in the presence of 10ng/ml rhHGF. The concentrations of mAbs used were: P1E6, 1/500 ascites; L230, 1/500; P5D2, 3.6 μg/ml. The morphogenetic phenotypes of colonies were photographed after 7 days of growth at high power magnification.
Scale bar denotes 100 μm


A
B
C
D
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F
G
H

the matrix. If a higher concentration of Ab was used, from 10 - 25μg/ml purified Ab (or 1/100 dilution of P1E6 and 1/250 dilution of L230), then although some dissociation was still seen in a small proportion of the colonies, the more profound effect was one of growth inhibition.

As with the formation of dissociated colonies, the growth inhibition by the mAbs was matrix specific, with high concentrations of the α_2 integrin Ab P1E6, having no effect on growth in fibrin matrices but an inhibitory effect in collagen (Figure 5.10.1. panels C and D), and the α_v blocking Ab L230 having an inhibitory effect in fibrin but not in collagen (data not shown). The β_1 blocking Ab P5D2 had an inhibitory effect on growth in both matrices, (Figure 5.10.1. panels E and F). The growth inhibitory effect of high concentrations of integrin blocking Abs on HB2 cells was seen when the cells were cultured both in standard medium alone and in the presence of rhHGF.

5.11. Effect of "non blocking" Abs to the α_2 integrin on rhHGF induced branching morphogenesis of HB2 cells.

As described above, the term "non blocking" simply refers to the fact that although the Abs are specific for a particular integrin subunit, they have not been shown to have any effect on the function of the integrin in terms of adhesion.

The "non blocking" mAb to the α_2 subunit, HAS 4, has been shown to react with an epitope outside the "A domain", but within the extracellular domain of the α_2 subunit, (Bosco Chan and Fiona Watt - personal communication). This Ab was initially used in our branching morphogenesis studies as a negative control, to show that a specific, but non-adhesion blocking mAb did not induce the formation of dissociated structures. As expected, HAS 4 did not induce the formation of dissociated structures, but surprisingly, its effect was to block branching morphogenesis, thus increasing the proportion of spherical cysts formed (Figure 5.11.1.A). This is more of a "true" block in rhHGF induced branching morphogenesis, as it reverts the phenotype to what is seen when the cells are cultured in media alone with no rhHGF added.

Figure 5.10.1.
Effect of high concentrations of blocking antibodies to α_2 and β_1 integrin subunits on growth of HB2 cells in collagen and fibrin gels.

HB2 cells were pre-treated with the anti-integrin mAbs: α_2 (P1E6, panels C/D); and β_1 (P5D2, panels E/F); or given no treatment (panels A/B), before embedding in collagen gels (left hand side); or fibrin gels (right hand side), and culturing in the presence of 10 ng/ml rhHGF. The concentration of mAbs used were: P1E6, 25 μg/ml; P5D2, 18 μg/ml. The morphogenetic phenotypes of colonies were photographed after 7 days of growth at low power magnification.
Scale bar denotes 200 μm

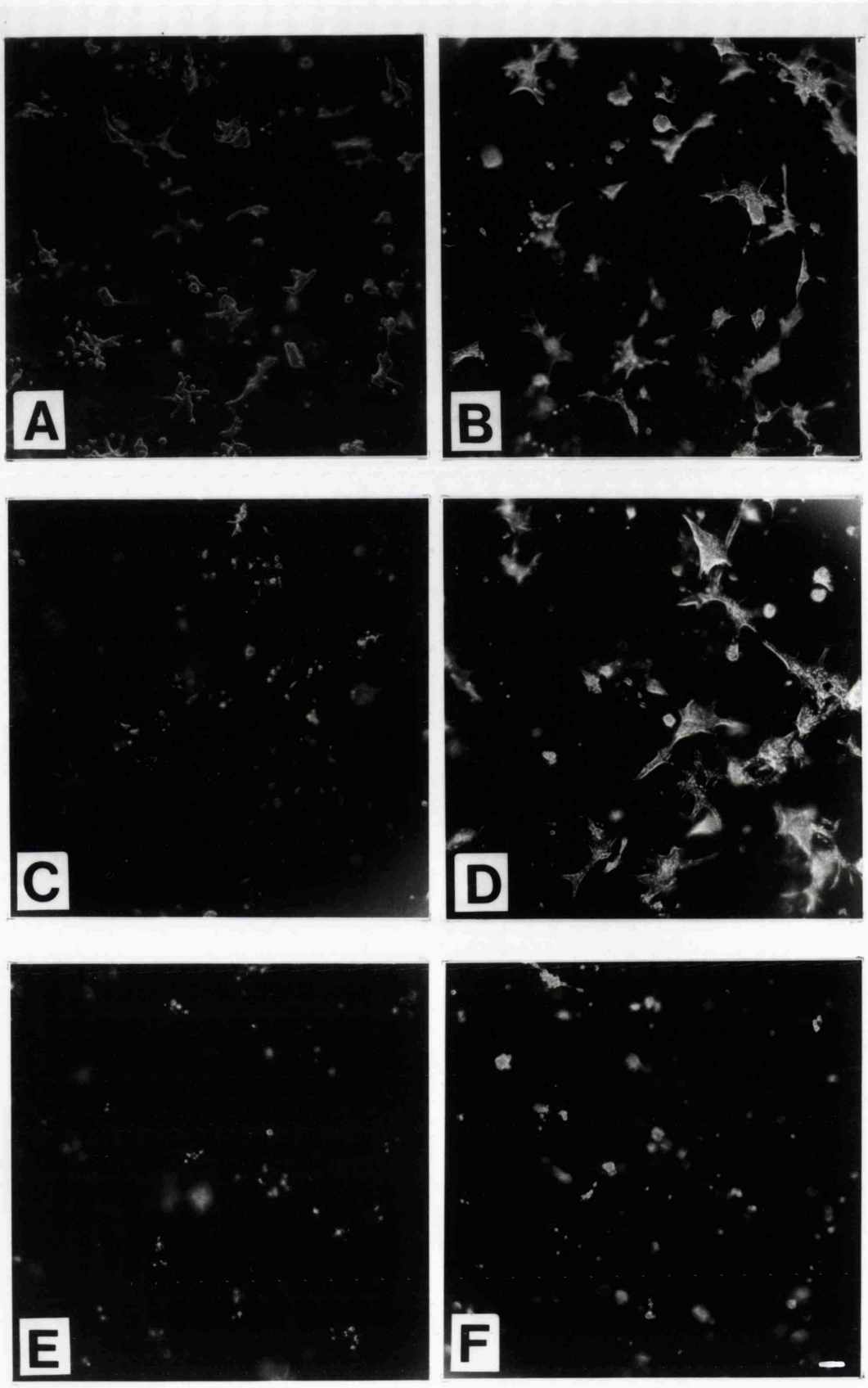

A
B
C
D
E
F

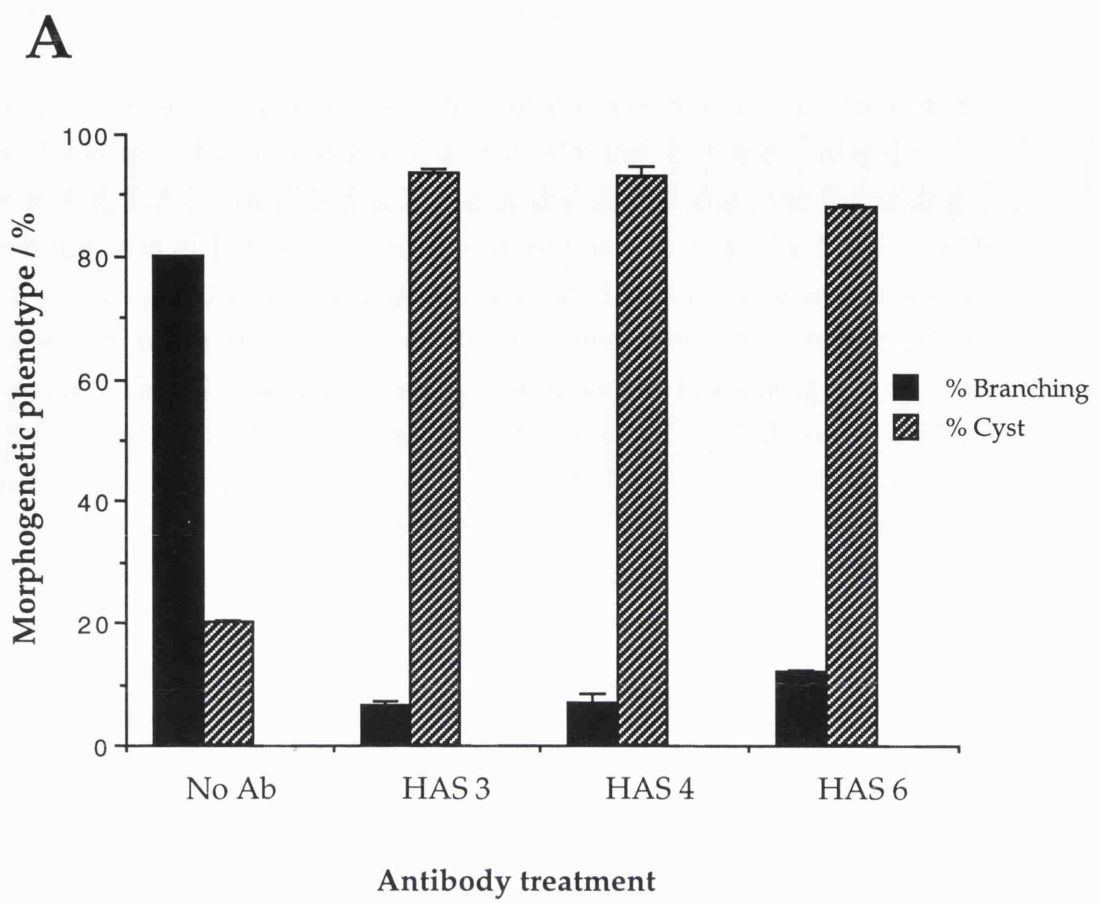
Figure 5.11.1.
A. Effect of "non-blocking" antibodies to the α_2 integrin subunit on rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

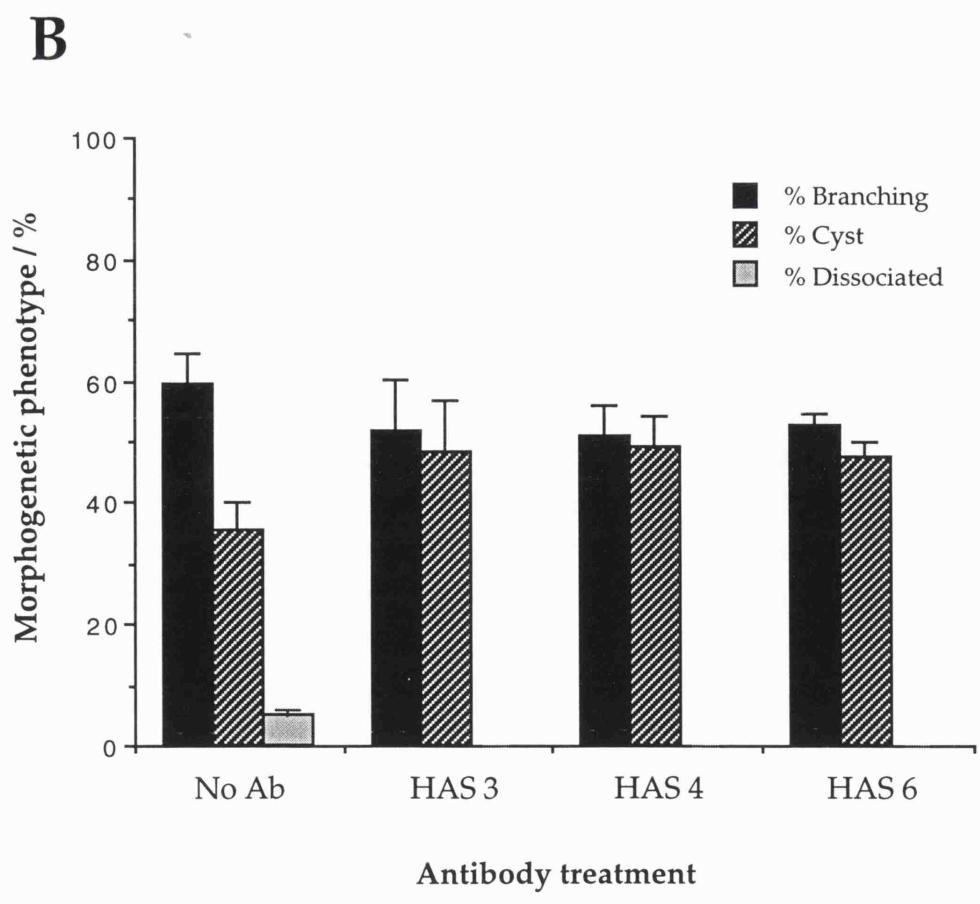
HB2 cells were pre-treated with the anti-α_2 integrin mAbs HAS 3, HAS 4, or HAS 6 at a concentration of 12μg/ml, or given no treatment, before embedding in collagen gels and culturing in the presence of 10ng/ml rhHGF. The morphogenetic phenotypes of colonies were counted after 7 days of growth.

B. Effect of "non-blocking" antibodies to the α_2 integrin subunit on rhHGF induced branching morphogenesis of HB2 cells in fibrin gels.

HB2 cells were pre-treated as in legend A above, before embedding in fibrin gels and culturing in the presence of 10ng/ml rhHGF. The morphogenetic phenotypes of colonies were counted after 7 days of growth.

The graphs show the results of typical experiments consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies in each morphogenetic group were expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.


A
100
80
60
40
20
0
Morphogenetic phenotype / %
% Branching
% Cyst
No Ab
HAS 3
HAS 4
HAS 6
Antibody treatment


B
100
80
60
40
20
0
Morphogenetic phenotype / %
% Branching
% Cyst
% Dissociated
No Ab
HAS 3
HAS 4
HAS 6
Antibody treatment

In order to ensure that this effect was not unique to this mAb, two other "non blocking" Abs mapping to the same domain in the α_2 integrin were tested; HAS 3 and HAS 6. These mAbs also blocked the branching morphogenesis of HB2 cells with the same phenotype as the HAS 4 mAb (Figure 5.11.1.A), thereby suggesting that the HAS 4 effect was not simply non-specific. In Figure 5.11.1.A. all the Abs were used at a concentration of 12μg/ml. The Abs have been shown to block the branching morphogenesis equally well at a concentration of 2.5μg/ml, and the HAS 4 effect has been shown to be dose dependent, being finally diluted out at a concentration of 0.01μg/ml (data not shown). The efficiency of the block in branching morphogenesis varies between 76 - 99% blocking, but the effect is consistently reproducible and striking. The blocking of rhHGF induced branching morphogenesis by the α_2 integrin Ab HAS 4 is shown pictorially in Figure 5.11.2. panels C and D (compare to 5.11.2. panels A and B).

Like the formation of dissociated structures induced by blocking mAbs, the blocking of branching morphogenesis by the HAS family of α_2 integrin mAbs was matrix specific and did not occur in fibrin, (Figure 5.11.1.B. - compare to 5.11.1.A). This provides further evidence that the effects of these mAbs are specifically mediated by the $\alpha_2\beta_1$ integrin, but are also dependent on the cell interaction with the collagen matrix.

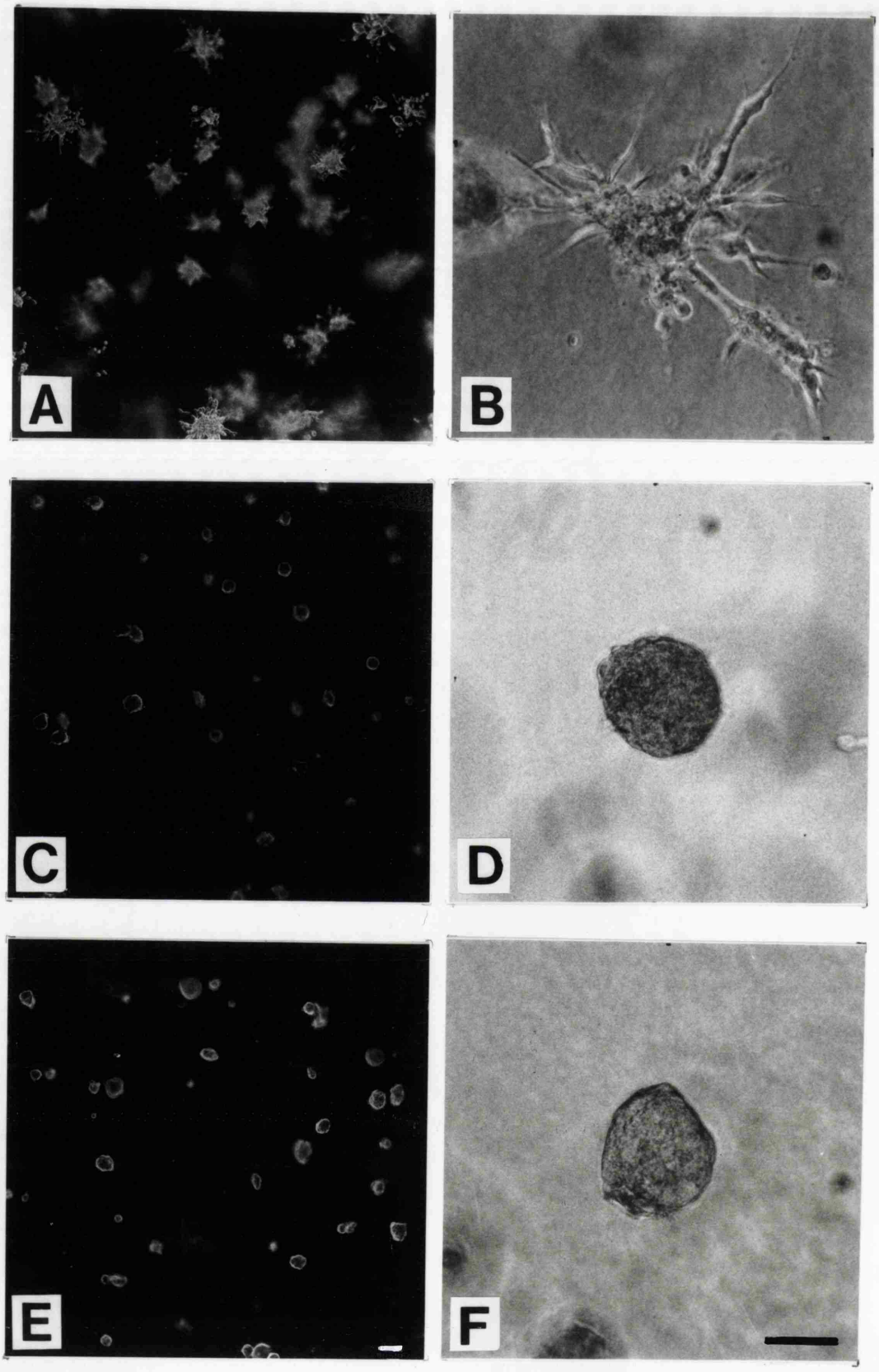
5.12. "Activating" mAbs to the β_1 integrin subunit also inhibit rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

The mAb to the β_1 integrin TS2/16, that is reported to activate the adhesion of cells to ECM by the induction of a conformational change in β_1 integrins, was also shown to block rhHGF induced branching morphogenesis of HB2 cells in collagen, with the same phenotype as the HAS family of α_2 "non-blocking" mAbs. This block in branching morphogenesis is shown pictorially in Figure 5.11.2. panels E and F, and graphically in Figure 5.12.1. As is the case with the HAS 4 Ab, the efficiency of blocking varies between experiments, from 67 - 98%, and is equally good at a concentration of 2.4μg/ml or 12μg/ml of TS2/16. A second activating mAb to the β_1 integrin, 8A2, also blocks branching morphogenesis of HB2 cells with the same phenotype, (Figure 5.12.1.).

Figure 5.11.2.
Effect of a "non blocking" antibody to the α_2 integrin subunit and an activating antibody to the β_1 integrin subunit on rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

HB2 cells were pre-treated with the anti-integrin mAbs: α_2, (HAS 4, panels C/D); and β_1, (TS2/16, panels E/F); or given no treatment, (panels A/B), before embedding in collagen gels and culturing in the presence of 10ng/ml rhHGF. The concentrations of mAb used were: HAS 4, 12 μg/ml; TS2/16, 2.4 μg/ml. The morphogenetic phenotypes of colonies were photographed after 7 days of growth at both low power (panels A/C/E) and high power (panels B/D/F) magnification.

Scale bar on photographs taken at low power denotes 200 μm
Scale bar on photographs taken at high power denotes 100 μm


A
B
C
D
E
F

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies in each morphogenetic group were expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

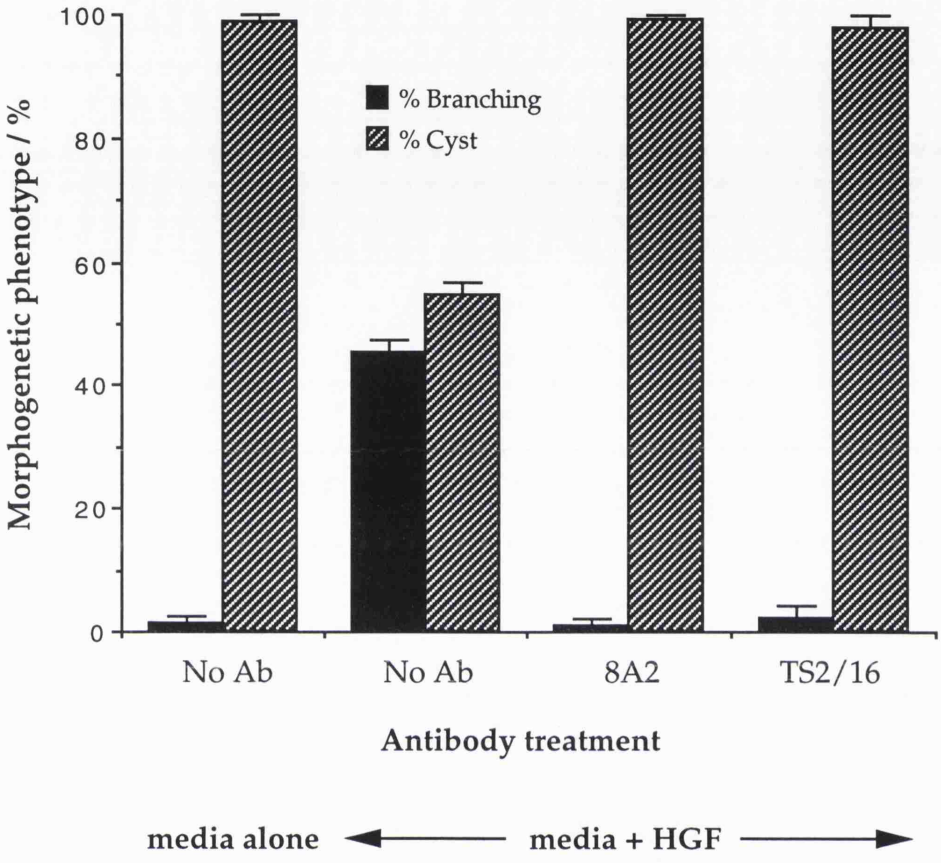


Figure 5.12.1
Effect of activating antibodies to the β1 integrin subunit on rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

Cells were pre-treated with the activating anti-β1 integrin mAbs 8A2 or TS2/16, at concentrations of 2.0 and 2.4 μg/ml respectively, or given no treatment, before embedding in collagen gels and culturing in the presence, or absence, of 10ng/ml rhHGF as indicated. The morphogenetic phenotypes of colonies were counted after 7 days of growth.

Figure 5.12.1. also illustrates the fact that the blocking of branching morphogenesis, induced with these Abs, reverts the morphogenetic phenotype to that seen in the absence of HGF stimulation, i.e. with media alone (as is also seen with the HAS family of Abs). Both the HAS 4 and the TS2/16 Abs can also inhibit the branching morphogenesis of HB2 cells induced by HFF-CM (data not shown).

5.13. The "activating" mAb to the β_1 integrin, TS2/16, but not the "non-blocking" mAb to the α_2 integrin, HAS 4, increases the adhesion of HB2 cells to collagen gels.

The blocking of branching morphogenesis by the TS2/16 and 8A2 β_1 activating mAbs, suggested that a possible mechanism for the block in branching morphogenesis might be to increase the $\alpha_2\beta_1$ mediated adhesion of the HB2 cells to collagen, thereby inhibiting the motility response of cells induced by rhHGF, which we hypothesise to be an integral part of the branching morphogenesis mechanism (see earlier discussion in Chapter 3).

It was therefore important to assess whether TS2/16 did indeed increase the adhesion of HB2 cells to collagen, and to verify that the mAb HAS 4, not previously shown to increase adhesion in any system, did not increase the adhesion of cells to collagen in this one. The effects of the TS2/16 and HAS 4 Abs on the adhesion of HB2 cells to polymerised collagen are shown in Figure 5.13.1. From this, it can be seen that TS2/16, at a concentration of 20μg/ml, does indeed increase the adhesion of HB2 cells to collagen; whereas HAS 4 does not significantly affect adhesion, even at the high concentration of 60μg/ml. This experiment has been repeated several times but HAS 4 has never been shown to have a significant effect on adhesion.

5.14. The "activating" mAb to the β_1 integrin, TS2/16, and the "non-blocking" mAb to the α_2 integrin, HAS 4, decrease the motility of HB2 cells on collagen gels.

Even though the HAS 4 antibody had no effect on the rate of adhesion of HB2 cells to collagen, it could inhibit motility by some other means, and thus block branching morphogenesis.

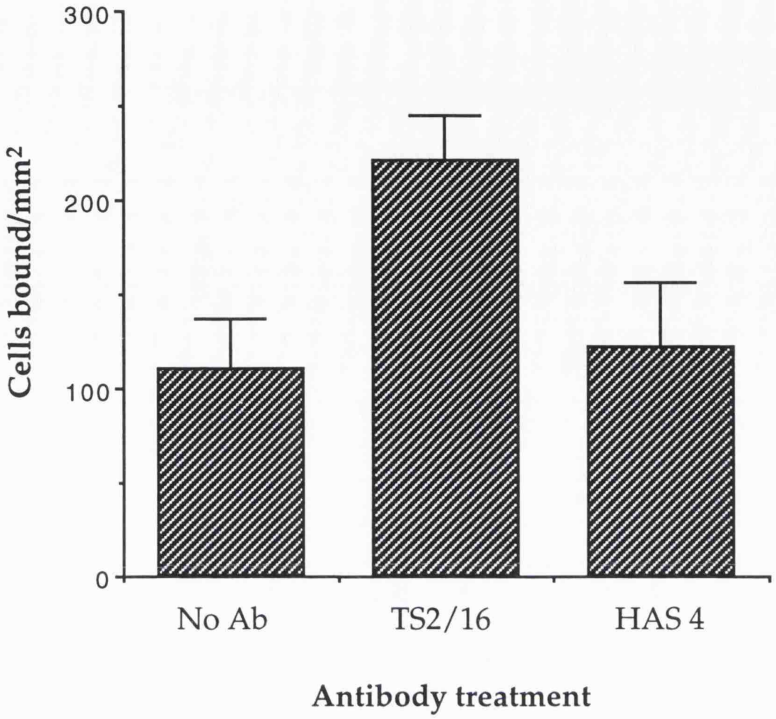


Figure 5.13.1.
Effect of the activating antibody to the β1 integrin subunit, TS2/16, and the HAS4 antibody to the α2 integrin subunit on the adhesion of HB2 cells to collagen gels.

$1.5x10^4$ HB2 cells were pre-treated with the anti-α2 integrin mAb HAS 4, the anti-β1 mAb TS2/16, or given no treatment, before cells were plated onto polymerised collagen in triplicate and allowed to adhere for 10 minutes. After the non-adherent cells were removd by washing, the number of cells bound/mm^2 was calculated as described in Materials and Methods. The concentration of mAbs used were: HAS 4, 60μg/ml; TS2/16, 20μg/ml.

The graph shows the results of a typical experiment. For each set of triplicates the mean number of cells bound/mm^2 was plotted, together with the standard error showing the deviation about the mean.

Time-lapse microscopy was used to measure the motility of HB2 cells on polymerised collagen as described in Materials and Methods, and the results are summarised in Table 5.14.1. The data show that the increase in cell motility induced by rhHGF (see Chapter 3), is indeed strongly inhibited not only by the activating β_1 integrin Ab TS2/16, but also by the HAS 4 Ab. Moreover, the inhibition is apparent whether a low (10 ng/ml), or high (20 ng/ml) concentration of rhHGF is used. A non-specific Ab (W632), to the MHC Class I surface molecule however, had no effect on the rhHGF induced motility of HB2 cells.

5.15. Mn^{2+} ions partially inhibit the rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

If one mechanism of inhibiting branching morphogenesis of HB2 cells is by increasing integrin-mediated adhesion to the matrix, then activating the function of integrins by some other method than by using Abs, should also inhibit branching morphogenesis. One extensively tested way of activating integrin function, is by treatment with the divalent metal cation Manganese $^{2+}$, (Mn^{2+}). Mn^{2+} ions are thought to activate integrin-mediated adhesion in a similar way to the activating Abs, i.e. by the induction of a conformational change in the integrin subunit. Pre-incubation of HB2 cells with different concentrations of Mn^{2+} ions rather than integrin Abs, before embedding in collagen gels, led to a partial but clear decrease in branching morphogenesis in a dose dependent manner (Figure 5.15.1). Unfortunately, a concentration of Mn^{2+} of more than 1 mM was toxic to the cells, so that the possibility that the inhibition of branching morphogenesis would be improved by further increasing the Mn^{2+} concentration could not be tested.

5.16. The "activating" β_1 mAb TS2/16 inhibits rhHGF induced branching morphogenesis of HB2 cells in fibrin gels.

Results using blocking antibodies suggest that the $\alpha_v\beta_1$ integrin is playing a role in branching morphogenesis in the fibrin system, and the $\alpha_2\beta_1$ in the collagen system. If, however, part of the mechanism of branching morphogenesis is dependent on HGF stimulation of motility

The mean speed of cells in 10 ng/ml rhHGF is 14.17 μm/hr. The calculated 95% confidence interval using a two-tailed t test of significance lies between 11.51 μm/hr and 16.83 μm/hr. Experimental means lying outside this range deviate sufficiently from the control to be deemed significantly different.

No statistical analysis has been carried out on the cells in 20 ng/ml rhHGF because data from only single experiments are shown.

Treatment	rhHGF concentration/ ng/ml	Average speed [1]/ µm/hour	Overall average speed [2]/ µm/hour
media alone	0	11.55 15.34 14.31 10.54	12.94
HGF	20	19.36	19.36
	15	17.83 18.93	18.38
	10	16.09 12.25	14.17
HGF + TS2/16 [3]	20	7.16	7.16
	10	5.08	5.08
HGF + HAS 4 [3]	20	10.36	10.36
	10	9.03	9.03
HGF + W632 [3]	10	15.65	15.65

Table 5.14.1.
Inhibition of rhHGF induced motility of HB2 cells on collagen by the "non-blocking" α_2 integrin antibody HAS 4 and the "activating" β_1 integrin antibody TS2/16.

$5x10^4$ HB2 cells were pre-incubated with the antibodies indicated, or given no treatment, before plating in standard growth medium onto 35 mm tissue culture dishes coated with 1 ml of polymerised collagen. The cells were allowed to attach and then stimulated with standard growth medium alone, or standard medium containing rhHGF at the concentration indicated. The motility of individual cells was monitored by time-lapse videomicroscopy as described in Materials and Methods.

Footnotes:

1. This column lists the average speed of 36 or 48 cells calculated for each condition in individual experiments.

2. This column shows the overall average speed of cells for a particular condition, calculated from all the experiments done.

3. All antibodies tested at a concentration of 15µg/ml.

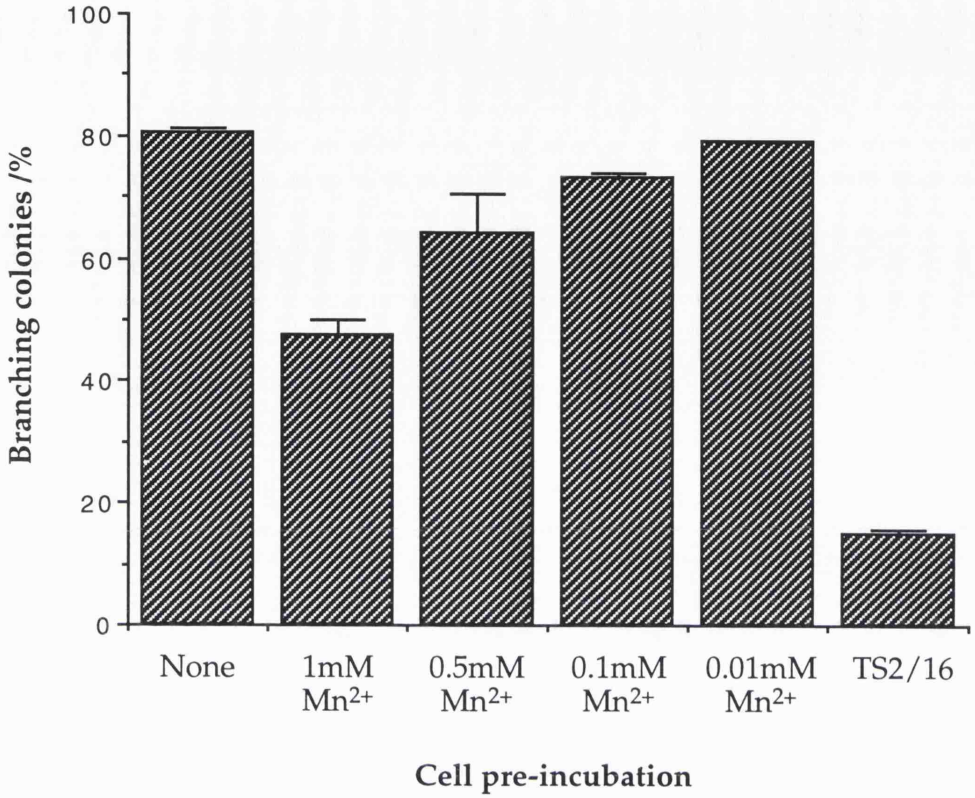


Figure 5.15.1.
Effect of Mn^{2+} ions on rhHGF induced branching morphogenesis of HB2 cells in collagen gels.

HB2 cells in Ca^{2+} and Mg^{2+} free E4 were pre-treated with various concentrations of Mn2+ ions, with 2.4μg/ml TS2/16, or given no treatment, before embedding in collagen gels and culturing in standard growth medium supplemented with 10 ng/ml rhHGF. The morphogenetic phenotypes of colonies were counted after 7 days of growth.

The histogram shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies branching was expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies in each morphogenetic group were expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

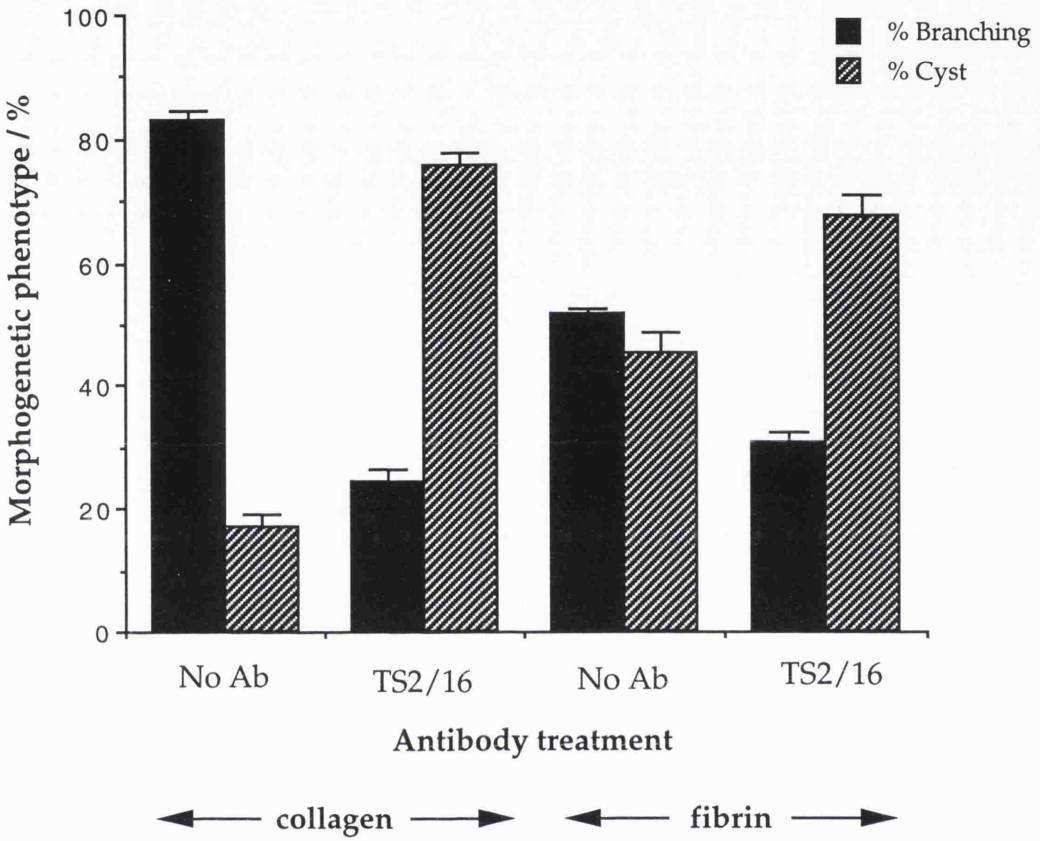


Figure 5.16.1.

Effect of an activating antibody to the β1 integrin subunit on rhHGF induced branching morphogenesis of HB2 cells in collagen and fibrin gels.

HB2 cells were pre-treated with the anti-β1 integrin mAb TS2/16 at a concentration of 2.4μg/ml, or given no treatment, before embedding in collagen or fibrin gels and culturing in the presence of 10ng/ml rhHGF. The morphogenetic phenotype of colonies were counted after 7 days.

in both the collagen and fibrin systems (see Chapter 3), then it might be predicted that the activating β_1 mAb TS2/16, which inhibits rhHGF induced motility and blocks branching morphogenesis in the collagen system, would also block branching morphogenesis in the fibrin system. This was indeed found to be the case, although in a similar way to the experiments carried out with the blocking β_1 Ab P5D2, the effect of the activating β_1 Ab is less potent in the fibrin system than the collagen. Results of a typical experiment are shown in Figure 5.16.1, where the TS2/16 Ab inhibits the rhHGF induced branching by 40% in fibrin and 70% in collagen.

DISCUSSION AND CONCLUSIONS. PART II.

The experiments described in Part II of this chapter examined the effects of specific integrin antibodies on branching morphogenesis of a human mammary epithelial cell line, and showed that integrins play an important functional role in both the growth of the HB2 human mammary epithelial cell line in matrix components, and also in the induction of branching morphogenesis and maintenance of cell-cell interactions within the branching structures. Moreover, we have shown that the integrins that are important for these roles, differ, depending on the matrix cells are grown in; the $\alpha_2\beta_1$ being important in the collagen system, and the $\alpha_v\beta_1$ in the fibrin.

The Abs used are shown to disrupt branching morphogenesis with one of two phenotypes:-
1. Reduced formation of compact branching structures, with the induction of "dissociated" colonies containing cells with reduced cell-cell interactions. This phenotype is induced with blocking Abs to the α_2 or β_1 integrin subunits in collagen (Figures 5.6.1, 5.6.2, 5.7.1, and 5.7.2), and blocking Abs to the α_v or β_1 integrin subunits in fibrin (Figures 5.9.1. and 5.9.2). To see this phenotype, the blocking Abs are used at relatively low concentrations (up to 5μg/ml). If their concentration is increased beyond a certain level (10-25μg/ml), then a profound growth inhibition of colonies is seen (Figure 5.10.1).

2. A block in branching morphogenesis by increasing the proportion of spherical cysts, and reducing dramatically the proportion of branching

colonies. This phenotype is seen with activating Abs to the β_1 integrin in collagen and fibrin (Figures 5.12.1, and 5.16.1) or, in collagen only, a set of Abs to the α_2 integrin that had previously been described as having "no functional effect" (Figures 5.11.1, and 5.11.2).

Each of these phenotypes will be discussed separately:-

1. Formation of "dissociated" colonies in collagen and fibrin gels in the presence of blocking Abs.

Role of the cell-matrix interaction.

The "dissociated" phenotype is seen in collagen gels when low concentrations of blocking Abs to the α_2 or β_1 integrin are used. We concluded from this that a major function of the $\alpha_2\beta_1$ integrin in branching morphogenesis, was to maintain cell-cell adhesion. This function of the $\alpha_2\beta_1$ in cell-cell adhesion could be mediated directly, at the cell-cell interface, with the $\alpha_2\beta_1$ integrin acting as a classical cell adhesion molecule; or indirectly, via the $\alpha_2\beta_1$ mediated cell interaction with the collagen matrix. Although the second possibility was the more likely, direct cell-cell interaction could not be discounted, especially since the $\alpha_2\beta_1$ had been reported to directly mediate cell-cell interaction via an interaction with the $\alpha_3\beta_1$ integrin (Symington, B.E. *et al.*, 1993). Also, in the mammary gland, the $\alpha_2\beta_1$ integrin is present in the expected position (at cell-cell junctions), for mediating cell-cell interactions (Berdichevsky, F. *et al.*, 1994b).

Good evidence was obtained to support the view that the role of the $\alpha_2\beta_1$ integrin in cell-cell interactions was mediated by an indirect mechanism; namely that the α_2 blocking Ab P1E6, did not induce dissociation of HB2 cells in fibrin gels (Figure 5.9.1.A). In this experiment, if the direct mechanism hypothesis was correct, the type of matrix would not be expected to have any effect on the ability of the α_2 blocking Ab to induce the dissociated phenotype, which was clearly not the case.

Turning this argument around, it could be said that good evidence for the indirect mechanism being correct, would be that the dissociated phenotype is only seen when the cell-matrix interaction is disrupted,

and therefore the integrins involved should vary depending on the matrix the cells are in contact with. Again, this was found to be the case, with the α_v integrin blocking Ab (L230), giving a dissociated phenotype only in the fibrin system, and the α_2 integrin Ab (P1E6), only in the collagen system (Figures 5.7.1 and 5.9.1).

The $\alpha_v\beta_1$ integrin recognises RGD (Arg-Gly-Asp) sequences in ligands (Dedhar, S. and Gray, V., 1990; Hynes, R.O., 1992) and has been shown to be a direct receptor for fibronectin, vitronectin, and fibrinogen (Dedhar, S. and Gray, V., 1990; Marshall, J.F. *et al.*, 1995). However, it is not thought that the $\alpha_v\beta_1$ integrin can act as a direct receptor for native collagen type I. It is known that binding of the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins to native type I collagen depends on the recognition of the tetrapeptide sequence DGEA (Asp-Gly-Glu-Ala) and requires collagen to be in a native configuration (Staatz, W.D. *et al.*, 1991; Gullberg, D. *et al.*, 1992). Heat denaturation of type I collagen disrupts binding by these integrins, while exposing cryptic RGD adhesive sites that can be ligated by $\alpha_v\beta_3$, and presumably, also by $\alpha_v\beta_1$ (Davis, G.E., 1992). Degradation of collagen is also thought to expose these cryptic RGD sites in native collagen (Montgomery, A.M. *et al.*, 1994). However, the recognition of RGD by $\alpha_v\beta_1$ and the absence of this peptide in native type I collagen, supports the fact that it is unlikely that $\alpha_v\beta_1$ is a direct receptor for native helical collagen type I. This is further supported by the results presented herein which showed that blocking α_v Abs did not inhibit adhesion of cells to polymerised collagen type I. This may also, of course depend on cell type.

If the "dissociated" phenotype only occurs when the cell-matrix adhesion is reduced, then this would provide an explanation as to why the blocking Ab to the α_3 integrin (P1B5) gave no dissociation in collagen gels (Figure 5.6.1 and 5.6.2). Even though the $\alpha_3\beta_1$ integrin is a potential collagen type I receptor (Hynes, R.O., 1992; Elices, M.J. *et al.*, 1991; Takada, Y. *et al.*, 1991), in adhesion assays using HB2 cells and polymerised collagen type I, the integrin mediated adhesion via the α_3 integrin was found to be very small; the majority of integrin mediated adhesion being via the α_2 integrin (Figure 5.8.1.C). Thus, since the $\alpha_3\beta_1$ integrin is not mediating adhesion of HB2 cells to collagen, according to the hypothesis, blocking its function would not be expected to induce a dissociated phenotype, which is in fact the result seen. The fact that the $\alpha_3\beta_1$

integrin is not acting as a major collagen type I receptor in this system, whereas it does in others (Elices, M.J. *et al.*, 1991), is in agreement with the suggestion by DiPersio, C.M. *et al.*, 1995, that the binding of the $\alpha_3\beta_1$ integrin to a particular ligand may be dependent on cell type.

We have been unable to show directly by sequential immunoprecipitation and Western blotting studies, that the $\alpha_v\beta_1$ integrin is expressed as a functional heterodimer by the HB2 cells. However, this is implied by the fact that blocking Abs to both the α_v and β_1 integrin subunits induce the same dissociated phenotype in fibrin gels. The effects of blocking Abs to other β integrin subunits that can heterodimerise with α_v to make fibrin receptors (e.g. β_3 and β_5), have not yet been tested. However, the $\alpha_v\beta_3$ integrin is not expressed in the mammary gland *in vivo* (Koukoulis, G.K. *et al.*, 1991; Anbazhagan, R. *et al.*, 1995), and it is extremely unlikely that this heterodimer is expressed by the HB2 cell line (John Marshall - personal communication). Therefore, this integrin is unlikely to be playing a role in branching morphogenesis in this *in vitro* system. The status of β_5 integrin expression in either the mammary gland *in vivo*, or in HB2 cells, is, as yet, unknown.

The "dissociated" phenotype is only induced in the presence of fibroblast secreted factors.

Interestingly, the appearance of dissociated structures is dependent on the cells being cultured in the presence of rhHGF (or HFF-CM, data not shown and not discussed here), and is not seen in the presence of unsupplemented growth media alone. A possible explanation for this, is that although the cell-cell interactions may be weaker, when cells are treated with blocking Abs in the absence of rhHGF, the dissociated phenotype only appears when these loosened cells are moved away from each other by the rhHGF stimulation of motility.

Alternatively, the rhHGF may also be acting to loosen cell-cell interactions as well as stimulate motility, so that when coupled in 3-dimensions with the cell-cell loosening effect of the blocking Abs, the cell-cell interactions are no longer retained and the dissociated phenotype is seen. It is important to note that the dissociated phenotype

must be a result of blocking Ab and rhHGF action in combination, as neither treatment alone leads to the formation of a significant proportion of dissociated colonies.

The second theory that HGF itself has the effect of loosening cell-cell interactions, is supported by the fact that rhHGF, in a 2-dimensional assay, induces a "scattered" phenotype in epithelial cells, where the cells no longer form classical epithelial islets, with tight cell-cell adhesions, but are more fibroblastic looking and more detached from each other. HGF has been reported to reduce the expression of E-Cadherin, and alter its distribution so that it is no longer concentrated at cell-cell contacts (Tannapfel, A. *et al.*, 1994).

As described earlier in Chapter 3, a scattered morphology is indeed induced in HB2 cells by rhHGF when the cells are cultured on collagen or fibrin in 2-dimensions, but not on tissue culture plastic. Interestingly, if this "scattered" response of HB2 cells is quantitated by counting colonies composed of single cells versus those composed of 2 or more cells, then it is seen that the α_2 blocking Ab, P1E6, has as much effect on reducing cell-cell association as rhHGF (data not shown). This suggests that either Abs blocking α_2 function, or HGF, can affect cell-cell association in 2-dimensions. On the other hand, in the 3-dimensional system neither treatment alone leads to cell separation. It seems likely that in 3-dimensional structures the scattering/motility response of HB2 cells to HGF alone, results in the formation of branching colonies, the effect on cell separation being too weak to force the breakdown of cell-cell interactions unless another factor such as a blocking integrin Ab is present. It should also be noted that the mechanism of action of the α_2 integrin blocking Abs and HGF on inducing cell-cell separation are likely to be different. As outlined above, we believe that the mechanism of induction of cell-separation by blocking integrin Abs is indirect, by virtue of the reduction in cell-matrix adhesion. However, in contrast we have shown in adhesion assays that HGF does not affect the adhesion of cells to collagen (data not shown), and therefore, that the HGF induced effect on cell-cell separation is likely to be mediated directly at the cell-cell interface (for example by involving the E-cadherin adhesion system), rather than indirectly by altering adhesion to matrix.

These results suggest that reduced α_2 integrin expression/function, in addition to the production of HGF, might potentially play an important role in the disruption of tissue architecture, and the initial stages of invasion in breast cancer development *in vivo*. This is especially so, given the reduction in α_2 integrin expression observed in a significant proportion of breast cancers *in vivo* (Zutter, M.M. *et al.*, 1990; Pignatelli, M. *et al.*, 1991; Zutter, M.M. *et al.*, 1993); and the correlation of increased levels of HGF in breast cancer patients with shorter overall survival (Yamashita, J. *et al.*, 1994), and also with invasive and metastatic disease (Taniguchi, T. *et al.*, 1994; Taniguchi, T. *et al.*, 1995).

A reduced dissociative response to blocking Abs is seen in fibrin compared to collagen.

As the appearance of the dissociated phenotype is dependent on the rhHGF stimulus as well as the blocking Ab function, this may well go some way to explain why the dissociative effects of the blocking β_1 Ab (P5D2), and blocking α_v Ab (L230) are smaller in fibrin than in collagen.

The rhHGF induced branching response in collagen can vary between 38% and 97% of colonies in different experiments. Obviously it is expected that there would be some experimental variation, but we have also seen more pronounced variations in the branching response, dependent on the batch of rhHGF and its method of storage. It is also a general observation in the collagen system, that the dissociative effect induced by the blocking Abs, is greatest when the branching response is the greatest. This seems reasonable if the generation of both the dissociated and the branching phenotypes are dependent on the motility response of the cells to rhHGF, a good response manifesting itself as a large proportion of branching or dissociated colonies, in the absence or presence of blocking Ab respectively.

In parallel experiments carried out in the collagen and fibrin systems, it is clear that the proportion of colonies that perform branching morphogenesis in response to rhHGF, is significantly higher (1.6 - 2 times), in the collagen system than the fibrin. (See Table below). In these same experiments where the blocking β_1 Ab P5D2 is used at a concentration of 3.6μg/ml, which induces the dissociated phenotype in

both systems, the proportion of colonies with a dissociated phenotype is 1.5 - 3.5 times higher in collagen than fibrin. Indeed in two out of three experiments, the relative reduction in the colonies branching in fibrin rather than collagen, is almost exactly the same as the relative reduction in the colonies dissociated in fibrin against collagen (see Table below).

Expt.	% Branching No Ab		Relative reduction in response.	% Dissociated P5D2/3.6µg/ml		Relative reduction in response.
	Coll	Fibrin	Fibrin/Coll	Coll	Fibrin	Fibrin/Coll
1	71.4	35.7	2.0	64.7	30.2	2.1
2	77.0	38.6	2.0	64.2	18.1	3.5
3	73.4	44.6	1.6	31.9	20.7	1.5

It therefore seems logical to suggest that the lower dissociative response of the HB2 cells in fibrin gels, can be accounted for simply by the reduced branching/motility response of cells to rhHGF, seen in these experiments.

The same explanation probably accounts for the lower dissociative response of HB2 cells to the α_v integrin blocking Ab in fibrin, when compared to the dissociative response seen with blocking α_2 Abs in collagen gels. However, because the Abs used are different in the two systems, it is impossible to directly compare, and it cannot be excluded that the two different Abs simply differ in their effectiveness at blocking integrin function.

With this last point in mind, it is to be expected that different blocking Abs would differ in their effectiveness to block Ab function. This is suggested by the fact that although the maximal percentage of colonies showing the dissociated phenotype in collagen, is very similar with all the α_2 and β_1 blocking Abs shown in Figure 5.7.1, the concentration of Ab required to achieve these maximal effects varies with the different Abs. The fact that the maximal effects are similar with the different Abs also implies that the induction of the dissociated phenotype is a saturable response, observed when a certain amount of integrin function has been blocked. If more integrin function is blocked, then rather than

increasing the number of dissociated colonies, the growth of cells is impaired.

Growth inhibition of HB2 cells in collagen and fibrin gels with high concentrations of blocking Abs.

We have shown that the induction of dissociated colonies with blocking Abs is matrix dependent, and that higher concentrations of the same Abs, rather than increasing the dissociative response, actually lead to a growth inhibition of cells, again in a matrix dependent fashion.

The experiments indicate that there is a very critical relationship between the level of integrin function, and therefore the level of integrin mediated cell-matrix interaction, and the behaviour of mammary epithelial cells. If the integrin function of HB2 cells and consequently the cell-matrix interaction, is inhibited up to a certain level by a relatively low concentration of blocking Abs, then the predominant effect is to reduce cell-cell interactions enough to lead to the loss of compact organised structures, with no obvious effect on cell growth. However, if the integrin function and consequently the cell-matrix interaction, is inhibited to a further threshold, or higher, then the predominant phenotype seen is the inhibition of cell growth.

In the experiments described in this chapter, it has been shown that treatment of cells with 2-5μg/ml of the appropriate blocking Ab, induces the maximum formation of dissociated colonies (Figure 5.7.1), and that 10-25μg/ml of the same Abs are required for the inhibition of cell growth to be the dominant phenotype (Figure 5.10.1). However, between the two extreme phenotypes of 60-70% of colonies that are dissociated, and up to 100% of cells that are growth inhibited, there are, of course, intermediate phenotypes, where some dissociated colonies are formed and some growth inhibition of cells occurs. This is illustrated in Figure 5.7.2. where it can be seen that although the dissociated phenotype is the most prevalent in panels A-H; the cells treated with 5μg/ml of the α_2 integrin Ab 6F1 (panels E/F), and 3.6μg/ml of the β_1 integrin Ab P5D2 (panels G/H), also show some growth inhibition, as compared to the cells treated with the α_2 Ab P1E6 (panels C/D), or the cells with no Ab treatment (panels A/B).

Results showing growth inhibition of normal mammary epithelial cells with anti-integrin Abs have also been seen by Howlett, A.R. *et al.*, 1995, although in this study the concentration of blocking Abs used to inhibit growth was significantly higher (100μg/ml). These authors showed that the blocking Abs are actually inducing apoptosis to occur at these high concentrations, and that breast cancer cell lines as opposed to normal cell lines are not growth inhibited by high concentrations of integrin Abs - an observation we have also made in our studies. This ability of breast cancer cell lines to grow and survive without the signals generated by integrins, when normal cells cannot, might be a mechanism by which the property of anchorage independent growth is developed by tumour cells. This is discussed in more detail in Chapter 6.

2. Blocking of branching morphogenesis by "activating" Abs to the β_1 integrin, or Abs to the α_2 integrin, previously described as having "no functional effect".

The blocking of branching morphogenesis, by increasing the proportion of spherical cysts and decreasing the proportion of branching colonies with these two different types of Abs, again demonstrates a role for the $\alpha_2\beta_1$ integrin in branching morphogenesis.

Mechanism of blocking branching morphogenesis.

The experiments described in this chapter suggest that the two different types of Abs; the "activating" and the "non-blocking", inhibit branching morphogenesis by different mechanisms, even though both have the ability to block the rhHGF induced motility of HB2 cells.

We have hypothesised that stimulation of motility plays a vital role in the mechanism of branching morphogenesis of HB2 cells. Therefore from this, the conclusion is easily drawn, that it is the inhibition of this HGF induced motility by both Abs, (Table 5.14.1), that leads ultimately to the block in branching morphogenesis. TS2/16 is thought to increase the adhesion of cells to matrices by inducing conformational changes in the β_1 integrin, leading to an increased affinity of the integrin-ligand interaction. It is clear that the TS2/16 Ab can indeed increase the

adhesion of HB2 cells to a collagen matrix (Figure 5.13.1), and presumably, thereby inhibit the motility of the cells. The HAS 4 Ab however, has no significant effect on the initial rate of adhesion of HB2 cells to collagen (Figure 5.13.1), showing that an increase in cell adhesion, *per se* via an increase in affinity, cannot be the only pathway leading to the inhibition of motility.

The mechanism of HAS 4 action is as yet unknown, although from the matrix specificity of the effect, it seems that the $\alpha_2\beta_1$-collagen interaction is important for its manifestation. It would be predicted that the $\alpha_2\beta_1$ integrin would play an important role in the motility of HB2 cells on a collagen matrix, by virtue of its being a collagen receptor. Such a role for the $\alpha_2\beta_1$ integrin has indeed been shown in other systems (Berdichevsky, F. *et al.*, 1992; Chen, J.D. *et al.*, 1993; Klemke, R.L. *et al.*, 1994). It could be speculated, therefore, that the HAS 4 Ab inhibits motility by affecting some function of the α_2 integrin (other than its affinity for ligand), that is important for motility. It is important to note that use of an adhesion assay to analyse the behaviour of cells, can only evaluate the "rate" of adhesion; a property that is dictated by the "affinity" of integrin for ligand. The adhesion assay does not measure alteration in the behaviour of cells after the integrin-ligand interaction has been established, i.e. post-receptor occupancy events. It is now well known (see general introduction), that post receptor occupancy events can lead to increased avidity of the integrin-ligand interaction, and hence increase the strength of adhesion of cells to matrix, without altering the monovalent affinity of integrins. These post-receptor occupancy events leading to an increase in avidity, are thought to be mainly associated with events that involve integrin-cytoskeleton interactions, including cytoskeletal organisation, the clustering of integrins at specialised adhesion sites and controlling integrin-mediated cell spreading. It is therefore possible that HAS 4 might inhibit motility by increasing the avidity of integrin-ligand interaction by such a post-receptor occupancy event, and if so, that this effect would not necessarily be detectable by the use of adhesion assays.

Interestingly, the alteration of α_2 integrin function without altering its affinity for ligand *per se* (as measured by adhesion assays), was also seen in a study by Zutter, M.M. *et al.*, 1995. These authors showed that

transfection of the α_2 integrin into a low expressing murine mammary cancer cell line, led to profound effects on the behaviour of cells (including the reduction of cell motility), without increasing integrin-mediated adhesion of cells to matrix as measured by adhesion assay. In conclusion, it therefore appears that alteration of the motility of epithelial cells can be achieved by other mechanisms in addition to the modulation of the initial affinity of $\alpha_2\beta_1$ integrin for ligand. Understanding these other mechanisms by which the $\alpha_2\beta_1$ (and presumably other integrins), can control cell motility is clearly an important area for further research.

Supporting evidence that the HAS 4 and TS2/16 effects, are due to the alteration of some fundamental cell property important for branching morphogenesis, such as motility, rather than by altering a factor related specifically to the HGF molecule itself, was provided by the observation that the basal level of branching (seen in up to 10% of colonies in the absence of HGF), and the branching morphogenesis induced by the unknown factor in HFF-CM (described in Chapters 3 and 4), could also be inhibited by both these Abs (data not shown).

Effect of Mn^{2+} ions.

The partial block in branching morphogenesis seen by treating the HB2 cells with Mn^{2+} ions (Figure 5.15.1) (which can activate integrins in a similar way to the Ab TS2/16), provides further support for the idea that increasing adhesion by increasing the affinity of integrins for matrix, is a method by which branching morphogenesis can at least be partially blocked.

Although 1 mM Mn^{2+} has been shown to induce optimal binding of cells to matrices in other studies (e.g. Masumoto, A. and Hemler, M.E., 1993); it is likely that the concentration of Mn^{2+} required for maximum binding will vary depending on the cell type and matrix involved. In our system adhesion assays were not carried out to assess the extent to which 1 mM Mn^{2+} treatment activated adhesion, and it may well be that a higher concentration of Mn^{2+} would be required, to get the maximum activation of integrin function, and therefore the maximum block in branching morphogenesis. However, because of the toxicity of the Mn^{2+},

concentrations higher than 1 mM could not be tested. It should also be borne in mind that the conditions of our assay mean that 1 mM Mn^{2+} is the concentration used to pre-incubate the cells before embedding them in the collagen matrix, upon which a 20-fold dilution of the cation occurs.

Effect of TS2/16 in fibrin gels.

It is interesting to note that the TS2/16 activating Ab also partially inhibits branching morphogenesis in a fibrin matrix (Figure 5.16.1). It is possible that the mechanism of this inhibition could be similar to that proposed for the collagen system, i.e. that TS2/16 activates the adhesion of the HB2 cells to fibrin (presumably via the $\alpha_v\beta_1$ integrin). This in turn would lead to the decreased motility of cells and the inhibition of branching morphogenesis. Confirmation of this hypothesis would require an examination of the effect of the TS2/16 Ab, on the adhesion and motility of HB2 cells on fibrin gels.

The effect of TS2/16 on inhibiting branching morphogenesis in fibrin gels, is not nearly as potent as its effect in collagen gels, in terms of the percentage reduction in branching colonies (branching is reduced by 40% in fibrin and 70% in collagen, Figure 5.16.1). The smaller effect of the TS2/16 in fibrin may be attributable to the mechanism of TS2/16 action. This antibody functions by inducing a conformational change in the β_1 integrin, to one that is in a better conformation to bind to extracellular matrix ligand. It may well be that the conformational change increases the interaction with the collagen matrix more than with the fibrin. This would mean that the cells would be induced to adhere better to collagen than fibrin, and that motility, and hence branching morphogenesis would be better inhibited in the collagen system than the fibrin.

Relation of the results obtained with the collagen matrix in vitro system, to the early stages of breast cancer malignancy in vivo.

In the early stages of malignancy in breast cancers (and many other types of carcinoma), the initial disruption of the organised tissue architecture, is followed (as the tumour becomes more aggressive), by the invasion of tumour cells into and through the collagenous stroma underlying the

basement membrane. This invasion process forms an essential part of the initial stages of metastasis.

Cell-cell separation of HB2 cells in the collagen matrix *in vitro* required the reduction of $\alpha_2\beta_1$ integrin function in addition to HGF (or HFF-CM) stimulation. Reduction of the α_2 integrin function in normal epithelial cells such as HB2 can be compared to the reduction in α_2 integrin expression commonly found in poorly differentiated and aggressive breast carcinomas. By analogy to the *in vitro* system using HB2 cells, a subsequent stimulation of the motility of breast carcinoma cells, showing reduced α_2 integrin expression, with HGF, could lead to the invagination of individual cells or small groups of cells into and through the collagenous stroma, and hence the initiation of tumour cell invasion. The HGF that stimulated this process could potentially be produced either by the tumour cells themselves, (as has been shown to occur in one murine mammary carcinoma cell line, Rahimi, N. *et al.*, 1996), or more likely by the stromal fibroblasts within the carcinoma. In this way, HGF could stimulate the invasion of carcinoma cells by either an autocrine or a paracrine mechanism.

In some more poorly differentiated breast carcinomas, the underlying basement membrane structure would have already been lost, but if it were still present as a barrier to the invading cells, production of proteases by tumour cells would be required to break it down before migration of cells through the stroma could occur. HGF is documented as having the ability to induce the secretion of proteases such as urokinase plasminogen activator (uPA) by epithelial cells (Pepper, M.S. *et al.*, 1992). It is therefore quite possible that HGF could stimulate the production of proteases by the tumour cells, and hence induce the disruption of the basement membrane.

This *in vitro* system also illustrates the importance for tumour cells to bypass the integrin-mediated signals transduced from the ECM, which effect the growth/survival of normal cells. Such a bypass could be achieved by constitutive activation, or overexpression, of the down-stream components of integrin signalling, many of which are proto-oncogenes. Constitutive activation of one or more of these components in tumour cells, could conceivably compensate for the reduced

expression of integrins (such as the $\alpha_2\beta_1$ integrin) which normally transduce such growth/survival signals. Whether constitutive activation of a single downstream component of integrin signalling is sufficient for bypassing of integrin-mediated growth/survival signals is discussed in the next chapter, focusing in particular on the ras oncogene.

It can be seen that in tumour cells, a reduction in α_2 expression alone, without a method of bypassing the growth/survival signals it transduces, is more likely to be deleterious rather than advantageous to tumour cells, by inhibiting growth and inducing cell death (as is the case with the normal HB2 cells treated with α_2 or β_1 blocking Abs *in vitro*). This situation may well reflect the proposed requirement for an accumulation of changes to occur before normal cells become fully tumorigenic.

CHAPTER 6: EFFECT OF OVER-EXPRESSION OF THE *V-HA-RAS* ONCOGENE ON MORPHOGENESIS OF HUMAN MAMMARY EPITHELIAL CELLS *IN VITRO*.

INTRODUCTION.

α_2 integrin expression is found to be reduced in breast cancers *in vivo*, where increased reduction in expression has been shown to correlate with increased grade of carcinoma, *i.e.* with increasing loss of tissue architecture (Zutter, M.M. *et al.*, 1990; Pignatelli, M. *et al.*, 1991). This reduction in α_2 integrin expression is also found in many breast cancer cell lines *in vitro*, and when these cell lines are grown in collagen gels, this reduction in expression correlates with a loss in ability of cells to form organised 3-dimensional structures (Berdichevsky, F. *et al.*, 1994b). As described in Chapter 5, $\alpha_2\beta_1$ integrin function has been shown to be important for the formation of organised morphogenetic structures by the non-tumorigenic human mammary epithelial cell line, HB2.

From these studies it appeared that in breast cancer development *in vivo*, a reduction in either α_2 integrin expression or function in cells, might play a role in the loss of tissue architecture that occurs at an early stage in tumorigenesis.

If changes in the $\alpha_2\beta_1$ integrin are important in the loss of tissue morphology and in the evolution of the breast cancer cell, then it is important to know which factors act to induce the loss of function or down-regulation of expression of this molecule. Oncogenes are obvious candidates, particularly in view of the observation that transformation of epithelial cells with oncogenes *in vitro*, often leads to their conversion to an undifferentiated fibroblastic/mesenchymal phenotype. Several *in vitro* studies have involved mammary epithelial cells, and it has been shown that the over-expression of c-erbB2, bcl-2, activated ras, c-fos and c-jun (Reichmann, E. *et al.*, 1992; D'Souza, B. *et al.*, 1993; Kinch, M.S. *et al.*, 1995; Lu, P.J. *et al.*, 1995; Fialka, I. *et al.*, 1996) can all lead to epithelial-mesenchymal conversion of mammary epithelial cells.

In trying to understand the molecular basis of this epithelial-mesenchymal conversion, most investigations have concentrated on the

role of E-cadherin and other components of epithelial specific cell-cell junctions, rather than on the $\alpha_2\beta_1$ integrin and cell-matrix interactions. In fact, the level of the $\alpha_2\beta_1$ integrin after epithelial-mesenchymal conversion has only been examined in the human mammary epithelial cell lines MTSV1-7 or HB2, which overexpress the *c-erbB2* (MTSV1-7 cells only), or *bcl-2* oncogenes. In both cases, a high level of expression of the transfected gene, was associated with an obvious reduction in the level of expression of the α_2 integrin subunit, (D'Souza, B. *et al.*, 1993; Lu, P.J. *et al.*, 1995). Moreover, in the *c-erbB2* transfectants of the MTSV1-7 cell line, there was a clear correlation between the level of expression of the proto-oncogene, the reduction in expression of the $\alpha_2\beta_1$ integrin (and E-cadherin, D'souza, B. and Taylor-Papadimitriou, J., 1994), the degree of epithelial-mesenchymal conversion, and the reduction in morphogenetic ability of the cells to form organised structures in collagen gels. It seemed, therefore, that in the epithelial-mesenchymal conversion induced by oncogenes, a whole spectrum of epithelial markers would be altered, and it was important to focus on changes that would effect epithelial specific cell-matrix adhesions, as well as components of the epithelial specific cell-cell junctions.

It had been shown by nuclear run on assays, that the reduction in expression of the α_2 integrin in the MTSV1-7 cells, seen at both the protein and mRNA level, and induced by c-erbB2 overexpression, was controlled at the level of transcription, (D'Souza, B. *et al.*, 1993). This result was confirmed by Ye, J.P. *et al.*, 1996, during *in vitro* transfections of HB2 and MTSV1-7 cells. These experiments showed that expression of a reporter gene, driven by a minimal α_2 promoter, was dramatically reduced when co-transfected with a plasmid expressing c-erbB2.

Signalling from c-erbB2 can occur via Grb2/Sos feeding into the ras pathway, as well as via the src kinase pathway (Ben-Levy, R. *et al.*, 1994; Muthuswamy, S.K. *et al.*, 1994). However, experimental evidence suggests that the c-erbB2 induced down-regulation of α_2 integrin expression in mammary epithelial cells occurs via the ras signalling pathway. This was shown by co-transfection of a v-Ha-ras expression plasmid, together with the α_2 promoter construct into HB2 cells, which resulted in down-regulation of reporter gene expression. Moreover, co-transfection of a dominant negative *ras* construct, inhibited the down-

regulation of α_2 integrin expression induced by the c-erbB2 expression plasmid (Ye, J.P. *et al.*, 1996). Both ras and c-erbB2 are also known to activate transcription factors such as c-fos, c-jun, Ets, NFkB and Sp1 (Ben-Levy, R. *et al.*, 1994; Derijard, B. *et al.*, 1994; Engelberg, D. *et al.*, 1994; Galang, C.K. *et al.*, 1996; Ye, J.P. *et al.*, 1996), which would be ultimately essential to mediate an effect on transcription.

From the *in vitro* transfection experiments, it appears that ras plays an important role in downstream signalling from c-erbB2, and in the down-regulation in expression of the α_2 integrin. However, in order to investigate the effect of ras overexpression on the actual behaviour of human mammary epithelial cells, HB2 cells that had been permanently transfected with a *v-Ha-ras* construct were obtained. These were then analysed for their ability to form organised structures in collagen gels, along with the expression level and functionality of the $\alpha_2\beta_1$ integrin.

RESULTS

6.1. Overexpression of the *v-Ha-ras* oncogene induces an epithelial-mesenchymal phenotypic conversion of HB2 cells.

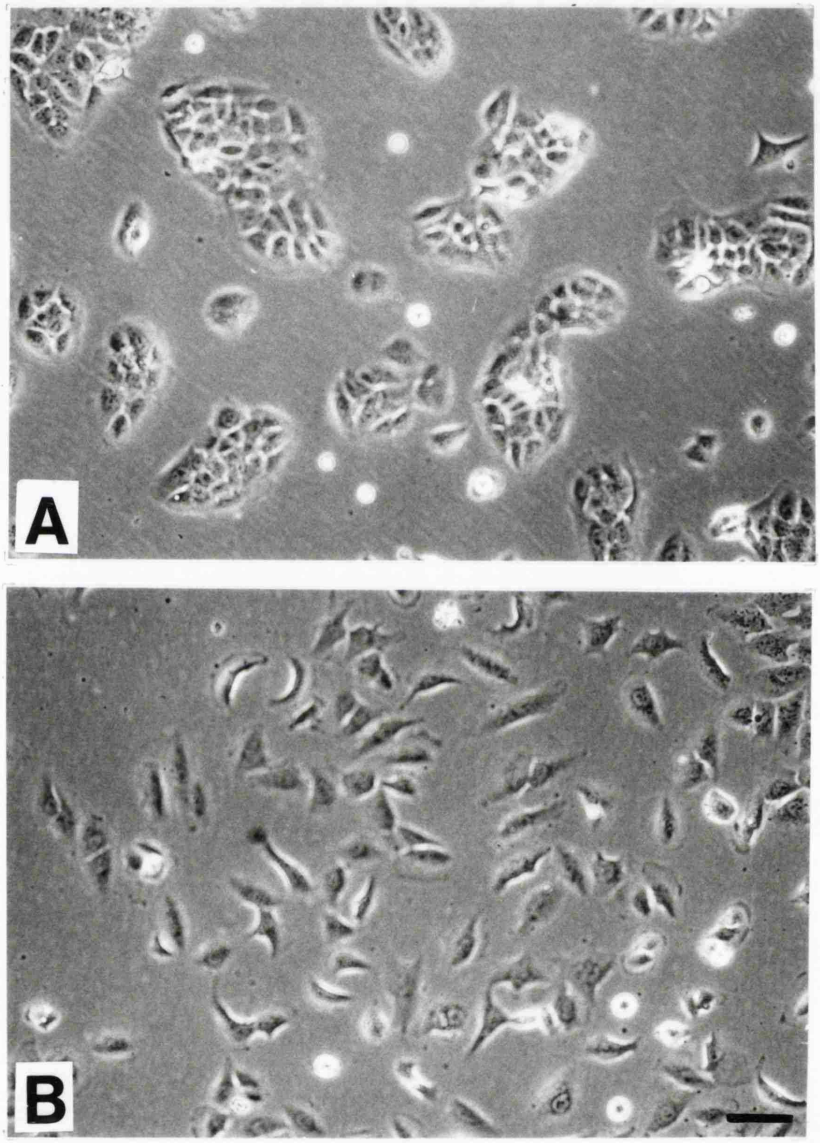
The HB2 cells overexpressing v-Ha-ras (HB2 ras), and the control transfectants expressing the neomycin selectable marker alone (HB2 neo), were produced in the laboratory of Paula Pitha Rowe, and generously given to us for our experiments.

When cultured on tissue culture plastic, HB2 ras cells showed a phenotypic change resembling epithelial-mesenchymal conversion (Figure 6.1.1. panel B), as compared to the neo control transfectants (Figure 6.1.1. panel A), and the HB2 parental cell line - which was indistinguishable in phenotype from the HB2 neo (data not shown). In this epithelial-mesenchymal conversion, the cells became more fibroblastic in appearance, and looked less polarised, with poorly defined cell-cell junctions, and a longer and less cuboidal cell shape.

Figure 6.1.1.
Overexpression of the *v-Ha-ras* oncogene induces an epithelial-mesenchymal conversion of HB2 cells.

HB2 (panel A) and HB2 ras (panel B) cells were cultured on tissue culture plastic in standard growth medium. The phenotypes of cells were photographed at high power magnification when the cells were still sub-confluent.

Scale bar denotes 100 μm


A
B

6.2. Overexpression of the *v-Ha-ras* oncogene in HB2 cells, is associated with a down regulation in expression of proteins involved in cell-cell and cell-matrix adhesion.

As indicated above, when the molecular basis of epithelial-mesenchymal conversion is studied, changes in the E-cadherin adhesion system are usually the focus of attention. However, studies looking at epithelial-mesenchymal conversion induced by overexpression of the bcl-2, or c-erbB2 proteins have shown that the level of expression of the α_2 integrin, as well as the E-cadherin is reduced. If c-erbB2 was mediating the down-regulation of the α_2 integrin through the ras pathway as is suggested, then it would be expected that the HB2 ras cells would also show reduced expression of the α_2 integrin.

Figure 6.2.1. shows that the level of expression of both the α_2 integrin and the E-cadherin protein were dramatically down regulated in the HB2 ras cells, as opposed to the HB2 neo control transfectants and the HB2 parental cell line. Interestingly, as was also observed in the c-erbB2 and bcl-2 transfectants, this down regulation in expression was specific for the α_2 integrin and was not seen with the α_3 integrin subunit.

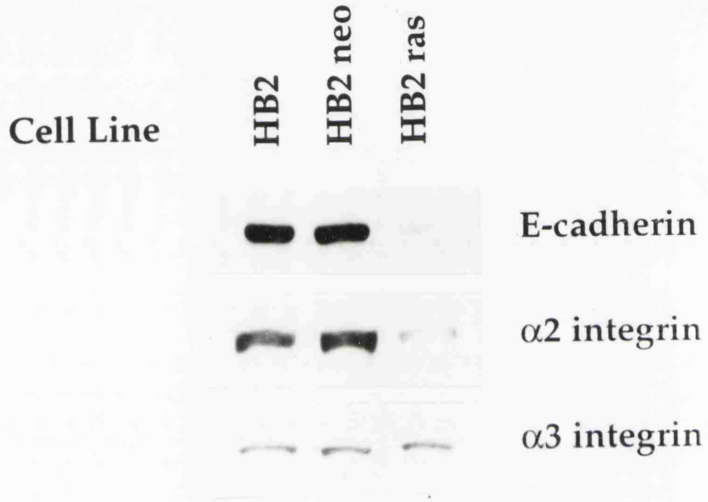
6.3. Reduced expression of the α_2 integrin correlated with reduced adhesion of HB2 ras cells to collagen type I.

It had already been shown that in HB2 cells, the $\alpha_2\beta_1$ integrin was the major integrin mediating the adhesion of cells to collagen type I. It was, therefore, predicted that the large reduction in expression of the α_2 integrin subunit in the HB2 ras transfectants, would result in decreased adhesion of the cells to polymerised collagen type I.

This was indeed the case, as measured in adhesion assays. An example of such an adhesion assay is shown in Figure 6.3.1, where the adhesion of HB2 ras cells to collagen type I was approximately 50% of the level seen with the HB2 neo control transfectants. Figure 6.3.1. also shows that the presence of the neomycin selectable marker gene alone in HB2 cells, did not significantly affect the adhesion of cells to collagen.

Figure 6.2.1.
Effect of overexpression of the *v-Ha-ras* oncogene on the expression of E-cadherin and the α_2 and α_3 integrin subunits in HB2 cells.

Cell extracts of HB2 cells and the HB2 neo and HB2 ras transfectants were made by lysis in buffer B. 50 µg of total protein was resolved by SDS-PAGE on a 7.5 % acrylamide gel under reducing conditions and Western blotted onto a nitro-cellulose membrane. The membrane was then probed with specific Abs to E-cadherin (HECD-1, 5 µg/ml), the α_2 integrin subunit (AB1936, 1/100 ascites) and the α_3 integrin subunit (DH4-B4, 1/1000 ascites) and detected by ECL.


Cell Line
HB2
HB2 neo
HB2 ras
E-cadherin
α2 integrin
α3 integrin

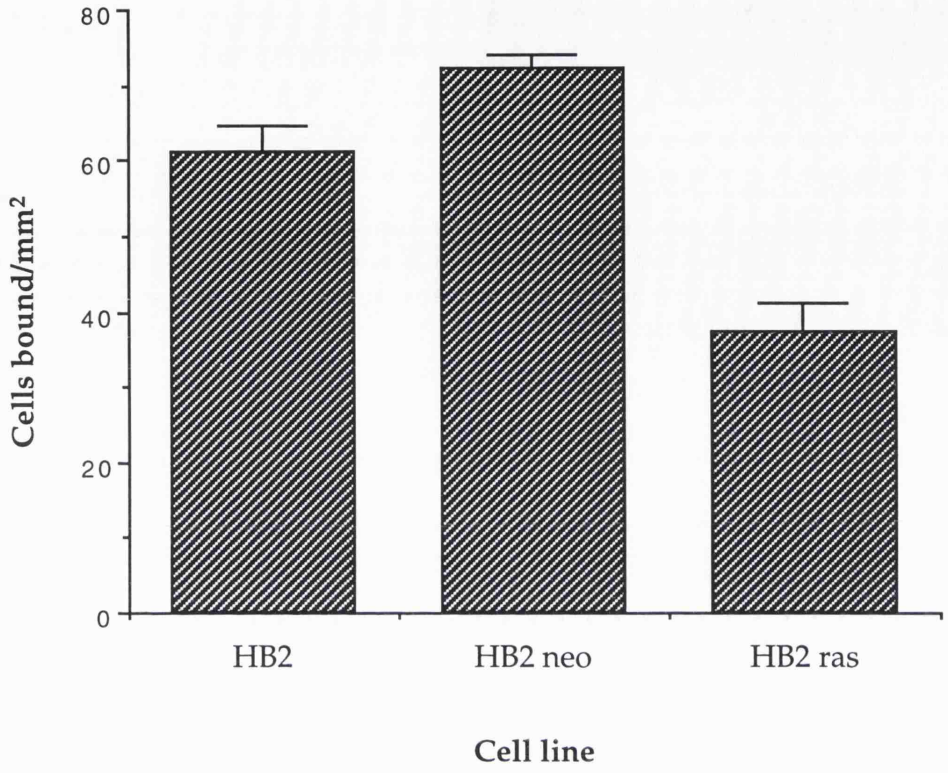


Figure 6.3.1.
Effect of overexpression of the *v-Ha-ras* oncogene on the adhesion of HB2 cells to collagen.

$1x10^4$ HB2 ras, HB2 neo, and HB2 parental cells were plated onto polymerised collagen in triplicate, and allowed to adhere for 15 minutes. After the non-adherent cells were removed by washing, the number of cells bound/mm^2 was calculated as described in Materials and Methods.

The graph shows the results of a typical experiment. For each set of triplicates the mean number of cells bound/mm^2 was plotted, together with the standard error showing the deviation about the mean. The coating concentration of polymerised collagen was 2.4 mg/ml.

6.4. Reduced expression of the α_2 integrin correlated with reduced formation of compact organised structures in collagen gels.

A vital role for functional α_2 integrin in the formation of compact organised structures in collagen has already been discussed. It was, therefore, predicted that the HB2 ras transfectants, that showed reduced expression of the α_2 integrin subunit, would also show a reduced potential for morphogenesis in collagen gels.

As expected, when HB2 ras cells were grown in collagen type I gels, the formation of compact organised structures was much reduced. The predominant phenotype seen was the formation of "dissociated" colonies with reduced cell-cell interactions (Figure 6.4.1. panels C/D), similar to those seen when the HB2 parental cells were treated with blocking Abs to the α_2 integrin in the presence of HGF (shown in Chapter 5). The control transfectants, HB2 neo, showed the same phenotype in collagen gels as the HB2 parental line, by forming compact, organised spherical cysts (Figure 6.4.1. panels A/B).

As concluded in Chapter 5, it seems that a reduction in α_2 integrin mediated cell-matrix interaction, leads to a reduction in cell-cell interactions in 3-dimensional morphogenetic structures. Interestingly, and in further support of this conclusion, when HB2 ras cells were cultured in fibrin gels (where the α_2 integrin would not be involved in adhesion to matrix), the dissociated phenotype was not seen, and spherical cysts were formed (Figure 6.4.2. panels C/D). The dissociated phenotype seen when HB2 ras cells were grown in collagen is shown for comparison (Figure 6.4.2 panels A/B).

6.5. Increased adhesive function of the remaining $\alpha_2\beta_1$ integrin in HB2 ras cells, led to a partial reversion of the dissociated phenotype in collagen gels.

From the results described above, it was concluded that the induction of a dissociated phenotype in collagen gels required either a reduction in $\alpha_2\beta_1$ integrin function, or a reduction in α_2 integrin expression.

Figure 6.4.1.
Morphogenetic phenotypes of HB2 neo and HB2 ras transfectants in collagen gels.

HB2 neo transfectants (panels A/B) and HB2 ras transfectants (panels C/D) were embedded in collagen gels and cultured in standard growth medium. The morphogenetic phenotypes of colonies were photographed after 7 days of growth, at both low power (panels A/C) and high power (panels B/D) magnification.

Scale bar on photographs taken at low power denotes 200 μm
Scale bar on photographs taken at high power denotes 100 μm

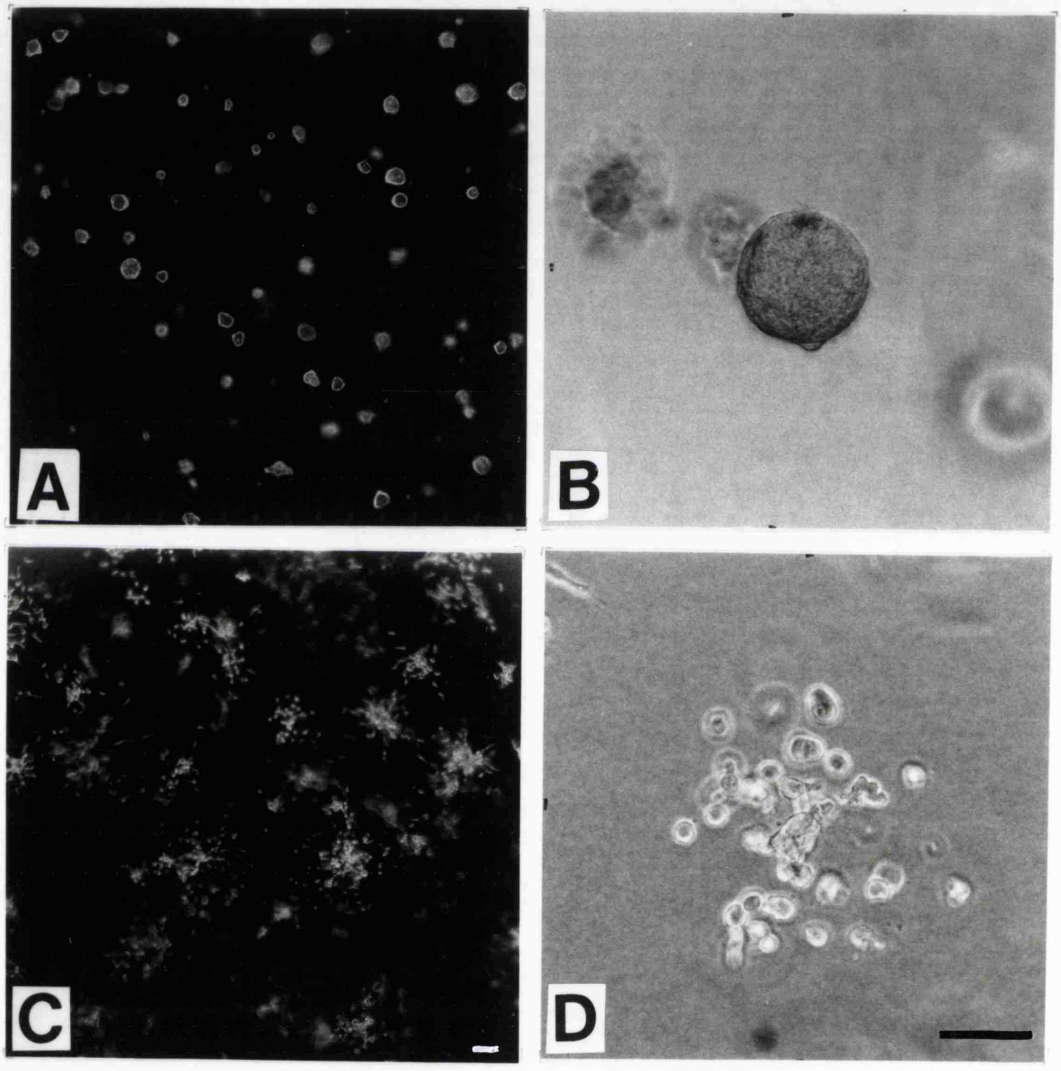
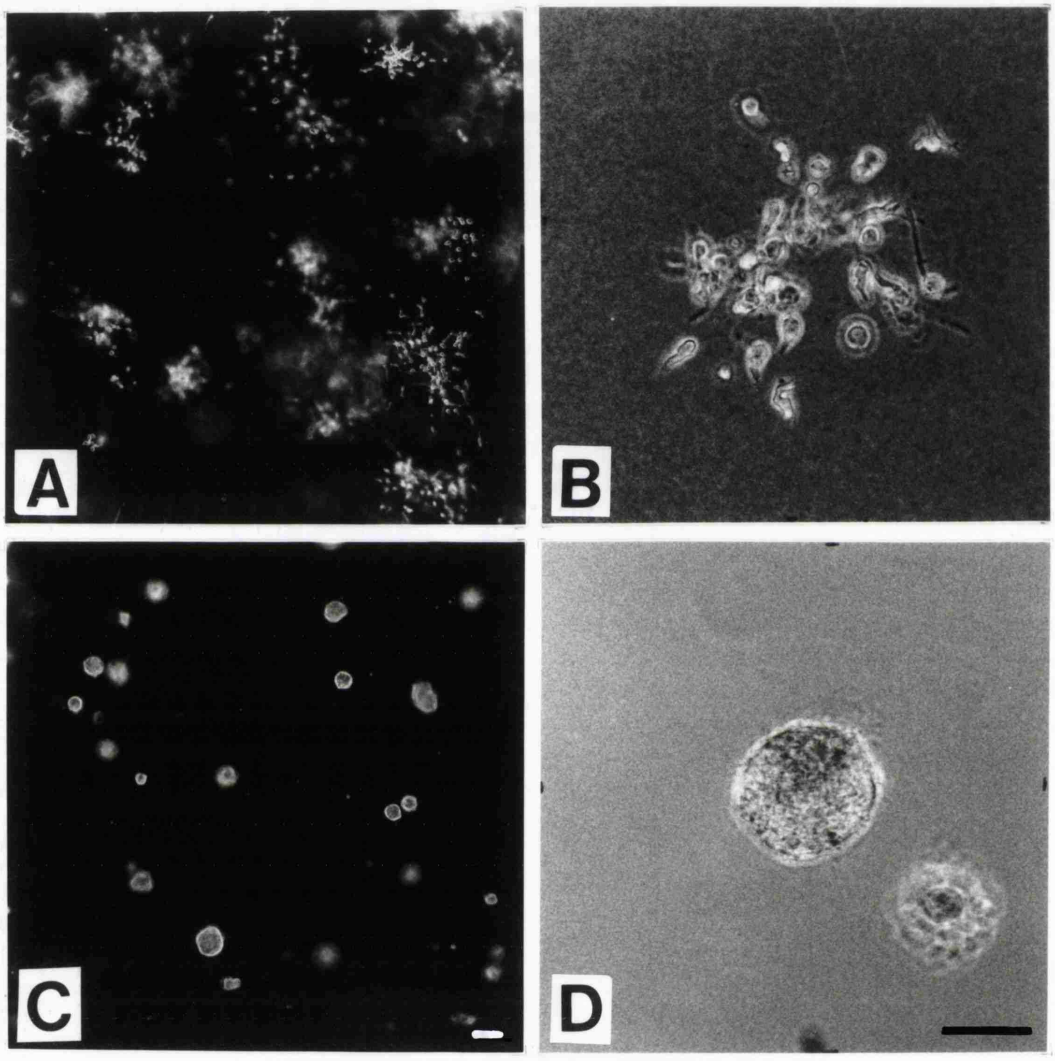

A
B
C
D

Figure 6.4.2.
Morphogenetic phenotype of HB2 ras cells in fibrin gels in comparison to collagen gels.

HB2 ras transfectants were embedded in collagen gels (panels A/B) or fibrin gels (panels C/D) and cultured in standard growth medium. The morphogenetic phenotypes of colonies were photographed after 7 days of growth, at both low power (panels A/C) and high power (panels B/D) magnification.

Scale bar on photographs taken at low power denotes 200 μm
Scale bar on photographs taken at high power denotes 100 μm


A
B
C
D

Confirmation that reduced $\alpha_2\beta_1$ integrin function is important for the formation of the dissociated phenotype, was obtained by showing that this phenotype of HB2 ras cells could be significantly reverted by activating the remaining $\alpha_2\beta_1$ integrin function using the β_1 activating Ab, TS2/16. Figure 6.5.1. shows that TS2/16 treatment significantly increased the adhesion of HB2 ras cells to a collagen matrix, and was therefore activating integrin function. Figures 6.5.2. and 6.5.3. show graphically and pictorially respectively, the reversion of the dissociated phenotype of HB2 ras cells in collagen gels when treated with TS2/16. This reversion took the form of a significant increase in the proportion of compact spherical cysts, (like those formed by the HB2 neo control cell line), while reducing the formation of dissociated colonies. The reversion of phenotype was not 100%, but was reproducibly seen at a level of between 60 - 75% in different experiments.

Low concentrations of the blocking Ab to the α_2 integrin subunit, P1E6, further reduced adhesion of the HB2 ras cells to collagen (Figure 6.5.1), even though there was already a relatively low level of adhesion due to the reduced expression of the α_2 integrin. However, interestingly, treatment of HB2 ras cells with this concentration of the P1E6 Ab, did not result in an increase in the number of dissociated colonies formed within collagen gels (Figure 6.5.2). This observation supports the results obtained in Chapter 5, which showed that 60-70% of colonies dissociated, was the maximum that could be obtained, before the reduction in integrin function reached the threshold point, at which further reduction in function was detrimental to cell growth.

6.6. Overexpression of the *v-Ha-ras* oncogene is not sufficient for integrin independent growth of HB2 cells.

It has been shown in this collagen gel *in vitro* system (data not shown), and by other investigators (Howlett, A.R. *et al.*, 1995) that breast cancer cell lines, unlike normal cell lines, have become independent of integrin mediated signals, thought to be required for cell survival in an extracellular matrix environment. This independence of breast cancer cell lines from integrin signalling for growth/survival, was shown by treatment of cells with high concentrations of blocking α_2 or β_1 integrin

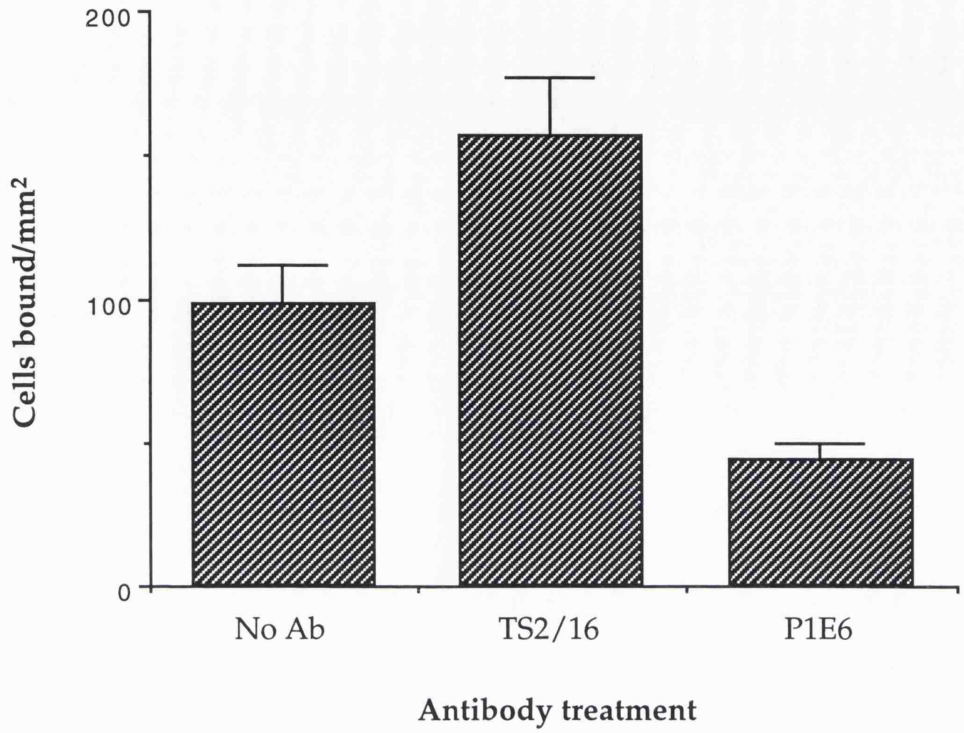


Figure 6.5.1.
Effect of the β1 integrin activating antibody TS2/16, and the α2 integrin blocking antibody P1E6, on the adhesion of HB2 ras cells to collagen.

HB2 ras cells were pre-treated with the anti β1 integrin activating mAb TS2/16, or the anti-α2 integrin blocking mAb P1E6, or given no treatment, before 1.5×10^4 cells were plated onto polymerised collagen in triplicate and allowed to adhere for 15 minutes. After the non-adherent cells were removed by washing, the number of cells bound/mm^2 was calculated as described in Materials and Methods. The concentration of mAbs used were: TS2/16, 20μg/ml; P1E6, 5μg/ml.

The graph shows the results of a typical experiment. For each set of triplicates the mean number of cells bound/mm^2 was plotted, together with the standard error showing the deviation about the mean. The coating concentration of polymerised collagen was 2.4 mg/ml.

The graph shows the results of a typical experiment consisting of duplicate samples for each condition. For each duplicate sample the proportion of colonies in each morphogenetic group were expressed as a percentage of the total number counted and the mean percentage and the standard error showing the deviation about the mean was plotted.

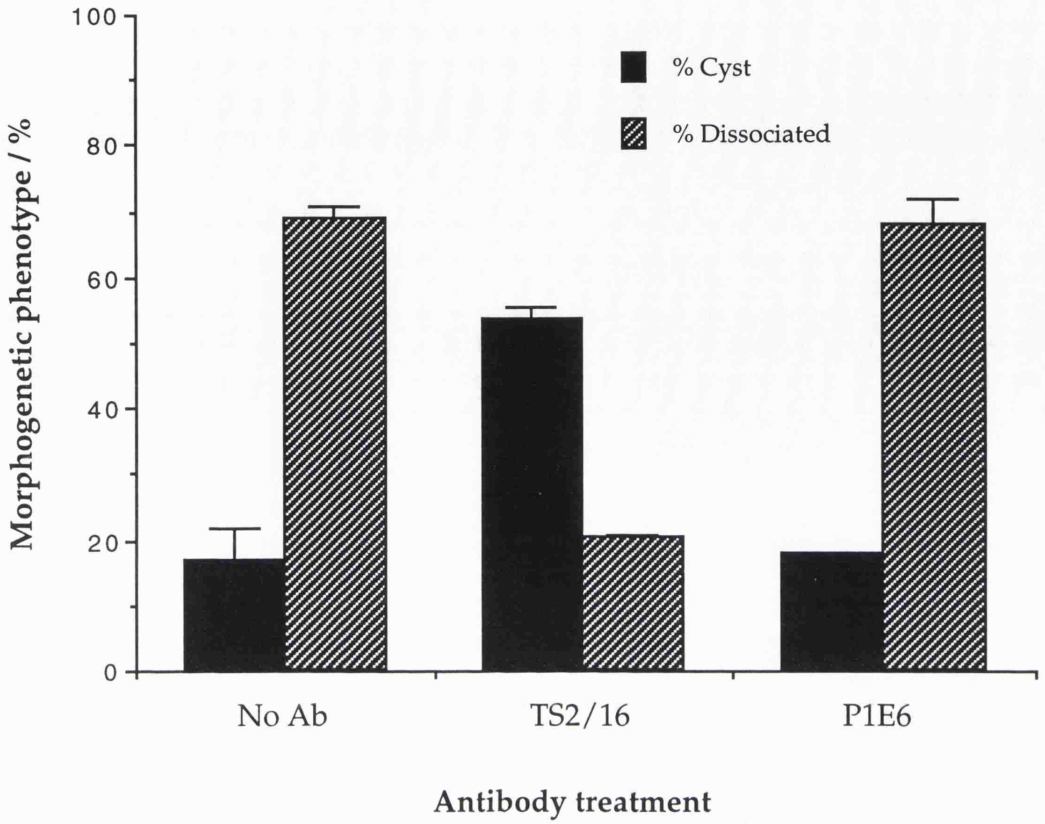


Figure 6.5.2.
Effect of the β1 integrin activating antibody TS2/16, and the α2 integrin blocking antibody P1E6 on the morphogenetic phenotype of HB2 ras cells in collagen gels.

HB2 ras cells were pre-treated with the anti-β1 integrin activating mAb TS2/16, or the anti-α2 integrin blocking mAb P1E6, or given no treatment, before embedding in collagen gels and culturing in standard growth medium. The concentrations of mAbs used were: TS2/16, 2.4μg/ml; P1E6, 1/200 ascites. The morphogenetic phenotypes of colonies were counted after 7 days of growth.

Figure 6.5.3.
Partial reversion of the dissociated morphogenetic phenotype of HB2 ras cells by treatment with the activating β_1 integrin antibody TS2/16.

HB2 ras transfectants were pre-treated with the anti-β_1 integrin activating mAb TS2/16 at a concentration of 2.4 μg/ml (panels E/F), or given no treatment (panels C/D), before embedding in collagen gels and culturing in standard growth medium. The morphogenetic phenotype of HB2 neo control transfectants with no antibody treatment are shown for comparison (panels A/B). The morphogenetic phenotypes of colonies were photographed after 7 days of growth at both low power (panels A/C/E) and high power (panels B/D/F) magnification.

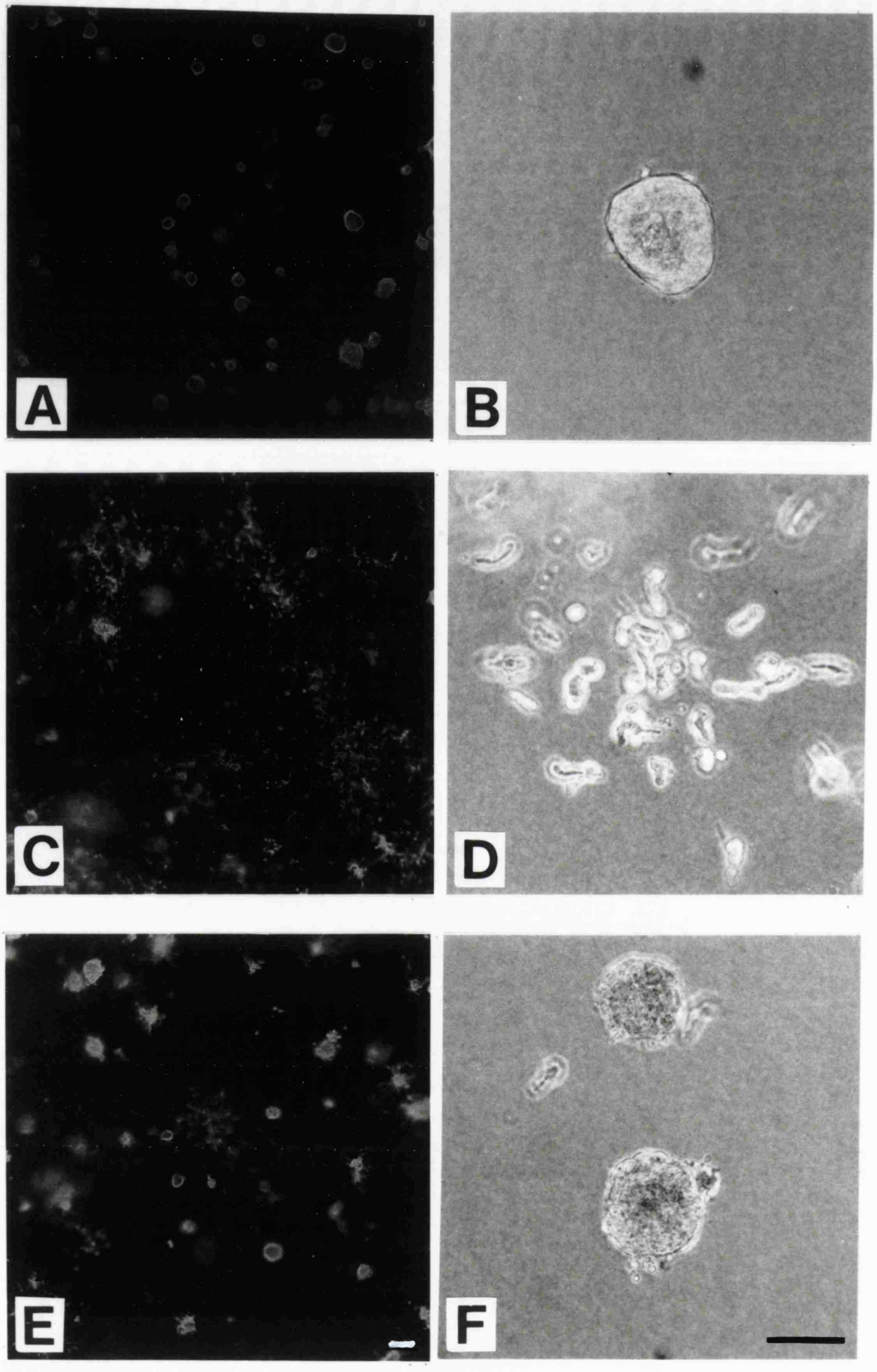
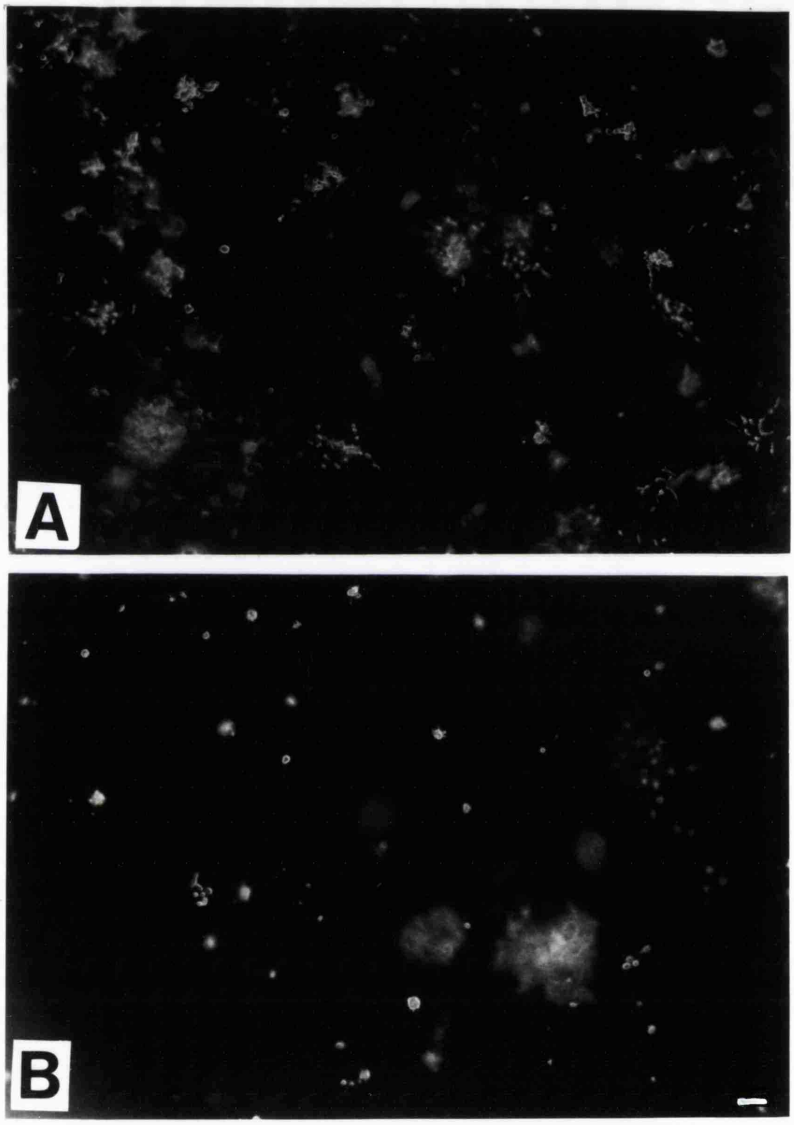

A
B
C
D
E
F

Figure 6.6.1.
Effect of high concentrations of blocking antibodies to the β_1 integrin subunit on the growth of HB2 ras transfectants in collagen gels.

HB2 ras cells were pre-treated with the anti-β_1 integrin antibody P5D2 at 18 μg/ml (panel B), or given no treatment (panel A), before embedding in collagen gels and culturing in standard growth medium. The morphogenetic phenotypes of colonies were photographed after 7 days of growth at low power magnification.


A
B

Abs. Under these conditions, growth of the breast cancer cell lines MCF-7, T47D, (data not shown), MCF7/9 and MDA-MB-435 (Howlett, A.R. *et al.*, 1995) was not inhibited; unlike that of non-tumorigenic cells such as HB2 (Figure 5.10.1), MTSV1-7 (data not shown), and HMT-3522 (Howlett, A.R. *et al.*, 1995). It therefore seems likely that an independence from integrin signalling/function for growth/survival plays an important part in the tumorigenic process.

HB2 ras cells were not independent of integrin signalling/function for growth/survival while cells were in contact with the ECM component collagen. This was shown by the fact that when HB2 ras cells were treated with high concentrations of α_2 (data not shown), or β_1 integrin blocking Abs (Figure 6.6.1), the growth of cells in collagen was severely inhibited. HB2 ras cells, however, had obtained the ability to grow in soft agar - the classical parameter for defining anchorage independent growth (data not shown).

Discussion and Conclusions.

The results presented in this chapter, show that the overexpression of the *v-Ha-ras* oncogene in HB2 human mammary epithelial cells has significant effects on the phenotype and behaviour of HB2 cells. These effects are:
1). The induction of an epithelial-mesenchymal phenotypic conversion (Figure 6.1.1).
2). Reduction in the level of expression of the α_2 (but not α_3) integrin subunit, and of E-cadherin (Figure 6.2.1).
3). Loss in ability of cells to form organised, compact, morphogenetic structures in collagen type I gels, with the predominant colony formed being that with the dissociated phenotype.
4). Anchorage independent growth in agar.

Overexpression of the *v-Ha-ras* oncogene is not sufficient for integrin independent growth/survival of HB2 cells while in contact with a collagen matrix.

Perhaps surprisingly, a complete block of the collagen-$\alpha_2\beta_1$ integrin interaction inhibited the growth of the HB2-ras transfectants in collagen

gels, in spite of the fact that these cells could form colonies in agar - the classical parameter used for the definition of anchorage independent growth. Clearly, the ability to proliferate in collagen gels is a more stringent parameter defining anchorage independent (integrin independent) growth than is growth in agar. Why this is so is not immediately obvious. Possibly, other collagen receptors on the epithelial cells (Wirl, G. and Pfaffle, M., 1988) may, in the absence of $\alpha_2\beta_1$ integrin signalling, induce growth inhibitory or apoptosis inducing signals. Further investigation into the differences in behaviour of the HB2-ras cells in agar and in collagen is clearly warranted. In this context, it is of interest that, unlike the situation with many other carcinomas, mutated *ras* is not found in breast cancer. Conceivably the marked down regulation of expression of the α_2 integrin subunit, which would result from constitutive activation of ras, would not allow cell survival, and other changes would be required for the cells to become independent of the growth promoting or apoptosis inhibitory signals generated by the integrin-collagen interaction.

The formation of the dissociated phenotype; is the α_2 integrin the whole story?

From the results described in this chapter, and also in Chapter 5, it can be concluded that $\alpha_2\beta_1$ integrin function and/or expression is vital for maintaining organised 3-dimensional morphogenetic structures. Either the reduction in function of the $\alpha_2\beta_1$ integrin (e.g. that induced in normal cells by treatment with a blocking Ab), or the reduction in expression of the α_2 integrin (e.g. as found in breast cancer cell lines, or normal cell lines transfected with oncogenes such as *c-erbB2* and *ras*), lead to the loss of ability of cells to form 3-dimensional organised structures and the formation of structures with a dissociated phenotype and reduced cell-cell interactions.

Interestingly, the results with the non-transfected HB2 cells described in Chapter 5 implied that down regulation of α_2 integrin expression or function was necessary, but not sufficient, to induce the dissociated phenotype, and that additional factors were also required, such as the presence of a motility factor like HGF. Such a motility signal might act to force apart the cell-cell contacts that were already loosened by either the

down regulation in expression or function of the $\alpha_2\beta_1$ integrin. Alternatively, such additional factors might involve those that induce alterations to cell-cell adhesion systems such as that mediated by the E-cadherin and its associated catenins. The E-cadherin and catenin molecules are excellent candidates to be disrupted in the generation of dissociated structures, particularly as a reduced expression of E-cadherin in breast cancers *in vivo* has been correlated with increasing grade of tumour and disruption of tissue architecture (Gamallo, C. *et al.*, 1993; Moll, R. *et al.*, 1993; Oka, H. *et al.*, 1993; Rasbridge, S.A. *et al.*, 1993). Such changes to the E-cadherin adhesion system might occur in an independent manner, or might equally occur as a result of the presence of a motility factor such as HGF. Changes to the E-cadherin via the latter pathway is a possibility because it has been shown in other epithelial systems that HGF can reduce E-cadherin expression and alter its cellular distribution so that it is no longer concentrated at cell-cell junctions (Tannapfel, A. *et al.*, 1994). Alternatively, HGF has been shown to increase the tyrosine phosphorylation status of β-catenin (Shibamoto, S. *et al.*, 1994) - a change shown to reduce β-catenin association with E-cadherin and the cytoskeleton, and hence reduce E-cadherin function (Kinch, M.S. *et al.*, 1995).

If a reduction in E-cadherin expression/function, as well as a reduction in α_2 integrin expression/function is required for the appearance of the dissociated phenotype, then this could explain the absence of the requirement for HGF to induce the dissociated phenotype in the HB2 ras transfectants (Figure 6.4.1) and breast cancer cell systems (data not shown, Berdichevsky, F. *et al.*, 1994a). This is because, unlike in the HB2 parental cell system where E-cadherin expression and function is present at a high level, in the *v-Ha-ras* oncogene transfected cells and the breast cancer cell lines, a low expression level of the E-cadherin (as well as the α_2 integrin), has been induced either by the overexpression of the *ras* oncogene or by the process of transformation, respectively.

On the other hand, if a motility signal is a fundamental requirement for dissociation, then the non-requirement for HGF to induce the dissociated phenotype in the HB2-ras transfectants might be explained by the fact that the HGF induced motility pathway is constitutively activated in the ras transfectants by virtue of the constitutive

overexpression of ras (Hartmann, G. *et al.*, 1994). In this case, however, it must also be borne in mind, that without more detailed analysis as to the effect of HGF or of the overexpression of ras on the expression/function of the E-cadherin and its associated catenins in this system, a role for the E-cadherin and its associated molecules in the motility/cell separation pathway controlled by HGF and ras is not discounted.

CHAPTER 7: CONCLUSIONS AND DISCUSSION.

7.1. CONCLUSIONS.

1. This thesis describes the development of an *in vitro* morphogenesis system wherein a non tumorigenic human mammary epithelial cell line (HB2) is cultured in 3-dimensions within collagen type I or fibrin matrices. In this system the mesenchymally induced process of branching morphogenesis, seen *in vivo*, can be reproduced by culturing the HB2 cells with conditioned medium from fibroblasts.

2. Using this *in vitro* system it has been shown that there are at least two distinct soluble factors secreted by fibroblasts that can induce branching morphogenesis of HB2 cells within collagen gels. The first of these secreted factors is HGF, and the second is an as yet unidentified and potentially novel factor secreted by human foreskin fibroblasts. Also, in more recent experiments carried out by Dan Baeckstrom, a third fibroblast secreted factor, heregulin, has been shown to stimulate branching morphogenesis of HB2 cells.

3. Preliminary characterisation of the potentially novel factor secreted by human foreskin fibroblasts, has shown that it is a 36 - 46 kDa, heat stable protein, with an active form that is unlikely to be glycosylated, or to contain inter- or intrachain disulphide bonds required for function.

4. Using this *in vitro* system an important role for integrin-mediated cell-matrix interaction was shown, both for cell-cell adhesion within the branching structures, and for the growth of cells within the matrix. In both cases the integrins involved were shown to be dependent on the matrix involved; with the $\alpha_2\beta_1$ being vital for growth and cell-cell interactions in the collagen gel system, and the $\alpha_v\beta_1$ being vital in the fibrin system. The observation that the integrins important for cell-cell adhesion were dependent on the matrix involved, showed that the mechanism of integrin involvement in cell-cell adhesion was indirect, as a result of their mediation of cell-matrix interactions, rather than a direct involvement at the cell-cell interface. We believe that this is the first convincing demonstration of such an indirect mechanism.

5. Evidence is presented that suggests that the stimulation of motility of cells may play an important part in the branching morphogenesis process and, moreover, that the $\alpha_2\beta_1$ integrin has a specific role in branching morphogenesis by way of regulating this motility. It was shown that increasing the affinity of the $\alpha_2\beta_1$ integrin-collagen interaction is one way in which motility can be inhibited and branching morphogenesis potentially blocked. However, there is another as yet uncharacterised mechanism by which the $\alpha_2\beta_1$ integrin can inhibit motility and hence potentially block branching morphogenesis, that does not involve altering the rate of initial adhesion of the cells to the matrix.

6. Overexpression of the oncogene *v-Ha-ras* in HB2 cells led to the disruption of cell-cell interactions in 3-dimensional morphogenetic structures. The overexpression of v-Ha-ras resulted in a reduced level of expression of the α_2 integrin subunit, and a consequent reduction of HB2 ras cell adhesion to a collagen type I matrix. Further evidence that the strength of the $\alpha_2\beta_1$ integrin-collagen interaction is involved in regulating cell-cell interaction in 3-dimensional structures within collagen gels, was obtained when the dissociated phenotype of the HB2 ras cells was significantly reverted by increasing the affinity of the remaining $\alpha_2\beta_1$ integrin for the collagen matrix by way of an activating β_1 integrin antibody.

7. Overexpression of the oncogene *v-Ha-ras* in HB2 cells conferred on the cells the ability to grow in soft agar. However, it was shown that the HB2 ras cells were still dependent on integrin-mediated signals for growth in a collagen type I matrix. These results show that although it is documented that breast cancer cells show properties of both anchorage independent growth (defined by ability to grow in soft agar) and integrin independent growth (defined by ability to grow in the absence of integrin function), these two properties are not equivalent to each other.

7.2. ROLE OF HGF IN THE DEVELOPMENT OF THE MAMMARY GLAND *IN VIVO*.

The work described in this thesis provided the first demonstration of the ability of HGF to induce branching morphogenesis of human mammary epithelial cells *in vitro*, (Berdichevsky, F. *et al.*, 1994b). This has now also been demonstrated for normal murine mammary epithelial cells by

Soriano, J.V. *et al.*, 1995, and by Brinkmann, V. *et al.*, 1995. The ability of HGF to induce branching morphogenesis in an *in vitro* system, suggested that it might play a role in the development of the mammary gland *in vivo*, where branching morphogenesis is an important event. When this thesis project was started there was not much experimental data about the expression status of HGF and its cognate receptor c-met in the mammary gland, and certainly none that directly examined the potential role for HGF in mammary gland development. However more recent data indicate that HGF does indeed play a role in branching morphogenesis *in vivo*.

The time course of HGF and c-*met* expression during mammary gland development has been analysed in at least three independent studies. Two of these studies (Yang, Y. *et al.*, 1995, and Niranjan, B. *et al.*, 1995) were carried out in the mouse, and one (Pepper, M.S. *et al.*, 1995) in the rat. In all three cases the expression profiles for HGF were very similar. It appears that HGF expression is highly controlled during mammary gland development, with high levels expressed in the virgin animal and in the initial stages of pregnancy, extremely low levels in late pregnancy and lactation, followed by large increases in expression during involution. The main discrepancy in these three studies concerns the expression of the c-met receptor. Pepper, M.S. *et al.*, 1995, and Niranjan, B. *et al.*, 1995, reported decreases and increases in expression that are concurrent with HGF, but Yang, Y. *et al.*, 1995 reported that the level of c-met expression does not change during development.

The study by Yang, Y. *et al.*, 1995, has also used *in situ* analysis to look at the spatial localisation of c-*met* and HGF in the mammary gland. This analysis showed HGF to be expressed in a thin layer of mesenchymal cells adjacent to the ductal epithelium, and c-*met* to be expressed in the basal epithelial cell layer of the ductal epithelium. This distribution had also been observed for other epithelial organs (Sonnenberg, E. *et al.*, 1993), and puts HGF and c-*met* in the correct cell types to mediate mesenchymal-epithelial interactions in the induction of branching morphogenesis. Having shown that HGF/c-*met* were expressed in the correct cells and at the correct time (in the virgin animal and at early pregnancy), to be involved in branching morphogenesis, this study also showed that HGF could stimulate branching morphogenesis of the

mammary gland in an organ culture system which, because experiments are carried out using complete explanted mammary glands, is more representative of the situation *in vivo* than other *in vitro* systems.

The high expression levels of HGF in the virgin/early pregnant animal, and at involution, not only correspond to times when extensive branching morphogenesis is taking place (virgin and early pregnancy), but also to the times when extensive ECM remodelling is occurring (involution). ECM remodelling is thought to be important both for the process of branching morphogenesis, and for involution, and is thought to involve the production of proteases. HGF has been shown to induce synthesis and secretion of proteases (Pepper, M.S. *et al.*, 1992) and it may well be that HGF not only provides the morphogenetic stimulus for branching, but also plays a role in tissue remodelling and ECM digestion. Such a role for HGF might also explain why HGF is down-regulated in late pregnancy and lactation. In these "functional" phases of development, lobular alveolar differentiation and milk protein synthesis are occurring, rather than radical structural alterations, and it is probably important that tissue remodelling is kept to a minimum and that the basement membrane of the mammary gland remains intact. This is supported by experiments by Streuli, C.H. *et al.*, 1991, who showed that expression of β-casein (one of the major milk proteins) by mammary epithelial cells required the presence of a laminin rich basement membrane. Indeed the removal of the basement membrane by proteolysis was shown to induce apoptosis of the cells and is proposed to be part of the mechanism by which involution is triggered *in vivo* (Boudreau, N. *et al.*, 1995). The expression profile of HGF indicates that it could well be involved in the degradation of the basement membrane at involution, and must, therefore, be down-regulated in order for lactation to occur. Another possible reason for HGF down-regulation at lactation and up-regulation at involution may be because HGF has also been shown to down-regulate the expression of milk proteins in organ culture (Yang, Y. *et al.*, 1995).

Thus it seems that HGF and c-*met* are present in the right cells at the right time to mediate branching morphogenesis *in vivo*, and indeed the induction of branching morphogenesis in an organ culture system and other *in vitro* systems, strongly supports a role for HGF in this process in

the mammary gland *in vivo*. However, it is probable that HGF is also involved in tissue remodelling of the mammary gland, both during the process of branching morphogenesis and in the breakdown of the basement membrane during involution, and may also act to down regulate milk protein expression.

Finally, it should be noted that HGF is a ubiquitous mesenchymal inducer of branching morphogenesis and other morphogenetic programmes. HGF has not only been shown to be expressed in the mesenchyme of many organs (Sonnenberg, E. *et al.*, 1993), but has actually been shown to play a role in the morphogenesis of the kidney and liver *in vivo* (Santos, O.F. *et al.*, 1994; Schmidt, C. *et al.*, 1995; Woolf, A.S. *et al.*, 1995), and in colon, kidney, pancreas, prostate and lung cell lines *in vitro* (Montesano, R. *et al.*, 1991b; Brinkmann, V. *et al.*, 1995). In mediating these broad effects, HGF is acting as an inductive and not an instructive mesenchymal effector, because the morphogenetic programmes induced are dependent on the type of epithelial cell concerned (Brinkmann, V. *et al.*, 1995). However, other instructive signals specific to the organ concerned, must be present in the mesenchyme. This is known to be the case, because for example, mammary gland epithelia develop features of salivary glands when cultured in the presence of salivary gland mesenchyme (Kratochwil, K., 1983).

7.3. ROLE OF INTEGRINS IN CELL-CELL ADHESION.

A principal finding of this thesis was to identify an important role for integrins in cell-cell adhesion. Interestingly, we found that this role was indirectly mediated, via the integrin interaction with ECM components, rather than directly mediated at the cell-cell interface, via for example integrins on one cell interacting with other integrins, or other proteins on an adjacent cell. As discussed below, a direct role for integrins in cell-cell adhesion is well documented; but we believe that the results presented in this thesis provide the first convincing demonstration of an indirect role for integrins in cell-cell adhesion via their control of cell-matrix interactions. The consequences of this indirect role of integrins in cell-cell adhesion meant that cell-cell associations in 3-dimensional morphogenetic structures were disrupted by integrin blocking Abs only

if these Abs had the ability to reduce the cell-matrix interaction. As a direct result of this phenomenon, the integrins involved varied depending on the ECM component present, with the $\alpha_2\beta_1$ being important in a collagen type I system and the $\alpha_v\beta_1$ in a fibrin system.

A role for integrins in cell-cell interactions has been well documented for the leukocyte integrins; where integrins on leukocytes interact with different molecules (usually members of the Ig superfamily) on another leukocyte, or a different cell type like endothelial cells (Hynes, R.O., 1992; Newham, P. and Humphries, M., 1996). A direct role for integrins in cell-cell interactions of non leukocytic cell types was initially suggested by the localisation of certain integrins at cell-cell contact regions *in vivo*. For example, the β_1 integrin subunit is found in such regions in keratinocytes (Larjava, H. *et al.*, 1990), and the $\alpha_2\beta_1$ and $\alpha_5\beta_1$ in endothelial cells (Lampugnani, M.G. *et al.*, 1991). In endothelial cells, the α_v integrin was also found to be localised at cell-cell contacts, although it was shown that it was not associated with its most common partner the β_3 subunit, and was therefore presumably associated with another partner such as β_1 or β_5, (Lampugnani, M.G. *et al.*, 1991).

More recent studies have suggested that the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins can directly mediate cell-cell adhesion. This cell-cell adhesion has been shown to be mediated either by homotypic interactions such as $\alpha_3\beta_1$-$\alpha_3\beta_1$ interactions seen in melanoma cells (Sriramarao, P. *et al.*, 1993), or heterotypic interactions of $\alpha_3\beta_1$-$\alpha_2\beta_1$ seen in keratinocytes (Symington, B.E. *et al.*, 1993), or the heterotypic interaction of the $\alpha_3\beta_1$ with an as yet unidentified non $\alpha_3\beta_1$ ligand in fibrosarcoma and renal carcinoma cells (Takeuchi, K. *et al.*, 1994). However, a direct role for integrins in cell-cell interaction has not been found in all cell systems, even when extensive analysis to identify such a role has been undertaken, (Weitzman, J.B. *et al.*, 1993; Weitzman, J.B. *et al.*, 1995).

In most of the experimental systems used to analyse the involvement of integrins in cell-cell interactions, cell-cell adhesion is usually assessed by observing the aggregation of cells in suspension, or the binding of cells in suspension to a confluent monolayer of cells, and assessing the effect of mAbs to various integrin subunits on these processes. Alternatively, biochemical assays are used, which do not actually look at the binding of

cells to other cells, but look at the ability of cells to bind to immobilised purified integrins on a dish, or *vice-versa* the binding of purified integrins to a monolayer of cells. Some studies have even involved the use of completely cell free systems, such as analysing interactions of purified $\alpha_3\beta_1$ integrin with $\alpha_2\beta_1$ (Symington, B.E. *et al.*, 1993), or affinity chromatography using $\alpha_3\beta_1$ integrin columns to pull out interacting molecules (in this case the $\alpha_3\beta_1$ integrin, Sriramarao, P. *et al.*, 1993) from a lysate of cell surface proteins. These types of analysis show quite convincingly that integrins have the ability to induce cell-cell aggregation, and directly mediate cell-cell interactions by interacting with the same or a different integrin, or even another ligand. However it is important to bear in mind that these assays do not represent very physiological conditions for cell types such as epithelial or endothelial cells, which *in vivo* would be part of a polarised monolayer, or a 3-dimensional structure, and in contact with ECM components or a basement membrane.

It is important that the potential role for integrins in cell-cell interactions should also be studied in systems which more accurately represent physiological situations. This has been done for keratinocytes, where the role of integrins in cell-cell adhesion has been studied on cells growing in polarised monolayers on tissue culture plastic, and also on ECM proteins; although it should be stressed that most adherent cells will lay down their own matrix in the absence of an exogenous matrix if cultured on tissue culture plastic or glass. In such experiments, Carter, W.G. *et al.*, 1990 showed that in HPV-transformed keratinocytes, Abs to the α_3 or β_1 integrins led to the partial disruption of cell-cell adhesion. In experiments by Larjava, H. *et al.*, 1990, it was shown that Abs to the β_1 subunit (but not any α subunits), led to the dissociation of a monolayer of keratinocytes, and the disruption of cell-cell interactions. However, this effect was only observed when the keratinocyte monolayers were cultured in low Ca^{2+} medium, under which conditions adherens junctions and desmosomes do not form. This leads to the conclusion that in normal high Ca^{2+} growth conditions, other junctional complexes have to be disrupted to get cell-cell dissociation, and agrees well with the results in this thesis which demonstrated that in order to get epithelial cell dissociation in a 3-dimensional culture system, a motility stimulus known to interfere with cell-cell adhesion (e.g. HGF) was required in

addition to blocking integrin Abs. Carter, W.G. *et al.*, 1990 also showed that the effect of antibodies on cell-cell association could be induced independently of an effect on cell-matrix interaction, and that integrins were therefore, directly mediating cell-cell adhesion. This was done by plating cells on vitronectin and showing that dissociation was still induced with the β_1 Ab, under which conditions keratinocyte interaction with vitronectin was unaffected because it was the $\alpha_v\beta_3$ and not a β_1 integrin that was involved in the cell-vitronectin interaction.

Tenchini, M.L. *et al.*, 1993 confirmed that Abs to the β_1 subunit could lead to cell-cell dissociation of keratinocytes in low Ca^{2+} medium. However these authors used two other assays to assess the effects of integrin Abs on cell-cell adhesion and in both cases saw no effect. These results led the authors to suggest that, although a direct role for integrins in cell-cell adhesion could not be ruled out, the loss in cell-cell association seen in the monolayer culture may be due to an indirect effect of inhibiting interaction of cells with their cell deposited matrix. This proposal that integrins can alter cell-cell interactions indirectly, rather than directly, via an alteration in the cell-matrix interaction has been demonstrated in this thesis. Moreover, this effect has been shown in a reasonably physiological system, whereby non-tumorigenic human mammary epithelial cells were cultured in 3-dimensions within collagen type I or fibrin gels. We believe that this is the first convincing demonstration of such an effect.

7.4. Role of integrins in the regulation of normal cell growth and apoptosis, and the potential of tumour cells to bypass these controls.

It is known that normal cells require adhesion to a substratum/ECM for cell survival (Meredith, J.J. *et al.*, 1993), and also for growth, by allowing the progression of cells through the G1 phase of the cell cycle and into S (Guadagno, T.M. and Assoian, R.K., 1991). The ability of integrins to mediate adhesion of cells to ECM components and to transduce signals from the ECM into cells, has made them prime candidates to mediate the control of these processes. In this thesis we have shown that integrins can indeed mediate the signals required for cell growth, and that different integrins are involved in mediating this signal depending

on the matrix with which the cells are in contact. However, it is not known whether the growth inhibition induced by abolishing the cell-matrix interaction is a result of inhibiting the proliferative signal, and/or inducing an apoptotic signal.

The adhesion dependent signalling pathway leading to growth was recently shown to involve the expression and activation of cyclins D1 and E, leading to the hyperphosphorylation of Rb (Zhu, X. *et al.*, 1996); and probably, also involves the activation of MAP-K and the subsequent regulation of other specific genes associated with mitogenesis. The signalling pathways that mediate apoptosis in cells are thought to involve the regulation of the expression of proteins, such as the induction of ICE (interleukin-1-β converting enzyme Miura, M. *et al.*, 1993), or Bax (Oltvai, Z.N. *et al.*, 1993), and the inhibition of bcl-2 expression (Korsmeyer, S.J., 1992).

For the process of growth inhibition described in this thesis, the induction of apoptosis is favoured as a mechanism: The HB2 cells were created by the immortalisation of primary milk epithelial cells with SV40 large T Ag that is known to bind and sequester Rb, thereby making the cyclin-regulated pathway unlikely to be operative. In support of this, it has been shown in other systems that apoptosis is induced on the deprivation of cells of ECM contact, (Meredith, J.J. *et al.*, 1993; Frisch, S.M. and Francis, H., 1994; Boudreau, N. *et al.*, 1995; Howlett, A.R. *et al.*, 1995; Re, F. *et al.*, 1994). Again it seems that the signals preventing cells from undergoing apoptosis are mediated by integrins. This is shown by the studies of Frisch, S.M. and Francis, H., 1994, and Howlett, A.R. *et al.*, 1995, using RGD peptides and blocking integrin Abs respectively to induce apoptosis of cells. The *in vitro* system used by Howlett, A.R. *et al.*, 1995 involved culturing of a human mammary epithelial cell line (MCF-10) in collagen type I and reconstituted basement membrane matrices. This work confirmed the results we had obtained by showing that the signals leading to the inhibition of apoptosis were mediated by different integrins in different matrices, with the $\alpha_2\beta_1$ being important in collagen and the $\alpha_3\beta_1$ important in reconstituted basement membrane.

Boudreau, N. *et al.*, 1995, looked at the mechanism of the induction of apoptosis in mammary epithelial cells deprived of basement membrane, and found that loss of ECM contact corresponded with the induction of the ICE protein (known to actively kill cells, Miura, M. *et al.*, 1993), and that inhibition of ICE activity prevented apoptosis. Other studies have shown that the process of apoptosis in the absence of ECM contact involves changes in expression of the Bax protein (Pullan, S. *et al.*, 1996, Merlo et al, submitted for publication).

As described above, another mechanism by which adhesion mediated growth could be affected is by the modulation of cyclin or MAP-K mediated growth pathways. It is possible that cross-talk between the pathways controlling apoptosis and cell growth may be required to decide the final fate of cells. This is suggested in a study by Boudreau, N. *et al.*, 1995, where it was shown that the suppression of apoptosis of mammary epithelial cells in contact with basement membrane was dependent on the withdrawal of cells from the cell cycle, accompanied by the down regulation of expression of c-myc and cyclin D1 (which are mediators of positive proliferative signals), and the absence of phophorylated Rb.

Just as it has been known for a long time that adhesion is required for the growth of normal cells, it is also known that tumour cells are independent/less dependent on anchorage for growth (Stoker, M. *et al.*, 1968). This property of anchorage independent growth of cells is usually assessed by an ability to grow in soft agar, i.e. to proliferate when no ECM components are present. The mechanism by which cells acquire this property is unknown, but it is possible that it involves a loss of response of tumour cells to the signals from the ECM that control proliferation and apoptosis of normal cells. As the ECM control of cell growth and apoptosis is thought to be mediated through signals generated by integrins, it is possible that the loss of response to the growth and/or apoptotic signals is mediated by the bypassing/overriding of integrin signals. Indeed, a study by Howlett, A.R. *et al.*, 1995, showed that breast cancer cell lines had acquired an independence from integrin signals for the growth of normal cells, a property termed "integrin-independent growth" in this thesis. This was done by treating cells with blocking Abs

to integrin subunits and showing that the growth of normal but not cancer cells in ECM was inhibited.

Although it is likely that by-passing integrin-mediated signals plays an important role in the development of anchorage independence of tumour cells, the results in this thesis suggest that the ability of cells to proliferate in collagen gels in the absence of a collagen-integrin interaction, is a more stringent parameter defining anchorage independent (integrin independent) growth than is growth in agar. It seems in this regard that certain breast cancer cell lines have acquired sufficient changes to enable anchorage independent growth as defined by both parameters, whereas the constitutive overexpression of v-Ha-ras in a non-tumorigenic human mammary epithelial cell line HB2, although sufficient to allow cells to grow in soft agar, is not sufficient to allow cells to proliferate and form 3-dimensional structures in collagen gels in the absence of a collagen-$\alpha_2\beta_1$-integrin interaction. This observation may reflect the requirement for multiple cellular changes in the development of full blown malignancy.

7.5. A SPECIFIC ROLE FOR THE $\alpha_2\beta_1$ INTEGRIN IN ORGANISED MORPHOGENESIS, CELL GROWTH, AND IN MALIGNANT PROGRESSION.

7.5.1. Role of the $\alpha_2\beta_1$ integrin in organised morphogenesis.

This thesis has directly shown a role for the $\alpha_2\beta_1$ integrin and in particular the strength of the interaction of this integrin with a collagen matrix, in determining the form of 3-dimensional morphogenetic structures adopted by a non tumorigenic human mammary epithelial cell line, HB2, when the soluble morphogenetic factor HGF is present. This was done by showing that functional blocking Abs to the α_2 or β_1 subunit which partially inhibited the interaction of HB2 cells with a collagen matrix, disrupted cell-cell interactions within organised morphogenetic structures leading to the formation of disrupted dissociated colonies of cells. On the other hand, modifications of the $\alpha_2\beta_1$-collagen interaction which decreased cell motility, resulted in the inhibition of the branching morphogenesis induced by HGF. The results suggested that this effect was a result of increasing the strength of the $\alpha_2\beta_1$-collagen interaction.

Further evidence for a role for the $\alpha_2\beta_1$ integrin and the strength of the interaction of this integrin with the collagen matrix in the determination of 3-dimensional form, was obtained by studies of the behaviour of HB2 cells transfected with and overexpressing the *v-Ha-ras* oncogene. The HB2 ras cells showed reduced α_2 integrin expression, a reduced adhesion to collagen, and a concurrent loss in ability to form organised 3-dimensional structures in collagen gels with dissociated structures being formed. However, increasing the strength of interaction of the remaining $\alpha_2\beta_1$ integrin with the collagen matrix with an activating Ab to the β_1 subunit, significantly reverted the dissociated phenotype of the HB2 ras cells to compact spherical cysts.

Studies by other groups have convincingly supported a role for the $\alpha_2\beta_1$ integrin in normal morphogenesis. Keely, P.J. *et al.*, 1995, used an antisense approach to reduce expression of the α_2 integrin in cells, rather than blocking Abs to reduce function. These authors showed that the reduction of α_2 integrin expression in the T47D well-differentiated breast cancer cell line, led to the reduction in adhesion of cells to collagen and the loss of ability to form organised structures in collagen gels. Moreover, Zutter, M.M. *et al.*, 1995, transfected the α_2 integrin back into a non-expressing poorly differentiated mouse mammary carcinoma line, and showed that this restored the ability of cells to form organised and highly branched duct like structures when grown in reconstituted basement membrane matrices.

7.5.2. Role of the $\alpha_2\beta_1$ integrin in cell growth.

Our studies, and those of Howlett, A.R. *et al.*, 1995, have shown that in normal cells a large reduction in $\alpha_2\beta_1$ integrin function (induced by high concentrations of blocking Abs) can lead to growth inhibition of cells; but that in breast cancer cell lines no such effect is seen and cells are no longer dependent on integrin mediated signals for growth, (Howlett, A.R. *et al.*, 1995). From these results it could be predicted that a large reduction in $\alpha_2\beta_1$ integrin expression could produce the same effects on cells as the high concentrations of blocking Abs. It is therefore extremely interesting that Keely, P.J. *et al.*, 1995, who have reduced α_2 expression by up to 70% in the breast cancer cell line T47D, report no adverse effects on growth in collagen; whereas Saelman, E.U. *et al.*, 1995, who also reduced the expression of the α_2 integrin by up to 70% by an antisense approach, in the normal MDCK cell line, report a marked inhibition of growth and induction of apoptosis in collagen. These antisense experiments therefore strongly support the conclusion that normal cells but not breast cancer cells are dependent on the $\alpha_2\beta_1$ integrin function for growth in collagen matrices.

7.5.3. Role of the $\alpha_2\beta_1$ in malignant progression/invasion and metastasis.

In the process of malignant progression it is important that cells become more motile, and that single cells or small groups invade the underlying stroma to initiate metastasis. In this thesis we have shown that the $\alpha_2\beta_1$ integrin is involved in the control of motility of normal epithelial cells and that a reduction in the function of the $\alpha_2\beta_1$ integrin, in addition to a motility stimulus, can lead to the dissociation of cells from each other and invasion into a collagen matrix - a process analogous to the first steps in invasion and metastasis. It is therefore interesting to note in this regard that both a reduction in function of the $\alpha_2\beta_1$ integrin (by way of its reduced expression), and also an increased expression of HGF have been shown to be associated with metastatic breast cancers (Arihiro, K. *et al.*, 1993; Taniguchi, T. *et al.*, 1994; Taniguchi, T. *et al.*, 1995).

A convincing demonstration of the role of the $\alpha_2\beta_1$ in motility, invasion and tumour progression was shown in the study by Zutter, M.M. *et al.*, 1995. These authors showed that the transfection of the α_2 integrin into a motile and invasive murine mammary carcinoma cell line, not only

converted its phenotype to being less motile and non-invasive, but also led to the reduction of tumorigenic potential both *in vitro* (by the abolition of growth in agar), and *in vivo* (by a significant reduction in the development of tumours in SCID mice).

7.6. Summary.

In summary, this thesis describes an *in vitro* experimental system that has been developed and used to study molecules involved in morphogenesis of human mammary epithelial cells. The system has been used to characterise a potentially novel motogenic factor secreted by HFFs that can induce branching morphogenesis of human mammary epithelial cells, and also to identify a known fibroblast factor, HGF, that can induce branching morphogenesis. In later experiments carried out by Dan Baeckstrom, another known fibroblast secreted factor, heregulin, was identified as being able to induce branching morphogenesis in the same system. Since HGF has been found to be a physiological factor inducing branching in the mouse mammary gland (Yang, Y. *et al.*, 1995), the *in vitro* system described herein may clearly be used to gain insight into molecular mechanisms underlying the development of form during the growth of the mammary tree.

Interestingly, in connection with this latter point, the *in vitro* system has also been used to identify a vital role for integrins in the processes of cell growth and morphogenesis. In particular the role of the $\alpha_2\beta_1$ integrin, the major integrin expressed in the mammary gland *in vivo*, has been analysed. These studies have demonstrated the importance of an interplay between the action of soluble morphogenetic factors and the strength of interaction of the relevant integrin with the extracellular matrix, in the development of 3-dimensional form by human mammary epithelial cells. Such an interplay between the expression and action of morphogenetic factors, and the level of expression and functionality of integrins, might clearly be important not only in the normal development of the branching human mammary tree during gestation, puberty and pregnancy, but also in the changes in organised morphology of the human breast, seen at an early stage in the development of breast cancers.

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