

Biophysical regulation of cell motility by adhesion ligands
and growth factors:
Effect of spatial presentation of the ligand

by

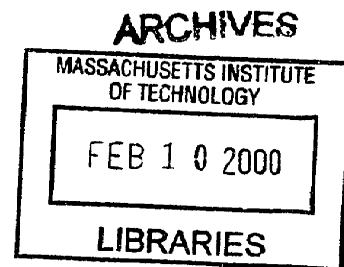
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Biophysical regulation of cell motility by adhesion ligands and growth factors: Effect of spatial presentation of the ligand

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on August 9, 1999 in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy in
Chemical Engineering

Abstract

A key problem in biomedical engineering today is in understanding the mechanisms which control cellular functions such as cell proliferation, migration and differentiation. The ability to engineer tissue replacements requires understanding of the interactions between the cell and its environment - the surface with which it interfaces and the fluid medium surrounding it. We are interested in designing a biologically inspired substrata which controls mammalian cell migration based on principles of receptor/ligand interactions involved in its regulation. Recent studies have shown that integrin cell surface receptors for the extracellular matrix (ECM) proteins initiate signaling cascades, some of which are in common with those initiated by growth factors. We have quantitatively investigated the potential synergy between growth factors and ECM ligands in governance of cell motility. In initial experiments using a model system of the ECM protein fibronectin and epidermal growth factor (EGF), we found that locomotion speed of a mouse fibroblast cell line is affected by combinations of EGF and fibronectin in diverse ways that can be accounted for by a biophysical model for migration. Following on these finding, we have designed a minimalistic artificial matrix using the linear peptide sequence, arginine-glycine-aspartic acid (RGD), derived from fibronectin as the adhesion ligand, conjugated to a protein-resistant poly (ethylene oxide) (PEO) surface. With this system, we have identified a role for the micro-level spatial presentation of the RGD peptide integrin ligand in stimulating migration. In addition, we have investigated the role of presentation of EGF as a soluble ligand in its governance of cell motility. We find that presentation of EGF in an autocrine manner in human mammary epithelial cells, where the cell simultaneously synthesizes the receptor and the ligand, results in the regulation of the directionality of cell motion. Formation of cell surface EGF/EGFR complexes in an autocrine manner causes an increased persistence of cell motion which is abrogated upon addition of EGF into the bulk extracellular media. These studies highlight the importance of quantitative deconstruction of a biological problem and have important ramifications for the rational design of cell receptor/ligand interactions to control cell behavior.

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Dedicated to
my grandmother,
Bimla Jain (Nona),
who I loved dearly

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Chapter 1

Introduction and Overview

1.1 Tissue Engineering

There have been significant advances in the field of tissue engineering in the last decade with the emergence of the theme of using cells and synthetic materials simultaneously for the repair and regeneration of human tissue. This burgeoning area of research has been motivated primarily by the scarcity of human donor tissues and organs experienced by transplantation surgeons for replacement of tissue/organ damage caused by conditions such as accident trauma or cancer (Cima et al., 1991; Langer and Vacanti, 1993; Hubbell, 1995; Langer, 1997; Langer and Vacanti, 1999; Mooney and Mikos, 1999). One of the approaches adopted by tissue engineers to solve this problem involves the use of synthetic biodegradable scaffolds to guide the reorganization of cells into a functional tissue. It is postulated that biodegradable scaffold synthesized in the right architecture and modified with the right set of biological cues will be able to guide the assembly of the cells seeded on the scaffold over time, and that the biodegradable scaffold will eventually degrade away and be replaced by the natural extracellular matrix secreted by the cells surrounding it (Figure 1.1).

It is not unreasonable to expect that neo-organ assembly may be achieved by understanding the principles behind embryonic development (Duband, 1990; Bronner-Fraser, 1993; Bronner-Fraser, 1995). During development, most cells proliferate at their sites of origin, then migrate along very well defined paths to their destination where they finally differentiate into the required phenotype. This whole process is under a very fine control

and is orchestrated by cues that the cells receive from their extracellular environment. The extracellular environment comprises the extracellular matrix (ECM) the cells are adhered to, their soluble environment and the other cells around them. Furthermore, the fate of the cells is also affected by biomechanical stimuli that they are subjected to. In short, development of a functional organ is highly complex and is under both spatial and temporal regulation by the extracellular environment of the cell.

Therefore, in order to use the principles of development in *in vitro* organogenesis, one of the key questions is to determine the environmental cues that the cell is exposed to and their spatio-temporal pattern of presentation. Based on that understanding then, synthetic polymer scaffolds may be patterned with the required biological cues that will then guide tissue assembly (Hubbell, 1995; Dillow and Tirrell, 1998; Hubbell, 1999; Hukins et al., 1999). It is beneficial to use a poorly adhesive background that would prevent non-specific adhesion of proteins and cells in the body thereby reducing the possibility of an immune reaction. Furthermore, it is highly desirable that the cells solely interact with the biological cues that are immobilized on the scaffold in order to have a controlled and well-regulated colonization and reorganization of the cells of interest.

Comprising the extracellular environment of the cells are specific extracellular matrix (ECM) proteins and growth factors which interact with specific receptors on the cell surface. These interactions generate intracellular signals which in turn regulate cell functions such as proliferation, migration and differentiation (Rozengurt, 1995; Matsumoto et al., 1995). While use of natural ECM proteins such as collagen to form the biodegradable scaffold has exhibited limited success, it does not pose a very commercially viable option for large-scale tissue reconstruction (Craig et al., 1995). Instead, use of short peptide sequences derived from the cell binding domains of whole ECM molecules which mimic the action of the large protein appear to be attractive alternatives for a couple of

reasons. First, obtaining large quantities of homogenous and contaminant-free proteins is difficult and expensive whereas short peptide sequences may be synthesized chemically in large scale. Second, large ECM proteins are prone to protease degradation, which is very often detrimental since some of the degradation products may be inflammatory.

Short peptide sequences from ECM proteins which are the sites of interaction between the cell and the whole protein, have been used widely in recent years to recapitulate the effect of the whole ECM protein. Specifically, the tri-peptide sequence, arg-gly-asp (RGD) first identified in the cell binding domain of fibronectin has been used in numerous biomedical studies to render a surface cell adhesive (Pierschbacher et al., 1994; Ruoslahti, 1996; Danilov and Juliano, 1989). Sequences derived from laminin (YIGSR) have also been used to modify a polymer scaffold (Massia and Hubbell, 1990). Polymer scaffolds have ranged from those made of polymethylmethacrylate, polyglycolic acid, polylactic acid, polyethylene oxide and natural polymers such as collagen and hyaluronic acid (Cao et al., 1998; Mooney et al., 1996; Mooney et al., 1995). They have been assembled with varying pore structures ranging from porous meshes and sponges to fibrous meshes and hydrogels. Although such composite materials comprising a polymeric material modified with a biological ligand have been shown to permit cell adhesion, the organization and densities of the biological moieties are far from being optimum.

Molecular signals of interest may be presented to the cell using a gene therapy approach as well. This entails inserting the gene of interest into the cell transiently with the hope that the encoded protein will influence cell behavior (Zwiebel et al., 1991). This mode of ligand presentation differs from the one described earlier where the ligand of interest is presented via the extracellular environment of the cell. A very interesting question then arises as to whether cell behavior is influenced by the mode of presentation of the ligand or whether the ligand supplied using either approach produces a similar cell response.

Therefore, in order to develop guidelines for tissue assembly from first principles, it is essential to perform a systematic and quantitative study of the cause-effect relationships between the various stimuli and the cell's response to them. While the field of tissue engineering offers tremendous promise, significant research effort is required in identifying the underlying basic principles that govern tissue organization and assembly which would then permit rational design of a biomaterial surface which supports a given cell function. In addition to aiding tissue regeneration and replacement, having the ability to reconstruct a functional tissue in vitro has other applications. We may be able to use functional tissues assembled in vitro as model systems for drug screening. Such multicellular assemblies may prove to be more physiologically relevant systems and hence may provide a more accurate response to a pharmacological agent, thereby aiding in the drug discovery process.

1.2 Thesis overview

This thesis is motivated largely by the problems and issues in the field of tissue engineering as mentioned in the last section. Specifically, we have studied the phenomena of cell migration and have determined the effect of mode of ligand presentation on cell motility.

To begin with, we have determined whether growth factor-stimulated cell motility is influenced by the quantity of extracellular ligand adsorbed on a surface. As a model system, we have used Fibronectin (Fn) as a representative ECM protein and Epidermal Growth Factor (EGF) as representative motility-affecting growth factor. We have used the well-studied wild type NR6 (WT NR6) cells as a model cell system. WT NR6 cells are a 3T3-derived murine fibroblastic cell line, which does not express endogenous EGF receptor (EGFR), but have been stably transfected with the human EGFR. We find that cell motility under the influence of EGF is strongly dependent on the amount of Fn on the surface. By quantifying the underlying biophysical phenomena of cell adhesion and cell

membrane extension activity, we have come up with an integrated model to explain the EGF-Fn mediated regulation of cell motility (Figure 1.2). (Described in Chapter 3)

Based on the above studies, it was apparent that in order to design a surface which specifically supports cell motility, adhesion ligand density needed to be optimized. We have used the sequence, arg-gly-asp (RGD) as a model adhesion ligand. The two parameters which characterize ligand presentation on a surface, average ligand density and average ligand cluster size, have been varied. Cell speed was quantified on varying densities and configurations of the adhesion ligand RGD. Using the biomaterial design approach described in the previous section, radiation cross-linked polyethylene oxide (PEO) gels were used to obtain a completely cell resistant background on the surface (Figure 1.3a). PEO molecules in a star configuration with 35 linear PEO arm on an average emanating from a central divinyl benzene core were used to present the RGD. Use of star PEO molecules permitted the presentation of ligands in a clustered format by modifying more than one PEO arm with the RGD peptide. Surfaces were synthesized with varying ligand surface densities and ligand cluster sizes (Figure 1.3b & 1.3c) since it had been hypothesized in previous studies that local receptor densities may affect cell behavior. We showed that a surface composed of RGD alone is able to support cell migration. The most important finding was that for the same average ligand surface density, the growth factor stimulated cell speed is greater when the ligand is presented in a clustered format as opposed to an uniform density. (Described in Chapter 4)

In order to study the effect of presentation of a soluble ligand, we next examined the motility of human mammary epithelial cells in response to exogenously added soluble EGF and autocrine EGF (EGF made endogenously by the same cell which also expresses the EGF receptor) (Figure 1.4). The single cell migration tracks were analyzed using a persistent random walk model. We observe differences in the speed and persistence of

cells transfected with various mutants of EGF as compared to their speeds and persistence in the presence of soluble EGF. We find that the mode of ligand presentation makes a significant difference to the motility behavior of the cell, hence demonstrating another method for regulation of cell behavior. (Described in Chapter 5)

We have used an engineering approach to address the biological problem of stimulated cell migration. An attempt has been made to understand the signals which affect the process and based on the knowledge of the architecture of the cell and the surface, have developed rules for the rational design of a bioartificial matrix. We have studied the importance of the presentation of ligands in both a substrate bound form and in the soluble phase and how this knowledge may be used to engineer a bioartificial matrix which would stimulate a desired cellular response.

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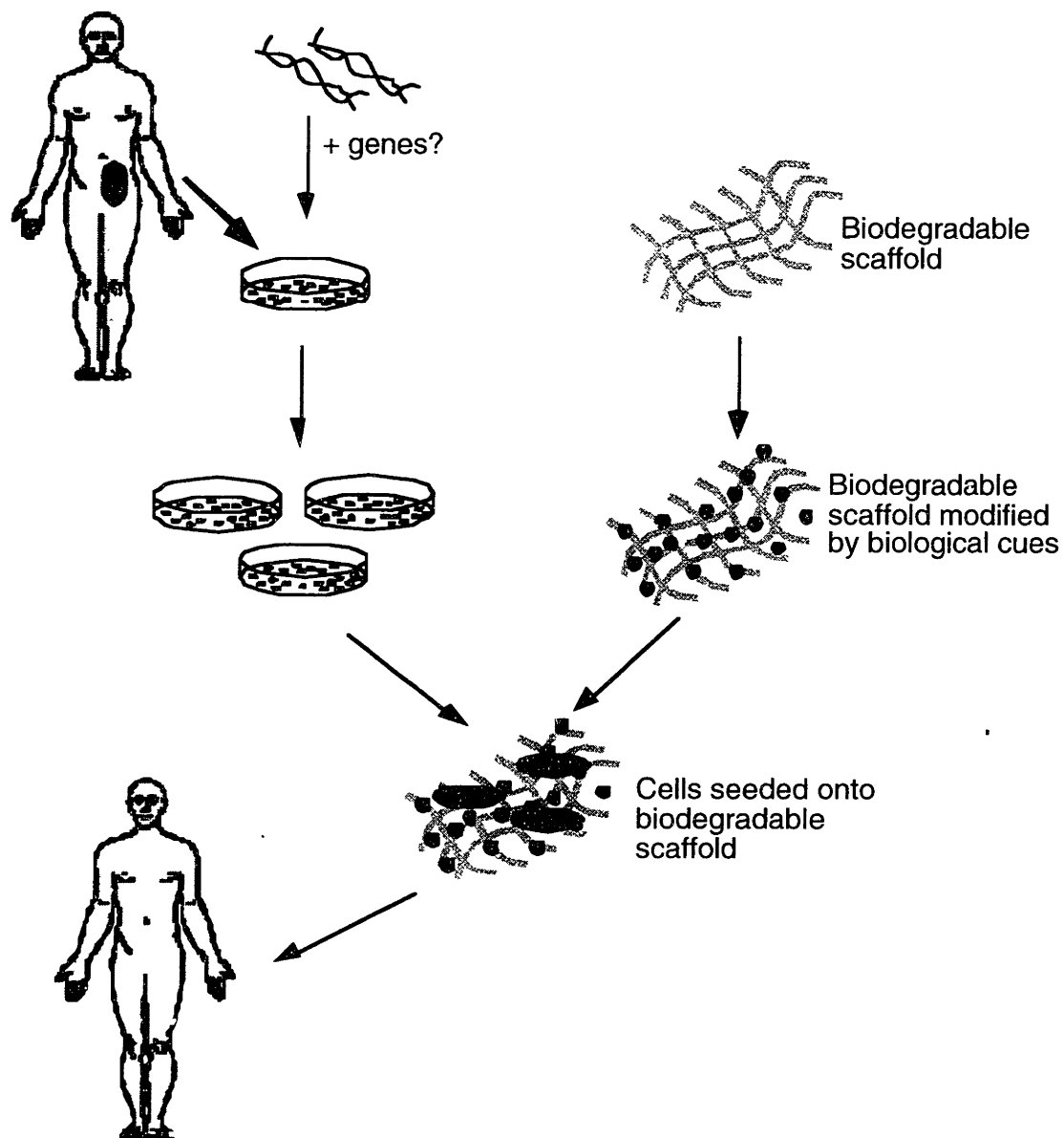


Figure 1.1 Schematic of the scaffold based tissue engineering approach. Briefly, primary cells are harvested from the body and grown in culture. They will then be seeded onto a biodegradable scaffold which has been modified by specific biological cues. The colonized scaffold will then be implanted into the patients body. It is hoped that the scaffold will eventually degrade away and be replaced by matrix secreted by the cells.

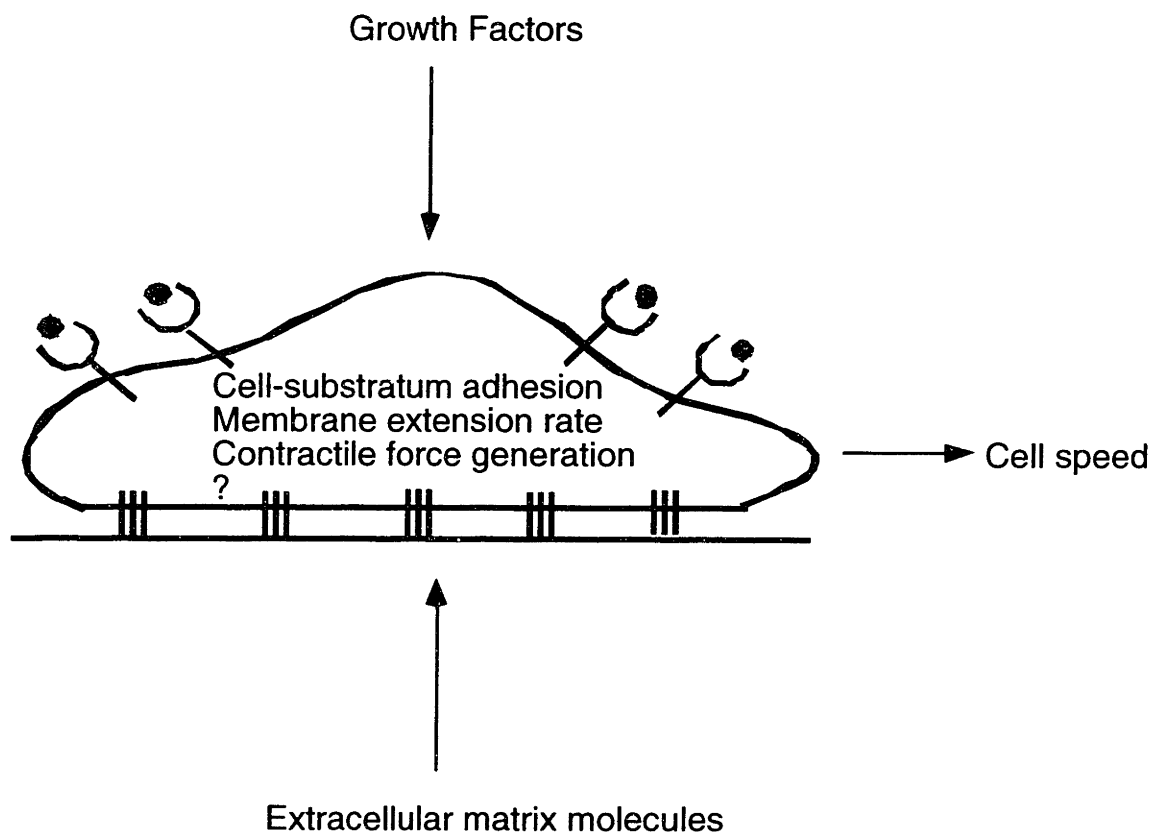


Figure 1.2 Cell speed may be influenced by growth factors and extracellular matrix proteins.

Some of the biophysical processes involved in the regulation of cell speed include the strength of cell-substratum adhesion, rate of cell membrane extension and the amount of contractile force generated within the cell.

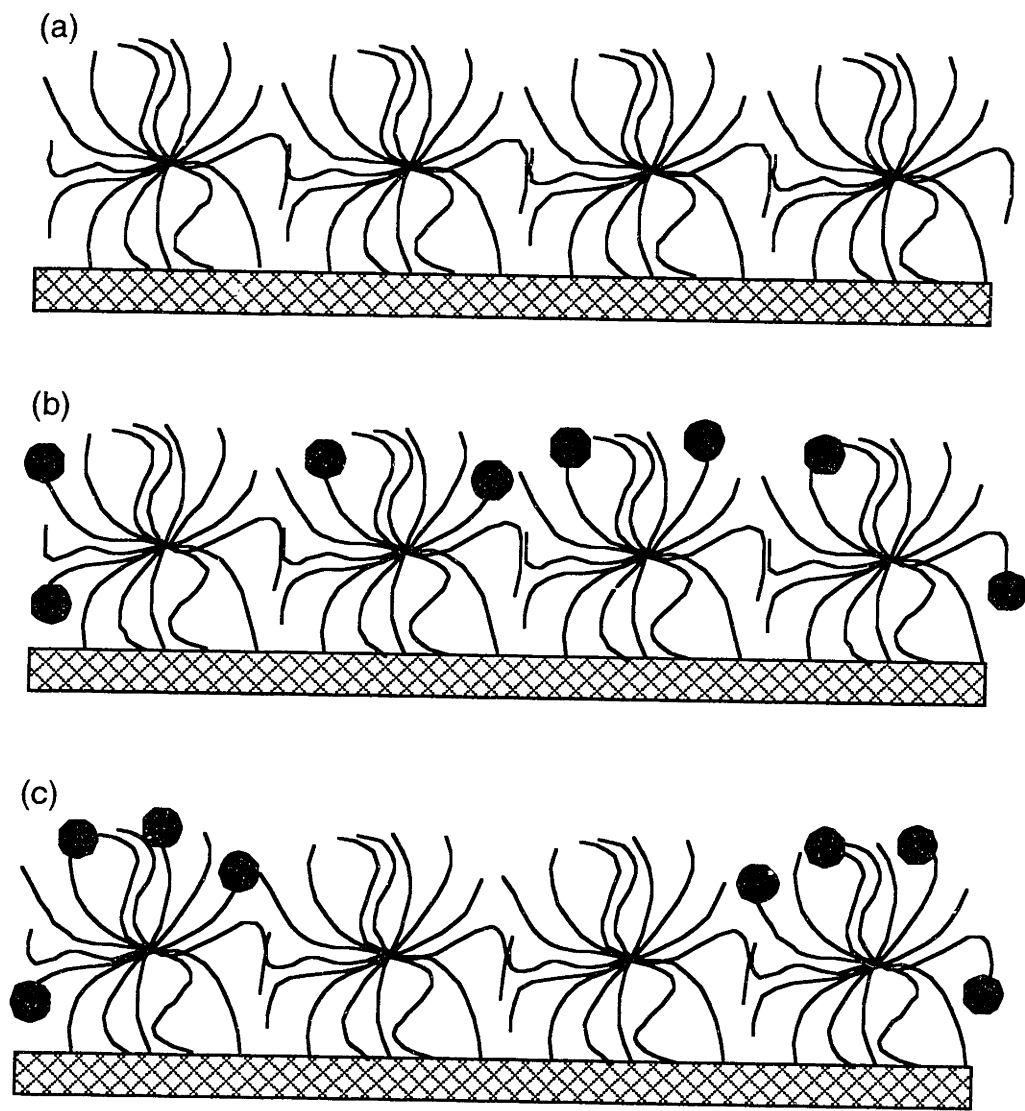


Figure 1.3 PEO molecules can be used to present a ligand in different spatial distributions.

(a) Unmodified star PEO molecules covalently immobilized on a surface can be used to render a surface non-adhesive; (b) Star PEO molecules can be modified by one or more ligands and used to present the ligand at a uniform density. (c) In addition, multiple arms of a single star PEO molecule may be modified resulting in presentation of the ligand in a clustered format.

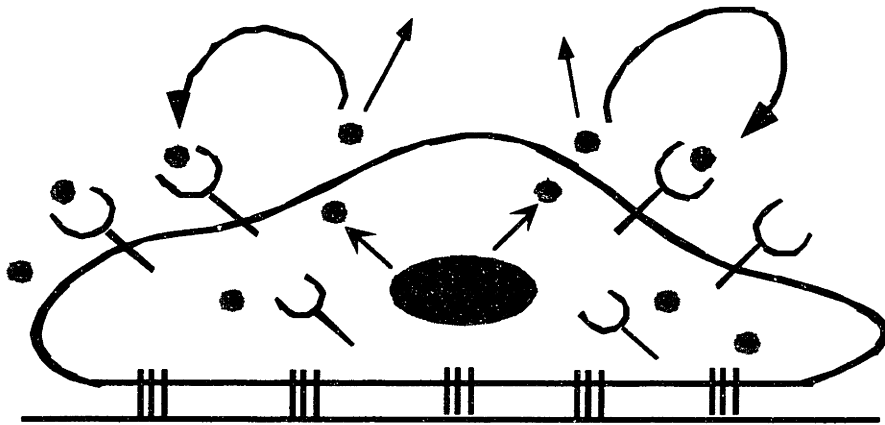


Figure 1.4 Autocrine cells synthesize the ligand as well as its receptor. The secreted ligands can either bind back to receptors on the cell surface or they can escape into the bulk media. Depending on parameters such as, the rate of ligand secretion and the receptor density on the cell surface, the probability of ligand capture by the cell that synthesized it can be determined.

Chapter 2

Background

2.1 Cell migration

The ability of cells to move plays a very important role during normal physiological processes and in many pathological conditions. Regulated cell motility is crucial during embryonic development where a delicate balance of positive and negative signals guides cells to their final destination to form mature tissues and organs. Cell motility continues to play a key role in the adult animal in many situations. Triggering of an immune response causes leukocyte activation and their migration to the site of distress. During wound healing, fibroblasts and keratinocytes migrate into the wounded site causing the surrounding skin to contract and leading finally to wound closure (Gailit and Clark, 1994; Racine-Samson et al., 1997). Angiogenesis and neo-vascularization is facilitated when endothelial cells and smooth muscle cells migrate out and finally differentiate to form blood vessels. Cancer metastasizes when some tumor cells from the original tumor site migrate and settle at an alternate site (Herlyn and Malkowicz, 1991; Stromblad and Cheresch, 1996; Brooks et al., 1998; Clezardin, 1998). As mentioned in Section 1.1, regulated cell motility will play a crucial role in tissue engineering in obtaining the required segregation and aggregation of cell types along specific regions of the guiding scaffold (Cima et al., 1991; Langer, 1997).

Cell motility has been studied extensively in the last few decades. A large body of knowledge now exists about molecules which regulate the motion of cells. Broadly, the two classes of molecules have been identified which are known to affect cell motion.

These are the extracellular matrix molecules which form the surface on or through which the cells move, and the growth factors, which are small polypeptides usually present in the soluble environment of the cell. While biochemical pathways involved in governing cell motility are now being uncovered at a rapid pace, as to how these biochemical signals finally integrate at a macroscopic level to produce cell motion is still largely unknown.

2.2 Integrins and minimal adhesion sequences

Integrins are a major class of cell surface receptors which mediate cell-extracellular matrix contacts (Albelda and Buck, 1990; Brooks et al., 1998; Hynes, 1992; Hynes, 1987; Varner and Cheresch, 1996; Stromblad and Cheresch, 1996). They are transmembrane heterodimeric proteins made of α and β subunits. At least 16 α subunits and 8 β subunits are known to date which can combine to form at least 20 different receptors. When first identified, they were thought to be simply sites of adhesion between the cell and the substratum. However, recent studies have revealed that integrins are actively involved in signal transduction within the cell and play an important role in affecting cell function (Schwartz, 1993; Schwartz and Ingber, 1994; Schlaepfer and Hunter, 1996; Schlaepfer and Hunter, 1998). Different $\alpha\beta$ dimers exhibit specific patterns of cell surface expression and are involved in interactions with a specific group of ligands.

Following ligand occupation, integrins have been found to colocalize with a number of cytoskeletal and signaling proteins at punctate sites known as focal adhesions (Akiyama, 1996; Defilippi et al., 1994; Gumbiner, 1996; Zanetti et al., 1994). These are thought to be points of anchorage between the cell and the surface as well as sites of convergence of signals from pathways initiated from other cell surface receptors such as growth factor receptors. Integrins interact with the actin cytoskeleton at these focal adhesions via cytoskeletal proteins such as talin, vinculin and α -actinin. The actin cytoskeletal network

plays a critical role in cell shape and cell function determination as a consequence of adhesion of the cell via integrins onto ECM proteins (Figure 2.1).

A number of different mechanisms of integrin activation have been suggested. These include modulation of the affinity between the integrin and its ligand as well as modulation of the avidity of interaction between integrins and their ligands by clustering of integrins on the cell surface (Schwartz et al., 1995; Longhurst and Jennings, 1998; Kolanus and Seed, 1997; Cary and Guan, 1999; Richardson and Parsons, 1995; Klemke et al., 1997; Miyamoto et al., 1995b; Chen et al., 1994c). Affinity of integrins for their ligand may be modulated by a conformational change mediated by their cytoplasmic domains. The mechanism of this effect on the integrin cytoplasmic tail is as yet poorly understood although there is some evidence that molecules such as protein kinase C (PKC), PI - 3 kinase, Rho and H-Ras may be involved indirectly. Regulatory proteins such as calreticulin, Rack1, endonexin and ILK, and cytoskeletal proteins such as talin, filamin and α -actinin have been shown to interact directly with the integrin cytoplasmic domain which might result in their affecting the affinity of interaction between the integrin and its ligand. It is not clear whether these proteins interact with the integrin in the absence of an integrin ligand and what is the exact mechanism of transference of this interaction into an affinity change at the extracellular domain of the integrins.

Another mechanism of integrin activation that has been suggested involves their forming clusters at the cell surface which then result in the recruitment of a plethora of regulatory and cytoskeletal proteins affecting cell function (Ward et al., 1994; Hato et al., 1998; Palecek et al., 1999; Rychly et al., 1998; Defilippi et al., 1994; Dogic et al., 1998; Zanetti et al., 1994). Clustering of integrins following ligand occupation has been demonstrated using microbeads coated with integrin ligands and use of bivalent antibodies against both extracellular and cytoplasmic domains of integrins. In an *in vivo* model of focal adhesion

assembly. latex beads coated with Fn or with monoclonal antibodies to the α_5 or β_1 subunit of integrin bind to cultured fibroblasts and induce accumulation of focal adhesion proteins on the cytoplasmic surface of the bead-cell interface (Craig and Johnson, 1996). It has been shown that in the absence of integrin clustering, fibroblasts fail to form focal contacts. Erb et al. demonstrated that small ligands such as the GRGDS peptide presented in the soluble form do not induce integrin clustering suggesting that multi-domain ECM molecules might present multiple sites for integrin ligation resulting in their clustering (Erb et al., 1997). Whether integrin clustering is essential for cellular functions such as cell adhesion and motility has not been demonstrated directly although the necessity of adhesion to ECM proteins indirectly suggests that clustering of integrins and not their occupancy alone might be important in the regulation of cell function. It has also been suggested that initial integrin occupancy increases their mobility/diffusivity resulting in greater probability of cluster formation and subsequent signal generation.

Integrins are involved in the regulation of behavior of many different cell types and physiological situations. They play a critical role in influencing cell motility and cell adhesion. In addition, survival of several cell types is dependent on their adhesion to a matrix with the adhesion often times being mediated by integrins. Changes in integrin expression levels has been shown to correlate with transformation of certain cell types to a metastatic state (Varner and Cheres, 1996; Brooks et al., 1998; Heino, 1996; Ruoslahti, 1992; Schwartz, 1993).

A wide variety of intracellular signals are activated by integrins which range from tyrosine and serine phosphorylation of signaling proteins, changes in phosphoinositide metabolism, activation of GTPases and changes in calcium fluxes. A critical component of signaling via integrins is the non-receptor tyrosine kinase, focal adhesion kinase (FAK) which is thought

to be recruited to sites of integrin clustering following ligand occupancy. Phosphorylated FAK binds src and consequently activates it. Activation of FAK-bound src then leads to the phosphorylation of additional sites on FAK ultimately causing the activation of the Ras/MAP kinase pathway. In addition, FAK has been shown to interact with PI - 3 kinase which plays an important role in cell survival via activation of akt (Defilippi et al., 1994; Akiyama, 1996; Dedhar and Hannigan, 1996; Miyamoto et al., 1995b; Schlaepfer and Hunter, 1997; Cary et al., 1998; Reiske et al., 1999).

There is compelling evidence to suggest that FAK activation is linked to the assembly of actin stress fibers and the focal adhesion complex. Members of the Rho-family of small GTPases, Rho, Rac and cdc42 are important regulators of actin cytoskeletal assembly and therefore it has been suggested that they may be involved in integrin-mediated cell adhesion. Rho is involved in actin stress fiber formation, rac mediates lamellipodia and membrane extension formation and cdc42 orchestrates filipodial extension. Their activation is sequential, with cdc42 activating Rac which then activates Rho. Integrin-induced Rho activation may mediate PIP₂ synthesis, an important intermediate of the EGFR signaling pathway(Cary et al., 1996; Ren et al., 1999; Leong et al., 1995; Wells et al., 1998; Ridley, 1995). Rho has been shown to activate Rho kinase leading to phosphorylation of myosin light chains (MLC) involved in the generation of contractile force within the cell. Generation of contractile force is coordinated with the assembly of the actin stress fiber network and focal adhesions. Klemke et al. reported the existence of a motility regulating signaling pathway directly downstream of MAP kinase with MAP kinase causing the phosphorylation and activation of MLC and MLC kinase involved in cell contraction (Klemke et al., 1997). These studies point towards multiple signaling cascades which might be involved in translating integrin occupancy into a motility response.

ECM proteins in general are modular in nature with specific domains of the molecule interacting with a particular cell surface receptor. Some of the commonly found domains are fibronectin type III domains, EGF-like domains, heparin-binding domains and collagen binding domains (Yamada et al., 1992). Extensive study of integrins and their ligands in recent years has led to the identification of specific ligands for many of the integrins. In most cases, the sequences involved in recognition by cell surface receptors are surprisingly short, suggesting the importance of linear amino acid sequences either alone or in combination to mediate processes such as cell adhesion. The first binding site to be identified was the arg-gly-asp (RGD) sequence present in fibronectin (Fn), vitronectin and variety of other adhesive proteins (Ruoslahti, 1996). It is present in a loop on the 10th type III repeat of FN (Ely et al., 1995) (Figure 2.2). This tri-peptide sequence is recognized by several integrins ($\alpha_5\beta_1$, $\alpha_{11b}\beta_3$ and all or most $\alpha_v\beta$ integrins) but not by most others. Recent studies wherein it was shown that the cell attachment activity of ECM molecules could be mimicked by short, immobilized, synthetic peptides containing the RGD sequence led to tremendous research in the development of RGD-containing artificial matrices to promote the interaction of cells (Koivunen et al., 1994; Pierschbacher et al., 1994). A short sequence in the 9th type III repeat in fibronectin has been identified as a “synergy site” aiding the interaction of the integrin with the RGD sequence, suggesting that the synergy site increases the affinity of the integrin for RGD (Mould et al., 1997). A recent study theoretically showed that application of force on the 10th Fibronectin type III repeat results in a conformational change in the loop containing the RGD sequence and increasing its accessibility to cell surface receptors (Krammer et al., 1999). Numerous studies with synthetic RGD peptides have confirmed its role in promoting cell adhesion (Glass et al., 1994; Massia and Hubbell, 1991; Massia and Hubbell, 1990; Danilov and Juliano, 1989; Zanetti et al., 1994). Its binding is specific to certain integrins on the cell surface. Soluble RGD inhibits the formation of focal contacts whereas substrate linked RGD sequences promote early focal contact formation in fibroblasts (Singer et al., 1987; Singer et al.,

1988). Singer et al. used the sequence GRGDS in their studies. Another cell binding sequence that was identified in laminin is YIGSR which binds to a 67 kD cell membrane bound receptor (Massia et al., 1993). The sequence YIGSR has also been covalently immobilized on glycoPhase glass and has been shown to render the surface adhesive for endothelial cells. Minimal adhesion peptide sequences such as RGD and YIGSR in solution can inhibit cellular migration and adhesion on the intact ECM molecules adsorbed onto the surface (Singer et al., 1987; Wu et al., 1994).

In some cases, migration on peptide fragments exhibit only part of the motility-stimulating activity of the intact protein, suggesting either that multiple sites are required for maximal activity or that steric presentation of the individual sequences is optimal in the intact molecule.

2.3 EGF and EGFR

The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that binds a family of ligands including the epidermal growth factor (EGF), transforming growth factor- α (TGF- α), betacellulin and heregulin (Wells, 1989). It has been shown to play an important role during wound healing and cancer metastasis (Aaronson, 1991; Brasken, 1991; Marti et al., 1989; Schultz et al., 1991). EGFR is expressed by a number of normal cell types including fibroblasts and epithelial cells. The signaling cascade activated upon EGFR activation is one of the most extensively studied pathways and is closely related to other motility-signaling pathways (Wells et al., 1998). Hence, the EGFR-EGF system is an ideal candidate for investigation of growth factor effects on cell motility.

A number of soluble growth factors such as epidermal growth factor (EGF) (Chen et al., 1994b; Chen et al., 1994a; Maldonado and Furcht, 1995; Gupta et al., 1994), platelet-derived growth factor (PDGF) (Wennstrom et al., 1994) and insulin-like growth factor

(IGF) (Bornfeldt et al., 1994) generate an enhanced mitogenic and motogenic response in diverse cell types. EGF, a high affinity ligand for EGFR induces cellular motility in numerous cell types (Fujii et al., 1995; Cha et al., 1996; Chen et al., 1994a). EGF increases the migration of keratinocytes as measured by the phagokinetic track assay and the *in vitro* wounded monolayer assay (Nanney et al., 1996; Cha et al., 1996). Increased expression levels of EGFR have been observed in cancerous cells with the suggestion that elevated levels of receptors make the tumors highly sensitive to very low levels of growth factors inducing enhanced metastasis and proliferation (Goustin et al., 1986; Turner et al., 1996; Seedorf, 1995; Wahl and Carpenter, 1987; Heino, 1996). EGF-dependent increases in cell proliferation and motility have also been shown to result in accelerated wound healing (Schultz et al., 1991)

EGF is synthesized as a transmembrane precursor which is proteolytically processed to release the mature growth factor external to the cell membrane (Figure 2.3a) (Pandiella et al., 1992; Massague and Pandiella, 1993). The EGF precursor has a large N-terminal domain, a transmembrane domain and a cytoplasmic domain. The precise mechanism by which mature EGF is released is largely unknown though it has been speculated that matrix metalloproteases may play a role in the proteolytic cleavage. The structure of mature EGF is very stable with three intra-molecular disulphide bonds and 2 anti-parallel beta sheets. EGF maintains its tertiary structure when subjected to heat, acidic pH and high concentrations of denaturing agents such as urea.

Wild type EGFR has five tyrosine sites in its cytoplasmic domain (Nanney et al., 1996; James and Bradshaw, 1984) which are trans-phosphorylated upon EGFR activation (Figure 2.3b). The phosphorylated tyrosine residues are sites of interaction with variety of signaling molecules and initiation of a number of signaling cascades regulating cell function (Figure 2.4). Pathways have been identified which are downstream of EGFR activation

and influence cell proliferation as well as cell motility (Riese and Stern, 1998; Ciardiello and Tortora, 1998; Pai and Tarnawski, 1998; Tarnawski and Jones, 1998; Seedorf, 1995; Castellino and Chao, 1996; Rijken et al., 1994; Aaronson et al., 1990; Rosner, 1990; Chen et al., 1994a; Xie et al., 1998; Chen et al., 1994b). EGF causes activation of MAP Kinase via the ras-raf pathway which is also involved in the regulation of the MLC phosphorylation, implicating it in contractile force generation within the cell (Klemke et al., 1997). Activated EGF phosphorylates phospholipase C- γ (PLC- γ) which in turn hydrolyzes PIP₂. IP₃ and DAG, the hydrolysis products of PIP₂ increase Ca²⁺ flux and activate PKC respectively. PIP₂ is also involved in gelsolin- and α -actinin- mediated activation of actin polymerization and stress fiber formation, thereby providing a direct mechanism of influencing cell motility (Chen et al., 1996; Sakisaka et al., 1997). It has been suggested that EGFR- mediated cytosolic calcium influx is not required for motility stimulation, but may be required for the activation of myosin-II and calpain activity which suggests a role for the calcium influx in influencing cell motility via its effect on rear detachment of the cell (Wells et al., 1998). EGFR activation results in enhanced lamellipod extension rates and membrane ruffling (Ridley, 1995; Segall et al., 1996; Chan et al., 1998; Bailly et al., 1998b; Wyckoff et al., 1998; Bailly et al., 1998a). It also causes short-term disassembly of focal adhesions in fibroblasts and decreased cell-substratum adhesion at short time scales (Xie et al., 1998; Deugnier et al., 1999). These studies demonstrate that EGFR activation can influence motility via different biophysical and biochemical means and strongly suggest to possible synergy with signaling pathways activated by integrin occupation in regulating cell motility.

2.4 Convergence of signals from integrins and growth factor receptors

In vivo, the cell surface receptors interact simultaneously with a large number of ligands, both soluble and surface-bound (Ruoslahti, 1991; Ruoslahti, 1992). Adhesiveness

between the cell and the substratum is modulated by both adhesive and anti-adhesive signals from the matrix-phase and solution-phase (Chiquet-Ehrismann, 1995; Saiki, 1997; Gotz et al., 1996). Both integrins and the EGFR have been found to co-localize with the actin cytoskeleton at sites of focal contact formation (Rijken et al., 1995; Nakamura et al., 1995). While signaling pathways have been identified which are common between those activated by growth factor receptors and integrins, their integration at a biophysical level remains as yet largely unexplored.

When integrins were first discovered, they were thought to be simply structural connectors which link the cell cytoskeleton with the external environment. However, since then it has become apparent that integrins are multifaceted and play a role both structurally and biochemically (Hynes, 1987; Hynes, 1992; Ingber, 1993). As mentioned in Section 2.2, a number of signaling pathways have been identified which are triggered by integrin activation. Most of these pathways had previously been studied in connection with their stimulation via growth factor receptors (Hannigan and Dedhar, 1997; Matsumoto et al., 1995; Schlaepfer and Hunter, 1998). This then has led to the suggestion that signaling via the integrins may just be another way of influencing cell function by conveying the information regarding the extracellular adhesive environment of the cell. And that the final cellular response is a consequence of the cell's interaction with its insoluble and soluble environment with the respective signals integrating to produce a net effect on cell behavior.

With this notion in mind, there have been many recent studies focusing on simultaneous stimulation of specific signaling pathways/intermediates by growth factors and integrins. Woodard et al. showed that a platelet derived growth factor -bb (PDGF-BB) gradient stimulated rat microvessel endothelial cell migration in an $\alpha_v\beta_3$ and RGD- dependent manner pointing towards synergistic regulation of cell motility by a growth factor and integrin (Woodard et al., 1998). It has also been shown that adhesion of fibroblasts and

endothelial cells to β_1 and α_5 antibodies stimulates tyrosine phosphorylation of the EGF receptor resulting in Erk-1/MAP kinase induction (Moro et al., 1998). Cytokines such as basic fibroblast growth factor, transforming growth factor- β_1 and LIF alter expression of $\alpha_5\beta_1$ integrin thereby affecting adhesive and migratory properties of endothelial cells during angiogenesis (Collo and Pepper, 1999). Occupancy of the insulin receptor in CHO cells leads to increased adhesiveness via the $\alpha_5\beta_1$ integrin suggesting cooperative interaction between the insulin receptor and the integrin which then causes formation of intracellular complexes containing insulin receptor kinase -1(IRS-1)and PI 3-kinase with enhanced activity of IRS-1(Guilherme et al., 1998).

It has been suggested that FAK may be involved in the integration of signals from integrins and growth factors (Dedhar and Hannigan, 1996; Hannigan and Dedhar, 1997; Schlaepfer and Hunter, 1997; Cary and Guan, 1999). Occupation and aggregation of β_1 and β_3 integrins induce tyrosine phosphorylation of p125FAK and tyrosine phosphorylation of paxillin and tensin, either by pp125FAK or by a FAK-stimulated tyrosine kinase. FAK is also rapidly tyrosine phosphorylated in response either to oncogenic transformation or to treatment of cells with a variety of growth factors and neuropeptides suggestive of a role for FAK in regulating the synergy between the signals generated from growth factors and integrin-ligands (Plopper et al., 1995; Ruoslahti, 1992; Schwartz and Ingber, 1994; Ruoslahti, 1996; Gilmore and Romer, 1996; Brunton et al., 1997). Recent work has shown that β_1 integrin- induced tyrosine phosphorylation activity can activate the mitogenic MAPK cascade though with differences in regulation from the MAPK cascade stimulated by growth factors (Roskelley et al., 1995; Roskelley and Bissell, 1995). In an interesting set of experiments, Maniero et al. demonstrated that activation of the EGFR by EGF causes tyrosine phosphorylation of the β_4 subunit of the $\alpha_6\beta_4$ integrin and leads to increased $\alpha_6\beta_4$ integrin-mediated migration towards laminins as measured in a modified Boyden chamber (Mainiero et al., 1996). Soluble EGF was also shown to stimulate human corneal epithelial

cell migration on fibronectin and GRGDSP peptide which was blocked by anti- α_5 antibodies (Maldonado and Furcht, 1995). Miyamoto et al. demonstrated that integrins and growth factors co-cluster using integrin ligand-coated beads leading to MAP kinase activation (Miyamoto et al., 1995a; Miyamoto et al., 1998). They have suggested that integrins and growth factors aggregate into large signaling and cytoskeletal complexes which support synergistic stimulation of signals generated by both kinds of receptors.

These reports strongly suggest interaction between growth factors and integrins in regulation of cell function. While their simultaneous effect on signaling molecules is being studied at many levels of complexity, how these signals integrate at a biophysical level to influence cell function such as motility remains largely unanswered.

It has been observed that fragments of specific domains derived from ECM proteins need not necessarily mimic the function of the intact protein. Recently there have been a number of contradictory reports of the effect of fragments derived from ECM molecules on mitogenesis, adhesion and motility (Chiquet-Ehrismann, 1995). End et al. reported that tenascin-C was mitogenic for several cell types and that this activity was associated with a region in FN type III domains (End et al., 1992). In contrast, Crossin reported that tenascin-C inhibited the mitogenic response of 3T3 fibroblasts stimulated by growth factors (Crossin, 1991). Giese et al. demonstrated that a human glioma-derived cell line exhibited contrasting migratory responses to tenascin deposited on the culture surface (Giese et al., 1996). By using specific antibodies to inhibit specific integrins, it has been suggested that different integrins bind with different affinities to ligands on tenascin causing contrasting cell responses (Prieto et al., 1993). Tenascin exhibits a distinct anti-adhesive activity as demonstrated by its inhibition of cell adhesion on fibronectin and laminin surfaces (Deryugina and Bourdon, 1996). This activity has been mapped on the tenascin molecule to the region of EGF-like repeats (Spring et al., 1989). A mechanism suggested involves

anti-adhesive signal generation from interaction of the EGF-like domains with cell surface receptors, possibly the EGFR, thereby counteracting the adhesive signal from other cell binding domains (Schulze et al., 1996; Engel, 1989; Panayotou et al., 1989; Prieto et al., 1992).

In wounds, proteolytic fragments of ECM molecules are often present due to the action of wound proteases (Clark, 1996). It was shown that such proteolytic fragments display biological activities that are absent or suppressed in the intact parent molecule (Alexander et al., 1996; Huhtala et al., 1995). For example, the central cell adhesive domain of FN displays potent chemotactic activity for monocytes, even though the intact molecule has little activity (Sottile et al., 1998; Wikner and Clark, 1988). Similarly, a fragment containing this cell adhesive domain can stimulate the synthesis of proteases such as collagenases even though the intact FN molecule does not (Werb et al., 1989). Cell proliferation can be stimulated in cells possessing EGF receptors by a fragment of laminin that includes EGF-like repeats at concentrations comparable to those of EGF itself (Panayotou et al., 1989). It is therefore possible that proteolytic degradation of laminin *in vivo* can lead to liberation of growth factor activity that stimulates growth in regions of tissue damage.

These studies suggest that the activity of an intact ECM protein is the result of synergy between signals from various domains. In particular, adhesion and motility may be mediated by signals from the integrin binding domain and the EGF-like domain. Alternatively, the cellular response obtained may depend on which domains of the adsorbed proteins are accessible for interaction with the cell surface receptors. The system which will be used in this study permits the presentation of individual domains both in solution or in matrix-bound form. Thus, by altering the surface density and configuration of the

ligands, it may be possible to mimic the ligand presentation in an intact ECM protein which can lead to the design of an artificial extracellular matrix.

2.5 Autocrine regulation of cell behavior

There exist many spatial modes of interaction of ligands with their receptors (Sporn and Roberts, 1992). They can interact in a paracrine manner which requires the receptor and the ligand be secreted by two different cell types and the secreted ligand then diffuses through the bulk extracellular media to interact with the receptor (Figure 2.5a). Juxtacrine interaction requires cell-cell contact with cell A expressing the ligand and cell B expressing its receptor (Figure 2.5b). Receptor ligand interactions where the same cell synthesizes the ligand and also expresses its receptor are termed as autocrine interactions (Figure 2.5c). Autocrine loops were first observed in tumor cells where it was found that transformation of a cell resulted in the overproduction of certain cytokines causing uncontrolled proliferation of cells (Sporn and Roberts, 1985; Liotta et al., 1991). However since then autocrine loops have been observed in homeostatic conditions as well suggesting that spatial regulation of receptor-ligand interactions works as another regulatory mechanism in controlling cell function (Sporn and Roberts, 1985; Hudson and McCawley, 1998).

Direct study of autocrine interactions between receptors and their ligands is non-trivial since the standard techniques employed to study receptor-ligand interactions fail because of the receptor and ligand being synthesized by the same cell type. Therefore, indirect methods for evaluating their effects need to be employed. These may include monitoring changes in cell function such as proliferation, differentiation and migration or changes in downstream signaling molecules within the cell or their subsequent effect on the extracellular environment such as a change in its pH (Oehrtman et al., 1998). Two possible scales of action of autocrine loops have been suggested (Forsten and Lauffenburger, 1991; Lauffenburger et al., 1995). One is a “community model” which suggests that the

autocrine ligand is secreted by the cell into the bulk media from where it then diffuses and binds to cell surface receptors (Figure 2.6a). According to this model, there would be no functional difference between a paracrine ligand and an autocrine ligand. An alternate model that has been proposed assumes a more “local” mode of action of autocrine loops, suggesting that an autocrine ligand will preferentially bind to the receptors on the cell that secreted it and that their initial sphere of influence is limited to the dimensions of a cell (Figure 2.6b). There has been recent theoretical and experimental evidence which supports the local model rather than a communal mode of action of autocrine loops.

Theoretical studies by Forsten and Lauffenburger have suggested that a ligand secreted by a cell has a greater probability of binding back to its own receptor than escaping into the bulk extracellular media (Forsten and Lauffenburger, 1992a; Forsten and Lauffenburger, 1992b). They also predicted that a lower concentration of an anti-receptor antibody will be required than that of an anti-ligand antibody in blocking the binding of the secreted ligand to the cell surface receptor. This prediction was borne out in subsequent experimental studies by Lauffenburger et al. who showed that a blocker antibody against the EGFR was at least 1000 times more effective than a decoy antibody against TGF- α in blocking the binding of autocrinely secreted TGF- α to the cell surface EGFR (Lauffenburger et al., 1998).

Recent studies by Wiley et al. strongly suggest a role for the mode of soluble ligand presentation in regulating cell function (Wiley et al., 1998). In order to understand the role of the membrane anchoring domain of EGF, Wiley et al. synthesized a set of EGFR ligands, one consisting of the mature EGF along with its cytoplasmic and transmembrane domain (EGF-Ct), and one with simply the mature form of EGF (sEGF). They expressed these constructs in an autocrine manner using a human mammary epithelial cell line which expressed the EGF receptor endogenously. They observed that removal of the membrane

anchoring domain of the EGF precursor resulted in an uninterrupted autocrine loop and have suggested that EGF in sEGF expressing cells may be able to signal in an intracrine manner. A blocking antibody which blocks the binding of ligands to the EGFR was unable to prevent the interaction of sEGF with EGFR causing continued proliferation and EGFR activation even in the presence of high concentrations of the antibody. In addition, the reorganization and differentiated structures formed by EGF-Ct expressing cells and sEGF expressing cells differed considerably with EGF-Ct expressing cells forming structures which closely resembled those formed by primary human mammary epithelial cells under EGF stimulation. This study strongly suggests that the effect of receptor activation on cell behavior was dependent on the spatial localization of the activated receptor-ligand complex. It remains to be determined whether soluble ligand presentation has an effect on cell motility or whether activation of the receptor in any manner elicits the same migratory response.

In a preliminary study by Rodgers and Lauffenburger, it was found that addition of a blocking antibody or soluble growth factor (EGF) into the bulk media resulted in a reduced persistence time relative to that of a truly autocrine cell. This result pointed to the role of autocrine ligands in regulating the directionality of cell motion and their role in providing another extracellular environment sensing system to the cell to fine tune its behavior appropriately.

2.6 Conceptual models of motility

Cell locomotion is a consequence of orchestrated processes of lamellipod extension, formation of new adhesive contacts with the substratum, and uropod contraction accompanied with cleavage of pre-existing adhesive contacts in the rear of the cell (Lauffenburger, 1991; Lackie et al., 1999). A pre-requisite for cell locomotion is the

existence of asymmetry in the strength of the adhesion bonds between the leading and trailing edges of the cell (Sheetz and Dai, 1996; Sheetz et al., 1998)

Single cell migration is considered analogous to a persistent random walk. It is characterized by cell speed S , which is the speed of locomotion, and by cell persistence time P , which is the time period over which the leading edge remains in the same direction. Migration of cells on a two-dimensional substrate has been shown to be very well described by the equation:

$$\langle d^2 \rangle = S^2 P [Pt - P^2 (1 - e^{-t/P})] \quad \text{Eq. (1)}$$

where $\langle d^2 \rangle$ is the average displacement of the cell after elapsed time of observation, t (Dickinson and Tranquillo, 1993; Dunn and Brown, 1987)(Figure 2.7).

Dimilla et al showed that there exists a biphasic relationship between the cell speed and the cell/substratum adhesive strength (Dimilla et al.,1991; Dimilla et al., 1992). Three regimes of cell-substratum adhesiveness have been identified (Figure 2.8). On highly adhesive surfaces, the cell is well-spread and immobilized resulting in impaired disruption of adhesions which then prevents locomotion. On weakly adhesive surfaces, cells are unable to generate the traction required for cell motility. It is only on surfaces with intermediate adhesive strengths that the cell is able to generate sufficient traction for it to be motile. The adhesiveness of the surface is relative to the amount of motile force generated within the cell and transmitted to the cell surface.

Dynamic adhesive interactions between the cell and the substratum are mediated by specific cell surface receptors and their respective ligands on the substratum. The adhesiveness of the surface as measured by adhesion assays such as the sedimentation assay and the radial flow chamber assay has been shown to be directly proportional to the surface concentration of the ECM protein adsorbed/covalently tethered onto the surface (Goodman and Majack,

1989; Dimilla et al., 1993; Palecek et al., 1997). Thus, the biphasic relationship mentioned earlier translates to that between the cell speed and the concentration of the adhesion ligand on the substratum. This has been validated experimentally by migration studies with EGF-stimulated WT NR6 cells on varying Amgel concentrations (Ware et al., 1998), with smooth muscle cells on varying concentration of fibronectin and type IV collagen (Dimilla et al., 1993) and with murine myoblasts on varying concentration of laminin (Goodman and Majack, 1989). Speed of human breast carcinoma cells on laminin and type I collagen varied in a biphasic manner with the expression level of $\alpha_2\beta_1$ integrin in these cells (Keely et al., 1995). Modulation of cell speed using mutant integrin receptors with varying affinities for the integrin adhesion ligand has also been demonstrated (Schmidt et al., 1995). Palecek et al tested integrin expression level, ECM ligand concentration and integrin-ligand affinity as modulators of cell-substratum adhesiveness thereby altering cell motility. It remains to be seen whether varying integrin-ligand avidity could be a possible mechanism of modulating the strength of cell-substratum adhesiveness (Palecek et al., 1997).

It has been suggested that the effect of growth factors on migration may be targeted to cell-substratum adhesiveness, lamellipod extension frequency and/or contractile force generation by the cell (Lauffenburger and Horwitz, 1996; Huttenlocher et al., 1996; Huttenlocher et al., 1995) (Figure 1.2). Welsh et al. observed lamellopodial retraction and concomitant cell shape changes in c'973 NR6 cells upon exposure to 50 nM soluble EGF suggesting that EGF attenuates cell adhesion (Welsh et al., 1991). Ware et al demonstrated that EGF regulated cell speed on varying Amgel (a natural extracellular matrix which consists of many ECM proteins) concentrations and reported that EGF affects persistence of cells in a manner that is inverse of its effect on cell speed with increased cell speed correlating with decreased persistence of the cells (Ware et al., 1998). Xie et al reported that addition of EGF decreases cell-substratum adhesion immediately after addition of

EGF (Xie et al., 1998). Therefore there is significant evidence linking EGF-EGFR interaction to biophysical means of cell motility regulation. It remains to be seen whether EGFR-mediated signals integrate with those generated via specific integrins to have a concerted effect on cell motility and biophysical mechanisms involved in its regulation.

2.7 Engineering surfaces for studying cell motility and adhesion

In an attempt to mimic the *in vivo* extracellular environment of the cell, extracellular matrix molecules have been adsorbed non-specifically over glass and plastic surfaces in numerous previous studies. Protein adsorption occurs via non-covalent interactions of hydrophobic domains of the protein with the substrate. This may result in varying degrees of denaturation of certain segments of the protein. The non-specific nature of the adsorption may cause certain functional domains to become inaccessible for cellular interaction. Such surfaces are also prone to change during the course of an experiment due to either desorption of the adsorbed protein or due to non-specific adsorption of unwanted proteins present in the media or those secreted by the cells. While such non-covalently modified surfaces provide an adhesive surface, they usually lack rigorous characterization and hence are unsuitable for investigating the effect of presentation of a ligand on cell behavior. It is essential to have the cells interacting solely with the ligands of interest and hence it is imperative that the results are not confounded by the surface adsorption of extracellular matrix proteins secreted by the cell. We propose to adopt a two step approach in the rational design of a bioartificial matrix. Firstly, we require a background surface which is completely resistant to cell and protein adhesion. To such an inert surface, ligands will be covalently tethered in a well-defined spatial configuration which will regulate cell behavior in a known manner (Figure 2.9)

The use of a surface with covalently modified polyethylene oxide (PEO) overcomes a lot of the problems due to non-specific adhesion of cells and proteins (Harris, 1992; Bergstrom et al., 1992). PEO-based hydrogels and polymer networks provide a highly non-adhesive surface for cell culture (Cima, 1994). They are also amenable to covalent modification by biological molecules. The adhesiveness of such surfaces was greatly enhanced upon covalent immobilization of minimal adhesion sequences such as RGD and YIGSR (Drumheller and Hubbell, 1994; Hubbell, 1999; Hubbell et al., 1991; Hubbell, 1995; Dillow and Tirrell, 1998). Surfaces with adhesion peptides immobilized on glycoPhase glass and dacron, and incorporated into polyurethane and polyacrylamide gels have been developed for potential applications in biomaterials. These covalently adhered peptides have been found necessary and sufficient for fibroblast adhesion, proliferation and migration on such surfaces, though detailed examinations of surface architecture for maximal cellular motility are lacking. Cell motility, as measured by fibroblast migration through porous membranes in response to chemoattractant PDGF, showed that polycarbonate membranes non-covalently coated with RGD peptide or FN were able to support motility whereas coating with a non-RGD peptide was unable to support cell migration (Glass et al., 1994).

It has been reported that average surface spacing of 440 nm is sufficient for spreading of primary human fibroblasts, though a spacing of 140 nm is required for focal contacts on surfaces with GRGDY linked to non-adhesive glass substrate by a terminal glycine (Massia and Hubbell, 1991). Pierschbacher and coworkers showed that primary dermal fibroblasts migrate as efficiently on RGD modified with a long hydrophobic tail which enables its adsorption onto surfaces, as they do on Fn (Glass et al., 1994). These previous studies validate the use of the minimum adhesion sequence RGD for creation of a migration-conducive surface. However, it is not clear from these studies that the

composition of the underlying surfaces is not changing over time due to desorption of the adsorbed RGD and due to adsorption of ECM synthesized by the cells.

Use of an immobilization scheme based on PEO provides control over the positioning of the ligands on the surface (Lopina et al., 1996; Kuhl and Griffith-Cima, 1996) (Figure 2.10). The chemistry of covalent immobilization of PEO has been studied extensively and is well characterized (Griffith and Lopina, 1998; Zalipsky et al., 1996; Zalipsky and Harris, 1997; Zalipsky et al., 1997; Cao et al., 1998; Bergstrom et al., 1992). The hydroxyl groups of the PEO molecule provide a ready site for covalent attachment of other molecules and surfaces (Harris, 1984). Use of the star version of PEO in which f PEO arms, each of molecular weight M_w , emanate from a central divinyl benzene core increases the availability and clustering of the hydroxyls. Control over ligand clustering and spacing can be achieved by altering ligand concentration and lengths of the PEO tether. It has been demonstrated that star PEO-tethered galactose units were able to recreate the high affinity form of the galactose - binding hepatocyte asialoglycoprotein receptor to serve as an adhesion receptor by optimizing the PEO arm length for obtaining tri-galactose clusters which are required for cooperativity between the receptor subunits for high affinity binding (Lopina et al., 1996). The biological activity of mouse EGF was retained when it was covalently coupled to aminosilane-modified glass via star PEO molecules. Tethered EGF was as effective as soluble EGF in eliciting DNA synthesis and cell rounding responses in primary rat hepatocytes under serum free conditions (Kuhl and Griffith-Cima, 1996).

Use of PEO as a tether between ligands and surfaces presents many advantages. PEO inhibits non-specific protein adsorption on the surface (Gombotz and Pettit, 1995; Harper et al., 1995). PEO chains are highly mobile and have a large exclusion volume in water (Jeon et al., 1991; Jeon and Andrade, 1991; Jeon and Andrade, 1992; Andrade et al., 1996). The tethered molecules closely simulate the behavior of free ligands in solution

without the disadvantage of diffusive loss with time. Their flexibility allows the ligands to adopt different conformations thus increasing the probability of binding at the active site on the cell surface receptor. PEO is an FDA-approved component of implantable therapeutic formulations and has been shown to be weakly immunogenic and non-toxic in *in vivo* studies. It is expected that the use of RGD-modified star PEO molecules, as opposed to RGD-modified linear PEO molecules, will mimic ligand clustering which in turn will stimulate integrin clustering.

2.8 References

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2.9 Figures

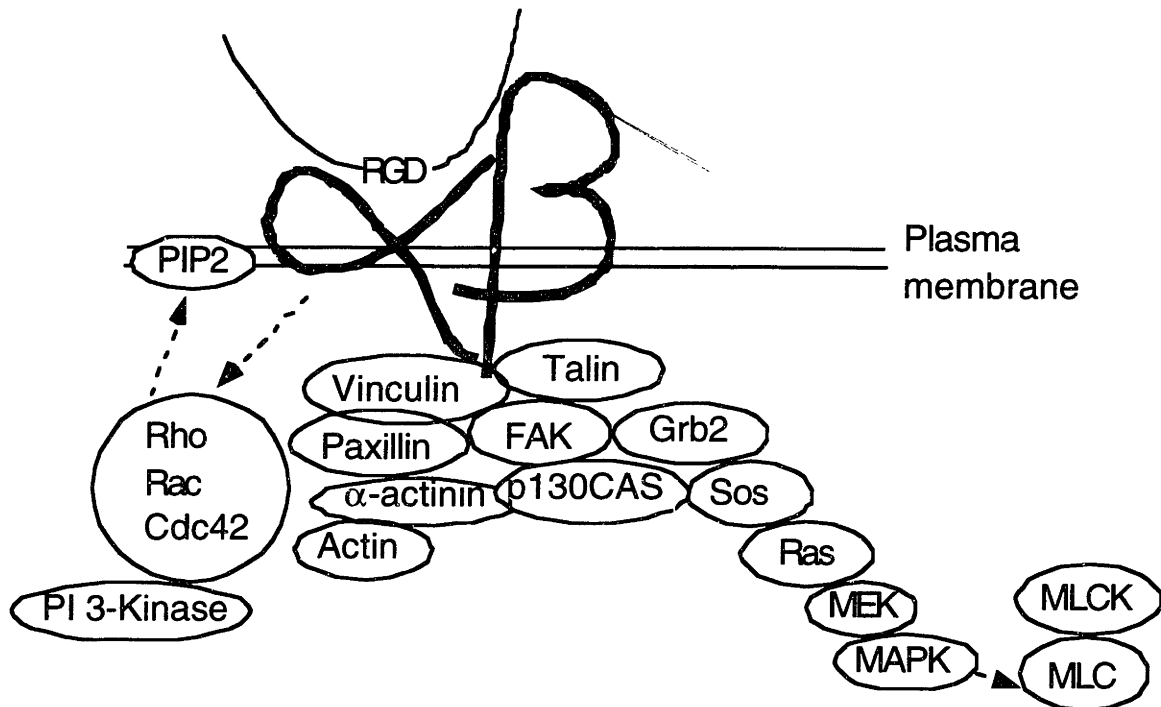
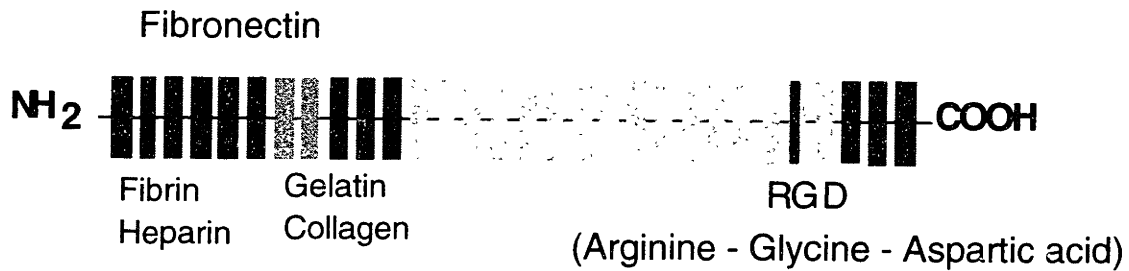


Figure 2.1 Intracellular interactions between integrins and the cytoskeletal and regulatory molecules.

Integrins have been shown to interact with a variety of regulatory and structural proteins both directly and indirectly. This figure shows some of the key molecules involved in motility regulation via integrins. These various pathways influence cell motility by modulating the underlying biophysical processes. Some of these molecules such as vinculin and FAK are thought to localize at focal complexes upon integrin aggregation.

(a)



(b)

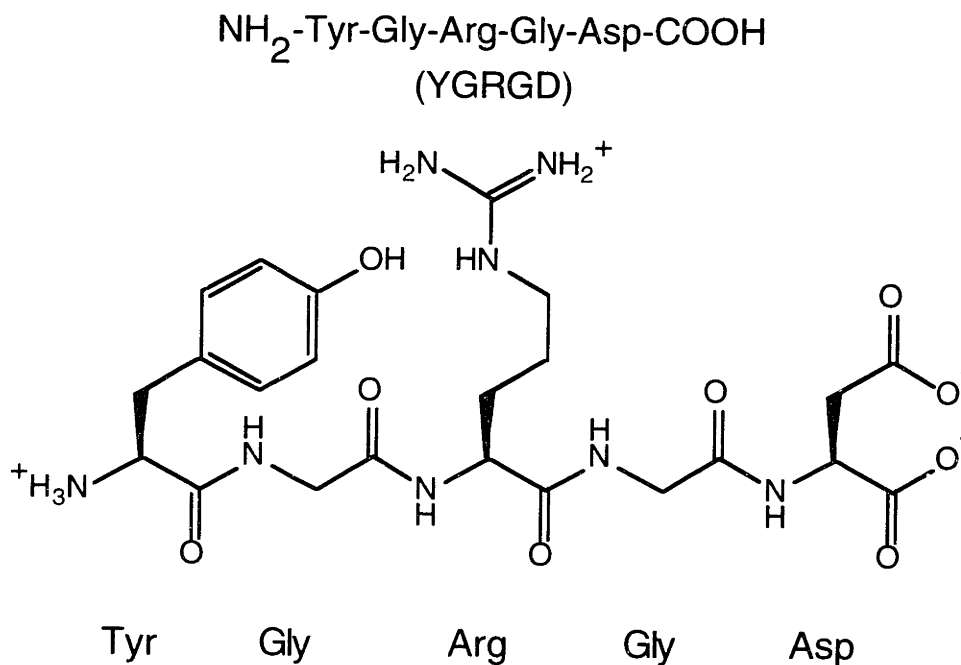


Figure 2.2 Schematic structure of fibronectin and its cell binding domain, arg-gly-asp sequence

Fibronectin is a multi-domain extracellular matrix protein with specific domains which bind to integrin receptors and other molecules. A three amino acid long sequence (RGD) has been identified from the 10th Fn type III repeat which is known to bind to specific cell surface integrins. The sequence YGRGD has been used in this study (Chapter 4).

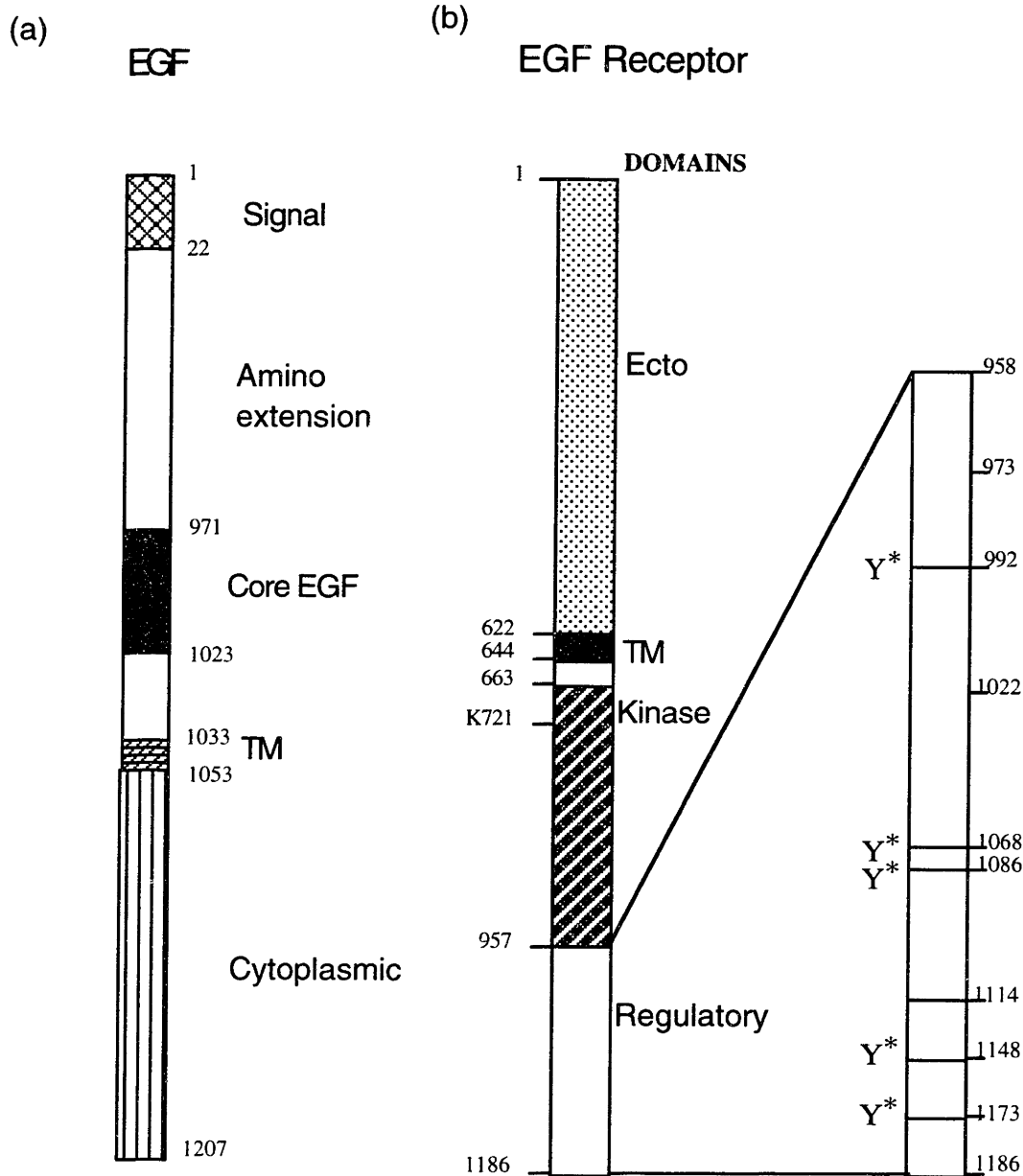


Figure 2.3 Schematic domain structure of EGFR and EGF.
 (a) Epidermal Growth Factor precursor. The wildtype transmembrane precursor is proteolytically processed to release the mature 53 amino acid long EGF. (b) Epidermal Growth Factor Receptor. Tyrosine residues which undergo phosphorylation upon receptor activation are shown with an asterisk. TM - transmembrane segment.

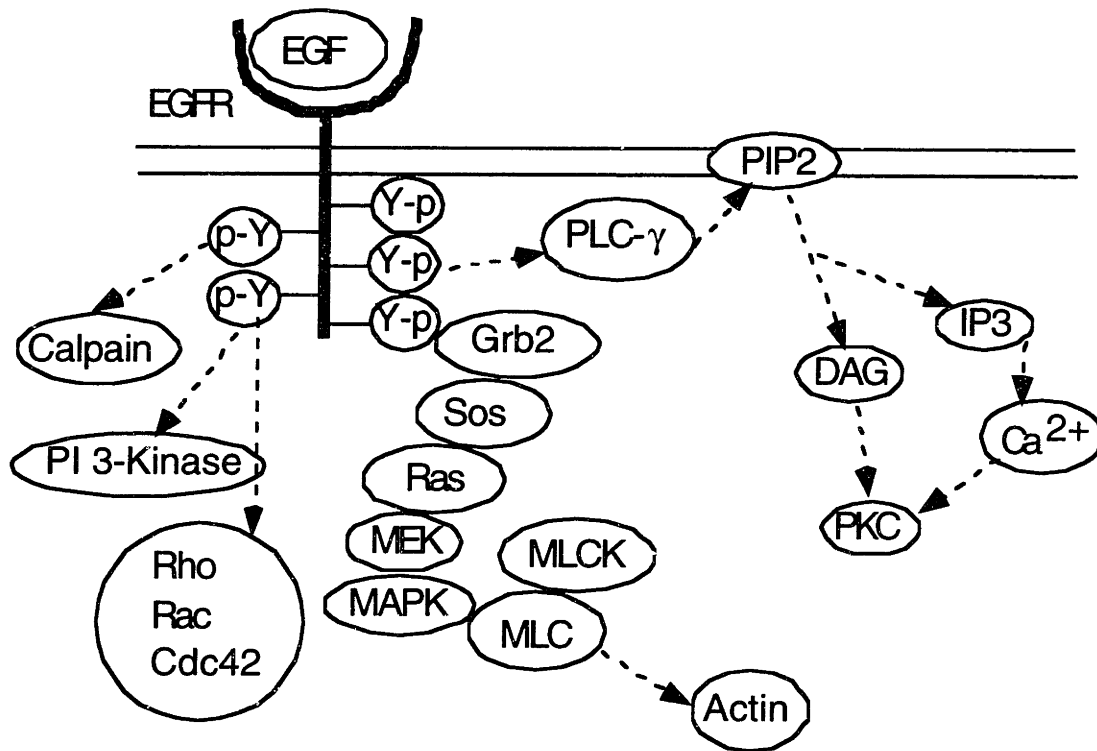


Figure 2.4 Signaling cascade activated by EGFR-EGF interaction

This figure shows some of the signaling molecules activated upon EGFR activation which have been implicated in regulation of cell motility. Some of the down stream messengers can be activated by more than one pathway. These various pathways then integrate to regulate cell motility via their effects on underlying biophysical processes such as cytoskeletal organization, generation of contractile force within the cell, adhesion, protrusion and release of attachments in the rear of the cell.

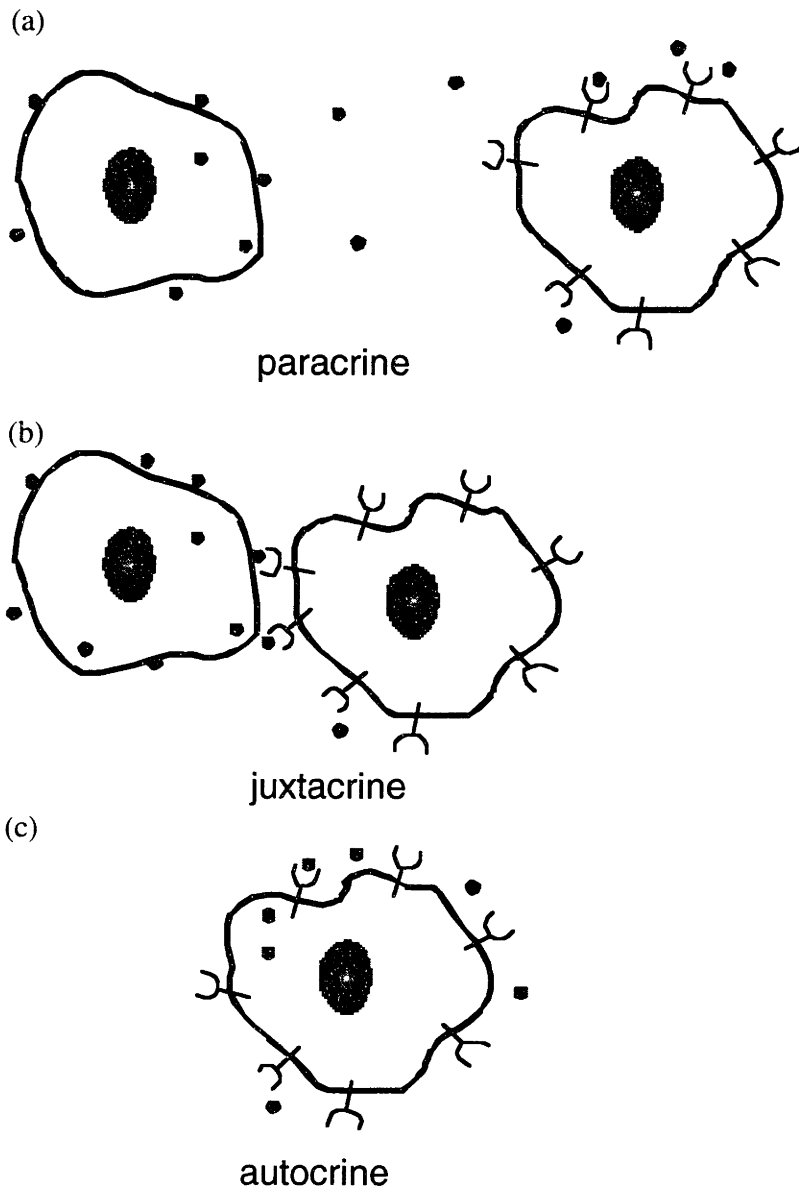
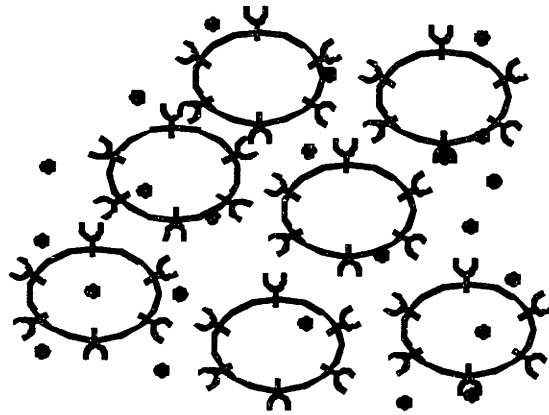


Figure 2.5 Modes of receptor-ligand interaction.

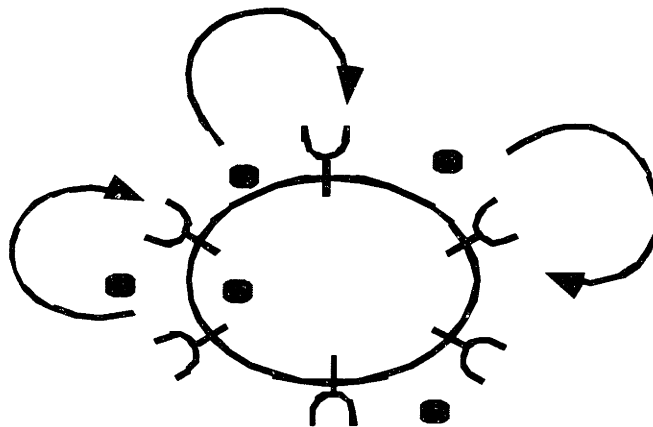
(a) Paracrine interaction. The ligand secreted by one cell type diffuses through the bulk extracellular media and binds to receptors on a neighboring cell type. (b) Juxtacrine interaction requires cell-cell contact. (c) Autocrine interactions require that the ligand and its receptor be synthesized by the same cell. (Adapted from Forsten and Lauffenburger, 1992a)

(a)



"Community" model

(b)



"Local" model

Figure 2.6 Models of autocrine signaling.

(a) The community model suggests that the ligand is secreted by the cell into the bulk extracellular media from where it then diffuses and binds to receptors on the cells. The secreted ligand has no preference for receptors on the cells that secreted it. (b) The local model assumes that the ligand has a greater probability of binding back to its own receptor than being secreted into the bulk extracellular media. The value of the probability is dictated by factors such as the the density of receptors on the cell surface, the ligand secretion rate and the binding constant between the receptor and the ligand.

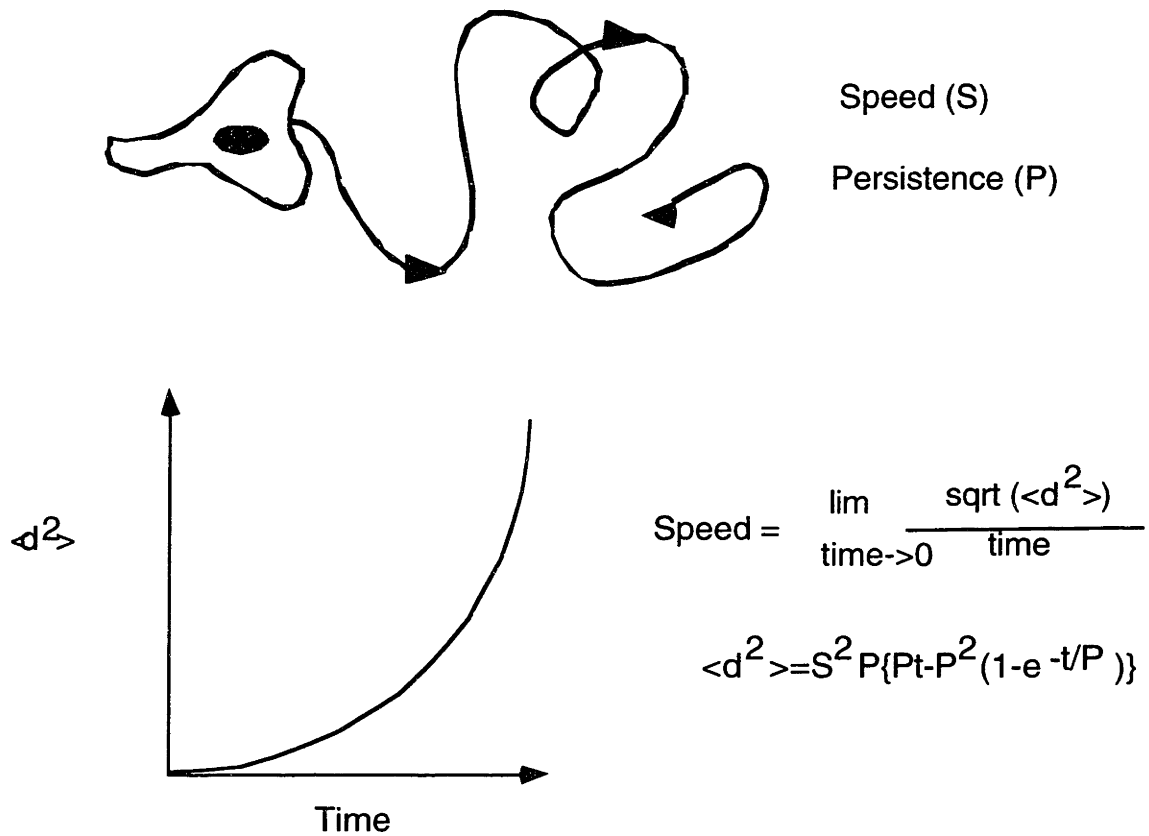


Figure 2.7 Analysis of cell motion.

A typical cell path may be modelled as a persistence random walk. A plot of average squared displacement versus time of a typical cell path may be fitted to obtain the values of the parameters of cell speed and persistence. Alternately, cell speed may be obtained independently using the root mean squared displacement of the cells and this value used while fitting the persistence random walk model to obtain the value of persistence time.

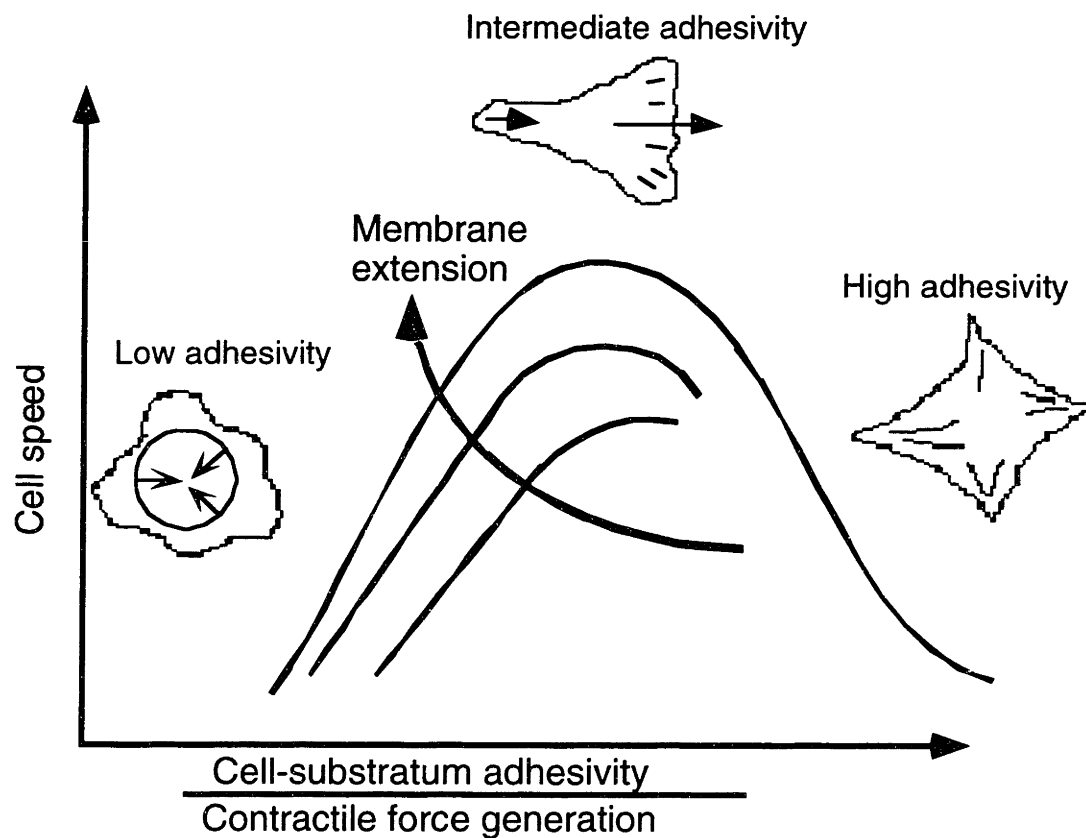


Figure 2.8 Biophysical regulation of cell motility.

Cell speed has a bi-phasic dependence on the ratio of cell-substratum adhesion and contractile force generation within the cell. When the cell is very weakly adhered on the substrate, it is unable to generate sufficient traction for locomotion. When the cell is very strongly adhered on the substrate, it is limited by the amount of contractile force that is generated within the cell, i.e., the cell is unable to break existing linkages to form new ones. It is only at intermediate values of this ratio that the cell is able to locomote with significant cell speeds.

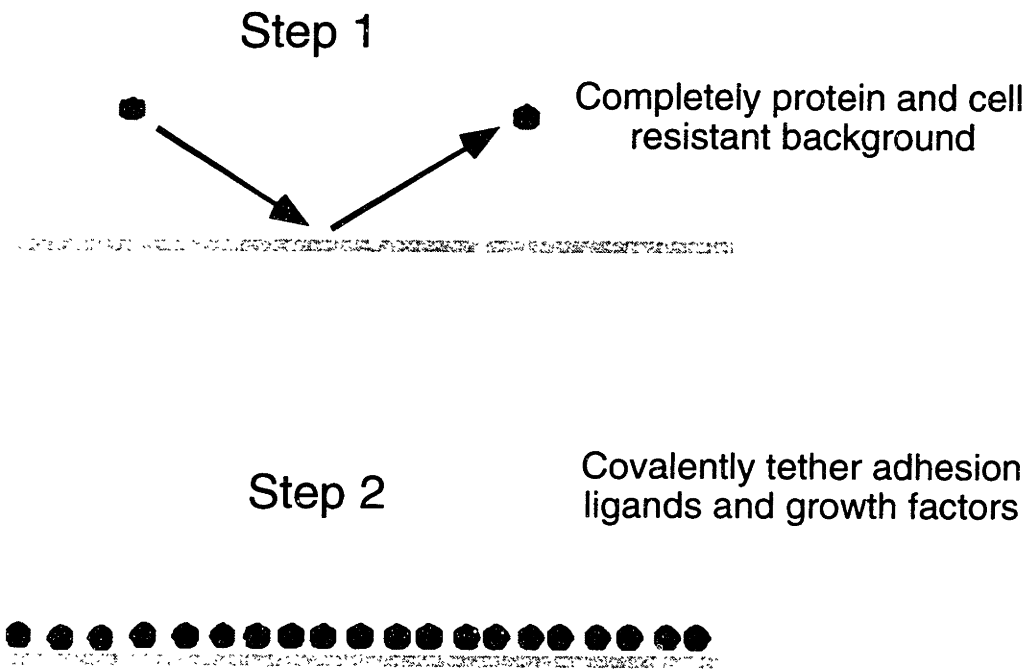


Figure 2.9 Design of surfaces which support a given cell function.
 A two step approach has been adopted, with first rendering the surface completely cell and protein resistant. Such an inert surface will then be rationally modified by adhesion ligands, growth factors and other molecules involved in influencing cell function. Parameters such as ligand density and spatial distribution will be optimized to obtain the desired response.

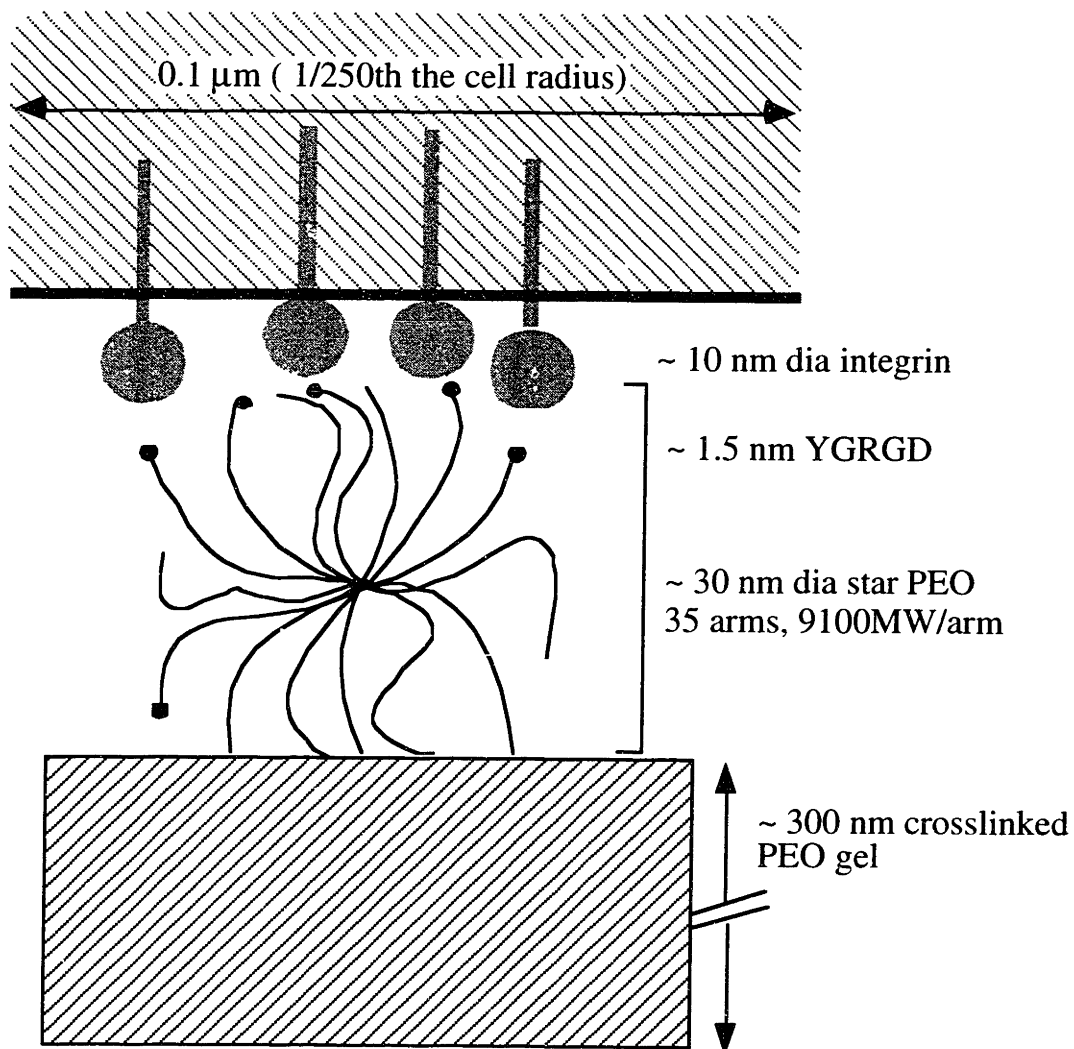


Figure 2.10 Schematic of star PEO system used drawn to scale.
 The star PEO molecule is covalently attached to a 300 nm thick radiation crosslinked PEO hydrogel using EDC-sulpho NHS chemistry. Assuming a hard sphere packing model of integrins onto a star PEO hemisphere, approximately 14 integrins may be packed onto a single star without steric hinderence.

Chapter 3

Fibronectin and EGF mediated regulation of cell speed

3.1 Introduction

As described in Section 2.4, there is significant overlap in the signaling molecules activated upon integrin and growth factor receptor occupation. In this set of studies, we wanted to determine whether there was an integration of effects of growth factors and ECM proteins in their regulation of cell motility. It was envisaged that results from this study would provide a rationale for the design of the minimalistic bioartificial matrix which specifically supports migration. As a model growth factor, we have used the epidermal growth factor (EGF), and Fibronectin as a model ECM protein.

Cell movement requires coordination of underlying biophysical processes including membrane extension and retraction, formation of new attachments at the cell front, generation of contractile force, and detachment of old attachments at the cell rear (Sheetz, 1994; Lauffenburger & Horwitz, 1996). It remains as yet largely unexplored as to how these processes are coordinated and regulated as an integrated system. A mathematical model has been constructed incorporating the key biophysical processes listed above, with some details of how they might depend on molecular-level properties (Lauffenburger, 1989; DiMilla et al., 1991). Experimental studies, both *in vitro* (Duband et al., 1991; DiMilla et al., 1993; Wu et al., 1994; Palecek et al., 1996; Huttenlocher et al., 1996; Condic & Letourneau, 1997) and *in vivo* (Ho et al., 1997), have been found to be consistent with predictions of this model concerning the effects of parameters characterizing

interactions of adhesion receptors and extracellular matrix ligands. But a simultaneous examination of the multiple biophysical processes is required to understand the integration of the external signals.

Growth factor and ECM effects on migration may operate by influencing cell-substratum adhesiveness, membrane activity, and/or contractile force generation (Lauffenburger & Horwitz, 1996). Growth factors such as EGF and PDGF enhance filopodia formation via *cdc42* (Kozma et al., 1995; Nobes & Hall, 1995). They also stimulate short term lamellipodial extension and membrane ruffling which requires activation of *rac* (Ridley & Hall, 1992). In addition to affecting adhesiveness (Palecek et al., 1997), integrin binding to ECM may influence membrane activity and contractile force generation through signaling intermediates such as mitogen-activated protein kinase (MAPK) (Klemke et al., 1997; Lin et al, 1997). These types of studies provide insight into the components involved in regulation of motility by growth factors and ECM. An essential next step is the investigation of how this regulation at the molecular level is coordinated through effects on the biophysical processes that integrate to yield locomotion.

We initially address this integration and coordination of regulation by examining how the effects of EGF and the ECM protein fibronectin (Fn) on fibroblast locomotion speed operate through combined changes in membrane activity and cell/substratum adhesion. We use NR6 murine fibroblast cells, a 3T3- derived cell line, transfected with wild type human EGFR (WT NR6 cells) as a well-characterized immortalized but nontransformed fibroblastic cell line, with EGF and Fn as well-characterized representatives of growth factor and ECM stimuli. We find that the effect of EGF on cell speed depends on the surface Fn concentration, with EGF able to either enhance or diminish locomotion. EGF reduces the strength of the cell-substratum adhesion at all Fn concentrations implying that adhesion effects alone do not fully account for the cell speed changes. EGF also affects

membrane extension activity -- similarly dependent on the surface Fn concentration but not precisely in parallel to its effects on cell speed. EGF diminishes, then enhances and finally has no effect on membrane extension activity with increasing Fn concentrations. For maximal migration, cells require both high membrane activity and optimal, intermediate cell-substratum adhesion permitting not only attachment at the cell front but also detachment at the cell rear. EGF and Fn together influence each of these processes, so that their net overall effect arises from the integration of their individual effects on each of the biophysical processes.

3.2 Experimental Design

3.2.1 Choice of Cell line

We have used wild type NR6 cells, a murine fibroblastic cell line which lacks endogenous EGF receptor and has been transfected with human EGFR as described previously. Briefly, the EGFR construct was cloned into the gag position of a Moloney MLV-derived vector using an SV40 early promoter-driven neomycin phosphotransferase gene in the env position as the selectable marker. The cells were cultured under a neomycin selection pressure (G418 350 $\mu\text{g/ml}$) to select for stable transfectants. The cells express physiological levels of the EGFR ($R_{\text{total}} \sim 100,000/\text{cell}$). This cell line provides an excellent system for the study of EGF effects since the expression of EGFR on these cells and the signaling pathways initiated upon EGFR activation have been extensively studied and well characterized. We also confirmed that these cells expressed $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins which are known to bind to the RGD sequence on fibronectin. Since NR6 cells lack endogenous EGFR, they can be transfected with variants of EGFR for further studies with mutant receptors if desired.

3.2.2 Choice of ECM and growth factor

Fibronectin was used as a model ECM protein since it has a wide tissue distribution and cell surface integrin receptors have been identified which bind to its specific sequences. Most importantly, the tri-peptide sequence, arg-gly-asp (RGD) was first identified from the cell binding domain of fibronectin and has since been studied extensively. Since in the later part of the study (Chapter 4), the RGD sequence would be used as an adhesion ligand, it was felt that fibronectin would serve as a good model ECM protein. In addition, since we were interested in determining the effect EGF on cell motility, fibronectin is a good choice since it does not contain any EGF-like domains which might be involved in interactions with the EGF receptors on the cell surface. Fibronectin was adsorbed onto glass surfaces in the range of concentrations ranging from 0.1 $\mu\text{g/ml}$ to 3 $\mu\text{g/ml}$ since earlier studies had shown that the FN adsorption isotherm was linear up approximately 10 $\mu\text{g/ml}$ FN coating concentrations. Four FN coating concentrations separated approximately equidistant on a logarithmic scale were chosen for this study.

The EGF-EGFR system was used as a model growth factor – receptor system since EGF is known to be motogenic and is ubiquitously expressed in fibroblasts. Also, there is a single isoform of EGFR expressed on the cell surface which interacts with EGF and hence simplifies studies involving receptor-ligand dynamics. EGF was used in the soluble form at a concentration of 25 nM which is approximately 10 times its K_D value to avoid growth factor depletion and in order to operate in a growth factor saturating regime. In addition, in order to compare our results with those reported by Ware et al where a concentration of 25 nM EGF had been used to study the motility of the NR6 cells on Amgel, we decided to continue using EGF at a concentration of 25 nM.

3.2.3 Choice of experimental methods

It was decided to plate cells in serum-free conditions initially in order to quiesce the cells and minimize the alteration of the underlying substrate by proteins usually present in serum. The medium was then changed to one containing HEPES buffer at 25 mM concentration (assay medium) in order maintain the pH in humidified air (in the absence of high CO₂ concentration). The assay medium also contained 1% dialyzed FBS in order to provide the cell with a minimum amount of serum proteins for long-term survival.

Cell-substratum adhesion strength was measured using the centrifugation assay as opposed to the flow chamber assay due to higher substrate requirements in the latter. The studies described in this chapter were to be compared with investigations on RGD-modified surfaces. Since the surface area of the RGD-modified surfaces was limited by the cost of the material, we decided to use the centrifugation assay since it requires fewer surfaces and surfaces of smaller sizes than those used in the flow chamber assays.

3.3 Materials and Methods

3.3.1 Cell culture

The generation of WT NR6 cells, a 3T3-derived murine fibroblastoid cell line lacking endogenous EGF receptor (EGFR), transfected with wild type human EGFR, has been described previously (Chen et al., 1994a; Pruss & Herschman, 1977). WT NR6 cells were cultured in MEM- α media supplemented with fetal bovine serum (FBS: 7.5%), penicillin (100 U/ml), streptomycin (200 mg/ml), non-essential amino acids (1 mM), sodium pyruvate (1 mM), glutamine (2 mM) and G418 (350 μ g/ml). Cells were passaged at subconfluence by trypsinization (0.25%, 1 mM EDTA) and incubated at 37 °C, 90% humidity and 5% CO₂. Assay medium used while performing the migration, adhesion, and membrane extension activity assays contained MEM- α with HEPES (25 mM), 1 gm/l BSA, 1% dialyzed FBS, penicillin (100 U/ml), streptomycin (200 mg/ml), non-essential amino acids (1 mM), sodium pyruvate (1 mM), glutamine (2 mM) and G418 (350 μ g/ml).

FACS analysis with the appropriate antibodies demonstrated the expression of the Fn receptors $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins on these cells (data not shown).

3.3.2 Surface preparation and substratum coating

Glass coverslips were acid-washed in 20% HNO₃ for 1 hr, rinsed with deionized water for 1 hr and silanized by exposure to hexamethyldisilazane vapor (Sigma Chemical Co.) for 30 min. at 200 °C. 18-mm diameter coverslips were used for migration and membrane extension activity assays, and 12-mm diameter coverslips were used for the adhesion assays. The coverslips were attached to the bottom of culture dishes using an optically clear adhesive (Norland Chemicals). Surfaces were then coated with Fn (Sigma Chemical Co.). 35-mm dishes for migration and membrane extension activity assay were coated with 2 ml of appropriate concentration of Fn in PBS and incubated at room temperature for 2 hr. Non-specific protein adhesion was blocked by subsequent incubation in 1% BSA for 1 hr. Dishes were washed thrice with PBS and stored at 4 °C. This protocol was also used to coat 24-well plates for the adhesion assay while maintaining a constant surface area to volume ratio.

3.3.3 Migration assay

Cell migration speed was measured using time-lapse videomicroscopy of single cells. 30,000 cells were plated onto 35-mm dishes in 2.5 ml serum free medium. 12 hr. post-seeding the medium was changed to 2.5 ml assay medium with or without 25 nM EGF and incubated at 37 °C in humidified air for 8 hr. At this cell density, soluble ligand concentration is relatively unchanged over a 24 hr period (Reddy et al., 1996). 3 ml mineral oil was added to the dish to prevent evaporation, and the dish was then placed in a heated stage insert for a Ludl 99S008 motorized stage on a Zeiss Axiovert 35 microscope. Cell boundaries and centroids were identified using image processing software developed by Engineering Technology Center (Mystic, CT) running under a LabVIEW (National

Instruments, Austin TX) and Concept Vi (Mystic, CT) environment. 5-10 cells per field in 10 different fields were scanned every 15 minutes for up to 20 hr. The x and y coordinates of the cell centroids were recorded every 15 minutes. Single cell speed is calculated by determining the total path length as measured by the total centroid displacement divided by the tracking time. The reported cell speed \pm s.e.m. for each condition is an average over 70-100 cells. For purposes of testing transient effects, cell speeds were calculated every 15 minutes by quantifying the centroid displacement every 15 minutes over a 20-hour period starting immediately after addition of EGF, or in the control case, immediately after the medium was changed to the assay medium; the cells had been incubated in serum-free conditions for 12 hours prior to addition of EGF. As will be presented below in the Results section, cell speed increased toward a plateau for 6-8 hours following addition of EGF. Hence all subsequent migration measurements were carried out following an 8-hour incubation period.

3.3.4 Adhesion assay

The adhesion assay was performed as previously described (Chu et al., 1994). Briefly, 24-well plates were plated with 20,000 cells per well in serum-free conditions for 12 hr. The medium was then changed to the assay medium with or without 25 nM EGF. Short-term adhesion with EGF was measured 30 min after addition of EGF. Adhesion was also measured at times comparable to the migration assay, 8 hr after addition of EGF. The wells were filled with medium and sealed using sealing tape avoiding air bubbles. The plates were then inverted and spun in a swing bucket SH-3000 rotor in a bench top Sorvall centrifuge for 10 min. at 25 °C at 400, 600 or 800g. During each experiment, one plate at 3 μ g/ml Fn coating concentration without EGF was kept at 1g and used as a control. Cell number was quantified by manually counting cells in a defined well area. At least 4 wells were used at each condition and 4 fields were counted per well with each field in the control containing 300-400 cells. The cell number per well was normalized to the average cell

number in the control well to obtain fraction adherent cells. The amount of centrifugal force required to detach 50% of the cells (F_{50}) was obtained from a plot of fraction adherent cells versus centrifugal force. The mean detachment force was calculated using the equation, $f = RCF * V * (\rho_c - \rho_m)$, where f is the force on a cell, RCF is the relative centrifugal force, V is the cell volume, ρ_c is the density of the cell, and ρ_m is the density of the medium.

3.3.5 Membrane extension assay

Incubation protocol followed was identical to that described for the migration assay. The cells were videotaped using a 32x objective. Cell outlines were obtained every 15 minutes for a 1 hour period. The protrusion area was defined as the additional area extended by the cell at time $t=t_n + 15$ minutes when overlaid on the cell outline at time $t = t_n$. An average of 4 such areas over an hour divided by 15 minutes was defined as the absolute protrusion rate. Fractional protrusion activity was defined as the average rate of change of cell protrusion area normalized to the average cell area. The retraction area was defined as the area which was retracted by the cell at time $t = t_n + 15$ minutes when overlaid on the cell outline at time $t = t_n$. Similarly, fractional retraction activity was also measured by calculating the absolute retraction rate divided by the average cell area. 20-30 cells were analyzed at each condition.

3.4 Results

3.4.1 EGF-stimulated cell migration requires a 6-8 hour induction period for maximal migratory response

WT NR6 cells exhibit increased membrane activity, reorganization of the actin cytoskeleton and disassembly of focal adhesions within 10 minutes of exposure to EGF (Xie et al., 1998). However, with prolonged exposure to EGF, these phenomena are less dramatic, and it has not been previously established how these changes correlate with the long-term

motility response to EGF. We have previously observed that mean-squared displacements of WT NR6 cells measured every 30 minutes in the presence of EGF on Amgel, a biologically active extracellular matrix, increase with time of exposure to EGF up to a maximal value (Ware et al., 1998). Hence, we decided to examine whether the effect of EGF on cellular motility on fibronectin is dependent on the time of exposure to EGF. Cells were plated on 1 $\mu\text{g/ml}$ Fn and incubated in serum-free medium for 12 hours before addition of EGF. We quantified cell speed over a 20 hour period after addition of EGF. Cell speed was calculated every 15 minutes by determining the change in cell centroid position every 15 minutes. The cell speed measured immediately after addition of EGF was lower than that in its absence. However, while the cell speed in the absence of EGF remained constant at its initial value of 18 $\mu\text{m/hr}$, cell speed in the presence of EGF increased steadily over time for the first ~400 minutes (~7 hours) after which it remained at its average maximal value of 40 $\mu\text{m/hr}$ for the rest of the course of the experiment (Figure 1). This indicates that there is an induction time of about 6-8 hours before a maximal migratory response is obtained in this cell type. Hence all subsequent cell migration measurements with EGF were performed after the cells had been exposed to EGF for 8 hours. For consistency, speed measurements in the absence of EGF were also performed following an 8-hour incubation.

3.4.2 EGF regulation of cell speed is dependent on the surface fibronectin concentration

Since haptokinetic and haptotactic motility are modulated by substratum density (Aznavoorian et al., 1996; Dickinson & Tranquillo, 1993; Keely et al., 1995), we investigated whether the effect of EGF on the motility was also dependent on the surface concentration of Fn. Cell speed was measured at varied surface Fn concentrations in the presence of saturating concentration of EGF and in the absence of EGF. The average cell speed of WT NR6 cells in the absence of EGF is roughly constant, independent of Fn

coating concentration, at approximately 18 $\mu\text{m/hr}$ (Figure 2). However, in the presence of EGF, cell speed demonstrated a biphasic dependence on the surface Fn concentration, with maximal cell speed of 42 $\mu\text{m/hr}$ occurring at an intermediate Fn concentration of 1 $\mu\text{g/ml}$. At the lowest Fn concentration of 0.1 $\mu\text{g/ml}$, cell speed in the presence of EGF actually was drastically reduced to 0.1 $\mu\text{m/hr}$ as compared to 16 $\mu\text{m/hr}$ in the absence of EGF. At Fn concentrations of 0.3 and 3 $\mu\text{g/ml}$, cell speeds in the presence of EGF were increased, though the increase was not as great as that observed at 1 $\mu\text{g/ml}$ Fn concentration. Thus, EGFR-mediated cell motility is strongly dependent on Fn concentration, suggesting an interaction of the growth factor-stimulated pathways for cell motility regulation and those initiated upon ECM binding. Qualitatively similar results have been reported in a study of migration of this cell type under the influence of EGF on varying concentrations of Amgel (Ware et al., 1998).

3.4.3 EGF reduces cell adhesion at all surface fibronectin concentrations

In theoretical models, cell speed is predicted to depend on biophysical processes such as cell-substratum adhesion, contractile force generation, and membrane extension activity (Lauffenburger, 1989; DiMilla et al., 1991). Palecek et al. (1997) demonstrated that the variation of CHO cell speed with cell-substratum affinity, integrin level, and ECM substratum concentration could be explained by a variation in the strength of the cell-substratum adhesion. It has also been reported that short-term EGF exposure of WT NR6 cells causes a dramatic change in their morphology resulting in decreased cell spread area and disassembly of focal adhesions (Ware et al., 1998; Welsh et al., 1991; Xie et al., 1998). Hence, we investigated whether the variation in EGF-stimulated migration on Fn could be explained by a variation in the cell-substratum adhesivity. The centrifugal force required to detach the cells is a measure of the strength of the cell-substratum adhesivity; the greater the F_{50} (see 'Materials and Methods' section), the stronger is the cell-substratum

adhesivity. The cell adhesivity both in the presence and absence of EGF increased with increasing Fn coating concentration (Figure 3). At each concentration of Fn, cell adhesion dropped substantially by 30 minutes after addition of EGF. By 8 hr after addition of EGF, the cell adhesivity had risen to a value which was still significantly lower than that in the absence of EGF. Decreased adhesivity was most dramatic at the lowest Fn concentration of 0.1 $\mu\text{g}/\text{ml}$, where the cell-substratum adhesivity did not rise significantly even 8 hours after addition of EGF following the 4 fold decrease in F_{50} 30 minutes after addition of EGF.

In order to determine how locomotion speed in the presence and absence of EGF correlate with cell adhesivity, speed was determined as a function of the mean detachment force at each experimental condition by eliminating the Fn concentration as a common variable (Figure 4). We observed that cell speed in the absence of EGF is not a function of cell adhesivity in the measured range of Fn concentration. However, under the influence of EGF, cell speed exhibits a biphasic dependence on cell adhesivity. Upon addition of EGF at 0.1 $\mu\text{g}/\text{ml}$ Fn concentration, the cell adhesivity dropped indicating that cells are so weakly adhered that they are unable to generate sufficient traction for locomotion. Cell morphology appeared rounded in the presence of EGF at this lowest surface Fn concentration, in contrast to well-spread cells in the absence of EGF (not shown). When the Fn concentration is increased to 3 $\mu\text{g}/\text{ml}$ in the presence of EGF, locomotion speed decreased from its maximal value of 42 $\mu\text{m}/\text{hr}$, indicating that the highly-adherent cells now are unable to generate sufficient contractile force to break cell-substratum bonds for detachment. At the Fn concentrations considered here, cell adhesion in the absence of EGF did not reach either extreme of cell-substratum adhesion levels where locomotion is completely inhibited. If adhesion was the sole physical process regulating cell speed in this situation, we would expect the plots of locomotion speed versus adhesivity in the presence and absence of EGF to collapse onto a single curve (Palecek et al., 1997). Instead, we

observe that EGF reduces adhesion at all Fn concentrations, but that along with this reduction in adhesion an increase occurs in the maximal locomotion speed in the presence of EGF. Hence, the changes in cell speed induced by EGF can be explained only in part by effects on Fn-mediated adhesion.

3.4.4 Fractional membrane protrusion and retraction activity varies with surface fibronectin concentration in the presence of EGF but not in its absence

Since adhesion alone cannot fully explain the effects of EGF and Fn on WT NR6 motility, we investigated their effects on the cell membrane activity. In order to further understand the increase in maximal cell speed in the presence of EGF, we quantified the average membrane protrusion and retraction activity in the presence and absence of EGF on varied surface Fn concentrations. Cell spread area was also quantified under all the above conditions. The cell spread area increased with increasing surface Fn density in the presence as well as the absence of EGF, although the cell area in the presence of EGF was lower than that in its absence at each of the Fn concentrations. At intermediate concentrations of 0.3 and 1 $\mu\text{g/ml}$, the absolute protrusion rate was significantly greater in the presence of EGF than that in its absence. At the lowest Fn concentration of 0.1 $\mu\text{g/ml}$ EGF drastically reduced the absolute protrusion rate. However at 3 $\mu\text{g/ml}$ of Fn, EGF had no significant effect on the absolute protrusion rate. Identical trends were observed in the absolute retraction rates (Table 1).

In order to gain better insight into the effect of EGF on membrane activity, the absolute protrusion and retraction rates were normalized to the respective cell spread areas to account for the EGF and Fn effects on spreading (see 'Materials and Methods' section). The fractional protrusion activity was essentially independent of Fn concentration in the absence of EGF (Figure 5). However, in the presence of EGF, the fractional protrusion activity

was the highest at Fn concentrations of 0.3 and 1.0 $\mu\text{g/ml}$ and was also significantly greater than that in the absence of EGF. At 0.1 $\mu\text{g/ml}$ Fn, EGF significantly decreased the fractional protrusion activity. There was no observable effect on activity in the presence and absence of EGF at the highest Fn concentration of 3 $\mu\text{g/ml}$. Fractional retraction activity was similar to the fractional protrusion activity (Table 1). Hence, we find that EGF alters membrane activity of these cells in a manner which again depends upon Fn concentration. However, it is important to note that the variation of membrane activity does not precisely parallel the variation of locomotion speed with Fn concentration in the presence of EGF. At 0.3 $\mu\text{g/ml}$ Fn, membrane activity is enhanced by EGF but locomotion speed is not. This discrepancy is significant for interpretation of the rate-limiting steps of migration, as will be discussed below.

3.5 Discussion

Cell migration is controlled by the concerted action of both growth factor receptors and integrins. We analyzed this integrated regulation at the level of underlying biophysical processes which govern cell movement, beginning with membrane protrusion activity and cell-substratum adhesion. Previously, there has been little information concerning how growth factors and extracellular matrix coordinately influence the biophysical processes that integrate to govern migration.

We find that sustained cell speed in the presence of EGF is a function of Fn concentration with the maximal cell speed occurring at intermediate levels. A surprising result is that, at low Fn coating concentration, addition of EGF reduces cell speed drastically to levels well below that in the absence of EGF. At the same time, our centrifuge assay showed that EGF decreases adhesion both acutely and at the longer time scales relevant to migration. It is important to note again that more extensive experimental protocols than are typically employed for cell migration and adhesion studies are necessary in order not to miss these

effects. For instance, if comparisons of cell locomotion speed in the absence and presence of EGF had been made here at only a single ECM concentration, we would have been led to an incomplete understanding of its effect. Similarly, if adhesion had been measured here by a simple washing assay alone in which minimal distractive forces are applied, we would have incorrectly concluded that EGF had no effect on adhesivity.

Because EGF did reduce cell-substratum adhesivity, we might have initially speculated that EGF induces motility by reducing adhesion to substratum, with a threshold being required for motility (Xie et al., 1998). However, upon analyzing cell speed as a function of adhesivity (Figure 4), it is clear that a change in adhesion alone cannot explain the variation in cell speed with concentration in the presence of EGF. Hence, we also quantified the membrane extension activity to determine whether this is an additional biophysical phenomenon which helps govern locomotion speed. We find that EGF does indeed increase the absolute membrane activity at intermediate concentrations and decreases the cell spread area at all the concentrations studied. The trends and values of the absolute protrusion rate and the absolute retraction rate are identical, indicating that overall cell spread area is maintained at a constant level during the course of cell body translocation despite noticeable changes in cell shape. This is consistent with previous reports of a relationship between events at the cell front and rear (Chen, 1979; Weber et al., 1995). Fractional protrusion and fractional retraction (*i.e.*, membrane activities normalized to spread area) are significantly elevated in the presence of EGF at intermediate concentrations, though their levels decrease at extremes of the substratum density.

Analyzing locomotion speed as a function of membrane activity by eliminating concentration as an independent variable, permits elucidation of how membrane activity and adhesivity integrate to yield locomotion (Figure 6). Locomotion speeds in the absence of EGF do not vary with fractional membrane protrusion activity. However, in the presence

of EGF locomotion speed is affected by fractional membrane protrusion activity, but differently within three sequential adhesivity regimes. At the lowest concentration, speed was the lowest along with the lowest membrane activity and adhesivity. At the slightly higher concentration of 0.3 $\mu\text{g/ml}$, speed increased with a concomitant increase in fractional membrane protrusion activity. When the concentration was further increased to 1.0 $\mu\text{g/ml}$, speed increased to its maximum value without a significant change in the fractional membrane protrusion activity -- suggesting that at 0.3 $\mu\text{g/ml}$ the cell speed had been limited by insufficient adhesive traction at the cell front. However, at the highest concentration, locomotion speed decreased accompanied with a decrease in fractional membrane protrusion activity, probably due to an inability of cells to detach dynamically from the substratum; alternatively, high concentrations may generate signals suppressing membrane activity.

A different angle from which to illustrate the mechanisms by which EGF and Fn coordinately govern locomotion is seen by analyzing speed as a function of adhesion strength, again eliminating concentration as an independent variable (Figure 7). We find that there is no significant variation in either speed or membrane activity in the absence of EGF (Figure 7a). However, both speed and membrane activity vary with adhesion strength in a biphasic manner in the presence of EGF (Figure 7b). At the low adhesion condition of 0.1 $\mu\text{g/ml}$, both membrane activity and locomotion speed are lower in the presence than in the absence of EGF, likely due to the inability of membrane protrusions to form stable attachments with the substratum. At 3 $\mu\text{g/ml}$, both membrane activity and locomotion speed are roughly similar in the presence and absence of EGF, probably due to the inability of cells to detach dynamically under this strong adhesion condition or alternatively due to signals suppressing membrane activity. At 0.3 $\mu\text{g/ml}$, however, membrane activity in the presence of EGF is highly stimulated while locomotion speed is not significantly increased. This discrepancy may be caused by an ability of extended

membrane to form a stable attachment but one that is not sufficiently strong in traction for contractile forces to give rise to cell body translocation. At the condition of 1 $\mu\text{g/ml}$, though, adhesivity has increased enough for this translocation to occur.

The increase in membrane extension activity with EGF at certain concentrations is consistent with the increased membrane ruffling and filopodia formation (Segall et al., 1996) accompanied with increased activity of rac and cdc42, respectively (Ridley & Hall, 1992). The initial decrease in adhesion upon addition of EGF is expected based on the observation that EGF causes short term disassembly of focal adhesions and loss of stress fibers and results in a rounded cell morphology (Welsh, et al., 1991; Xie et al., 1998). It remains to be determined exactly what causes this decrease in effective adhesion; possible candidates include integrin downregulation, affinity decrease in integrin-ECM interactions, induced proteolysis of adhesion components, or increased contractile force generated within the cell. We quantified $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin expression in the absence of EGF, 30 minutes after exposure to EGF and 8 hours after exposure to EGF and found no variation in their expression (data not shown): this strongly suggests that downregulation of at least $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins is not involved. Future work will define the cell signals that alter integrin function to accomplish the biophysical process of membrane extension.

We note that a rigorous investigation of the time-dependence of growth factor-induced cell motility has not been reported. Knowing when cells start to move and how long it takes to reach maximal speed is required to link biochemical events to the physiological consequences. Activity of signaling pathways, often assayed within minutes of growth factor exposure, usually declines with prolonged EGF exposure (Chen, et al., 1994b; Waters et al., 1996). We found that despite the diminished membrane activity over time cell motility continued to increase significantly, not approaching maximal speed until 6-8 hours after EGF addition (Figure 1). Clear implications are that motility must be studied

either after an induction period or over an extended period of time of which the induction time is a small fraction, and that candidate causal signaling activities must be assayed during active motility, unless it is the acute activities -- which may be ultimately unrelated to sustained migration -- that are of central focus. The gradual increase in motility suggests cellular adaptation or reprogramming for maximal responsiveness to EGF. That cell locomotion can be noted even at the earliest time periods, however, does suggest that the basic response and motility machinery are in place and, at least, partially activatable. New protein synthesis or transcription of specific genes are speculated as being important for full response. This would be consistent with observations that low doses of actinomycin-D block cell motility (Bauer et al., 1992; Chen, et al., 1994a; Gordon & Staley, 1990).

The finding (Figure 2) that migration speed in the absence of exogenous EGF is essentially independent of coating concentration is an interesting finding in itself, although consistent with at least one recent report (Ware et al., 1998). This kind of situation has not been investigated in detail in previous literature. One reason is that studies of cell migration are typically performed in the presence of substantial levels of serum or a protein-containing medium even when not exploring effects of specific growth factors. Another is that studies of cell migration generally do not explore function over a wide range of substratum ligand concentrations. An intriguing possible explanation is that at low levels of soluble exogenous stimuli, the diverse effects of integrin-mediated signals (e.g., force generation (Klemke et al., 1997), affinity modulation (Hughes et al., 1997), and membrane activity (Lin et al., 1997) coordinate intracellularly to maintain a relatively constant balance of membrane extension activity, adhesion, and force generation.

We believe that our study provides new insight into the integration of signaling pathways initiated from growth factors and ECM through the biophysical readouts of cell migration, cell-substratum adhesion, and membrane activity. We suggest that the regulation of

locomotion by Fn and EGF overall can be understood in terms of membrane extension activity, attachment, and detachment as providing rate-limiting steps in sequential regimes of increasing cell/substratum adhesivity (see Figures 6 and 7). Membrane extension is limiting at lowest adhesivity, then membrane attachment for traction is limiting at intermediate adhesivity, and finally, membrane detachment is limiting at highest adhesivity. Whether other cellular factors such as contractile force, front-versus-rear asymmetry, and mechanical properties are additionally affected by synergistic regulation by EGF and Fn -- or, more generally, growth factors and ECM -- remains to be investigated, as they are also implicated in the theoretical models for cell migration (Lauffenburger, 1989; DiMilla et al., 1991). We believe that biophysical analysis analogous to that presented here offers a useful framework for understanding the action of various components involved in and regulated by biochemical signaling pathways.

3.6 References

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3.7 Figures

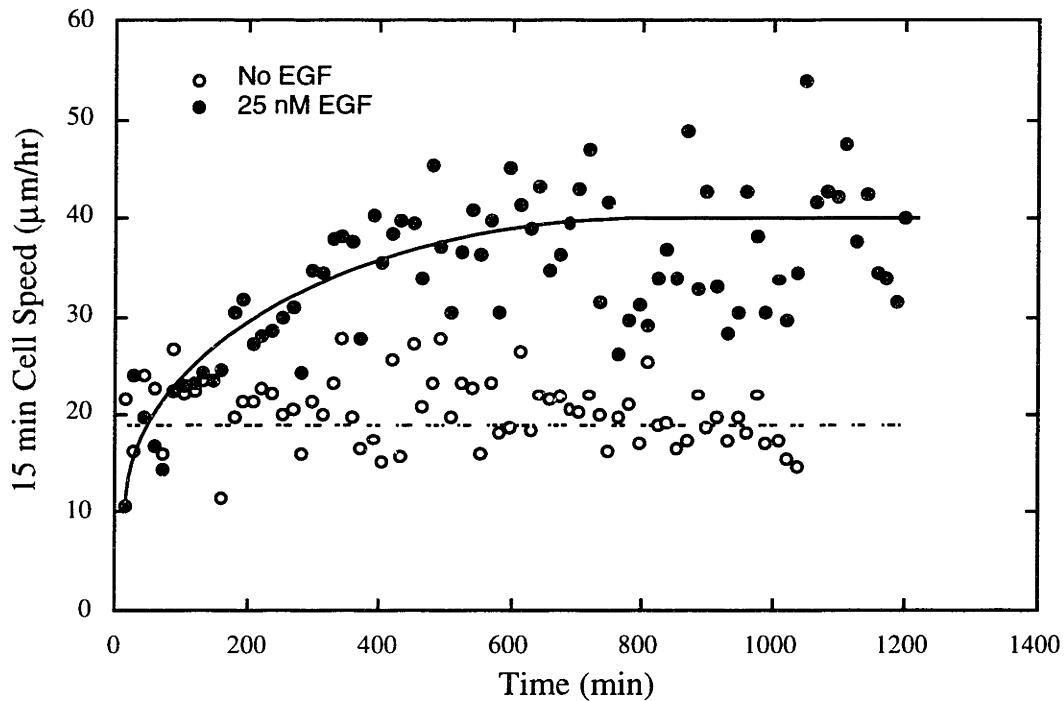


Figure 3.1 EGF-enhanced motility increases over an initial 6-8 hour period.

The effect of EGF on 15-minute cell speeds of WT NR6 cells was determined as a function of time on 1 µg/ml Fn coating concentration. Mean centroid displacements were calculated every 15 minutes immediately upon addition of EGF for 20 hours. Cells were plated in serum-free conditions for 12 hours before addition of EGF.

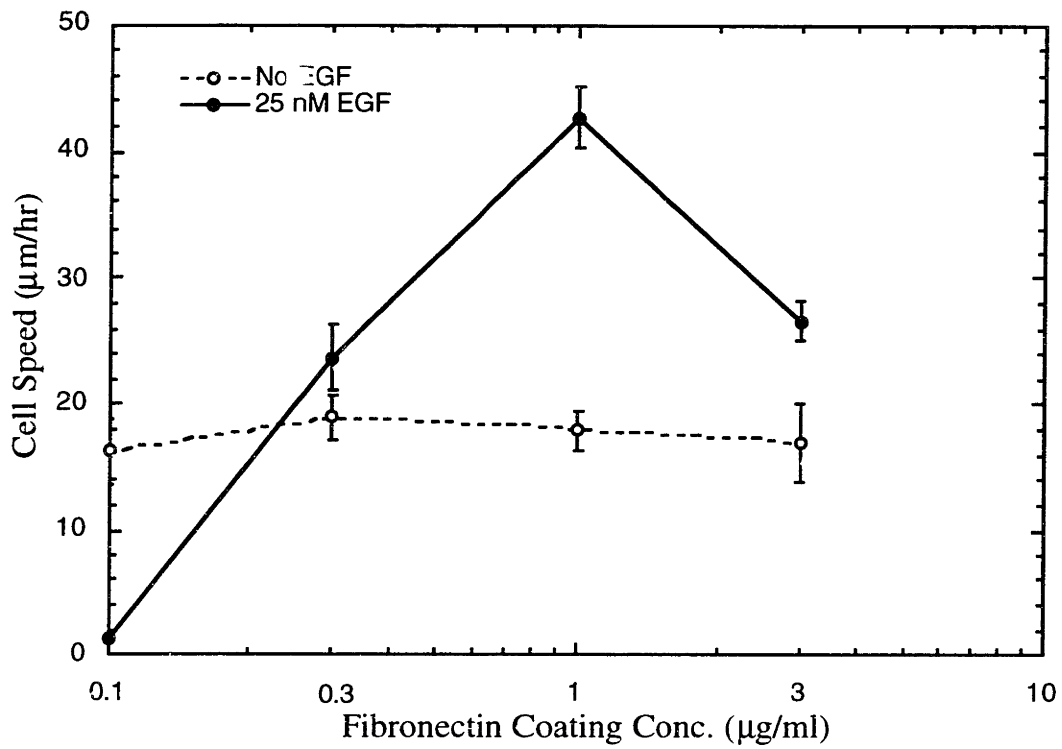


Figure 3.2 EGF differentially affects cell speed depending on fibronectin concentration.

Single cells were tracked for 12 hours after incubation with (filled circles) or without (open circles) EGF for 8 hours on varying surface concentrations of Fn. The cell speeds are an average of 70-100 cells at each experimental condition over a 12-hour time period.

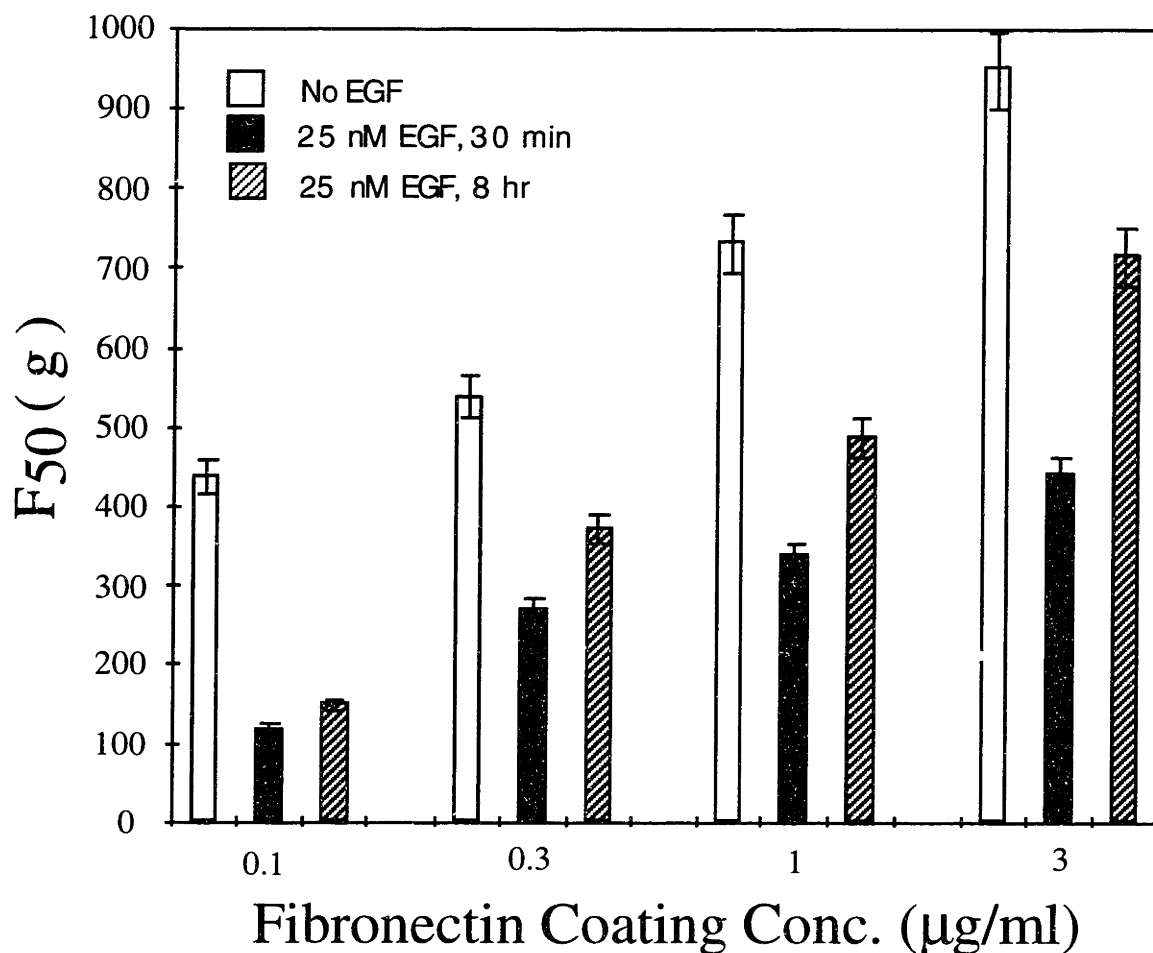


Figure 3.3 Cell adhesiveness is altered by both fibronectin concentration and EGF.

Cells were plated in serum free conditions on varying surface concentration of Fn in 24 well plates. In the absence of EGF, centrifugal force required to detach 50 % of the plated cells (F_{50} in g units) was determined 12 hours post plating (open bars). EGF was added 12 hours after plating and F_{50} was determined 30 minutes after addition of EGF (filled bars). Adhesion was also quantified after 8 hours of incubation with EGF (hatched bars). F_{50} was calculated from plots of fraction adherent cells as a function of centrifugal force (data not shown). These experiments were performed at centrifugation forces of 400g, 600g and 800g. Bars represent standard errors.

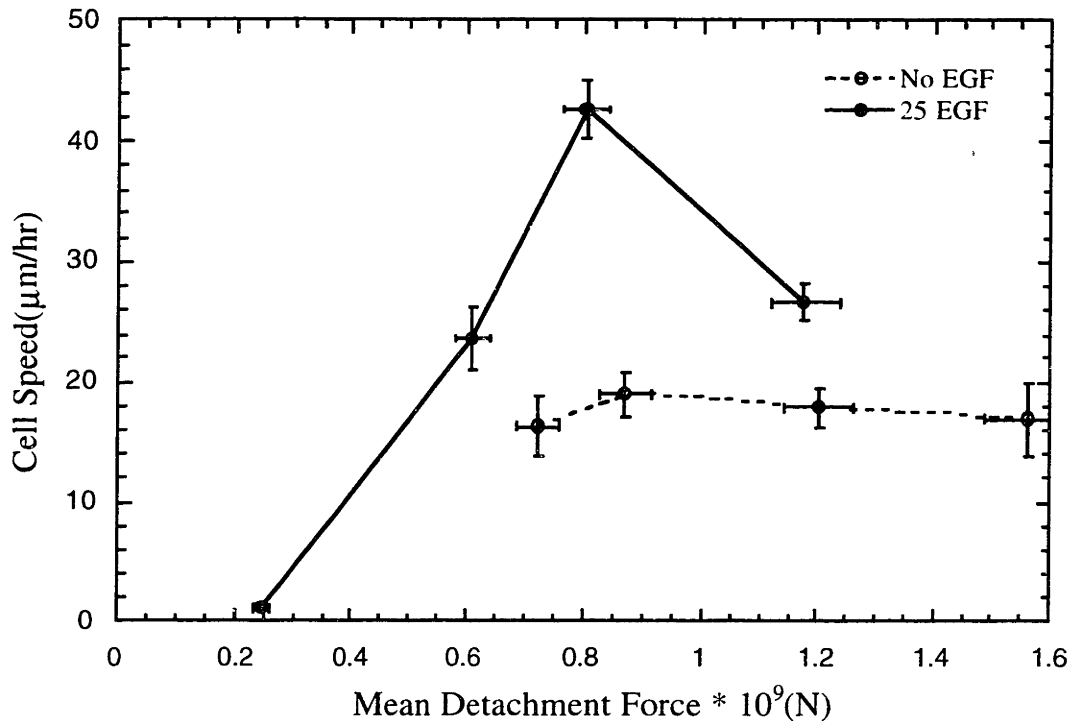


Figure 3.4 Cell speed as a function of mean detachment force.
 Data from Figures 3.2 and 3.3 are replotted by eliminating the F_n concentration as a common variable. Mean detachment force is calculated from values of F_{50} (see 'Materials and Methods' section). Open circles represent no EGF preincubation, filled circles represent 8 hour incubation in 25 nM EGF.

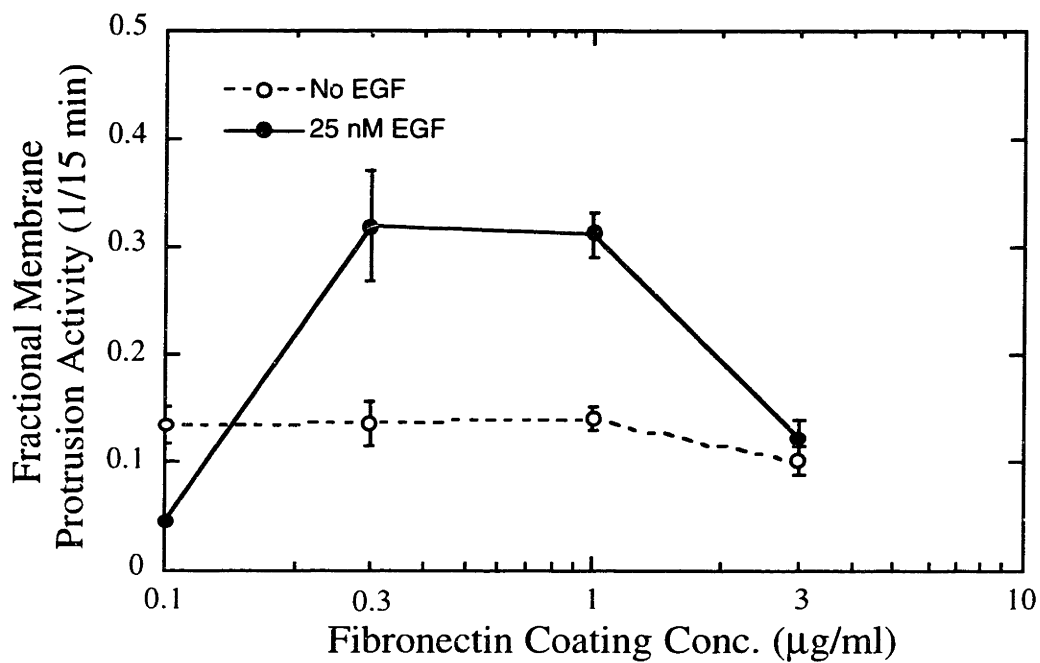


Figure 3.5 Membrane activity is altered by EGF depending on Fn concentration.

Fractional membrane protrusion activity was determined as a function of Fn coating density with (filled circles) and without (open circles) EGF. Fractional membrane protrusion activity was calculated by normalizing the average absolute protrusion rate (see Table 1) by the average cell area at each experimental condition. Bars represent standard errors.

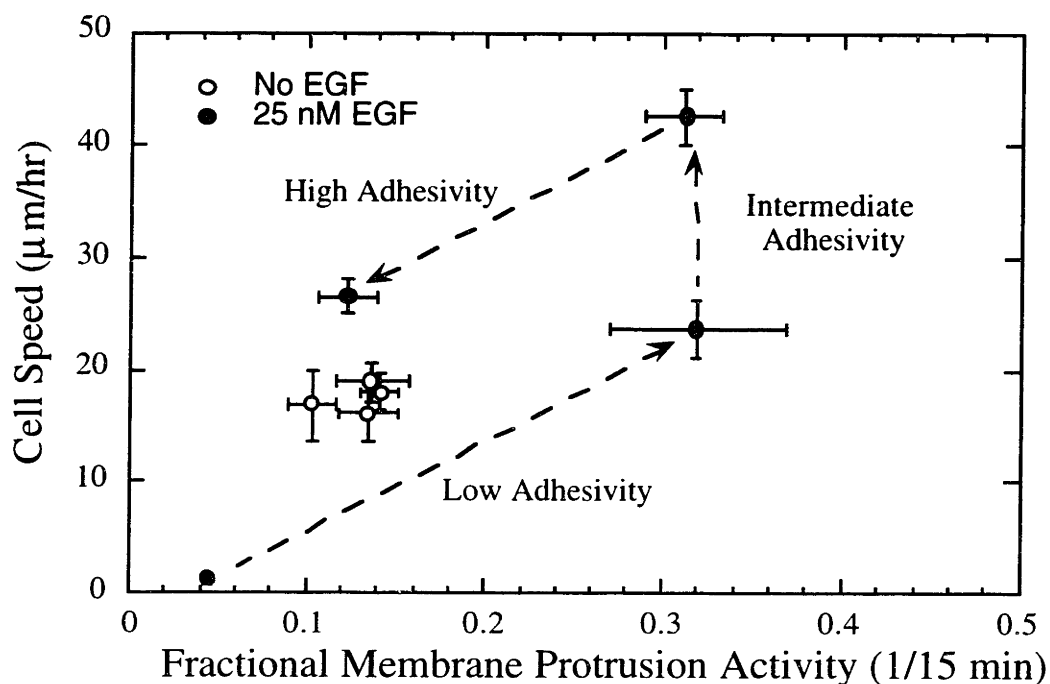


Figure 3.6 Cell speed can be considered a function of fractional membrane protrusion activity.

Data from Figures 2 and 5 were used to calculate the variation of cell speed with fractional membrane protrusion activity by eliminating the Fn concentration as a common variable. Cell speed in the absence of EGF (open circles) does not show significant variations with fractional membrane protrusion activity. Cell speed in the presence of EGF (filled circles) varies with fractional membrane protrusion activity through three regimes of adhesivity.

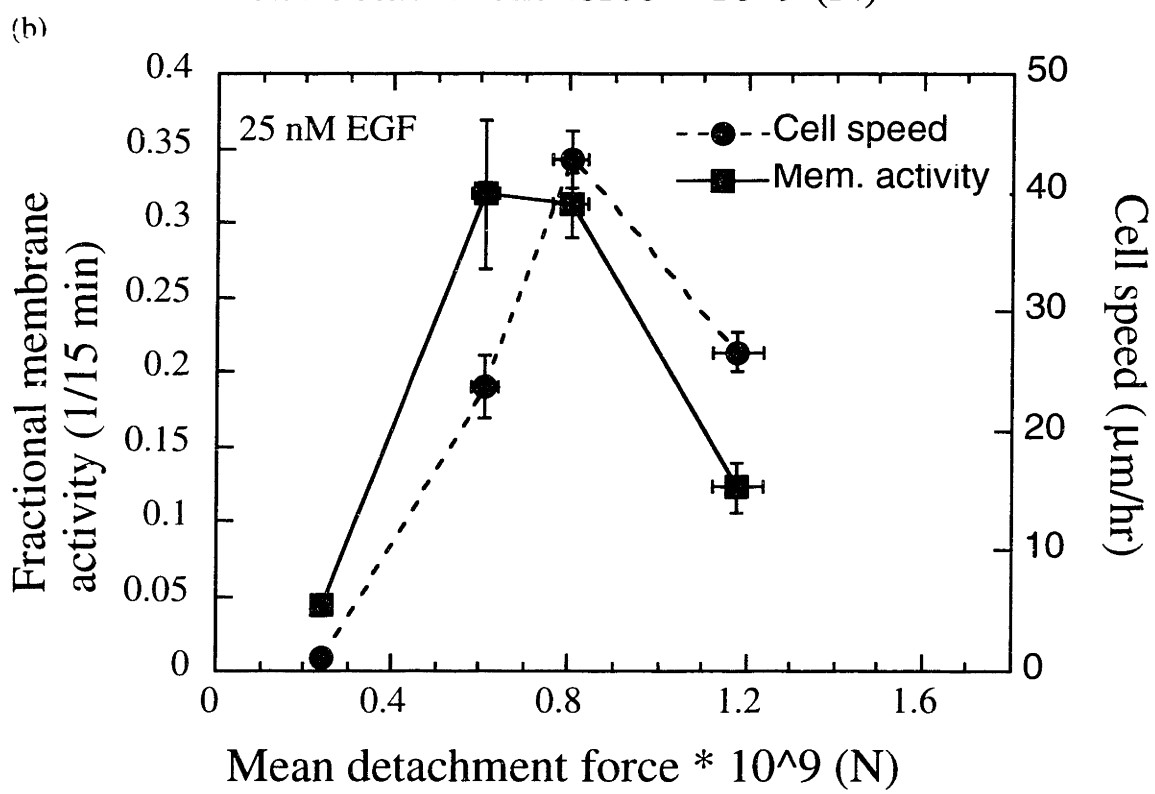
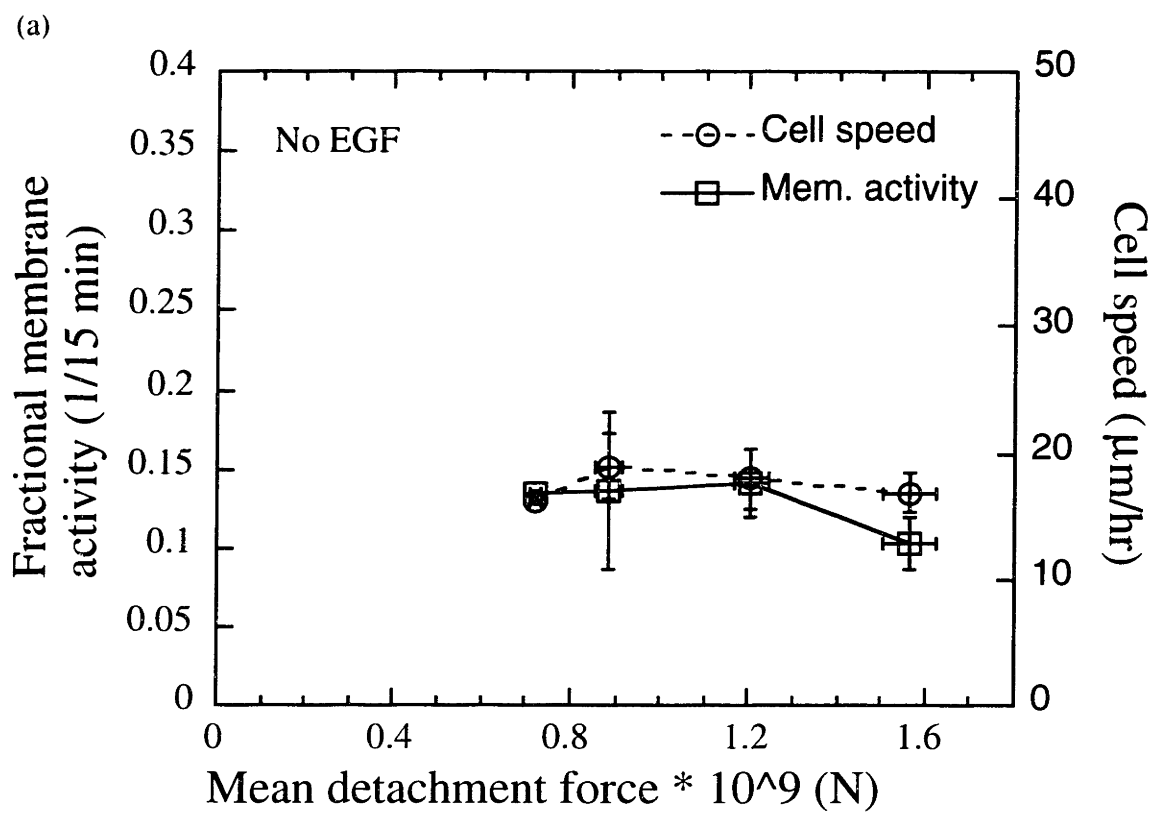


Figure 3.7 Cell speed and fractional membrane protrusion activity are modulated in a biphasic manner by the strength of cell-substratum adhesion.

(a) Cell speed (open squares) and fractional membrane protrusion (open circles) activity do not vary significantly with the strength of cell-substratum adhesion in the absence of EGF. (b) In the presence of EGF, both cell speed (filled squares) and fractional membrane protrusion activity (filled circles) vary with cell/substratum adhesiveness in a biphasic manner. High membrane activity as well as optimal adhesion are essential for maximal cell speed.

Table 3.1 Effect of EGF and Fn on cell area changes

Fibronectin Concentration (mg/ml)	Cell Spread Area (mm ²)		Absolute Protrusion Rate (mm ² /15 min)		Absolute Retraction Rate (mm ² /15 min)	
	0 nM EGF	25 nM EGF	0 nM EGF	25 nM EGF	0 nM EGF	25 nM EGF
0.1	790 ± 26	249 ± 12	107 ± 13	11 ± 0.5	96 ± 10	11 ± 0.5
0.3	1247 ± 29	857 ± 52	170 ± 26	273 ± 42	148 ± 27	262 ± 28
1.0	1352 ± 42	982 ± 35	190 ± 14	306 ± 21	170 ± 9	303 ± 28
3.0	1908 ± 68	1623 ± 39	196 ± 25	199 ± 28	213 ± 30	205 ± 30

Cell spread area was measured by outlining cells every 15 minutes over an hour interval. 20-30 cells were analyzed at each condition. Absolute protrusion and retraction rates were measured over a one hour interval by calculating the change in cell spread area between subsequent cell outlines every 15 minutes. Refer to 'Materials and Methods' section for the description of individual measurements. The errors represent s.e.m.

Chapter 4

RGD mediated regulation of cell motility

4.1 Introduction

Studies described in Chapter 3 showed that signals initiated by the interaction of EGF and Fn with the cell influence the motility response of the cell in a synergistic manner (Maheshwari et al., 1999). The results suggested that in the design of a bioartificial matrix which would specifically support cell motility, it is imperative to optimize the density of the adhesion ligand immobilized on the surface and that the density of the adhesion ligand would depend on the concentration of the growth factor in the bulk medium. In this chapter, we describe our efforts towards designing a minimalistic surface which supports cell motility using the tripeptide sequence arg-gly-asp (RGD) as the adhesion ligand of choice. We have based our design procedure on the structural characteristics exhibited by cell surface integrin receptors in their interactions with ECM proteins and have attempted to optimize adhesion ligand presentation on the surface.

As described in section 2.2, integrins play an important role in regulating various cellular processes such as cell migration, tissue organization during embryonic development, cell response during wound healing and inflammation, and metastasis of tumor cells. They provide not only a structural link between the actin cytoskeleton within the cell and the surrounding ECM, but are also involved in initiation of various signal transduction cascades within the cell which are common with those activated by growth factors. Integrins have been implicated in regulation of physical processes involved in cell migration including formation of new adhesions in the front of the cell, detachment of existing linkages in the rear of the cell and in regulation of cell migration via cell-substratum adhesion. They have been known to synergize with growth factors in the regulation of cellular

functions such as cell motility and proliferation, and activation of regulatory proteins such as MAP kinase and focal adhesion kinase (FAK).

Interaction of integrins with ECM proteins is involved in the regulation of cell motility both during normal physiological processes such as wound healing and embryonic development, as well as during pathological conditions such as cancer metastasis (Hynes, 1992; Schlaepfer and Hunter, 1998; Schwartz and Ingber, 1994; Schwartz, 1997; Schwartz and Baron, 1999). Effect of motility of a cell on an ECM protein is very closely related to its adhesive interaction with it. It has been shown both theoretically and experimentally that cell speed exhibits a biphasic dependence on the strength of cell-substratum adhesion of a number of cell types (eg. Chinese Hamster Ovary cells, endothelial cells and fibroblasts) (Dimilla et al., 1991; Dimilla et al., 1992; Dimilla et al., 1993; Palecek et al., 1997; Wu et al., 1994; Ware et al., 1998). Variation in the strength of adhesion between the cell and the surface might be achieved in many ways which include but are not limited to a change in the occupancy of integrins on the cell surface (by either a change in the number of integrins expressed on the cell surface or by a change in the surface density of ECM ligands which interact with the integrins), change in the affinity of the integrin for the ECM protein, or a change in the avidity of interaction between the integrin and the ECM protein. Palecek et al showed that integrin-mediated cell motility can be modulated by modulating integrin affinity, ECM surface density and integrin expression on the cell surface (Palecek et al., 1997). It has also been suggested that the strength of interaction between ECM proteins and integrins may be regulated by affecting the avidity of interaction which might be achieved by clustering of integrins. Yauch et al suggested that ligand binding to an integrin results in increased mobility of the integrin which is pro-adhesive since it permits formation of integrin clusters (Yauch et al., 1997).

Integrins are thought to be activated in many different ways ranging from change in the conformation of the integrin to clustering of integrins on the cell surface following receptor occupation (Schwartz et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996). There is strong evidence to suggest that

integrin receptors while interacting with ECM proteins are present in a clustered format on the cell surface and that clustering of integrins results in the assembly of a protein complex associated with the cytoplasmic domain of the integrins (Shattil, 1995; Hato et al., 1998). This multi-protein complex supports the assembly of the actin cytoskeleton which provides rigidity to the cell body and is involved in recruiting molecules involved in intracellular signaling such as FAK and that clustering of integrins is essential for a number of normal physiological processes of adherent cells. It has been suggested that sites of integrin clustering might also be sites of convergence of signals from growth factor receptors and integrins since certain growth factor receptors have been found to be co-clustered with integrins (Miyamoto et al., 1996).

While it is clear that integrin occupation is important for cell function, it is largely unknown whether controlling the spatial presentation of integrin ligands at length scales of integrin clusters affects cell behaviors such as motility. We hypothesize that presentation of an integrin ligand in a clustered format may result in more efficient clustering of the ligand-occupied cell surface integrins compared to the same surface density of ligand presented randomly, thereby affecting cellular processes such as motility. In this study, we have used the RGD (arg-gly-asp) peptide motif derived from the cell-binding domain of fibronectin as the integrin ligand of choice (Ruoslahti, 1996). The RGD sequence has been shown to interact with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. We have used surfaces covalently grafted with varying surface densities and spatial distributions of RGD in order to determine whether there exists an optimal configuration of adhesion ligand immobilization which supports cell motility. We have also investigated the role of the epidermal growth factor (EGF), a well studied growth factor known to affect cell motility, in governing the locomotion of cells on RGD-modified surfaces (Wells, 1989). Attempt has been made to cluster integrins by presenting the ligand in a clustered format on a two dimensional surface. In order to achieve interactions solely between the cell and the ligand of choice, we have used a polyethylene oxide-based hydrogel as an inert background surface (Griffith and Lopina, 1998; Lopina et al., 1996; Zalipsky, 1995; Sofia and Merrill, 1998). PEO resists protein adhesion as has been shown in number of previously published studies. RGD has been covalently

tethered to the inert background using PEO molecules in a star configuration which allows nanoscale patterning of the ligand. We have examined the effect of both growth factor-stimulated motility as well as basal motility of NR6 murine fibroblast cells, a 3T3- derived cell line, transfected with wildtype human EGFR (WT NR6 cells) as a well-characterized immortalized but nontransformed fibroblastic cell line. We find that cell speed strongly depends on both the average ligand density on the surface as well as the average cluster size of ligand presentation. The effect of RGD presentation on cell motility is mediated via its effect on the strength of cell-substratum adhesion and re-organization of the actin cytoskeletal network. Our results indicate that presentation of the adhesion ligand in a clustered format is more effective in supporting cell motility than presentation of the same amount of ligand in an uniform manner. This work demonstrates the use of well defined and well characterized surfaces for quantitative study of cell function and provides insight into possible rules for the rational design of biomaterial surfaces which would support specific cell function.

4.2 Experimental design

4.2.1 Choice of substrate

The approach adopted to develop the minimalistic matrix is described in Figure 2.10. Briefly, we required a background which was cell- and protein- resistant over long periods of time (> 24 hr). Such a surface would then be modified by adhesion ligand to render the surface cell adhesive. Polyethylene oxide-based matrices proved ideal for this purpose. We have used PEO molecules in a star configuration to present the adhesion ligand. Star PEO molecules provide a versatile system to present a ligand in varying spatial arrangements. By varying the number of arms modified by the ligand per star molecule and varying the fraction of modified star molecules immobilized on a surface, control on the average ligand cluster size and average surface ligand density can be achieved.

As the adhesion ligand on choice, we have used the sequence YGRGD. This sequence contains the RGD peptide motif along with a tyrosine residue which provides an iodination site for quantitation of RGD density on the surface. The glycine residue has been added as a spacer. The choice of the

cluster sizes and average densities used in this study were dictated by the chemistry of modification and the availability of the star PEO molecule of certain specificities from the manufacturer.

4.2.2 Choice of cell line and growth factor

We have continued to use the WT NR6 cells which were also used in the study described in chapter 3. Since the WT NR6 cells provide a well characterized system to study the simultaneous effects of growth factors and ECM proteins, they were ideal for this study. We have used the epidermal growth factor (EGF) as the model growth factor. Since we wanted to compare the results of this study with those obtained using fibronectin as a model ECM protein, we have used EGF at a concentration of 25 nM.

4.2.3 Choice of experimental methods

The migration and adhesion assay protocols described in Chapter 3 were followed in this study as well in order to provide a direct comparison with the results of the previous study.

4.3 Materials and methods

4.3.1 Cell culture

The generation of WT NR6 cells, a 3T3-derived murine fibroblastoid cell line lacking endogenous EGF receptor (EGFR), transfected with wild type human EGFR, has been described previously (Pruss and Herschman, 1977; Chen et al., 1994). WT NR6 cells were cultured in MEM- α media supplemented with fetal bovine serum (FBS; 7.5%), penicillin (100 U/ml), streptomycin (200 mg/ml), non-essential amino acids (1 mM), sodium pyruvate (1 mM), glutamine (2 mM) and G418 (350 μ g/ml). Cells were passaged at subconfluence by trypsinization (0.25%, 1 mM EDTA) and incubated at 37 oC, 90% humidity and 5% CO₂. Assay medium used while performing the migration, adhesion, and membrane extension activity assays contained MEM- α with HEPES (25 mM), 1 gm/l BSA, 1% dialyzed FBS, penicillin (100 U/ml), streptomycin (200 mg/ml), non-essential amino acids (1 mM), sodium pyruvate (1 mM), glutamine (2 mM) and G418 (350 μ g/ml).

FACS analysis with the appropriate antibodies demonstrated the expression of RGD binding receptors $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins on these cells (data not shown).

4.3.2 Peptide sequence

The sequence Tyr-Gly-Arg-Gly-Asp (YGRGD) was used as the adhesion ligand (Massia and Hubbell, 1990). The custom-synthesized peptide was procured from American Peptide Company with the end groups on Arg and Asp protected (YGR(pmc)GD(tBu)). Soluble peptide with the sequence GRGDSP (Gibco BRL, MD) was used to test specificity of interaction of the cells with the RGD modified surfaces. Briefly, GRGDSP peptide dissolved in PBS was added at a final concentration of 2mM to cells plated on RGD-modified surfaces for 12 hours in serum free media. The media was again changed to RGD peptide-free media 1 hour later.

4.3.3 Surface preparation

The methodology of surface preparation and brief details of its characterization are described in Appendix A1. \bar{n}_c refers to the average cluster size of RGD i.e., the number of RGD peptides tethered to a single star PEO molecule.

4.3.4 Migration assay

Cell migration speed was measured using time-lapse videomicroscopy of single cells. 30,000 cells were plated onto 35-mm dishes in 2.5 ml serum-free medium. 12 hr. post-seeding the medium was changed to 2.5 ml assay medium with or without 25 nM EGF and incubated at 37 °C in humidified air for 8 hr. At this cell density, soluble ligand concentration is relatively unchanged over a 24 hr period (Reddy et al., 1994). 3 ml mineral oil was added to the dish to prevent evaporation, and the dish was then placed in a heated stage insert for a Ludl 99S008 motorized stage on a Zeiss Axiovert 35 microscope. Cell boundaries and centroids were identified using image processing software developed by Engineering Technology Center (Mystic, CT) running under a LabVIEW (National Instruments, Austin TX) and Concept Vi (Mystic, CT) environment. 5-10 cells per field in 10

different fields were scanned every 15 minutes for up to 12 hr. The x and y coordinates of the cell centroids were recorded every 15 minutes. Single cell speed is calculated by determining the total path length as measured by the total centroid displacement divided by the tracking time. The reported cell speed \pm s.e.m. for each condition is an average over 70-100 cells.

4.3.5 Adhesion assay

The adhesion assay was performed as previously described (Chu et al., 1994). Briefly, 24-well plates were plated with 20,000 cells per well in serum-free conditions for 12 hr. The medium was then changed to the assay medium with or without 25 nM EGF. Adhesion was also measured at times comparable to the migration assay, 8 hr after addition of EGF. The wells were filled with medium and sealed using sealing tape avoiding air bubbles. The plates were then inverted and spun in a swing bucket SH-3000 rotor in a bench top Sorvall centrifuge for 10 min. at 25 °C at 800g. During each experiment, one plate at the highest cluster size and highest average surface RGD density without EGF was kept at 1g and used as a control. Cell number was quantified by manually counting cells in a defined well area. At least 4 wells were used at each condition and 4 fields were counted per well with each field in the control containing 300-400 cells. The cell number per well was normalized to the average cell number in the control well to obtain fraction adherent cells.

4.3.6 Actin and vinculin staining

For actin filament and vinculin visualization, cells plated on derivatized 18 mm glass coverslips were rinsed with PBS and fixed using 3.7 % paraformaldehyde. The cells were then rinsed with PBS and permeabilized by incubation for 3 minutes using 0.1 % Triton X-100 (Sigma Chemical Co., St. Louis, MO). After rinsing twice in PBS, the cells were incubated for 45 minutes at room temperature in a humidified chamber with 1:100 dilution of monoclonal mouse anti-vinculin antibody (Sigma Chemical Co.). The samples were then rinsed with PBS and incubated in a mixture of rhodamine-phalloidin (1:200 dilution, Sigma Chemical Co.) and Alexa 488-conjugated goat anti-mouse secondary antibody (1:50, Molecular Probes, OR) in PBS for 45 minutes in a dark humidified

chamber. The samples were then thoroughly washed and mounted on glass slides using Prolong Antifade solution (Molecular Probes, OR). The preparations were then visualized on a Zeiss 35 inverted fluorescence microscope and images captured using a Hamamatsu (place) cooled CCD camera on OpenLab imaging software (place). For the fibronectin controls, cells were plated on coverslips coated with 1 $\mu\text{g}/\text{ml}$ fibronectin coating concentration and blocked with 1 % BSA in PBS as described previously. Approximately 250 cells were scored from 4 coverslips to quantify cells with well formed actin stress fibers.

4.4 Results

4.4.1 Cell speed depends on average surface density of RGD

In order to achieve interactions solely between the cell and the adhesion ligand covalently tethered on the surface, we required an inert background that completely resisted cell and protein adhesion. Glass coverslips covalently modified by radiation-crosslinked PEO hydrogels provided the required cell resistance. No cell adhesion was obtained when cells were plated on such surfaces in media containing 7.5% fetal bovine serum (Figure 4.2). Furthermore, even when these surfaces were coated with 10 $\mu\text{g}/\text{ml}$ fibronectin, no cell adhesion was observed which suggested that PEO hydrogels may prevent adhesion of the cell to proteins secreted by it (data not shown). The inert surfaces were then covalently modified with a peptide with sequence YGRGD which contains the RGD adhesion motif. The RGD peptide was presented using PEO molecules in a star configuration (Figure 4.1). Surfaces with varying average ligand density ($1000 - 10^5$ RGD molecules/ μm^2) on the surface and average cluster sizes ($\bar{n}_{cl} = 1, 5, 9$) were synthesized. The synthesis and detailed characterization of the surfaces used in this study is described elsewhere (Brown, 1999).

Addition of soluble RGD peptide (GRGDSP at a concentration of 2 mM) abrogated cell adhesion to the RGD-modified surfaces which was restored upon removal of the soluble peptide suggesting that cell adhesion to RGD-modified surfaces is mediated via the RGD peptide (data not shown). Addition of a nonsense peptide did not alter cell adhesion to the RGD surfaces (data not shown). Cell speed

was quantified by time-lapse videomicroscopy of single cells over a 12 hour time period in the presence and absence of soluble EGF. We find that the RGD peptide alone is able to support cell motility. At each RGD cluster size, cell speeds increased with increase in the average RGD surface density (Figure 4.3). The increase was significantly more pronounced in the presence of EGF (Figure 4.3b). As seen from Figure 4.3a, the maximal cell speed on the RGD-modified surfaces were comparable with that obtained on the surfaces coated with Fn. At $\bar{n}_{cl} = 5$ and 9, cell speeds appeared to reach a plateau of 16 $\mu\text{m/hr}$ in the absence of EGF and 31 $\mu\text{m/hr}$ in the presence of EGF. We expect that increasing the average surface RGD density further will result in an increase in the of cell speed even at $\bar{n}_{cl} = 1$ to the plateau values obtained at the higher cluster sizes.

4.4.2 Cell speed depends on the average cluster size of RGD

At a given value of the average RGD surface density, increasing the cluster size of RGD presentation, increased the cell speed, both in the presence and absence of soluble EGF. For example, at an average RGD surface density of 10^4 molecules/ μm^2 , increasing \bar{n}_{cl} from 1, to 5 to 9 increased the cell speed from 1.2 to 8.8 to 14.6 $\mu\text{m/hr}$ in the absence of EGF, and from 1.9 to 13.9 to 29.7 $\mu\text{m/hr}$ in the presence of EGF. These results indicate that adhesion ligand presentation has a significant effect on the basal speeds and growth factor-stimulated speeds of NR6 fibroblasts, and that both parameters characterizing ligand presentation, the average surface density and local cluster size affect cell speed. In addition, there appears to be an upper bound to the maximum cell speed achievable which is regulated by \exists EGF (Figure 4.3).

4.4.3 Cell/substratum adhesion strength depends on characteristics of RGD ligand presentation

In earlier theoretical and experimental studies, it has been shown that cell speed may be limited by the strength of cell-substratum adhesion. Therefore, in order to determine whether the effect of RGD presentation on cell speed is mediated via its effect on cell-substratum adhesion, we quantified cell-substratum adhesion using the centrifugation adhesion assay. Fraction adherent cells is a population

measure of the strength of cell-substratum adhesion. The greater the value of fraction adherent cells, the greater is the strength of adhesion between the cell and the surface. As expected, it was found that increasing the average surface density of the adhesion ligand, increased the strength of cell adhesion at each of the cluster sizes. This trend was also observed in the presence of soluble EGF. However, addition of soluble EGF decreased the strength of the cell-substratum adhesion at each of the surface conditions. This decrease of adhesion in the presence of EGF is consistent with earlier studies on natural ECM molecules such as fibronectin and amgel. At $\bar{n}_{cl} = 5$ and 9, the strength of cell-substratum adhesion appears to be approaching a plateau suggesting that further increase in the average RGD surface density may not result in significant increase in the strength of cell-substratum adhesion. We observe that the same strength of cell-substratum adhesion may be obtained at a lower average surface RGD density when RGD is presented in a clustered format. For example, in order to have 30% cells adherent in the presence of EGF following centrifugation, an average surface RGD density of 7400, 61650, and $>10^6$ molecules/ μm^2 is required when RGD is presented in clusters of $\bar{n}_{cl} = 9, 5$ and 1 respectively. Similarly, in the absence of EGF, 4170, 50120 and $\sim 10^5$ RGD molecules/ μm^2 are required when using clusters of 9, 5 and 1 RGD/star PEO respectively to have 15% cells left adherent following centrifugation (Figure 4.4).

4.4.4 Actin stress fiber formation depends on characteristics of RGD ligand presentation

Cell adhesion and motility on RGD alone is dependent on its average surface density and local cluster size. In order to determine whether these parameters which characterize RGD presentation affect the cytoskeletal organization within the cell, actin filaments were visualized. In addition, the cells were also stained for vinculin which has been suggested to represent the presence of focal adhesions. We found that increasing the average RGD surface density increased the fraction of cells which exhibited stress fibers (Figure 4.5a). In addition, for the same average RGD density, when RGD was presented in a clustered format, a significantly higher fraction of cells exhibited well-formed stress fibers and focal contacts. Characteristic punctate vinculin staining was also almost completely

abrogated on surfaces with $\bar{n}_{cl} = 1$, while those with $\bar{n}_{cl} = 9$ exhibited significant vinculin stains (Figure 4.5b). This suggests that presentation of the RGD ligand in a clustered format is able to form focal contacts in a more effective manner than when the ligand is presented at a uniform density. While cell adhesion was observed on surfaces with $\bar{n}_{cl} = 1$, these surfaces were unable to support stress fiber formation suggesting that presentation of RGD in a manner which prevents clustering of integrins also prevents formation of stress fibers.

4.5 Discussion

We have attempted to determine the role of integrin clustering in regulating cell motility by presenting the cell with covalently tethered clustered integrin ligand and have gained insight into the underlying biophysical processes that might be affected by such integrin occupation. We have used surfaces covalently patterned at the length scale of integrins ($\sim 12\text{nm}$ in diameter) and ECM molecules. Earlier studies using microcontact printing and photolithographic techniques presented islands of high density of ECM molecules which were a few microns in diameter (Mrksich et al., 1996; Chen et al., 1997). Effects of integrin clustering have also been studied using microbeads varying in diameter from 1 - 10 μm coated with ECM proteins and as well as using bivalent antibodies as crosslinking agents (Miyamoto et al., 1995; Hato et al., 1998). The star PEO molecules used in this study on an average have 35 arms with a molecular weight of 9100 per arm which corresponds to an average radius of 18 nm (Brown, 1999). Assuming hard sphere packing of integrins, approximately 14 integrins can be packed on a star hemisphere without steric hindrance which corresponds well to the maximum cluster size of 9 integrin ligands per star PEO molecule that we have achieved. The technique used here presents the ligand in a covalently tethered manner on a well-defined two dimensional surface and permits the variation of the average ligand surface density and the average cluster size of ligand presentation.

The unmodified PEO surfaces maintain cell resistance over time periods greater than 24 hours. Since the RGD ligand is tethered covalently to the surface, we expect the surface to maintain its integrity

over the time course of an experiment (~ 24hrs). Use of surfaces with adsorbed protein have the drawback that the surface may be modified due to proteins desorbing off the surface and new protein synthesized by the cell being deposited on it over the course of the experiment. Hence, the system used in this study is ideal for a quantitative study of specific surface-bound ligand and cell-surface receptor interactions involved in a given cell function.

We find that ligand presentation affects cell speed as well as the strength of cell-substratum adhesion. In order to determine whether the effect of adhesion ligand presentation on cell speed was mediated via its effect on the strength of cell-substratum adhesion, the data obtained in Figures 4.3 and 4.4 were re-analyzed with cell speed as a function of cell-substratum adhesion (Figure 4.6). We find that the plots of cell speed versus strength of cell-substratum adhesion collapsed onto two curves - one in the absence of EGF and the other in the presence of EGF. The curve obtained in the presence of EGF is shifted to the left of the curve in the absence of EGF which captures the effect of decreased adhesion obtained in the presence of EGF. The fact that the plots of cell speed versus cell-substratum adhesion at the three cluster sizes collapsed onto a single curve indicates that presentation of the adhesion ligand in a clustered format regulates cell speed by solely regulating the strength of cell-substratum adhesion. Addition of EGF shifts the curve to the left and also increases the maximal cell speed that is obtained under the given surface conditions, suggesting that EGF-regulated cell speed continues to be limited by the strength of cell-substratum adhesion, but in a regime that is now governed by EGF and is different from that in the absence of EGF. Interestingly, the plots of cell speed versus cell-substratum adhesion on varying surface fibronectin concentrations both in the presence and absence of EGF obtained previously, follow very similar trends as those observed in this study. This reveals that cell speed on the RGD surfaces is governed by cell-substratum adhesion in a very similar manner to that on varying fibronectin surface concentrations. The maximal speed observed on fibronectin coated surfaces is 42 $\mu\text{m/hr}$ which is higher than that observed on RGD - derivatized surfaces (31 $\mu\text{m/hr}$) possibly because adhesion may be limiting or domains other than the RGD sequence on fibronectin may be required to attain a complete response. In addition, we suggest

that the differences in the compliance of the fibronectin-adsorbed surfaces and the PEO hydrogels might contribute to differences in the amount of traction and contractile force transmitted from the surface to the cell resulting in variations in the maximal speed attainable. In addition, here we have presented RGD on a flexible PEO tether which might affect the rigidity of adhesion between the cell and the surface, also contributing to the speeds exhibited by the cells on these synthetic surfaces.

As can be seen from Figure 4.3a, cell speeds on surfaces coated with Fn in the absence of EGF do not change substantially in the range of ligand densities investigated. It is predicted by the DiMilla model of cell motility that extremes of adhesion would result in a fall in cell speeds. From the data shown in Figure 4.3a, it may be hypothesized that like trend observed on surfaces modified with RGD, a further decrease in the density of Fn may result in a decrease in cell speeds. The differences in the cell speeds observed on Fn and those on the surfaces with $\bar{n}_{cl} = 9$ in the same average ligand density range are not statistically significant. However, at each of the average densities investigated, cell speeds on fibronectin appear to be at least as high as those on surfaces modified by RGD. In the presence of EGF however, we note that in the same range of average ligand densities, cell speeds on Fn show significantly higher variations than those seen on the RGD-modified surfaces (Figure 4.3b). At an average density of approximately 2500 molecules/ μm^2 , cell speed on surfaces modified with 9 RGD/star are significantly greater than that on Fn alone. In addition, at an average density of around 10^4 molecules/ μm^2 , cell speeds on Fn are 1.5 times that expected on RGD-modified surfaces with $\bar{n}_{cl} = 9$. This strongly suggests differences in the manner in which EGF-mediated signals interact with those initiated upon binding of Fn and those initiated by binding of clustered RGD alone to their respective cell surface receptors.

As has been observed with natural ECM proteins, increasing the average ligand surface density increased the cell speed of NR6 fibroblasts on the synthetic surfaces. We do not observe a biphasic dependence of cell speed on the average ligand surface density as has been observed in previous studies. It may be because significantly strong cell-substratum adhesion has not been achieved in the

range of RGD densities investigated, which would result in reduced cell speeds due to contractile force generation within the cell becoming rate limiting. At the higher ligand density (10^5 RGD molecules/ μm^2), we have ligand far in excess of the number of integrins on the cell surface, we suggest that higher cell adhesions cannot be achieved with the YGRGD peptide. Surfaces modified with other sequences such as GRGDSP and cyclic RGD peptides which exhibit greater affinity for integrins or the use of the synergy sequence along with the RGD peptide may result in a further increase in the strength of cell-substratum adhesion. Use of such sequences may result in the reduction of the average ligand surface density to achieve similar cell speeds since the same amount of adhesion may be obtained at a lower density of the ligand. In addition, increasing the cluster size of the ligand may also result in increasing adhesion further in the range of average densities used in this study. We are unable to achieve higher cluster sizes with the given system due to limitations of the chemistry involved in surface synthesis.

While direct visualization of integrin clusters using immunofluorescence is not easily feasible at this scale, indirect evidence suggests that clustering of the integrin ligand induces clustering of the corresponding integrins. The observation that at the average ligand surface density, a greater fraction of cells plated on RGD presented in a clustered format form well-defined stress fibers and exhibit vinculin staining suggests that clustered integrins may be able to recruit molecules involved in stress fiber formation such as those of the Rho family of GTPases in a more effective manner and that a minimum cluster size of integrins may be required for the recruitment (Chrzanowska-Wodnicka and Burridge, 1996; Burridge and Chrzanowska-Wodnicka, 1996; Wells et al., 1998; Keely et al., 1998). Presentation of a single RGD on one star PEO molecule which is approximately 35 nm in diameter may restrict the clustering of ligand-bound integrins. Hence in the absence of a minimum cluster of integrins, assembly of the actin cytoskeletal network may be inhibited.

Massia et al. reported that an inter-ligand distance of 140 nm (60 RGD/ μm^2) is essential for the formation of focal contacts visualized using IRM while cell adhesion measured after 4 hours was

achieved at a spacing of 440 nm (6 RGD/ μm^2) using RGD as the adhesion ligand (Massia and Hubbell, 1991). In this study, the minimum average RGD density investigated was 1000 RGD/ μm^2 . This density of RGD supported cell adhesion at all the cluster sizes investigated, but much reduced actin organization was observed at $\bar{n}_{c1} = 1$. It is not clear from the study published by Massia et al. that the background adhesion in the absence of the RGD peptide was negligible and that proteins secreted by the cells and those present in the albumin present in the media did not contribute to the cell adhesion and may also account for the discrepancy in the RGD densities between the two studies. In addition, since we use multi-valent star PEO as the tether for RGD, we do not have any control over the orientation of the RGD-modified arm. It has been suggested that PEO arms exhibit significant mobilities and hence we expect that a significant fraction of RGD-modified arms per star will be available for interaction with the cell though the reduction in the cluster size of the RGD may result in a reduction of the probability of RGD accessibility.

We find that clustering of the RGD ligand and hence clustering of the integrins is able to support pathways that stimulate motility more effectively than presentation of a uniform density of the ligand. Results from Figure 4.6 suggest that clustering of the ligand provides stronger adhesion and that the effects of ligand presentation on cell speed are mediated via the effects on adhesion. In this case, cell speed has been modulated by altering the avidity of interaction of the integrins. Punctate multivalent interactions are able to provide stronger adhesion than the same number of uniformly distributed interactions. This may be due to both biophysical and biochemical reasons. The RGD ligand has a 1000-fold lower affinity for integrins than does fibronectin. Hence increasing the avidity of the interaction may result in an increase in the local affinity of the RGD cluster. In addition, a cluster of integrins may be able to recruit focal adhesion proteins more effectively thereby establishing the required network for optimal cell rigidity and adhesion.

Results from this study suggest that nanoscale pattern of ligand presentation significantly affects cell function. Use of such well-defined surfaces will allow further studies of specific domains of proteins

and design of a minimalistic artificial matrix by simultaneous different peptide fragments. In addition, questions regarding co-clustering of growth factor receptors and integrins may be addressed by tethering growth factors and integrin ligands to the same star PEO molecule.

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4.7 Figures

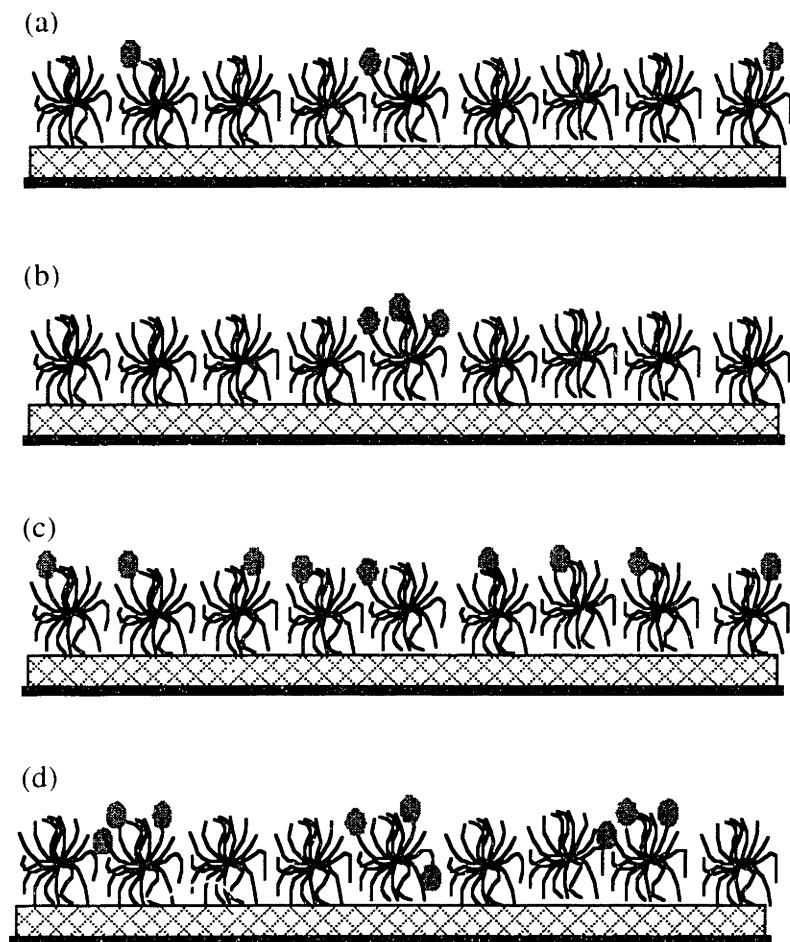
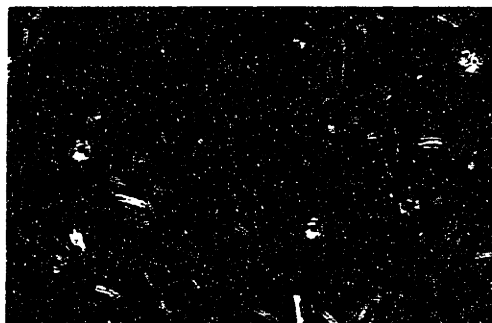


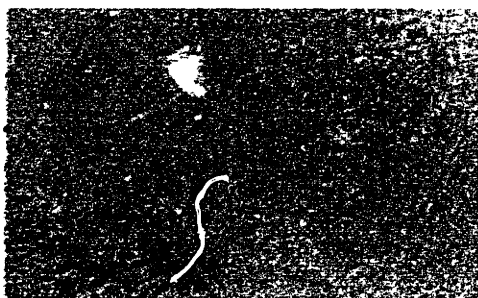
Figure 4.1 Varying spatial distribution of RGD using star PEO molecules.

Control over average cluster size may be obtained by varying the number of RGD-modified arms per star molecule. Average surface density can be varied by changing the number of RGD modified stars immobilized on the surface. (a) &(b), and (c) &(d) have the same average surface density but (b) and (d) have a higher ligand cluster size than (a) and (c) respectively. (c) and (d) have a higher average surface density than (a) and (b).

(a)



(b)

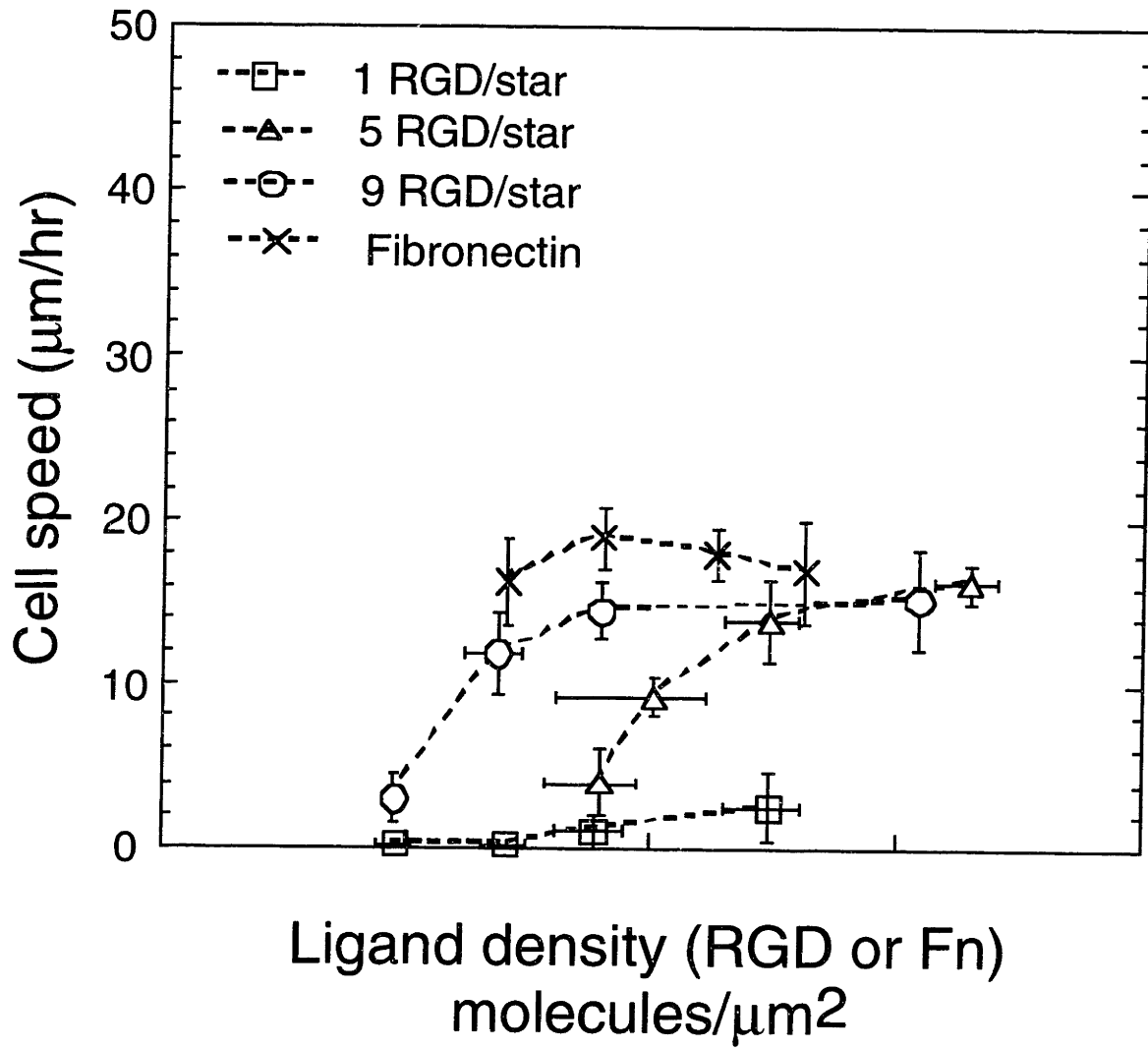


(c)



Figure 4.2. RGD modified star PEO molecules support cell adhesion. WT NR6 cells were plated in serum free media for 12 hours on (a) 1 $\mu\text{g}/\text{ml}$ fibronectin, (b) unmodified PEO surfaces, and (c) RGD modified star PEO surfaces.

(a)



(b)

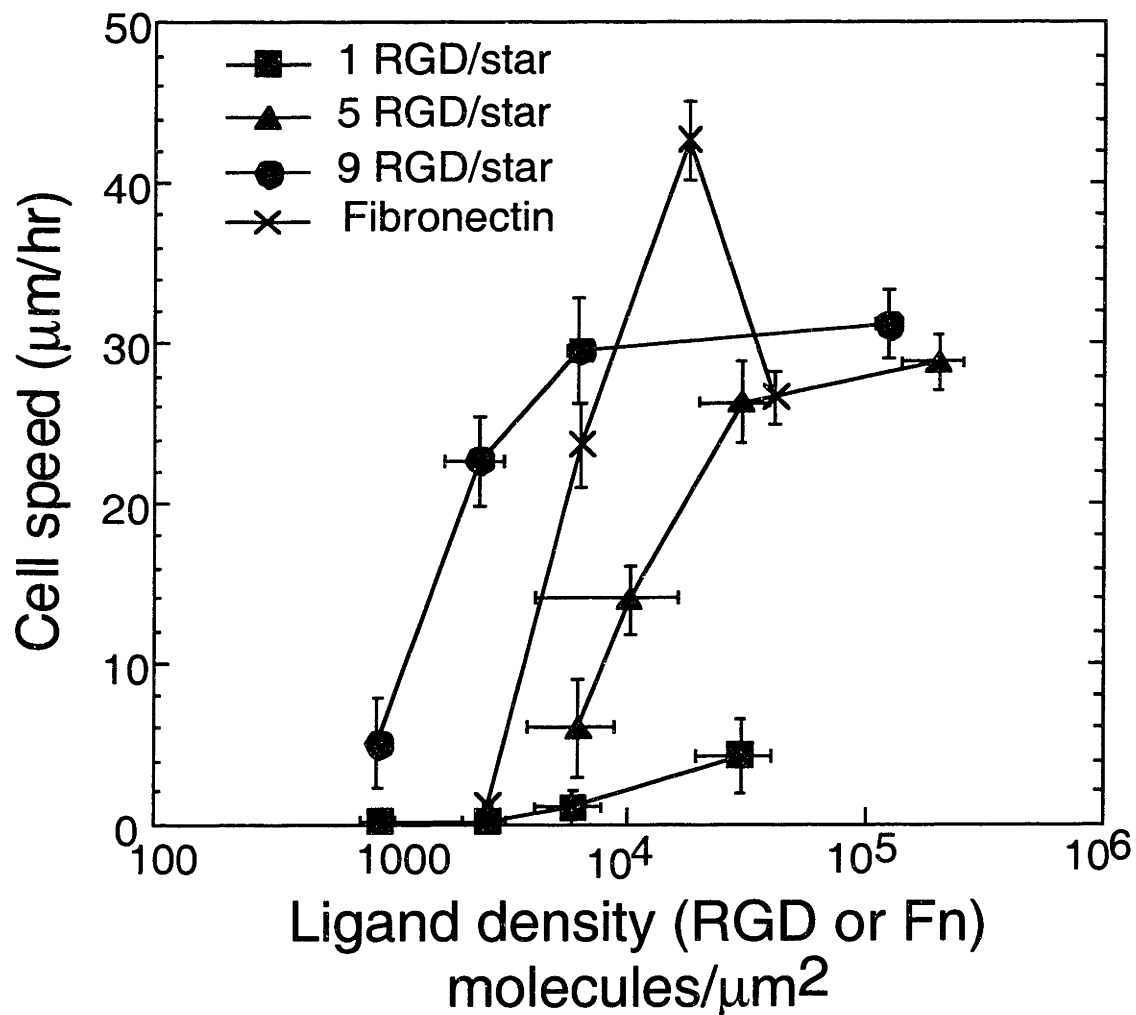


Figure 4.3 Cell speed depends on spatial presentation of RGD.

Cell speed was quantified using timelapse videomicroscopy of single cells in the (a) absence (dashed lines and empty symbols) and (b) presence (solid lines and filled symbols) of EGF. Three cluster sizes of 1 (squares), 5 (triangles) and 9 (circles) per star PEO molecule were used. The crosses refer to cell speeds on fibronectin (data obtained from A. Asthagiri (Personal communication)). Each point is an average of 50-70 cells. Error bars represent \pm s.e.m.

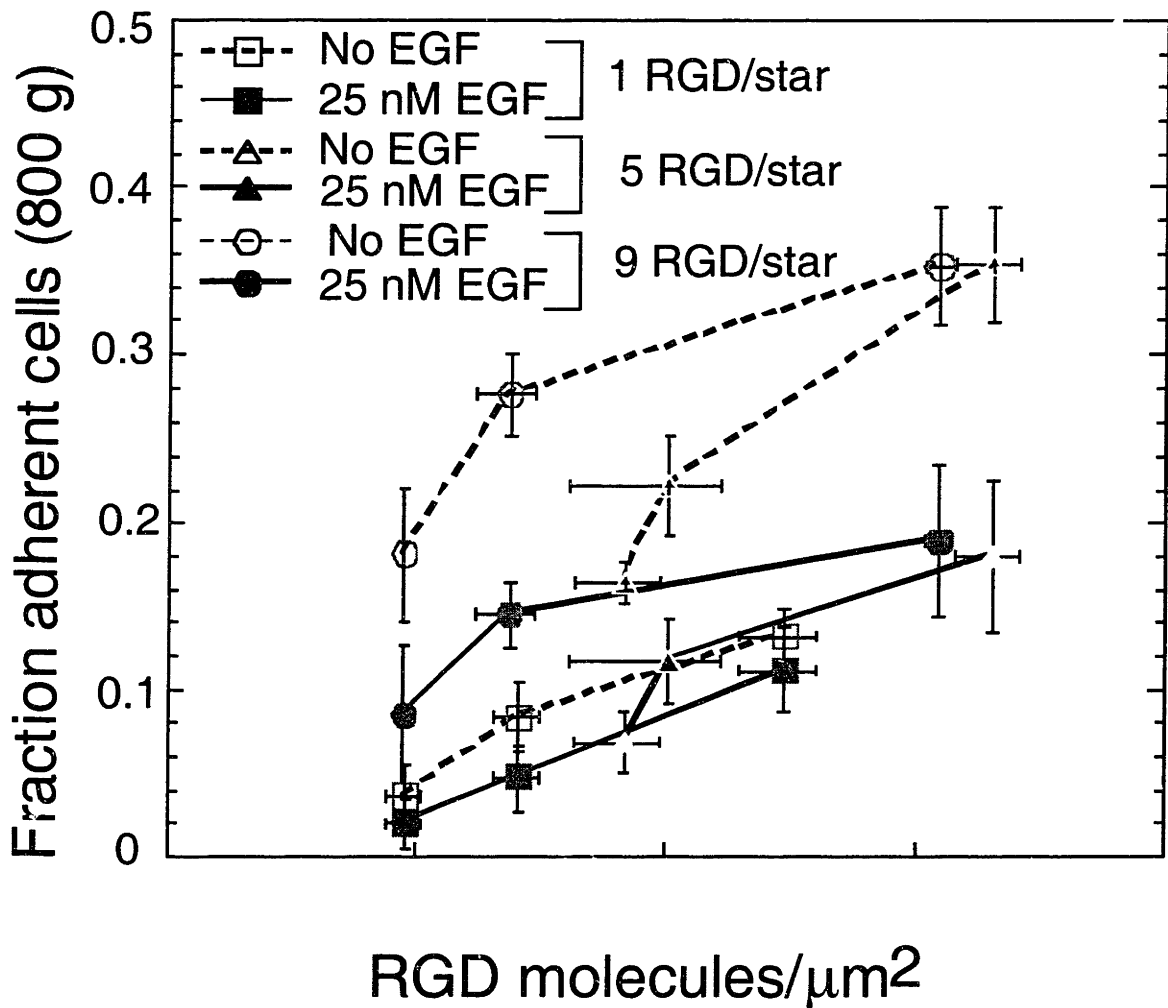
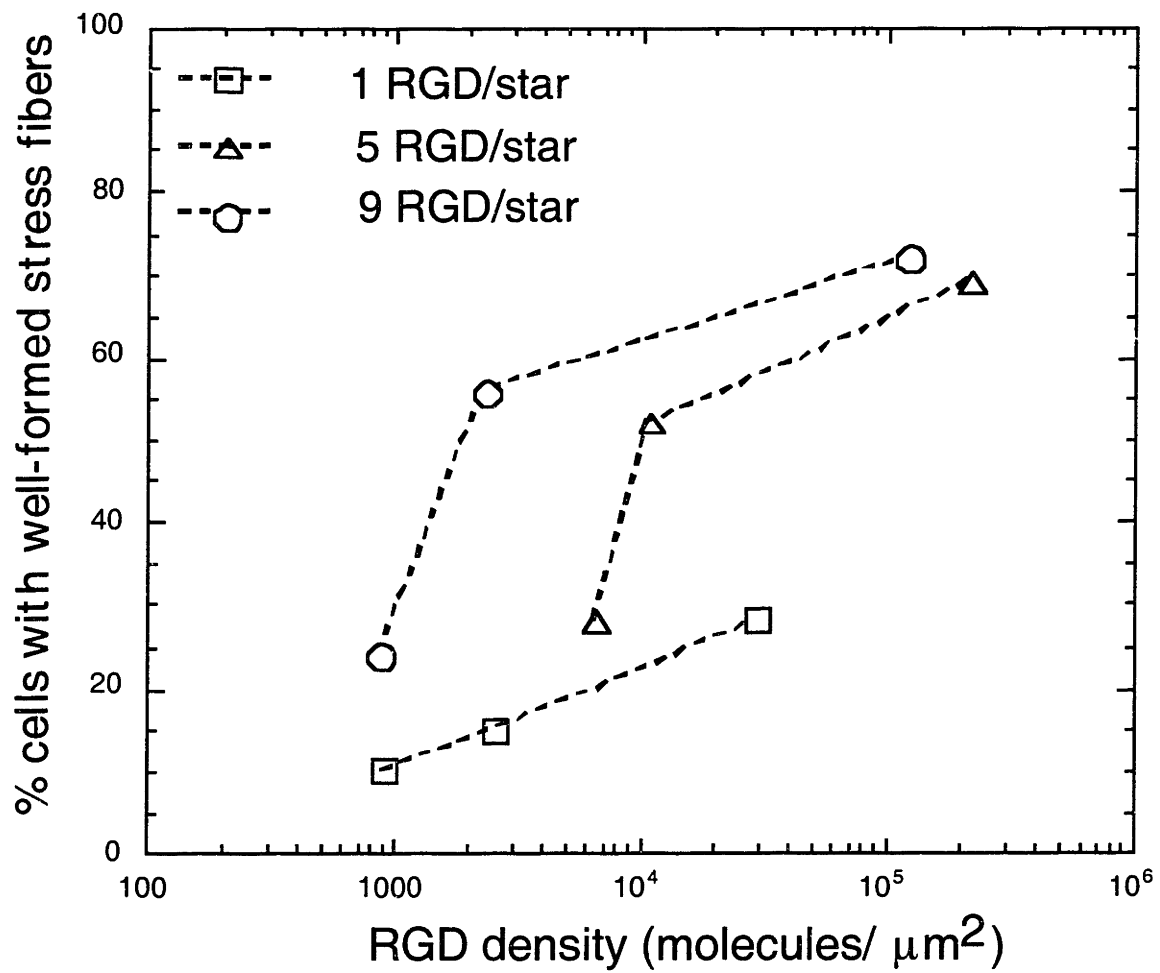


Figure 4.4 Strength of cell substratum adhesion depends on spatial presentation of RGD.

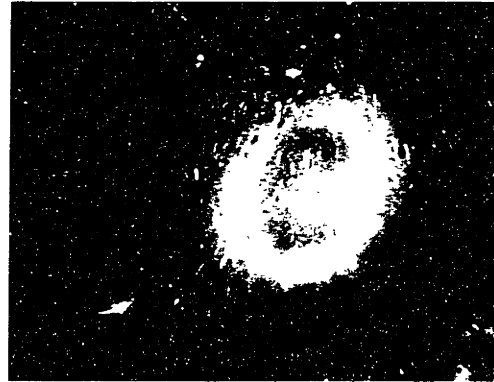
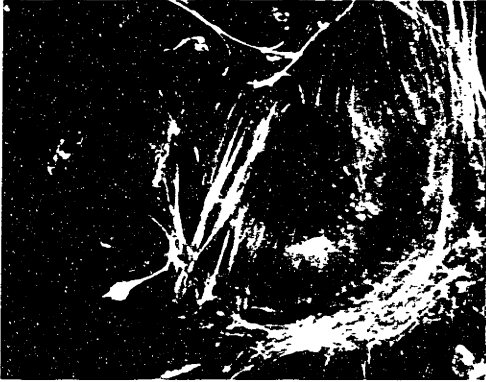
Cell-substratum adhesion was quantified using the centrifugation assay. Three cluster sizes of 1 (squares), 5 (triangles) and 9 (circles) per star PEO molecule were used. Experiments were done in the presence (solid lines and filled symbols) and absence (dashed lines and empty symbols) of EGF. In the experiments done in the presence of EGF, cells were incubated with 25 nM EGF for 8 hr before centrifugation. Error bars represent standard deviations from 3 independent experiments.

(a)

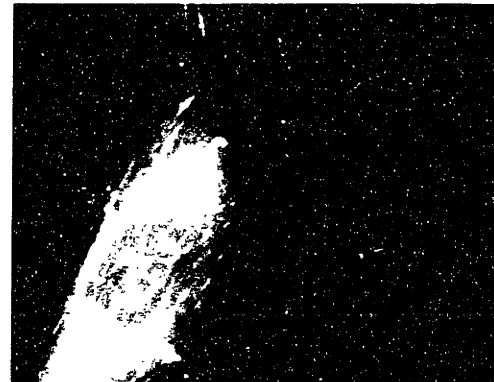
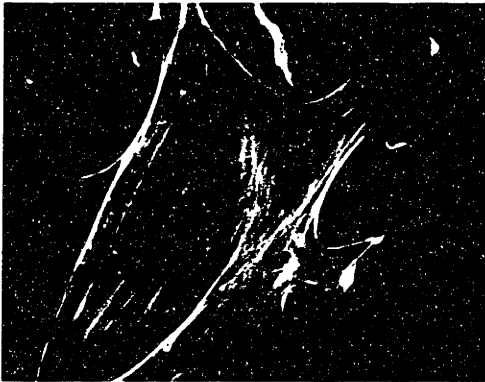


(b)

(i)



(ii)



(iii)

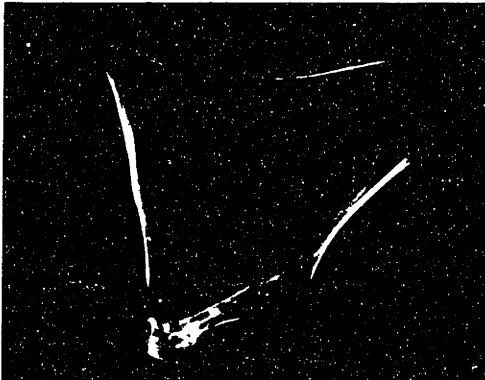


Figure 4.5 Spatial arrangement of RGD affects cytoskeletal organization.

(a) Cells were scored for actin stress fiber formation on varying RGD spatial arrangements. Approximately 140 cells from 3 independent experiments were scored at each condition. (b) Actin stress fibers and vinculin stains were visualized on varying RGD densities and cluster sizes. Shown here are typical stains from cells plated on surfaces coated with (i) 1 $\mu\text{g}/\text{ml}$ Fibronectin, (ii) 100 % RGD modified star PEO molecules with $n_{cl} = 9$, and, (iii) 100 % RGD modified star PEO molecules with $n_{cl} = 0.8$.

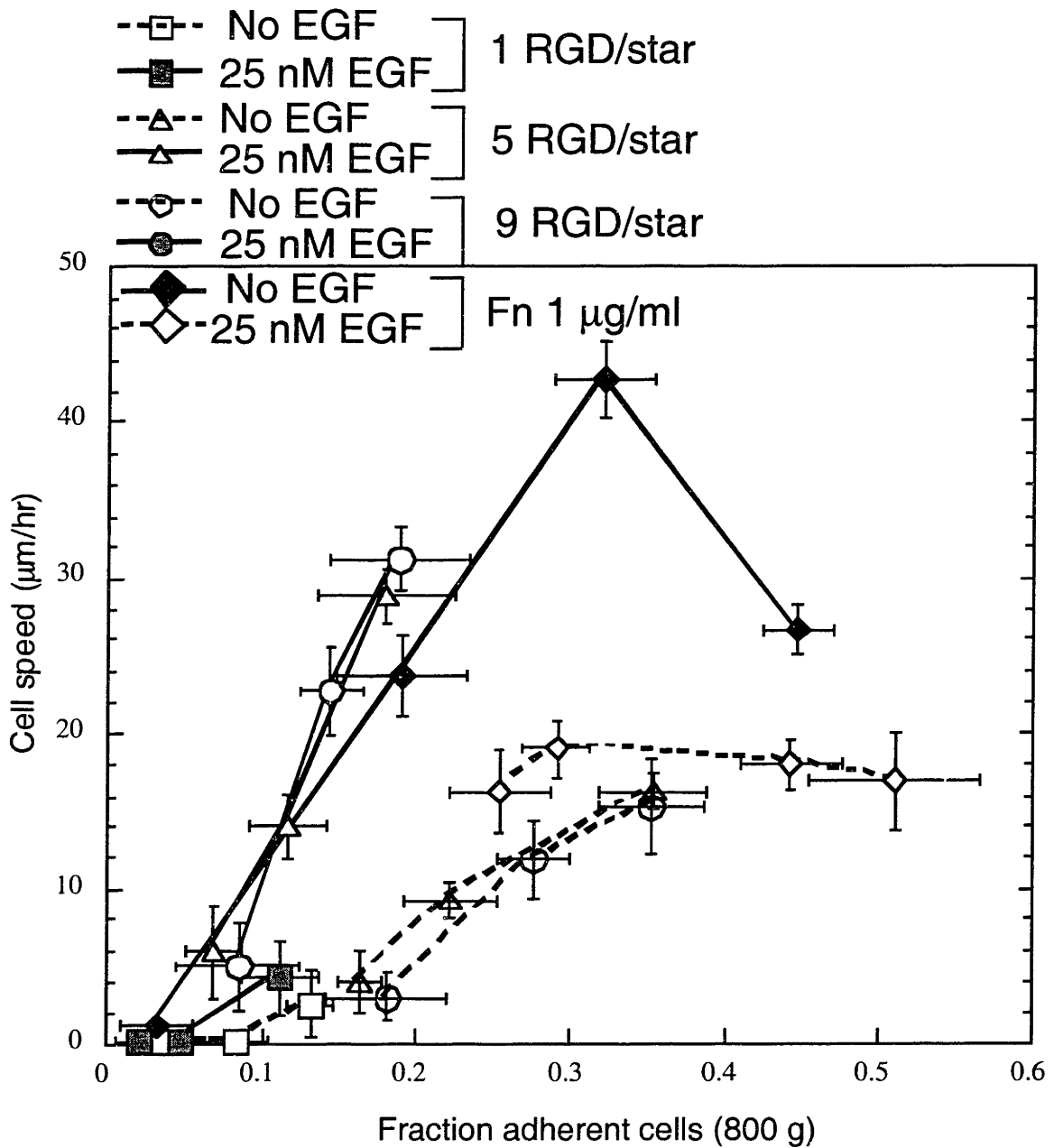


Figure 4.6 Cell speed is regulated via cell-substratum adhesion. Data from Figures 4.3 and 4.4. were re-analyzed with cell speed as a function of cell substratum adhesion. Cell speed data on fibronectin in the presence (filled diamonds) and absence (empty diamonds) of EGF from Figure 3.7 is super-imposed.

Chapter 5

Regulation of cell motility via autocrine loops

5.1 Introduction

In the studies described in earlier chapters, we had examined the effect of presentation of an adhesion ligand on cell motility. In this chapter, we describe investigations of the mode of presentation of a soluble ligand on cell motility. As described in Section 2.5, receptors and ligands can interact in many different ways which include paracrine, juxtacrine, endocrine and autocrine interactions. Here, we have determined the role of autocrine presentation of a ligand on cell motility. We have used the EGF-EGFR system as the receptor-ligand pair of interest. Presentation of a growth factor in an autocrine manner offers an alternative to its delivery by exogenous injection. By having the expression of the growth factor regulated by a controllable promoter, it can be turned on at the required instance. Since it will be produced by the cell in situ, problems associated with exogenous delivery of the ligand such as loss of the ligand due to diffusion and degradation can be avoided. Furthermore, modifying the cell using this gene therapy approach can be used in conjunction with the modification of a biomaterial surface to stimulate a required cell behavior. Hence, in order to ultimately achieve these technological objectives, it is essential to determine whether presentation of the ligand in an autocrine manner affects cell function.

Autocrine receptor/ligand “loops” were first identified in tumor cells where it was found that transformation of cells resulted in over-expression of certain cytokines which caused unregulated proliferation of the tumor cells. However, since then they have been identified

in numerous physiological situations, for example, during wound healing and tissue reorganization during development and in parts of the female reproductive system (Sporn and Roberts, 1985; Sporn and Roberts, 1992). While it is clear that autocrine loops play a crucial role in regulation of cell function, it is still unclear whether their effects on cell function are different from the effect of a ligand supplied exogenously. It has been suggested that the activation of signaling molecules may be influenced by their spatial distribution pointing to a significant role of mode of presentation of a ligand in affecting cell function (Carraway and Carraway, 1995).

Our studies are motivated by recent results published by Wiley et al. showing that mode of presentation of EGF affected cell proliferation and differentiation of human mammary epithelial cells (HMECs) (Wiley et al., 1998). EGF is synthesized as a transmembrane precursor which is then proteolytically cleaved and processed to release the 53 amino acid long mature ligand (Massague and Pandiella, 1993). While there exist multiple EGFR ligands with varied levels of tissue expression, whether the various ligands play distinct roles physiologically remains largely unknown (Dempsey et al., 1997). It has been postulated that each of the EGFR ligands may have a distinct compartmentalized sorting pattern within the cell and be expressed in a distinct pattern on the cell surface thereby regulating cell function in a ligand-specific manner.

In order to understand the role of the membrane-anchoring domain of EGF, Wiley et al synthesized a set of EGFR ligands, one consisting of the mature EGF along with its cytoplasmic and transmembrane domain (EGF-Ct), and one with simply the mature form of EGF (sEGF) (Figure 5.1). They expressed these constructs in an autocrine manner using a human mammary epithelial cell line which expressed the EGF receptor endogenously. They observed that removal of the membrane-anchoring domain of the EGF precursor resulted in an uninterrupted autocrine loop stimulating cell proliferation; this indicates that

EGF in cells which express EGF may be able to signal in an “intracrine” manner. A blocking antibody which blocks the binding of ligands to the EGFR was unable to prevent the interaction of sEGF with EGFR causing continued proliferation and EGFR activation even in the presence of high concentrations of the antibody (Figure 5.2). In addition, the organized structures formed by cells expressing EGF-Ct and those expressing sEGF upon prolonged culture on matrigel differed considerably. EGF-Ct expressing cells formed structures which closely resembled those formed by primary human mammary epithelial cells under EGF stimulation showing physiologically relevant lobular and ductal aspects. Cells expressing sEGF on the other hand continued to proliferate colonies lacking any significant organization. This indicates either that cell surface EGF-EGFR complexes are essential for the requisite organization; or that the spatial distribution of the EGF-EGFR complexes on the cell surface may be involved in providing the cell with information about its extracellular environment, thereby affecting cell organization. This study strongly suggests that the effect of receptor activation on cell behavior was dependent on the spatial localization of the activated receptor-ligand complex.

In this study, we have examined the role of autocrine ligands in governing cell motility. We find that EGF-EGFR interacting in an autocrine manner with EGF being expressed as a transmembrane precursor, modulates the directionality of motion of the cells which results in an enhanced persistence of motion. This highly persistent motion is lost when soluble EGF is supplied to the cells in the bulk extracellular media. We suggest that secretion of EGF as a transmembrane precursor in an autocrine loop results in a spatial localization of EGF-EGFR complexes causing a greater asymmetry of the downstream signal across the cell surface. This asymmetry then drives the cell towards a highly persistent motion. This study demonstrates that the mode of ligand presentation is critical to the cell’s response to it and that a fine control over cell behavior may be obtained by modulating the presentation of a soluble ligand.

5.2 Experimental design

5.2.1 Choice of cell lines

The wild-type human mammary epithelial cells (WT HMECs) and human mammary epithelial cells expressing EGF-Ct and sEGF constructs were used in this study since they presented a well-characterized cell system which had been engineered to express EGF in different ways. The WT HMECs are a good control cell line to study the effects of EGF-EGFR interaction since they express endogenous EGFR but lack the expression of EGF. EGF-Ct expressors express EGF as a transmembrane precursor which is proteolytically cleaved and processed to release the mature ligand and hence closely resembles a physiological EGF-EGFR autocrine cell system. sEGF expressing HMECs synthesize only the 53 amino acid long secreted form of EGF which is not associated with the cell membrane since it lacks the transmembrane domain of the EGF precursor. Thus, this system allows us to investigate the effect of the membrane-associated region of the EGF precursor as well as the effect of the mode of soluble ligand presentation on cell motility.

5.2.2 Choice of substrate and growth factor

Since in this study, we were interested in determining the effect of presentation of the soluble ligand, we chose not to vary the substrate that the cell was on in order to avoid complications of another variable. All studies were done on tissue culture plastic.

Exogenous EGF was added at a concentration of 2 nM since that is approximately equal to its K_D value and this concentration was used in the study published by Wiley et al. Since we wanted to correlate our results with their study, we continued to use EGF at a concentration of 2 nM.

5.2.3 Choice of experimental methods

Cells were tracked every 10 minutes for a 6 hour time period because of the high speeds exhibited by this cell type. After the 6 hour period, very few of the original cells remained on the screen since they had migrated away from the field of view, so it was not productive to track cells for time periods longer than 6 hours.

5.3 Materials and Methods

5.3.1 Cell lines and cell culture

Human mammary epithelial cells (HMECs) expressing sEGF and EGF-Ct and the wild type (WT) HMECs were obtained from Prof. H. Steve Wiley at University of Utah, Salt Lake City (Stampfer and Yaswen, 1993; Stampfer et al., 1993). The construction and characterization of sEGF and EGF-Ct has been described previously (Wiley et al., 1998). Briefly, the sEGF and EGF-Ct constructs were packaged using the MFG retroviral vector and transfected into wild type HMECs. The cells were cultured in DFCI-1 media as previously described (Band and Sager, 1989). Monoclonal antibody 225 mAb against the EGFR was also obtained from Prof. Wiley.

5.3.2 Migration assay

Cell migration speed was measured using time-lapse videomicroscopy of single cells. 30,000 cells were plated onto 35-mm dishes in 2.5 ml serum-free medium. 20 hr. post-seeding the medium was changed to 2.5 ml assay medium with or without 2 nM EGF and incubated at 37 °C in humidified air for 2 hr. At this cell density, soluble ligand concentration is relatively unchanged over a 24 hr period (Reddy et al., 1994). 3 ml mineral oil were added to the dish to prevent evaporation, and the dish was then placed in a heated stage insert for a Ludl 99S008 motorized stage on a Zeiss Axiovert 35 microscope. Cell boundaries and centroids were identified using image-processing software developed by Engineering Technology Center (Mystic, CT) running under a LabVIEW (National

Instruments, Austin TX) and Concept Vi (Mystic, CT) environment. 5-10 cells per field in 10 different fields were scanned every 10 minutes for up to 6 hr. The x and y coordinates of the cell centroids were recorded every 10 minutes.

5.3.3 Analysis of cell paths

Single cell speed was calculated by determining the total root mean squares path length as measured by the total centroid displacement, divided by the tracking time (Maheshwari and Lauffenburger, 1998; Dunn and Brown, 1987). The reported cell speed \pm s.e.m. for each condition is an average over 200-250 cells. In order to calculate the persistence time, the cell paths were fit to a persistent random walk model. Average squared displacements were calculated for each cell using a method of non-overlapping intervals. The plot of average squared displacements versus time for each cell was fit to the persistence random walk model using the value of speed of cell calculated independently, to obtain a value of persistence time of the particular cell. This value averaged over 120-150 cells was reported as the persistence time \pm s.e.m. at the given conditions. Cell paths less than 4 hours long were not included in the calculation of persistence time but were included in the calculation of cell speeds.

5.4 Results

5.4.1 EGF presented in an autocrine manner regulates cell motility locally

The experiments described in this sub-section were performed by our collaborator, Prof. H. Steve Wiley at the University of Utah. In order to determine whether autocrine loops regulate cell motility via a community or local mode, WT HMECs and EGF-Ct expressing cells were cultured simultaneously in the same culture dish. The WT HMECs were labeled green and the EGF-Ct expressing cells were labeled red using fluorescent dyes. Their motion was then tracked over a period of 4 hours. It was observed that while cells

expressing EGF-Ct exhibited significant motion, the WT HMECs did not appear to move significantly from their original locations in this time period (Figure 5.3a). However, exogenous addition of EGF into the media resulted in stimulating the motility of the WT HMECs confirming that their motility-stimulating machinery was intact (Figure 5.3b). The observation that EGF produced in an autocrine manner in cells expressing EGF-Ct was unable to stimulate motogenesis in WT HMECs supports the local mode of action of autocrine loop since operation via a community model would require that both the WT HMECs and the EGF-Ct expressors be stimulated equally by the secreted EGF.

5.4.2 Cell speed is not affected by mode of presentation of the ligand forming extracellular complexes

Cell speed of the various cell types was quantified using time lapse videomicroscopy of single cells. We found that cells expressing EGF-Ct exhibited cell speeds of 118 $\mu\text{m/hr}$ which were significantly higher than those of cells expressing sEGF (77 $\mu\text{m/hr}$) and the WT HMECs (54 $\mu\text{m/hr}$). Addition of 2 nM exogenous EGF increased the cells speeds of sEGF expressors and WT HMECs to 92 $\mu\text{m/hr}$, while the speeds of cells expressing EGF-Ct continued to be 120 $\mu\text{m/hr}$. The difference between the speeds of the three cell types in the presence of exogenously added EGF was not statistically significant. Addition of 225 nM Ab, which blocks the binding of EGF to its receptor by binding with EGFR, significantly inhibited the motion of cells expressing EGF-Ct and those expressing sEGF indicating that the increase in cell speed was mediated via the EGFR. These experiments demonstrated that locomotion speed of cells expressing EGF-Ct is unaffected by the mode of presentation of EGF, that is, these cells continue to move at high speeds under EGF stimulation regardless of whether EGF was supplied to the cell in an autocrine manner or added exogenously. However, cells expressing sEGF were unable to stimulate cell motility to levels of those expressing EGF-Ct in the absence of exogenously added EGF suggesting that intracellular EGF-EGFR complexes may not be sufficient to fully activate the motility-

stimulating machinery of the cell. Addition of exogenous EGF to sEGF-expressing cells results in formation of extracellular EGF-EGFR complexes which are now able to completely stimulate the motility of these cells (Figure 5.4).

5.4.3 Directionality of cell motion is affected by mode of presentation of the ligand

When the reconstructed cell paths of cells expressing EGF-Ct were re-plotted in the presence and absence of exogenously added EGF, they appeared to be significantly different when examined visually. We re-plotted the cells paths in a Windrose format and observed that cells expressing EGF-Ct appeared to travel significantly longer vectorial distances from their sites of origin as compared to their paths in the presence of exogenously added EGF (Figure 5.5). While no significant differences had been observed in the speeds to these cells under the two conditions of ligand presentation, there appeared to be a dramatic effect on the directionality of motion of the cells depending on the manner in which EGF was presented to the cells.

Single cell motion has been analyzed in terms of cell speed, which is a measure of how fast the cell moves, and persistence, which is the time a cell continues to move in a particular direction before changing direction by 60 degrees. Cell motion has been previously modeled as persistent random walk (Dunn and Brown, 1987). The persistence time contains information regarding how the cell senses its external environment. By quantitation of the persistence time of the cell, it is expected that invaluable information regarding the role of autocrine loops in particular and ligand presentation in general will be obtained. In order to quantify the effect of EGF presentation on the directionality of motion of the three cell types, we analyzed the cell path as persistent random walk and obtained the parameter of persistence time under the different conditions. We observed that ligand presentation had a significant effect on the directionality of motion of the cells.

Autocrine cells expressing EGF-Ct exhibited a persistence time of 50 min. which was significantly higher than that of sEGF expressors and WT HMECs. Addition of exogenous EGF abrogated the highly directional motion of cells expressing EGF-Ct and reduced the persistence time to 16 min. The persistence time of WT HMECs and sEGF-expressing cells did not change significantly upon addition of exogenous EGF into the extracellular medium. Blocking the binding of EGF to EGFR using 225 mAb resulted in reduced persistence times of less than 10 min. for each of the three cell types (Figure 5.6).

5.5. Discussion

In this study we have examined the effect of method of presentation of a soluble growth factor on cell motility. We find that the cell motility response is indeed sensitive to the manner in which the cell receives the ligand. In particular we find that EGF synthesized as a transmembrane precursor in an autocrine manner regulates the directionality of motion of the cell in addition to stimulating an enhanced speed response.

Parameters such as the rate of ligand secretion, density of cell surface receptors and the overall cell density will influence the time it takes for the secreted ligand to accumulate in the bulk extracellular medium thereby influencing cells other than ones that secreted it (Lauffenburger et al., 1995). The finding that EGF secreted by cells expressing EGF-Ct was unable to stimulate the motility of WT HMECs in the same culture dish while increasing their own motility strongly suggests the local nature of action of autocrine loops. The experiment shown in Figure 5.3 was conducted over a time period of 4 hours. Assuming a diffusivity of 10^{-6} cm²/sec for EGF in media, the time scale for EGF to diffuse over a distance of 100 μ m is approximately 2 min. which confirms that the lack of EGFR activation on WT HMECs is not due to diffusion limitation. This result is in agreement with earlier theoretical studies where it has been shown that an autocrine ligand released by a cell has a greater probability of binding back to its own receptor than being released into

the bulk media (Forsten and Lauffenburger, 1992a). It has also been shown experimentally that a significantly higher concentration of a decoy antibody is required than that of a blocker antibody to block the interaction of an autocrine ligand with its receptor demonstrating that a cell surface receptor is able to capture a released ligand in an efficient manner (Forsten and Lauffenburger, 1992b; Lauffenburger et al., 1998). However,

The observation that speed of cells expressing EGF-Ct was higher than that of cells expressing sEGF suggests a difference in the manner in which intracellular and extracellular complexes affect cell motility. It has been previously suggested that sEGF may be involved in the formation of intracellular complexes. While cells expressing sEGF proliferated to the same extent as those expressing EGF-Ct, there were considerable differences in the organization of multicellular structures formed by the 2 cell types. Cells expressing EGF-Ct formed ductal and lobular structures, while sEGF expressing cells proliferated in colonies indicating that cells expressing EGF-Ct may function as “cell sonars”, with the spatial distribution of the receptor-ligand complexes transmitting information about the extracellular environment of the cell. The varied motility response of EGF-Ct and sEGF expressing cells suggests that these differences in locomotion may also contribute to differences in the organization of these cells.

Furthermore, these findings point towards divergence in motogenic and mitogenic signaling pathways at a cellular compartment level suggesting that proliferation may be stimulated intracellularly whereas a complete migratory response requires cell surface EGF-EGFR complexes. This may be due to greater probability of direct/indirect association of the activated EGFR with membrane-bound regulatory molecules such as Ras and PIP₂. In addition, since cell motility requires cytoskeletal dynamics, with actin being one of the key players, surface EGF-EGFR complexes might be more amenable to the formation of associations with integrins at sites of focal adhesion, which have been proposed as possible

sites of convergence of signals from growth factor receptors and integrins. The observation that 225 mAb was able to block the motion of sEGF expressors further supports the argument that cell surface complexes rather than intracellular EGF-EGFR complexes are principally involved in motility regulation of these cells (Oehrtman et al., 1998). Haugh et al. have observed differences in the ability of cell surface and internalized EGF-EGFR complexes to stimulate PIP_2 hydrolysis (Haugh et al., 1999). They determined the ability of TGF- α and EGF to stimulate EGFR activation and the activation of PLC- γ 1, known to be involved in motility stimulated by growth factors (Chen et al., 1994). The ability of TGF- α and EGF to stimulate EGFR and PLC- γ 1 activity correlated linearly with the number of receptor-ligand complexes. However, the amount of PIP_2 hydrolysis obtained, a signal downstream of PLC- γ 1 activation, depended upon the total number of cell surface complexes and was independent of the internal complexes. Their results strongly suggest a role for compartmentalization of signaling molecules involved in motogenesis and support our observation of differences in the motility response of cell surface (EGF-Ct) and internal (sEGF) EGF-EGFR complexes.

We report here that EGF synthesized as a transmembrane precursor in an autocrine manner is able to regulate the directionality of motion of human mammary epithelial cells. There is a five-fold decrease in the persistence time of cells expressing EGF-Ct upon stimulation by exogenously added EGF. We suggest that a possible mechanisms for such directional regulation might be the existence of an asymmetry of the number of receptor-ligand complexes across the length of the cell causing a biased motion of the cells as has been suggested during motion up a chemotactic gradient (Tranquillo and Lauffenburger, 1987; Tranquillo et al., 1988). The asymmetry in the complex numbers may be created in a number of ways. Some possible mechanisms include 1) a spatially regulated secretion of the autocrine EGF to the cell surface, and 2) spatial asymmetry at the level of release of the mature ligand from the membrane-bound precursor – this may be achieved at the level of

the protease involved in the cleavage and release of EGF or at the level of the protease activator. We propose that addition of soluble EGF into the bulk media then abrogates that asymmetry resulting in a significant reduction in the directional motion of the cell. Cells expressing sEGF do not exhibit highly directional cell paths possibly due to the inability of intracellular EGF-EGFR complexes to be secreted in a spatially regulated manner. This result may also suggest an involvement of the transmembrane domain of the EGF precursor in directing its secretion or that of the associated proteases to the cell surface. Since sEGF lacks the membrane-spanning domain, its secretion may no longer be spatially regulated and hence precludes the formation of spatially regulated EGF-EGFR surface complexes.

Our study addresses the effect of an autocrine ligand using the indirect readout of cell motility in terms of the underlying phenomenological properties of cell speed and persistence. It points to the role of membrane-associated autocrine ligands in regulation of directionality of cell motion. Figure 5.7 shows the effect of pathlength of the cells on the mode of ligand presentation. The greater displacements of cells expressing EGF-Ct from their original location is well captured using the parameter of pathlength which is the product of the cell speed and its persistence. Cells expressing EGF-Ct have the longest path length of approximately 100 μm which decreased to 30 μm upon addition of 2 nM EGF into the bulk media. The WT HMECs and the sEGF expressing cells have pathlengths of 11 μm and 20 μm respectively which do not change drastically upon addition of EGF.

Parent et al. have shown that directional motion towards a chemoattractant is regulated by spatially restricted activation of a G-protein system (Parent et al., 1998). Chan et al. demonstrated that EGF stimulation resulted in increased actin nucleation and filament number in the leading edge of cells which suggests another level at which directionality of motion may be regulated (Chan et al., 1998). These studies coupled with our results

demonstrate the complexity in the mechanisms which are involved in the regulation of cell motility. We see here that cell motility may be controlled by modulating the presentation of the soluble growth factor. In principle, it may be envisaged that directional control of cell motion can be obtained by transiently stimulating the expression of an autocrine growth factor coupled with macroscopic patterning of the underlying substrata to obtain highly efficient motility along well defined tracks.

5.6 References

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5.7 Figures

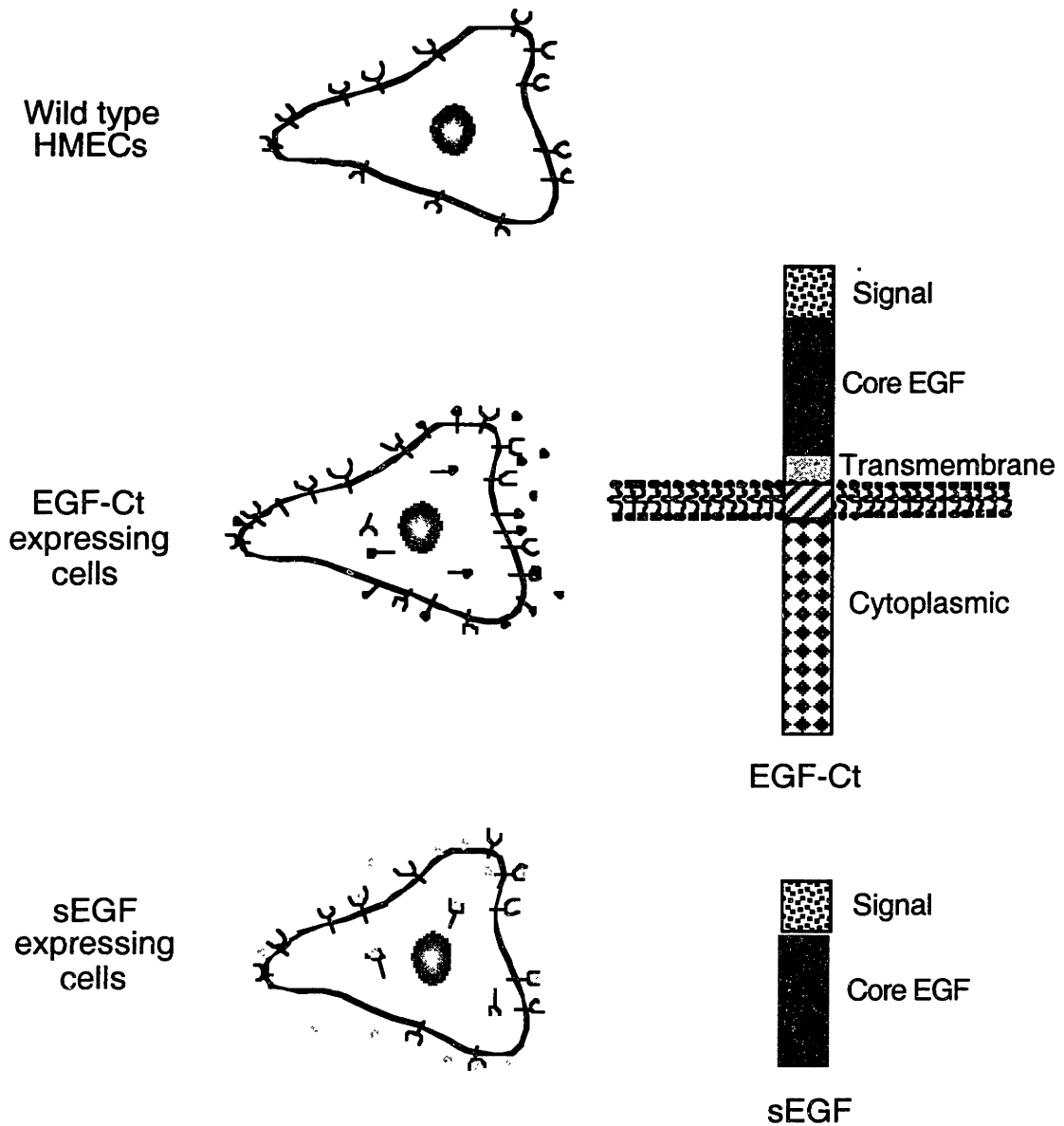


Figure 5.1 Schematic representation of the cell types used in this study. (a) Wild type Human Mammary Epithelial Cells (WT HMECs) express EGFR endogenously. They also express TGF- α and amphiregulin, but lack the expression of EGF. (b) EGF-Ct expressing cells are WT HMECs expressing the EGF-Ct construct. EGF-Ct has the sequence of WT human EGF precursor but lacks the N-terminal extracellular domain. (c) sEGF expressing cells are WT HMECs expressing the sEGF construct which is simply the 53 amino acid long mature EGF without the N-terminal, C-terminal or the transmembrane domain of EGF precursor (Wiley et al., 1998).

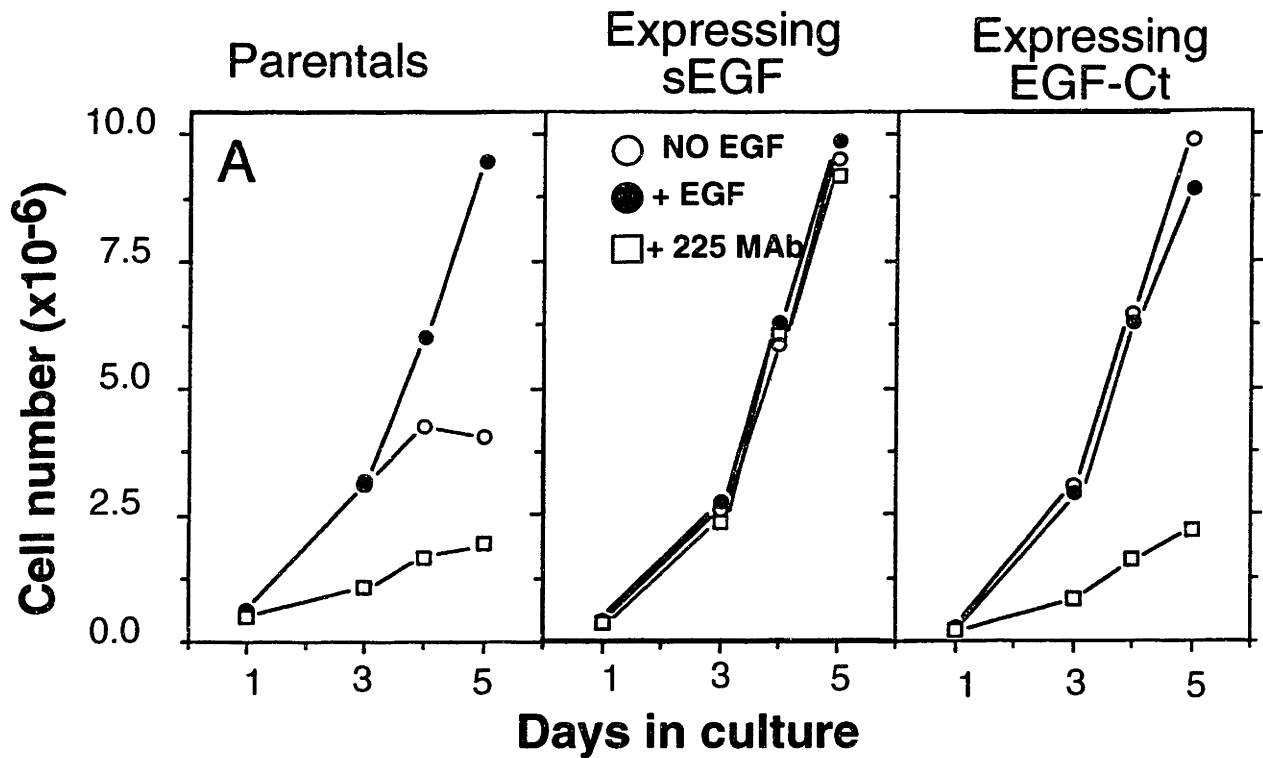


Figure 5.2 Autocrine signaling by sEGF cannot be interrupted. Equal number of each cell type was plated into dishes without EGF (empty circles) or either 20 nM EGF (filled circles) or 10 μ g/ml 225mAb (empty squares). Cell number was determined in duplicate at the indicated times. The media was changed every day. (Adapted from Wiley et al., 1998)

(a)



(b)

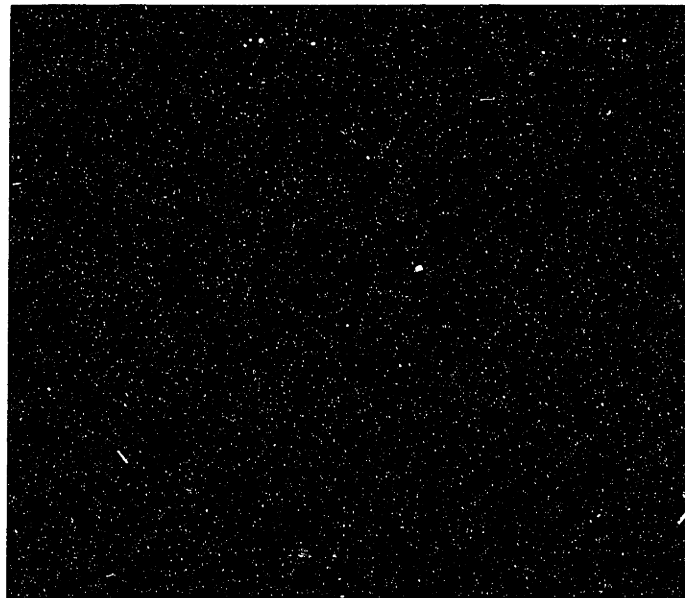


Figure 5.3 Co-culture of fluorescently labeled WT HMECs and EGF-Ct expressing cells.

WT cells are labeled green and EGF-Ct expressors are labeled red. (a) Co-culture in the absence of exogenously added EGF. Cell motion has been re-constructed by overlaying subsequent frames captured every 10 minutes. The EGF-Ct expressors exhibit significant motion while WT HMECs do not. (b) Co-culture in the presence of 2 nm exogenously added EGF. Addition of EGF results in stimulation of motion of the WT HMECs.

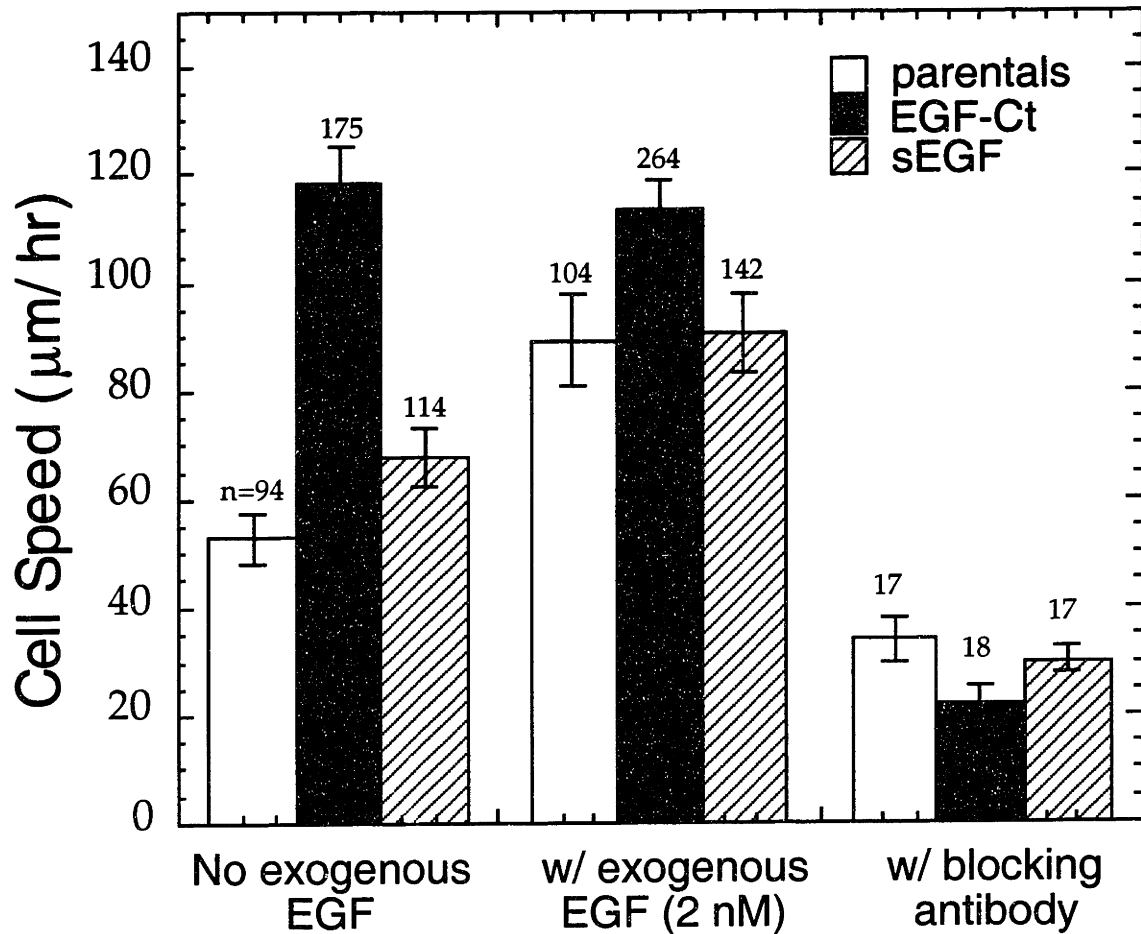


Figure 5.4 Effect of ligand presentation on cell speed.

Filled bars, hatched bars and blank bars represent the cell speeds of EGF-Ct expressing cells, sEGF expressing cells and WT HMECs respectively. Cell speeds of the three cell types were monitored in the absence of exogenous EGF, in the presence of 2 nM exogenously added EGF and in the presence of 10 µg/ml 225 mAB blocking antibody. Errors represent +/- s.e.m.

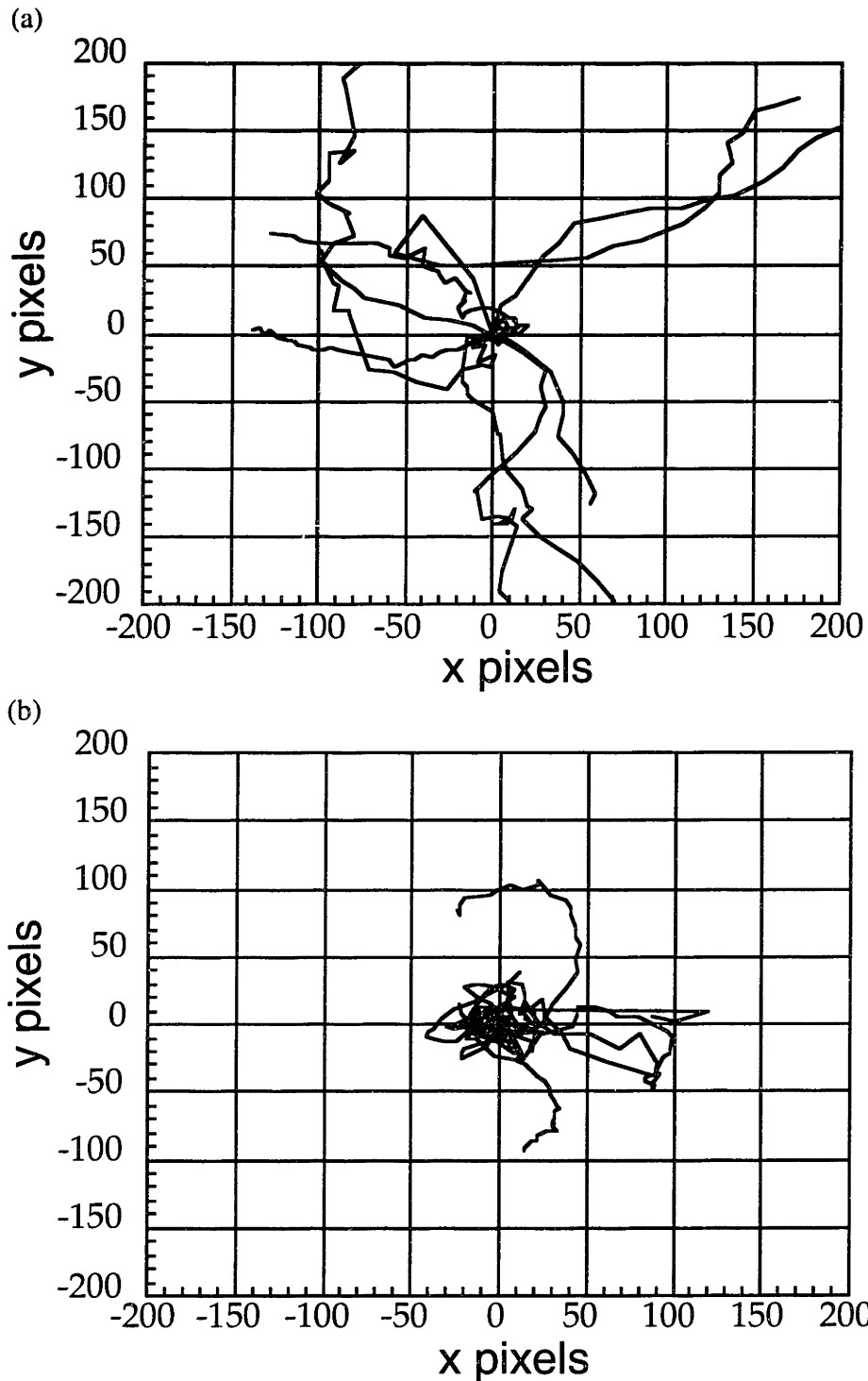


Figure 5.5 Effect of ligand presentation on cell tracks of EGF-Ct expressing cells.

(a) Typical cell paths of EGF-Ct expressors in the absence of exogenously added EGF replotted such that all cells start from the origin. These paths are of cells tracked over a period of 4 1/2 hours. (b) Cell paths of cells expressing EGF-Ct in the presence of 2 nM exogenously added EGF plotted such that all cells start from the origin. Cells shown in (a) and (b) have the same average cell speed but have significantly different patterns of motion.

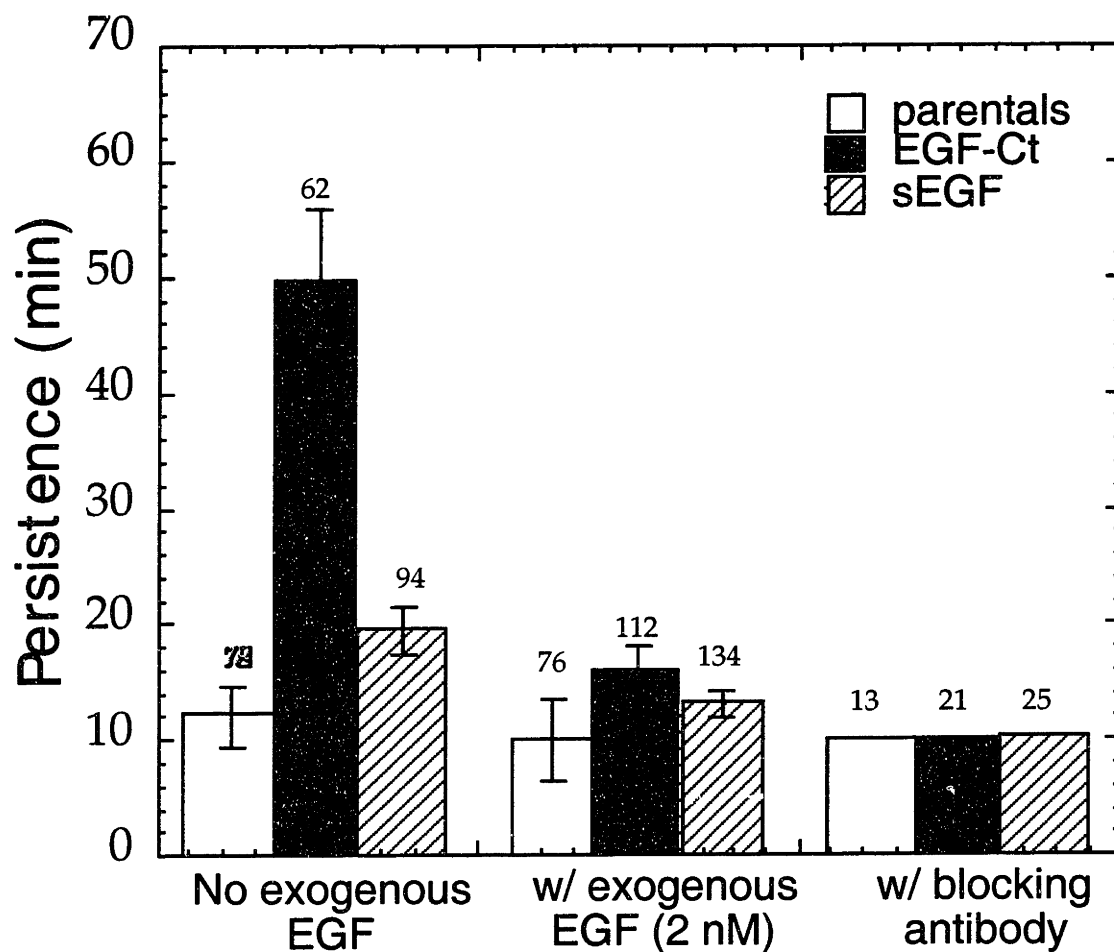


Figure 5.6 Effect of ligand presentation on persistence time.

The cell tracks were analyzed using a persistence random walk model to obtain the value of persistence time. Filled bars, hatched bars and blank bars represent the cell speeds of EGF-Ct expressing cells, sEGF expressing cells and WT HMECs respectively.

Persistence times of the three cell types were calculated in the absence of exogenous EGF, in the presence of 2 nM exogenously added EGF and in the presence of 10 $\mu\text{g/ml}$ 225 mAB blocking antibody. Errors represent +/- s.e.m.

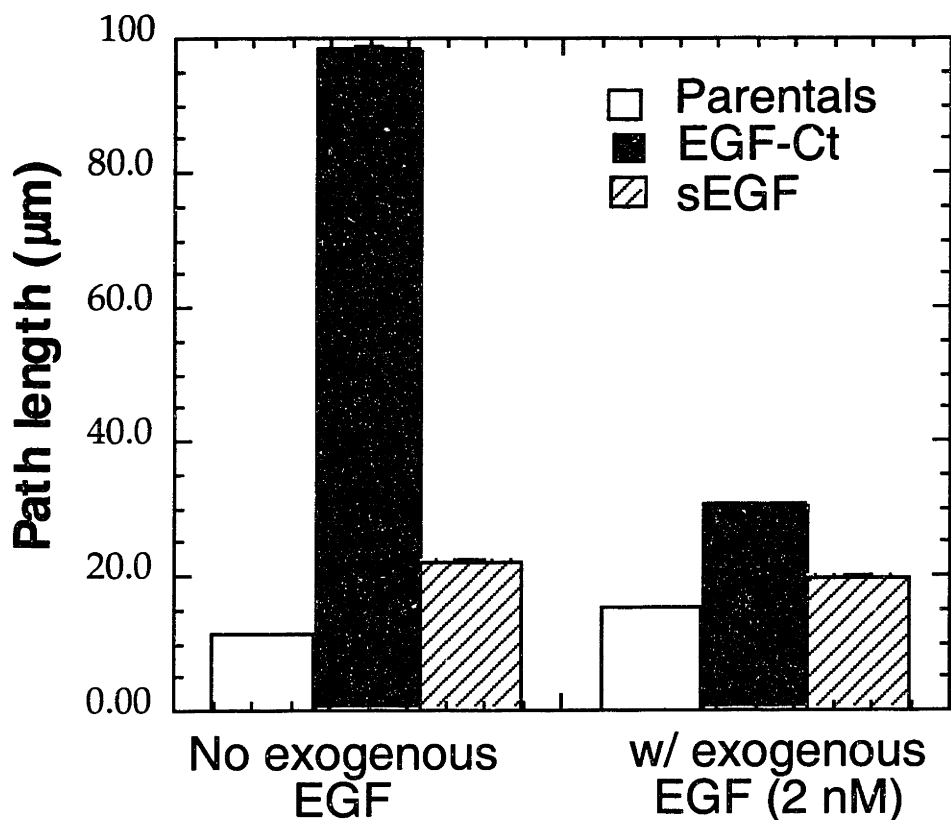


Figure 5.7 Effect of ligand presentation on cell path length.

Path length is the product speed and persistence of a cell under a given set of conditions. Filled bars, hatched bars and blank bars represent the cell speeds of EGF-Ct expressing cells, sEGF expressing cells and WT HMECs respectively. Path lengths were calculated in the presence and absence of exogenously added EGF.

Chapter 6

Future Directions

Studies conducted as a part of this thesis have shown that growth factors and ECM proteins synergize in their regulation of cell motility and that a cell is sensitive to the mode of presentation of a ligand - surface-bound as well as soluble. We have demonstrated that significant insight into cell motion may be gained by performing a systematic and quantitative study of the underlying phenomenological properties in terms of the biophysical processes affecting them.

Studies using the model ECM protein Fibronectin and EGF as the growth factor revealed that the biophysical processes of cell-substratum adhesion and membrane activity were involved in the regulation of motility. Additional insight into the regulation of cell motility can be gained by quantifying the amount of contractile force that is generated within the cell under these conditions. We have used a saturating concentration of EGF in the bulk fluid media in the studies described in Chapter 3. A more complete picture of EGF- and Fn- mediated regulation of cell motility may be gained by determining a dose-dependent response of EGF under varying surface concentrations of Fn. These experiments may also provide guidelines for the development of a minimalistic matrix where growth factors and adhesion ligands are both covalently tethered onto a surface.

Use of star PEO-based matrices provided a very versatile system for the study of effect of presentation of the adhesion ligand, RGD. It is hoped that this system will allow us to

answer a number of fundamental questions in cell biology and will help firm the foundations of the field of tissue engineering. It will be very interesting to re-construct an ECM by simultaneously immobilizing peptides that mimic specific domains of natural ECM proteins. For example, using the synergy sequence present on fibronectin along with the RGD sequence would be a facile extension of the current study. This will help determine whether even lower surface densities of RGD can be used to obtain the required amount of cell-substratum adhesion at a given cluster size. Similarly, variations of the flanking sequences of RGD which confer a higher affinity to the peptide might be used to determine the effect of ligand affinity and presentation on cell motion.

Another aspect of cell-surface interactions that has not been addressed by this thesis is the effect of compliance of the surface in regulating cell function. The star PEO molecules used in this study present the ligand on a long and flexible tether and are immobilized on a PEO-based hydrogel. These characteristics of the system may affect the amount of contractile force that the cell is able to generate on such a surface and hence limit the cell speeds observed. In order to address this problem, it is essential to carry out similar studies on surfaces with varying degrees of compliance which may be obtained by varying the tether length of the PEO molecule used to present the adhesion ligand as well as by varying the degree of crosslinking of the underlying PEO hydrogel.

The studies conducted in this thesis have been of a macroscopic nature and have involved determining cell function at the level of a single cell. A possible direction of further investigations may be to establish the effect of ligand presentation on the activity of the regulatory molecules within the cell involved in cell motility. Some candidate signaling proteins may be FAK, MAP kinase and the Rho family of GTPases. Modifications of the current star PEO-based system may be required since using current chemistry to synthesize surfaces of the required surface area is not cost-efficient. The star PEO

molecules used in this study exhibit a significant polydispersity in the number of linear PEO molecules present on a single star PEO molecule. For more rigorous quantitative studies, it is recommended that star PEO molecules with lower variation be used.

Investigations on the effects of presentation of an autocrine ligand on cell motility revealed that EGF presented as a transmembrane precursor is involved in the regulation of the directionality of cell motion. This observation opens several avenues of further investigation. Determination of a precise molecular level mechanism for the regulation of cell directionality is required before this mode of regulation of cell motility may be exploited for tissue engineering purposes. A possible line of investigation involves examining the sorting and trafficking of the synthesized receptors and ligands along with the cell surface distribution of endopeptidases that are involved in the release of the mature ligand from the membrane-bound precursors. In addition the set of human mammary epithelial cell lines used in the studies in Chapter 5 provide an excellent system for the investigation of the pathways involved in the regulation of motility and mitosis since there are differences in the effect of intracellular complexes in stimulating migration and proliferation. In order to rationally design experiments that might help in determination of a mechanism for the regulation of directionality of cell motion by autocrine ligands, it will be very helpful to develop a mathematical model that takes into account the compartmentalization of the ligand and the receptor along with some elements of the downstream signaling molecules involved in the regulation of cell motility.

In the studies described in Chapters 3 and 4, the soluble environment of the cells was maintained constant while the underlying substrata was varied systematically. On the other hand, in the investigations carried out in Chapter 5, the soluble environment of the cell was varied while keeping the substrate that the cells were adhered to unchanged. In order to ultimately use different modes of ligand presentation in affecting cell motility, the

two systems will need to be combined where presentation of the soluble ligand as well as that of the immobilized ligand will be varied simultaneously. In addition, further complexity can be built into the system by simultaneously tethering growth factors and adhesion ligands on the surface. Using tethers such as the star PEO molecules permits simultaneous presentation of multiple ligands in varied spatial configurations. Furthermore, similar approaches may be used to study other cellular phenomena such as cell proliferation and differentiation. Combination of such investigations is essential to ultimately achieve a complete understanding and control over specific cell behavior.

In conclusion, the results of these studies demonstrate the importance of applying quantitative engineering principles for understanding cell behavior and have uncovered several possible lines of future investigations.

Appendix A1

Preparation of RGD modified surfaces

(Adapted from Brown, 1999)

Step 1 Preparation of inert background

Step 1.1 Preparation of glass substrates

18 mm glass coverslips (#1 thickness, from VWR Scientific) were cleaned by immersion for 30 minutes, first in a 50 vol. % solution of hydrochloric acid in methanol, then in a 50 vol. % solution of sulfuric acid in water, followed by rinsing several times in water. The coverslips were then immersed in anhydrous methanol, containing 4 mg/ml O-[2-(Trimethoxy silyl)-ethyl]-O'-methyl-polyethylene glycol 5,000 (PEG-silane, Fluka) and 5 vol% water. Surfaces were rinsed three times in methanol, and cured at 50 °C for at least 1 hour.

Step 1.2 Electron-beam irradiation of PEO solutions

Amino-functional PEG hydrogels were covalently grafted to aminated coverslips by immersing the coverslips in an aqueous solution of bis (polyoxyethylene bis(amine)) (M.W. = 20,000, hereafter referred to as PEG-NH₂, Sigma Chemical Company), 50 mg/ml in deionized distilled water in a 100 mm glass petri dish (Corning). Immediately prior to irradiation, the excess solution was drained from the dish, leaving only a thin film of PEG-NH₂ solution at the surfaces of the coverslips.

Cross-linking of the PEG-NH₂ solution was achieved by exposure to electron beam irradiation, at a total dosage of 2 Mrad. The source was a 3 MeV Van de Graaf generator, which delivers radiation at a rate of 250,000 rad/second. Because of the large number of steps in the preparation of clustered ligand surfaces, it was not practical to handle all of the gels as sterile after they are prepared. After irradiation, substrates were rinsed briefly in deionised, distilled water, and the petri dishes were sealed with Parafilm™, and stored at 4 °C. Hydrogel-grafted coverslips were used within 30 days after preparation. Prior to use in cell-based assays, surfaces were sterilized by light spraying with a 70% solution of ethanol in water.

Step 2 Preparation and activation of star PEO molecules

Step 2.1 PEO functionalization

As received from the suppliers, star PEO molecules are terminated by hydroxyl groups. The end groups of the PEO tethers were first converted to the carboxyl functional group which could be made to react, both with the ligand to be immobilized, and with the substrate, by a method similar to that described by Royer (Royer, 1987).

Step 2.2 Peptide preparation

The RGD peptide used in this work, YGRGD is shown schematically in Figure 2.2. As can be seen from the structure, there are charged guanidine and carboxyl groups in the side chains of arginine and aspartic acid respectively, which are capable of participating in the chemical reaction desired at the N- and C-termini. These amino acids are therefore supplied with protected arginine and aspartic acid side chains (YGR(Pmc)GD(tBu), American Peptide Company), which can be removed after the synthesis reaction, based on recommendations of Lin et al. (Lin et al., 1994). The tyrosine residue was supplied unprotected as a site for radiolabeling; unprotected tyrosine does not participate in any of the activation or coupling reactions.

The peptide was labeled for quantitation by iodination of the tyrosine residue, using a variation on the method of Massia et al (Massia and Hubbell, 1990a; Massia and Hubbell, 1990b). Briefly, 20 μ l of a 1 mg/ml solution of protected peptide, dissolved in 2-[N-morpholinoethane sulfonic acid] buffer (hereafter referred to as MES buffer, Sigma Chemical Company) was reacted with 2 mCi Na¹²⁵I using the Iodobead method (Markwell, 1982). for 15 minutes at room temperature. Iodination protocol is given in Appendix A. Labeled peptide was separated from free iodine by reverse-phase chromatography, loading the mixture on a C₁₈ Sep-Pak cartridge (Waters), which had been equilibrated prior to loading, first with a solution of trifluoroacetic acid (TFA) and water in methanol (1 vol%: 19 vol%: 80 vol%, respectively), and then with PBS. Unincorporated iodine was flushed first from the cartridge using a 1% solution of TFA in water, then the peptide was fractionated using successive solutions of TFA and water in methanol, from to 1:89:10 to 1:79:20, and so on, increasing the methanol concentration by 10 vol% each time) until a

mixture of composition 1:19:80 was reached. The fractions containing peptide were pooled.

The radiolabeled peptide solution was adjusted to a pH of 6 by the addition of 1N sodium hydroxide, and stored at 4 °C until use. Because of the believed instability of iodinated species (Knoche, 1991) (radiolysis of water molecules generates free radicals, which in turn may attack the already weak carbon-iodine bond), the RGD samples were typically used within a week of iodination.

Step 2.3 PEO-RGD conjugation

Star PEO-carboxylate was dissolved in MES buffer, pH = 6.1 to a concentration of $7e-7$ mol of (total) chain ends per ml. Chain ends were activated by adding a 1.5-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Pierce Chemical) and a 2-fold molar excess of N-hydroxysulfosuccinimide (sulfo-NHS) (Pierce Chemical) over (total number of) chain ends, with stirring, for 20 min.

RGD peptide was added to the reaction mixture, in the molar ratios indicated in Table A1.1. The conjugation reaction was allowed to proceed for 12 hours. The PEO-ligand conjugate was separated from unreacted ligand, excess crosslinker and buffer salts by dialysis against distilled water, with 4 to 6 water changes until the radioactivity detected in the water decreased to background (Pierce dialysis cassettes, MWCO = 10,000). Dialysis against water typically causes the sample solution to take on a relatively large volume of water; the volume after dialysis is usually 250 to 300% of the initial volume. The product was frozen at -20 °C, lyophilized and stored at -20 °C until further use. Quantitation of the product was achieved by dissolving a known mass of PEO-RGD conjugate in PBS, and measuring its activity on a Packard gamma counter. Conjugation results are shown in Figure A1.1.

Step 3 Immobilization of star PEO to PEO hydrogels

Step 3.1 Surface immobilization

In order to achieve the desired variation in spacing between ligand clusters, PEO-ligand conjugates were immobilized on gel substrates from solutions containing a mixture of unmodified and ligand-modified star PEO. 5 % w/v solutions of the PEO-RGD conjugates

were prepared in MES buffer. At the same time, a 5 % w/v solution of unmodified star PEO was prepared. Carboxyl-functional chain ends were activated with EDC and Sulfo-NHS, as before. The two solutions were mixed, in the proportions shown in Table 4.2. Then 125 μ l aliquots of activated PEO-RGD were coupled to each substrate (18 mm hydrogel-grafted glass coverslip), and the coupling was allowed to proceed for at least 12 hours in a humid chamber at room temperature.

Substrates were rinsed several times in deionized water, then immersed for four hours in a 50 mM solution of Tris-HCl, pH = 6.1, to block any remaining activated chain ends. Finally, coverslips were rinsed again in water. Deprotection of the arginine and aspartic acid side chains was performed by immersion of substrates in a cleavage mixture known as "Reagent K" (King et al., 1990) for one hour. To determine the ligand density and cluster spacing, bioactive surfaces were prepared using iodinated RGD peptide. Three coverslips at each cluster size and spacing were crushed and their activity measured on a Packard gamma counter. Results are shown in Figure A1.2

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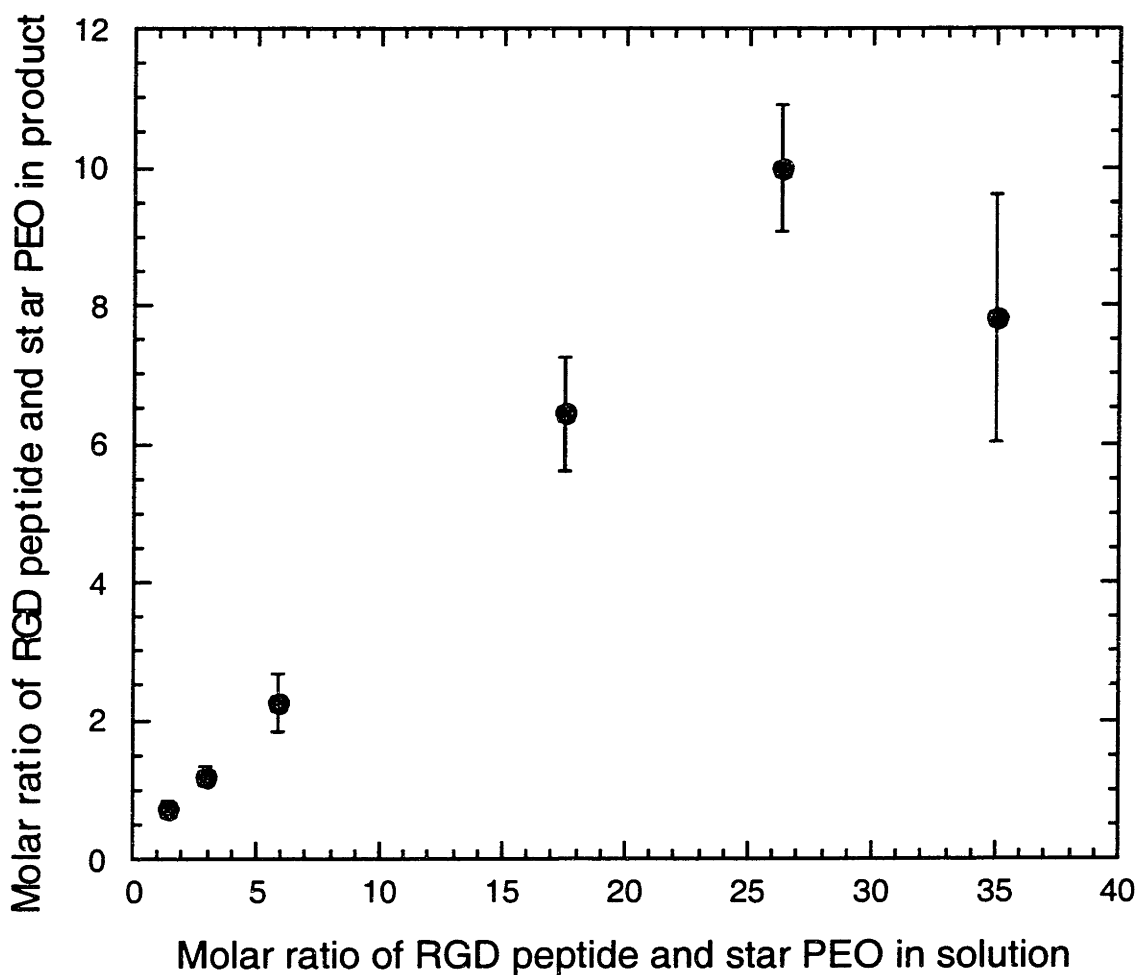


Figure A1.1 Generation of varying RGD cluster sizes.

Varying average cluster sizes (n_{cl}) of RGD per star PEO molecule may be obtained by varying the molar ratio of RGD peptide and star PEO molecules in solution. ECD-sulpho NHS is used as the zero length crosslinker. $n_{cl} = 0.8, 5$ and 9 were used in the cell studies described in Chapter 4.

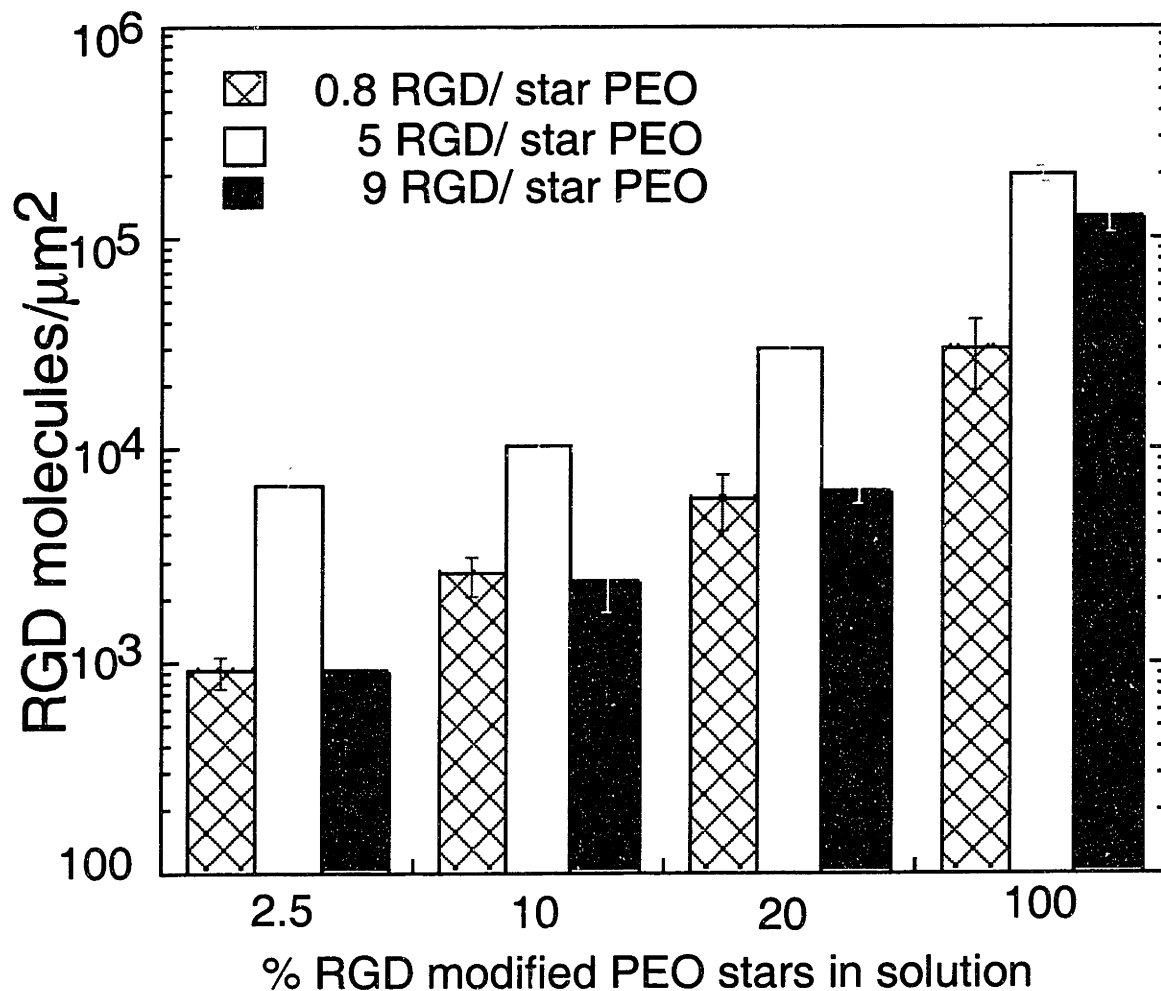


Figure A1.2 Average surface densities of RGD modified surfaces used. Varying RGD surface densities can be obtained by varying the ratio of RGD-modified to unmodified star PEO molecules at each cluster size. Star PEO molecules were covalently tethered to the PEO hydrogel surface using EDC- sulpho NHS as the zero length crosslinker.

Table A1.1 RGD-PEO ratios during conjugation in aqueous buffer

SAMPLE	MOL RATIO RGD:PEO	PEO MASS (mg)	QUANTITY OF PEO (mol)	RGD MASS (mg)	QUANTITY OF RGD (mol)
#1	1.5	30	5.36E-08	0.117	1.34E-07
#2	3	30	5.36E-08	0.234	2.68E-07
#3	6	30	5.36E-08	0.469	5.36E-07
#4	18	30	5.36E-08	1.406	1.61E-06
#5	26	30	5.36E-08	2.109	2.41E-06
#6	35	30	5.36E-08	2.812	3.21E-06

Table A1.2 Modified-unmodified PEO ratios during immobilization

SAMPLE	[PEO-RGD*], mg/ml	[PEO], mg/ml	% PEO-RGD*	Vol. (PEO-RGD*), ml	Vol. (PEO), ml
# 1	50	0	100	0.750	0.000
# 2	10	40	20	0.150	0.600
# 3	5	45	10	0.075	0.675
# 4	2.5	47.5	5	0.038	0.713
# 5	1	49	2	0.015	0.735

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