TENASCIN CYTOTACTIN EGF-LIKE REPEATS - NOVEL MATRIKINE LIGANDS FOR THE EPIDERMAL GROWTH FACTOR RECEPTOR

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Select epidermal growth factor (EGF)-like (EGFL) repeats of human tenascin cytotactin can stimulate EGF receptor (EGFR) signaling, but activation requires micromolar concentrations of soluble EGFL repeats in contrast to subnanomolar concentrations of EGF. Using *in silico* homology modeling techniques, we generated a structure for one such repeat, the 14th EGFL repeat (Ten14). Ten14 assumes a tight EGF-like fold with truncated loops, consistent with circular dichroism studies. We generated bound structures for Ten14 with EGFR using two different approaches, resulting in two distinctly different conformations. Normal mode analysis of both structures indicated that the binding pocket of EGFR exhibits significantly higher mobility in Ten14-EGFR complex compared to the EGF-EGFR complex; we attributed this to loss of key high-affinity interactions within the Ten14-EGFR complex. We proved the efficacy of our *in silico* models by *in vitro* experiments. Surface plasmon resonance measurements yielded equilibrium constant K_D of 74μM for Ten14, approximately three orders of magnitude weaker than that of EGF. In accordance with our predicted bound models, Ten14 in monomeric form does not bind EGFR with sufficient stability to induce degradation of receptor, or undergo EGFR-mediated internalization. This transient interaction of Ten14 with the receptor on the cell surface is in marked contrast to other EGFR ligands which cause EGFR transit through, and signaling from intracellular locales in addition to cell surface signaling.

We investigated whether Ten14-mediated surface restriction of EGFR resulted in altered cellular responses compared to EGF. Activation of PLCγ and m-calpain, molecules associated with migration, were noted even at sub-saturating doses of Ten14. However, activation of ERK/MAPK, p90RSK and Elk1, factors affecting proliferation, remained low even at high Ten14 concentrations. Similar activation profiles were observed for EGF-treated cells at 4^{o}C, a maneuver that limits receptor internalization. We demonstrated a direct concurrent effect of such altered signaling on overall biophysical responses - sustained migration was observed at lower levels of Ten14 that activated PLCγ, but proliferation remained basal.

We present a novel class of EGFR ligands that can potentially signal as a part of the matrix, triggering select signaling cascades leading to a directed cellular response from an otherwise pleiotropic receptor.

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DEDICATED TO

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1.0 INTRODUCTION

1.1 THE EXTACELLULAR MATRIX

The extracellular environment is one of the most critical and exhaustive sources of stimuli for a cell. The cell receives signals for basic functions such as survival, development, proliferation, migration, and differentiation through myriad soluble and insoluble factors from the extracellular environment (Nelson and Bissell, 2006). These factors activate one or more receptors on the cell surface, which can be temporally and spatially modulated to achieve a specific response. Although considerable knowledge has been gained over the past few decades of how these triggers are translated to cellular responses, we still do not have a complete understanding of how the initiating extracellular cues are parsed so that a particular cellular behavior may be elicited based upon temporal and spatial considerations.

The extracellular milieu consists of both soluble and insoluble factors that are released by cells due to external cues, and the action of these factors may be modulated in a manner so that they act either in a paracrine fashion, autocrine fashion, or both (Rozengurt, 1999). How a mode of action of a ligand is decided upon depends upon several factors, and the response can be altered based on whether the signaling occurs during physiological settings such as normal wound healing or pathological conditions such as tumor progression.

In addition to soluble factors such as chemokines, cytokines and growth factors (the latter including epidermal growth factor (EGF) and transforming growth factor alpha (TGFα), the extracellular matrix (ECM) also has an insoluble fraction of proteins that play key roles in supporting cells within tissues and maintaining important cellular functions. These insoluble matrix components are typically large proteins consisting of multi-subunit complexes, and are composed mainly of fibrous glycoproteins and proteoglycans, which are a subset of modified glycoproteins with heavy carbohydrate side-chains (Hay, 1981). ECM proteins include fibrous components including collagens, laminins and fibronectin and much heavier multimeric proteins including the tenascins and agrins (Dityatev and Schachner, 2006; Hay, 1981; Jones and Jones, 2000a; Midwood et al., 2006; Miyazaki, 2006). The ECM proteins are secreted largely by fibroblasts, and the type of extracellular proteins being secreted depends upon the physio-pathological condition around the cell (Powell et al., 1999). In some cases (as seen with syndecans), proteoglycans may also be inserted into the plasma membrane where they act as co-receptors in order to facilitate the interaction of conventional cell surface receptors with other proteins in the ECM (Alexopoulou et al., 2007). The ECM components can potentially bind both soluble proteins such as growth factors and chemokines secreted into the ECM, as well as signal a number of different cell types via specific cell surface receptors that are specific to each family of ligands.

In the past few decades, the focus of ECM research has largely been on the more ubiquitously expressed proteins such as collagen and fibronectin, leading to substantial understanding of their mode of action and decryption of intracellular signaling pathways triggered by these ECM proteins (Vogel et al., 2006; Yoneda et al., 2007). Recently however, tenascin cytotactin (tenascin C) has come to the fore as an important ECM component, mainly

due to its temporally and spatially restricted pattern of expression during development, angiogenesis and wound-healing, and dysregulated expression in pathophysiological situations such as cancer metastasis and in hypertropic scars (Dalkowski et al., 1999; Orend and Chiquet-Ehrismann, 2006). Although tenascin C has been implicated in numerous other pathologies, the signal transduction cascades involved in its function have remained poorly characterized. In order to better understand how tenascin C function, the individual domains in tenascin C structure need to be investigated.

1.2 TENASCIN CYTOTACTIN AND ITS EGF-LIKE REPEATS DOMAIN

Tenascin cytotactin (known also as hexabrachion, cytotactin, neuronectin, myotendinous antigen and glial/mesenchymal extracellular matrix protein) is the first member of the tenascin family of ECM proteins that has four other members (Tenascin R, Tenascin W, Tenascin X and Tenascin Y). It was first discovered when investigating different aspects of cell, developmental and tumor biology by independent groups (Jones and Jones, 2000a). Structurally, human tenascin C (TN-C) is a large oligomeric glycoprotein composed of three identical subunits or multiples thereof covalently linked at the N-terminal end, with each subunit between 190 and 300kDa (Conway and Parry, 1991) (Redick and Schwarzbauer, 1995). For formation of the final multi-meric structure, single subunits are first arranged into trimers via formation of disulfide bonds in the proximal tenascin-assembly domain, followed by linkage of trimers to form hexamers directed by homophilic interactions at the N-terminal region, as seen in the rotary image in Figure 1A (Kammerer et al., 1998; Luczak et al., 1998). Each subunit of human tenascin C is composed of an N-terminal tenascin assembly domain, a 14.5 EGF-like (EGFL) repeat array, anywhere

between 11 and 18 fibronectin type III (FNIII) domains (of which up to nine may be alternatively spliced, and eight conserved) and a terminal fibrinogen-like globe (Figure 1C). Each of these domains have been implicated in several physiological and pathological conditions, influencing responses ranging from neuronal outgrowth, cell attachment and spreading to stimulating expression of matrix metalloproteinases and binding heparin. Interestingly, heterotypic hexamers of tenascin are also found, consisting of trimers from two different variants of TN-C with alternatively spliced fibronectin regions, or from trimers of different members of the tenascin family, adding to the functional complexity of the macromolecule (Chiquet-Ehrismann et al., 1991).

Since the EGFL repeats are focus of this work, it is imperative to delve into them to gain a better understanding of their role vis-à-vis TN-C. The EGFL repeat array of TN-C consists of 14.5 EGFL repeats, each one 31 amino acids in length (Figure 1B). The approximate consensus sequence for EGFL repeats in TN-C is Cys-x-Cys-x(5)-Gly-x(2)-Cys, with 'x' being any residue with the length in brackets. However, EGFL repeats within laminin are slightly longer with an extra set of cysteines at the C-terminal end, and have a consensus sequence of Cys-x(1,2)-Cys-x(5)-Gly-x(2)-Cys-x(2)-Cys-x(3,4)-[PheTyrTrp]-x(3,15)-Cys (Baumgartner et al., 1996; Engel, 1989; Stetefeld et al., 1996). EGFL repeats form disulfide bonds between cysteines in the 1-3, 2-4, 5-6 conformation (for longer peptides such as laminin that have additional cysteines, 7-8 are also bonded in addition to the first six), and this binding conformation is conserved in all members of EGF-like family of proteins such as EGF, TGFα and amphiregulin, thereby giving them a closed and tightly packed structure (Carpenter and Cohen, 1990; Cooke et al., 1987). In addition to TN-C and laminin, EGFL repeats are also found within other proteins such as agrin, perlecan and even integrins (Selander-Sunnerhagen et al., 1992; Takagi et al., 2001; Tamkun et

al., 1986). However, as opposed to EGFL repeats found in all these proteins and others such as the Notch family of receptors and coagulation factors IX and X, TN-C EGFL repeats lack the acidic residues that are required to bind calcium (Handford et al., 1990). This suggests another function for these EGFL.

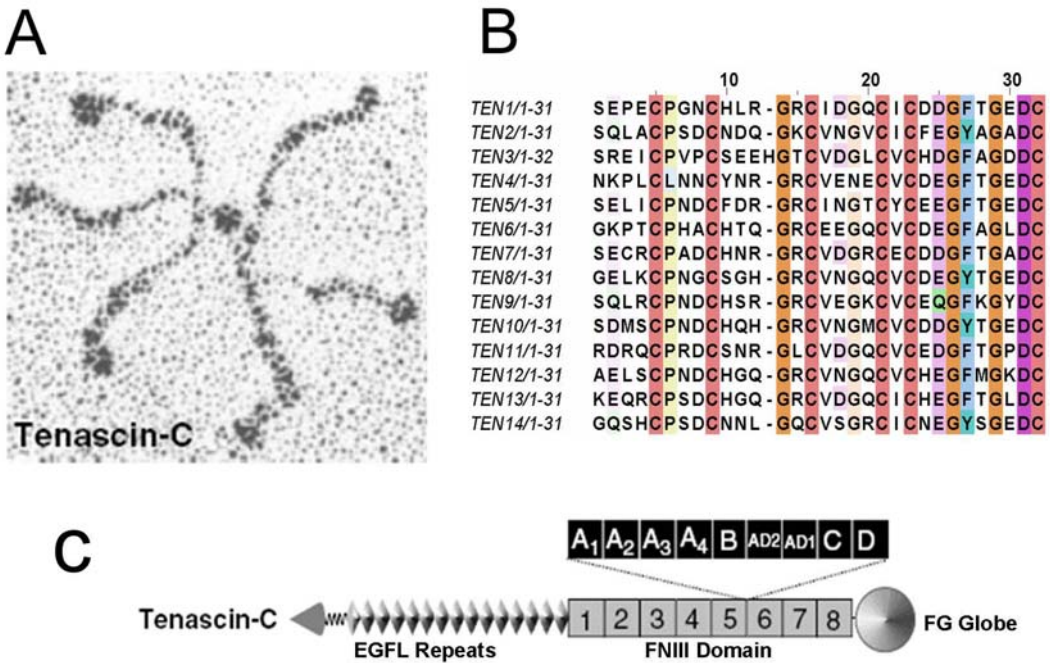

Figure 1. Human Tenascin C and Its Structural Domains

A. Rotary electron microscope image showing the hexabrachion structure of tenascin C. It has six identical arms extending radially out from a central tenascin assembly domain.

B. CLUSTALW analysis was performed on all the 14 repeats of tenascin C. Colored bars indicate conserved residues among the repeats.

C. Each lobe of tenascin C is composed of an N-terminal assembly domain, an EGFL repeat array, an alternatively spliced FNIII domain and a terminal fibrinogen globe.

During the early nineties, some postulated that EGFL repeats mediated physiological effects either directly or indirectly through EGFR, although no direct evidence was presented (Prieto et al., 1992; Spring et al., 1989; Thiery and Boyer, 1992). Interestingly, human tenascin C causes the clustering of EGF receptors, and also primes cells to the mitogenic effects of EGF (Jones et al., 1997; Jones and Jones, 2000b). In fact, in stromo-epithelial interactions, EGF induces the expression of tenascin C through EGFR (Sakai et al., 1995a; Sakai et al., 1995b). Recently, however, researchers have successfully demonstrated that select EGFL repeats of human tenascin C can bind and activate the EGF receptor (EGFR) (Swindle et al., 2001). Similar studies were performed with EGFL repeats of laminin, showing that they could be processed and released in vivo by matrix metalloproteinases (MMP), and could bind and activate EGFR (Schenk et al., 2003). Although EGFL repeats interact with EGFR, the physical binding of EGFL repeats with receptor and structural domains involved in this binding have not been identified and characterized to date. Additionally, it is also not well understood what effects such binding might have on the overall response in terms of modulating cell behavior. A general understanding of the EGF receptor and how it binds the EGF family of ligands may shed light on the mode of action of EGFL repeats.

1.3 STRUCTURAL AND FUNCTIONAL OVERVIEW OF THE EPIDERMAL GROWTH FACTOR RECEPTOR AND ITS LIGANDS

The epidermal growth factor family, which includes EGF, TGFα, amphiregulin, neuregulins, HB-EGF and betacellulin, represents one of the most important classes of receptor ligands that mediate numerous cellular functions including migration, proliferation, differentiation and

apoptosis by binding to the ErbB family of receptor tyrosine kinases (Wells, 1999). These ligands bind to either homo- or heterodimers of the ErbB receptors, thereby activating a number of signaling cascades leading to directed cellular responses. As mentioned earlier, all members of the EGF family of ligands contain six cysteines that are crosslinked via disulfide linkages. This intramolecular binding confers a closed tightly packed structure to the peptide, and is critical for receptor recognition and binding.

EGFR belongs to the ErbB family of receptors that includes ErbB2 (Her2), ErbB3 and ErbB4. Active EGFR functions as a dimer, partnering with another subunit of EGFR or even with another member of the ErbB family (Schlessinger, 2000; Wells, 1999). EGF and TGFα are the most well characterized ligand for EGFR; they bind with extremely high affinity to the receptor, activating nearly seven key tyrosine residues in intracellular kinase domain of the receptor (Zhou et al., 1993). Upon ligand binding, inactive EGFR residing in caveolae and invaginated membrane microdomains dimerize and migrate into clathrin coated pits, where they are then internalized in an adaptin-dependent fashion. In the endosomal compartment, EGFR may be either degraded or recycled, depending upon ligand properties. EGF remains bound to EGFR even in late endosomes, earmarking the receptor for degradation. Unlike EGF however, TGFα dissociates from EGFR in a pH dependent manner, leading to recycling of receptor (French et al., 1995).

In addition to EGF and TGFα, EGFR also binds betacellulin, amphiregulin, heparin-binding EGF, epiregulin, and other virally encoded factors {Harris, 2003 #799(Citri and Yarden, 2006; Jones et al., 1999). As mentioned earlier, independent researchers have shown that EGFL repeats of tenascin C or laminin-5 can also bind the receptor and activate it (Schenk et al., 2003; Swindle et al., 2001). In order to better understand the binding of such a variegated class of

ligands to EGFR, it is important to identify and assess the intra-molecular residue interactions between the receptor and its ligands, and recognize important structural binding motifs that may be required for ligand identification.

As mentioned previously, both EGF and TGFα bind EGFR with affinities in the low nanomolar levels. Considerable work has been done in identifying important residues in EGF and TGFα that contribute to this high affinity. Mutational analysis indicates that His10, Tyr13, Leu26, Arg41 and Leu47 in EGF and corresponding residues in TGFα are extremely important for the binding of ligand to EGFR (Campion et al., 1993; French et al., 1995; Reddy et al., 1996a; van de Poll et al., 1998). In addition, Tyr38, Gly40 and Arg45 seem to be important for endosomal binding of these ligands (French et al., 1995; Lenferink et al., 2000).

High resolution crystal structures of both ligands bound to the extracellular domain of EGFR shed considerable light on critical receptor-ligand interactions (Garrett et al., 2002; Ogiso et al., 2002). Both EGF and TGFα have three loops, A-C, with each loop containing critical residues for interaction with EGFR. A number of notable residue interactions occur (for example Arg125 of EGFR with Glu27 of TGFα) that are specific to each ligand and contribute to overall binding of receptor to ligand. However, when observations from crystal structures for both ligands bound to EGFR are coalesced, three structurally conserved regions in EGFR that are crucial for ligands identification and binding can be identified that seem to be critical for high affinity ligands to bind EGFR (Figure 2).

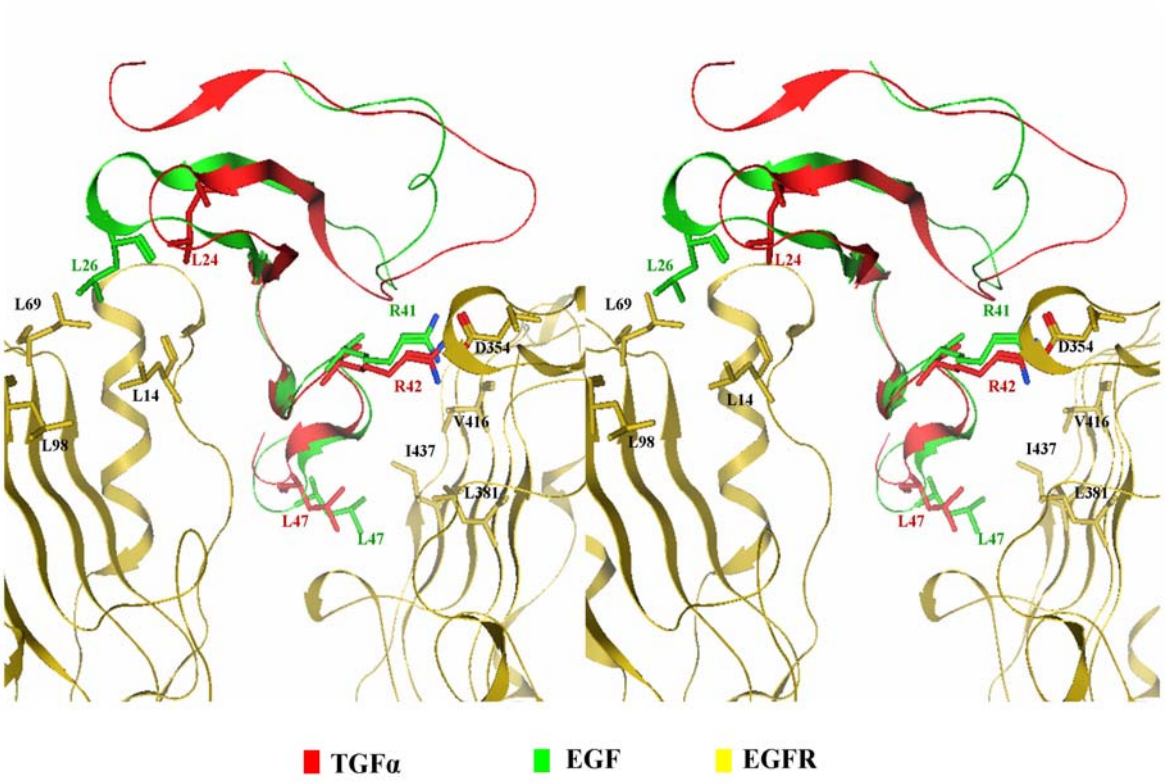

Figure 2. Docking of EGF and TGFα onto EGFR in the ligand-binding pocket

Stereo views show EGF (green) and TGFα (red) docked onto EGFR (yellow) at the ligand binding pocket. Important interactions between ligand and receptor have been indicated. Leu26 (Leu24 in TGFα) interacts with the hydrophobic pocket formed by Leu14, Leu69 and Leu98 of EGFR. Favorable hydrophobic interactions are also established between Leu47 of EGF (Leu47 in TGFα) and the pocket formed by Leu381, Val416 and Ile437 of EGFR. Arg41 of EGF (Arg42 in TGFα) anchors the ligand to the EGFR binding pocket by forming a high-affinity salt bridge with Asp354 of the receptor.

Firstly, a hydrophobic pocket is formed in domain I by Leu14, Leu69 and Leu98 of EGFR that accommodates the conserved leucine in loop B of ligand (Leu26 of EGF and Leu24 of TGFα). Secondly, another hydrophobic pocket is formed in domain III by Leu382, Phe412,

Val417 and Ile438 which interacts with the highly conserved leucine at the C-terminal end of the ligand near loop C (Leu47 in EGF; Leu48 in TGFα). Finally, a crucial and highly favorable hydrophilic interaction is established between Asp354 of EGFR and a highly conserved arginine in loop C of ligand (Arg41 in EGF; Arg42 in TGFα). These observations are well corroborated by numerous mutational analyses performed by researchers, a notable few of which were cited in the previous section, and others which were left out for brevity. We contend that the fulfillment of these three critical interactions with receptor would be defining factors for any peptide to qualify as a ligand for EGFR. Conversely, we also expect that any binding partner for EGFR would presumably establish at least one if not all of the aforementioned inter-residue contacts with the receptor.

Although EGFL repeats of tenascin C and laminin in soluble form do have the capacity to bind and activate EGFR, it is not known how this interaction would occur, or what pathways might be triggered downstream of EGFR upon such stimulus. Since EGFL repeats of TN-C are significantly shorter in length as compared to prototypical ligands such as EGF, with truncations in loops A and B, and being part of the ECM severely restricts their mode of presentation, it is surprising that they even bind and activate EGFR. Also, such binding must have some physiological and functional relevance, given that TN-C and numerous other ECM proteins present these arrays of EGFL repeats that have been conserved through evolution. In this context, at least TN-C and few other ECM proteins have been found to exhibit expression patterns that coincide with EGFR production. Cutaneous wound healing is a process involving the expression of TN-C, laminin and EGFR, and may shed light on the physiological mode of action of EGFL repeats.

1.4 CUTANEOUS WOUND HEALING

Cutaneous wound healing is a complex phenomenon, involving several soluble and insoluble factors, and requires the orchestration of distinct yet overlapping phases (Singer and Clark, 1999). Upon wounding, the haemostatic phase initiates the healing response. A fibrin clot is formed by the infiltration of platelets that, in addition to forming an external covering over the wound area and a provisional matrix, also release a number of chemokines such as platelet derived growth factor (PDGF) and transforming growth factor β (TGFβ) which recruit macrophages and fibroblasts into the wound bed (Heldin C-H and B, 1996). Infiltrating neutrophils clear the wound of cellular debris and act as a non-specific antimicrobial response and recruit macrophages into the wound (Brown, 1995). In addition to macrophages, monocytes that have also arrived at the wound area transform into primary macrophages and clear the wound. A host of pro-inflammatory cytokines including interferon γ (INFγ), transforming growth factor beta (TGFβ) and chemokines such as TGFα, EGF and vascular endothelial growth factor (VEGF) are released that recruit fibroblasts and endothelial cells (Martin, 1997). Epithelialization, the next phase in wound healing, involves the involution of the epidermal layer, mediated by interactions between the receptors on the surface of epithelial cells and the provisional basement membrane made up of immature collagen (collagen I), vitronectin and fibronectin (Larjava et al., 1993). It is not fully understood how the involution of the epidermal layer occurs, but local release of growth factors (EGF, TGFα and keratinocyte growth factor) and increased expression of these growth factor receptors may be contributing factors (Abraham JA, 1996; Werner et al., 1994). The granulation and neovascularization phases overlap the epithelialization phase, during which a huge influx of fibroblasts, macrophages and endothelial cells is observed in the wound bed (TK, 1980). During this phase, occurring typically between

days four and seven post initial injury, fibroblasts secrete ample amounts of extracellular matrix (ECM) components such as collagen and laminin, establishing a provisional matrix that facilitates fibroplasia and angiogenesis (Clark et al., 1995). Newly formed new capillaries invaginate the wound, providing nutrients and oxygen required for cell metabolism (Madri JA, 1996). Growth factors continue to be expressed by macrophages, constantly stimulating fibroblasts to migrate and establish the growing stroma, and endothelial cells to develop mature blood vessels (Clark et al., 1982). During the remodeling phase, the provisional matrix is slowly replaced by highly collagenous scar tissue. The scar tissue is generated mainly thorough mature fibroblasts (myofibroblasts), which selectively produce small amounts of collagen over an extended period of time (Desmoulière A, 1996). In addition, myofibroblasts and endothelial cells release matrix proteinases that gradually degrade the provisional matrix. At the end of the remodeling phase, fibroblasts, endothelial cells and macrophages in the wound regress by undergoing apoptosis (Ilan et al., 1998). Angiogenesis ceases though the action of anti-angiogenic factors such as angiostatin and components in the matrix such as thrombospondins (Folkman, 1997; Guo et al., 1997) and ELR-negative CXCR3 chemokines (Bodnar et al., 2006). Finally, the matrix is reorganized, leading to wound contraction and resolution. Due the formation of collagen bundles, the wound gradually contacts and gains tensile strength (Mignatti P, 1996). At the end of the resolution phase, the wound has healed completely; however it regains only 70-80% of the tensile strength of normal unwounded skin (Bailey et al., 1975).

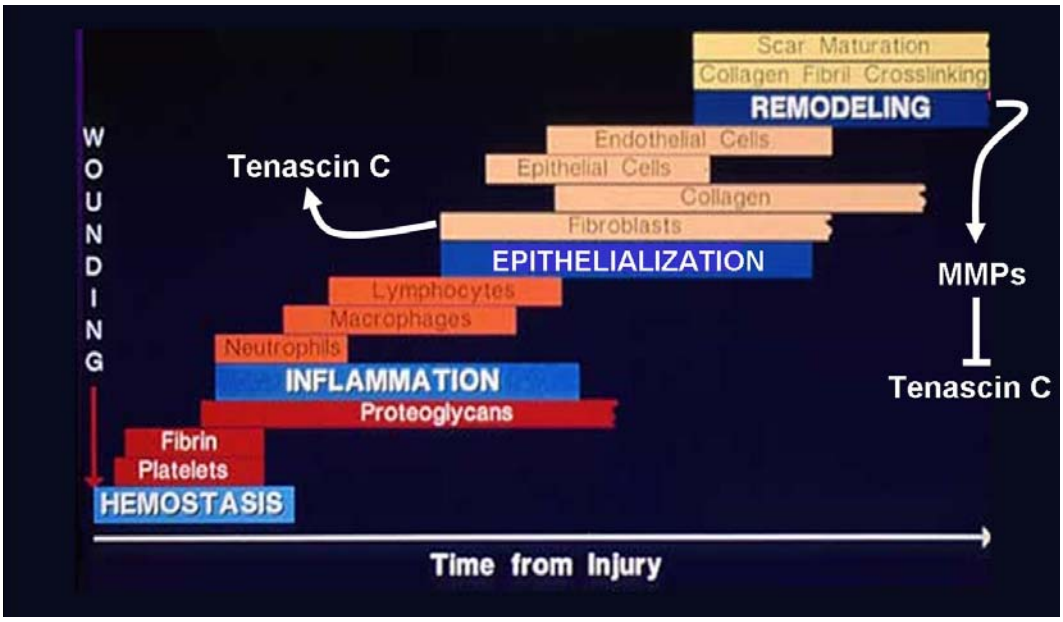

Figure 3. Phases in Cutaneous Wound Healing

The schematic depicts the various overlapping phases of cutaneous wound healing. As mentioned, a number of growth factors, cytokines, chemokines and major ECM components. Of them, tenascin C is unique in its highly regulated spatiotemporal pattern of expression. Tenascin C is expressed at the leading edge of the healing wound during the epithelialization phase, secreted predominantly by myofibroblasts in the stroma. However, by the start of the remodeling phase, tenascin C is degraded, presumably through the action of various matrix metalloproteinases (MMPs) secreted during this phase.

Tenascin C is interesting in that it has a unique expression pattern as compared to all the other major ECM components expressed during wound healing, including collagen, fibrin, laminin and fibronectin. In normal skin, TN-C expression is found to be at extremely low levels and appears diffusely through the dermal layer (Sakakura and Kusano, 1991). However, during wound healing, TN-C is found to be upregulated during phases requiring significant migration of cells, albeit it expression appears to be strictly regulated both spatially and temporally (Tran et

al., 2004). For example, a marked increase of TN-C is observed only at the leading edge of migrating epithelial cells during the epithelialization phase. It is also overexpressed at the leading edge of infiltrating fibroblasts and endothelial cells during the initial stages of granulation and neovascularization (Phipps et al., 2002). However, as the remodeling phase progresses, TN-C levels diminish gradually, regressing to basal levels by the end of the remodeling phase (Tran et al., 2005). Although the functional aspect of TN-C in this context is not understood, this regulated expression pattern is unique as compared to other ECM molecules, and suggests a potential role for TN-C in regulating migratory events during its expression.

With such a spatio-temporally regulated protein like TN-C, it would be interesting to delineate the role of EGFL repeats and how they modulate EGFR signal transduction pathways so as to contribute towards the overall effect that is observed with TN-C. Specifically, it is important to characterize the signal transduction pathway that could potentially contribute towards eliciting cell migration and proliferation through activation of EGFR via EGFL repeats. However, in order to do so, a complete understanding of the intracellular EGFR pathways is essential, and will pave the way for postulating the potential mode of action of EGFL repeats as part of TN-C in pathophysiological settings.

1.5 THE EGFR SIGNAL TRANSDUCTION PATHWAY

EGFR plays a crucial role in all the steps during cell migration and proliferation. As mentioned earlier, EGFR undergoes autophosphorylation upon ligand binding at various residues in the kinase domain causing homo or hetero-dimerization with other erbB family members (Wells, 1989). Activated EGFR is quickly internalized into endosomes in a clathrin-dependent manner

(Jiang et al., 2003). The occupancy and kinase activity of the receptor are determining factors of whether EGFR is degraded in lysosomes or recycled to the cell surface for another round of activation. A myriad of signaling cascades are activated downstream of EGFR, resulting in migration, proliferation, cell survival, cell cycle progression and differentiation (Carpenter, 2000; Schlessinger, 2000; Yarden and Sliwkowski, 2001). We can compartmentalize EGF dependent signaling downstream of the EGFR into two major but discrete pathways – one involving signal transduction molecules that contribute significantly to migration and the other that leads predominantly to proliferation (Chen et al., 1994a; Chen et al., 1994b). This compartmentalization of signaling occurs because of the restricted availability of the two main substrates of EGFR, namely extracellular signal regulated kinse/mitogen activated protein kinase (ERK/MAPK) and phospholipase C gamma (PLCγ) that mediate proliferation and migration respectively(Chou et al., 2003; Chou et al., 2002; Haugh et al., 1999a; Xue and Lucocq, 1998) .

1.5.1 The 'Proliferation' Cascade

Mitogenesis is an important component of nearly all pathophysiological processes including wound healing and tumorigenesis. Growth factor mediated proliferation via EGFR occurs when these factors are present in the media for at least 10 hours during the G1 phase of cell division. In order for the cell cycle to progress into the S phase of cell division, kinase activity of EGFR is required (Chen et al., 1987; Glenney et al., 1988; Moolenaar et al., 1988). However, EGFR-mediated mitogenesis seems to require only a threshold level of signaling via ERK/MAPK pathway downstream of EGFR over an extended period of time (Vieira et al., 1996). Although the sustained activation of ERK by endosomal EGFR seems to be important for potent mitogenesis, internalization deficient mutants of EGFR have also been shown to trigger

proliferation (Reddy et al., 1996a; Reddy et al., 1994; Reddy et al., 1996b). Thus, mitogenesis can be driven by surface or internalized EGFR.

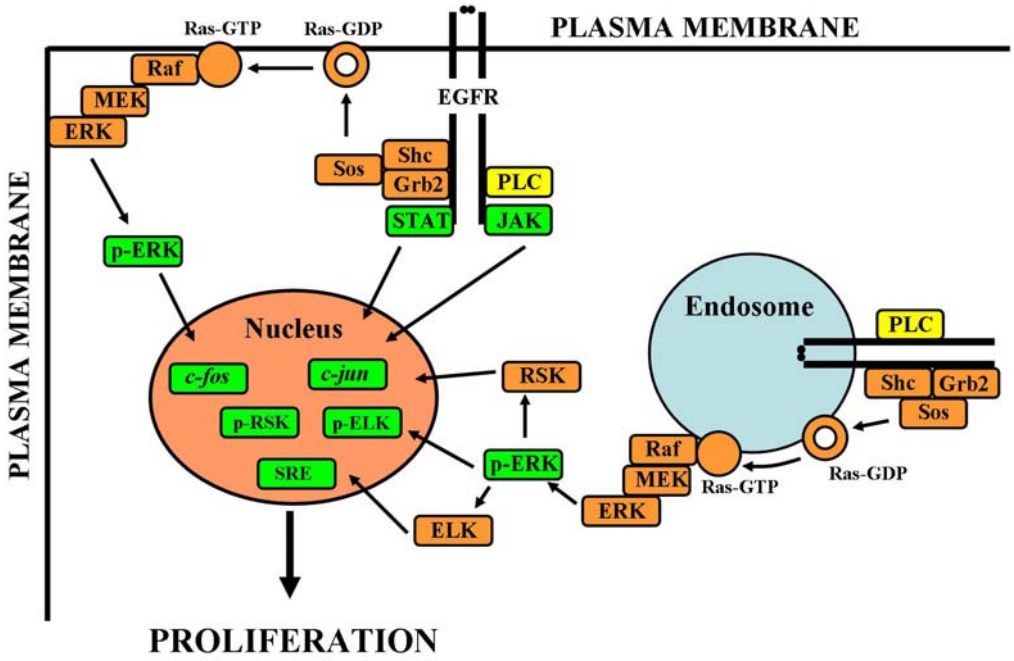

Figure 4. The EGFR 'Proliferation' Pathway

Upon binding ligand, the intracellular kinase domain of EGFR is phosphorylated, leading to the activation of a number of intracellular molecules, including PLCγ1. However, PLCγ1 exerts its effects mainly through PIP2 which is found only at the plasma membrane. However, ERK can be phosphorylated by the Ras-Raf-MEK pathway from both the cell surface and via active EGFR in the endosomal compartment. Activation of intracellular ERK leads to robust phosphorylation of transcription factors ELK and p90RSK, which then translocate to the nucleus. Other transcription factors such as serum response element (SRE), *c-fos*, *c-jun*, and STATs are also activated and translocate to the nucleus where they initiate the process of proliferation.

The proliferation arm for EGFR signaling mainly includes the signaling from the ERK/MAPK pathway which is activated by the small GTPase $p21^{ras}$ through EGFR from both the surface and endosomal compartments (Figure 4) (Haugh et al., 1999a). Activated ERK then dimerizes and relocates to the nucleus phosphorylating E26-like protein 1 (Elk1), a transcriptional regulator belonging to the ETS family of oncogenes that influences the activation of c-fos and c-jun (Gille et al., 1995). In addition, active ERK also causes the phosphorylation of p90-subunit of the ribosomal S6 kinase protein (p90RSK), which then translocates to the nucleus and regulates the activity of transcription factor cAMP response element binding protein (CREB). Signal transducers and activators of transcription type 3 (STAT3) can be directly phosphorylated by EGFR and/or activated through intermediary kinases such as Src oncoprotein and Janus Kinase (JAK). Active STAT3 can then cause transcription of a number of cell cycle regulation genes including c-myc by direct translocation to the nucleus or activation of ERK/MAPK (Bowman et al., 2000) (Brunet et al., 1999; Hochholdinger et al., 1999; Jones and Kazlauskas, 2001).

1.5.2 The 'Migration' Cascade

Cell migration is a cyclic process, starting with cell polarization and protrusion in the direction of migration in response to a migration-promoting agent, followed by cell contraction and rear de-adhesion (Horwitz and Webb, 2003; Ridley et al., 2003). During cell protrusion, lammelipodia and filopodia are stabilized by the formation of adhesion complexes via transmembrane integrin receptors linked to the cytoskeleton (Kamei et al., 1999). These adhesions also serve as traction sites for the cell as it contracts and moves forward with de-adhesion at the rear end completing the cycle (Friedl and Brocker, 2000). The cycle involves concerted action of a number of

molecules, synchronized in spatio-temporal manner. Active PLCγ1 is required for the Rho GTPase cell division cycle 42 (Cdc42) mediated polarization and formation of filopodium (Chou et al., 2003). Active Cdc42 and Rac are essential for defining the cell front (Etienne-Manneville and Hall, 2002; Srinivasan et al., 2003). Gelsolin, also activated by a PLCγ1 dependent pathway is required for cytoskeletal rearrangement and protrusion (Chen et al., 1996a; Chou et al., 2002). MLCK activation required for cell contraction is mediated by a protein kinase C delta (PKCδ) pathway (Iwabu et al., 2004). Activation of m-calpain by membrane associated ERK leads to cleavage of several focal adhesion proteins including focal adhesion kinase (FAK) and integrins, resulting in rear detachment (Glading et al., 2000b; Glading et al., 2002; Glading et al., 2001).

The migration arm of EGFR signal transduction pathway involves PLCγ1, phosphotidylinositol 3-kinase (PI3K) and phosphotidyl inositol (4,5)-bisphosphate (PIP2) (Figure 5). Since PIP2 is restricted to the cell surface, cell motility is actuated by EGFR dependent mechanisms instantiated at or near the cell surface. Phosphorylated PLCγ1 hydrolyses PIP2 to form 2 second messengers – membrane bound diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3) which is released into the cytoplasm. DAG activates protein kinase C δ (PKCδ) which in turn activates myosin light chain kinase (MLCK) involved in cell contraction (Iwabu et al., 2004). PKCδ also attenuates EGFR signaling through a feedback mechanism (Chen et al., 1996a; Welsh et al., 1991). IP3 causes the release of intracellular calcium from the endoplasmic reticulum, stimulating a number of migratory pathways. PIP2 also binds to a number of actin modifying proteins such as profilin and gelsolin that reorganize the actin cytoskeleton (Carpenter and Ji, 1999; Chen et al., 1996b). PIP2 can also be activated by PI3K to form phosphotidyl inositol (1,4,5) trisphosphate (PIP3), which then activates a number of proteins that restructure the actin cytoskeleton. PI3K also regulates a number of other

mechanisms aiding cell migration (Ridley et al., 2003). Active ERK at the cell surface phosphorylates m-calpain, an intracellular protease that cleaves focal adhesion at the rear of the cell (Glading et al., 2001).

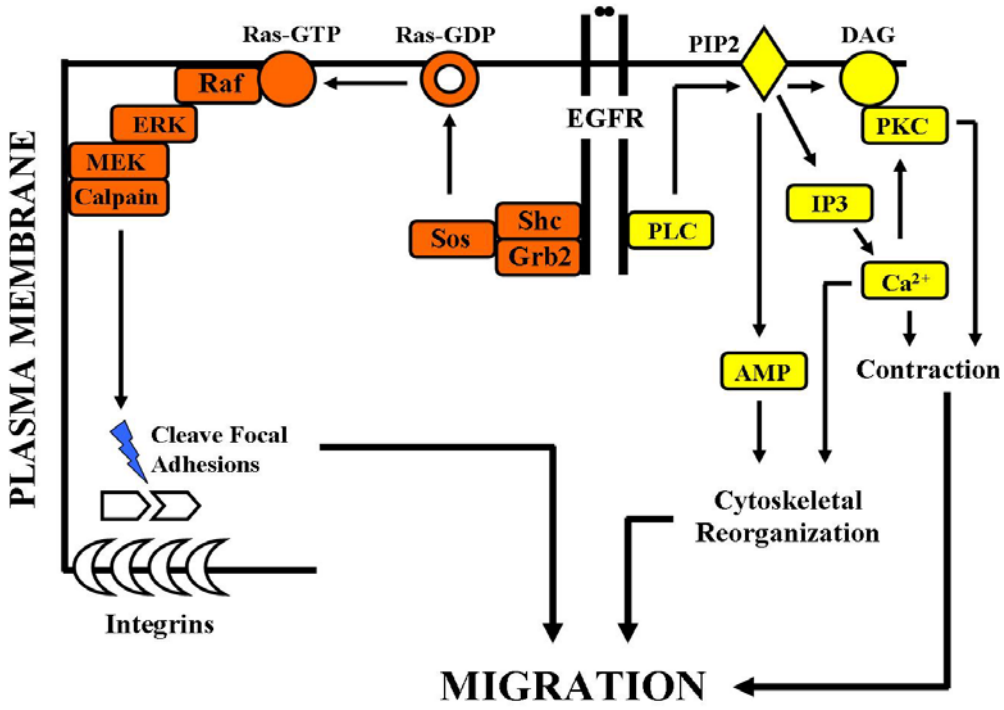

Figure 5. The EGFR 'Migration' Pathway

The migration pathway downstream of EGFR is mainly triggered at the plasma membrane. Activated PLCγ1 hydrolyzes surface-restricted PIP2 to release secondary messengers IP3 and DAG. PIP2 hydrolysis also releases several actin modifying proteins that, along with the IP3-driven calcium flux, lead to cytoskeletal reorganization. Also, PKC is activated which leads to cell contraction and also contributes to negatively regulate EGFR activation. On the other hand, pools of ERK at the cell surface are activated by Shc, leading to the activation of m-calpain. Active m-calpain cleaves focal adhesions that are linked to integrins, leading to rear de-adhesion.

The signaling pathways described above are with reference to high affinity EGFR ligands such as EGF and TGFα. It would be interesting to assess how these signaling pathways might be modulated in response to EGFL repeats of TN-C. Also, the overall biophysical response to EGFL repeat signaling could shed light on the possible function of these matrix-restricted peptides in vivo, and provide an explanation for the yet unknown role of TN-C in physiological and pathological scenarios that involves EGFR and its soluble ligands.

1.6 SIGNIFICANCE - TUMOR BIOLOGY AND DEVELOPMENT

Embryogenesis and development are processes that recapitulate some of the important cellular modalities that are observed during wound healing, in particular migration of different cell types into areas where cell involution is required (Burgess and Schroeder, 1979; Oligny, 2001). Not surprisingly then, an overexpression of TN-C is also observed during different phases of embryogenesis and organogenesis well documented in overall development, neuronal development in particular (Chiquet-Ehrismann et al., 1986; Porcionatto, 2006; Riou et al., 1992). Although the role of the FNIII repeats during organogenesis has been relatively well elucidated, the function of EGFL repeats has been thought to regulate interactions of other domains of TN-C with cell adhesion molecules (Jones and Jones, 2000a). However, given the capacity of EGFL repeats to bind EGFR, the EGFL repeats may very well play an alternative role by signaling select EGFR cascades, thus adding another dimension to their overall function and the function of proteins that present these repeats.

Migration is the most important aspect of cancer progression and metastasis. In order to metastasize, the primary tumor should be able to first transition from an epithelial to a mesenchymal phenotype, and then traverse to the destination tissue (Lee et al., 2006). In addition, an angiogenic response needs to be stimulated at the secondary site for the tumor to be established. Tenascin C has been well documented as a prognosis factor for metastatic tumors in a number of organs including breast, prostate, colon, etc. and has been found to be selectively upregulated selective at the mesenchymal margin, and has also been implicated in regulating epithelial-mesenchymal transitioning. Given that EGFR is upregulated in almost all metastatic tumors, the idea that EGFL repeats could modulate EGFR pathways in the context of metastatic tumorigenesis needs to be seriously considered and carefully evaluated. An elucidation of the binding mode of EGFL repeats and the identification of intracellular pathways that these repeats trigger downstream of EGFR may help in characterization of the ECM proteins that present these repeats in an evolutionarily conserved manner.

1.7 SUMMARY

Select EGFL repeats of human tenascin C and laminin have been shown to bind and activate EGFR. However, as compared to canonical soluble ligands such as EGF and TGFα, EGFL repeats are significantly truncated and probably bind as part of the extracellular matrix, thereby potentially restricting their mode of action. This distinguishes EGFL repeats from other ligands for EGFR, and these matrikines may have a mode of action that is distinct from other soluble ligands. Select EGFL repeats of tenascin C bind to EGFR with lower affinity, but the mode of action of these repeats has not been characterized. We hypothesize that

1. The low affinity of EGFL repeats is due primarily to an altered mode of binding to EGFR, driven by deletions and substitutions of key residues that bind to the receptor.
2. This altered binding action of EGFL repeats leads to differential activation of signaling cascades downstream of EGFR as opposed to typical soluble ligands, leading to a distinct cellular response as compared to EGF.

This work has broad implications for EGFL repeat signaling in numerous physiological and pathological processes that involve EGFR and tenascin C and other such ECM proteins that encode EGFL repeats.

2.0 TENASCIN CYTOTACTIN EPIDERMAL GROWTH FACTOR-LIKE REPEAT BINDS EPIDERMAL GROWTH FACTOR RECEPTOR WITH LOW AFFINITY

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2.1 ABSTRACT

Select epidermal growth factor (EGF)-like (EGFL) repeats of human tenascin cytotactin (tenascin C) can stimulate EGF receptor (EGFR) signaling, but activation requires micromolar concentrations of soluble EGFL repeats in contrast to subnanomolar concentrations of classical growth factors such as EGF. Using *in silico* homology modeling techniques, we generated a structure for one such repeat, the 14th EGFL repeat (Ten14). Ten14 assumes a tight EGF-like fold with truncated loops, consistent with circular dichroism studies. We generated bound structures for Ten14 with EGFR using two different approaches, resulting in two distinctly different conformations. Normal mode analysis of both structures indicated that the binding pocket of EGFR exhibits a significantly higher mobility in Ten14-EGFR complex compared to that of the EGF-EGFR complex; we hypothesized this may be attributed to loss of key high-affinity interactions within the Ten14-EGFR complex. We proved the efficacy of our *in silico* models by *in vitro* experiments. Surface plasmon resonance measurements yielded equilibrium constant K_D of 74μM for Ten14, approximately three orders of magnitude weaker than that of EGF. In accordance with our predicted bound models, Ten14 in monomeric form does not bind EGFR with sufficient stability so as to induce degradation of receptor, or undergo EGFR-mediated internalization over either the short (20min) or long (48h) term. This transient interaction with the receptor on the cell surface is in marked contrast to other EGFR ligands which cause EGFR transit through, and signaling from intracellular locales in addition to cell surface signaling.

2.2 INTRODUCTION

EGFR belongs to the ErbB family of Type I receptor tyrosine kinases, and plays an integral role in regulating cellular functions (Wells, 1999; Wells, 2000). Active EGFR signals from the cell surface and intracellularly as it is internalized; intracellular signaling is qualitatively distinct from surface signaling and likely promotes proliferation over migration (Haugh et al., 1999a; Haugh et al., 1999b; Pennock and Wang, 2003). The activity of EGFR is shut off by dephosphorylation when unliganded, and over a longer term by lysosomal degradation secondary to internalization. Thus, persistence and subcellular localization of receptor occupancy would impact cellular response from EGFR activation.

EGFR is activated by the (EGF)-like (EGFL) family of soluble ligands which includes EGF, transforming growth factor α (TGFα), heparin-binding EGF, amphiregulin, and a number of virally encoded factors (Citri and Yarden, 2006). These peptides are characterized by an EGFL domain consisting of a sequence distribution of six cysteines that form disulfide bridges, giving them a tight and closely packed structure (Carpenter and Cohen, 1990). They bind the extracellular (EC) domains I and III of EGFR with very high affinity (Kim et al., 2002); the physiological affinities of EGF and TGFα for EGFR are in the very low nanomolar range (Wells, 1999). EGFL domains are present in other proteins families, including a number of extracellular matrix (ECM) proteins (tenascin, fibrillin 1, del1, laminin, thrombospondin 1), and are arranged typically as an array of sequential EGFL repeats (Hohenester and Engel, 2002). These EGFL repeats have disulphide bonds similar to EGF, and this intra-molecular cross-linking of the cysteines is essential for function (Zanuttin et al., 2004). With few exceptions, little is known about the function of these matrix EGFL repeat domains.

Human tenascin C is an ECM protein re-expressed during normal tissue regeneration, and implicated in tumor progression (Tsunoda et al., 2003). Interestingly, its expression profile coincides with active cell migration and proliferation, properties similar to those elicited by EGFR activation (Chen et al., 1994a; Jones and Jones, 2000a). It is a hexabrachion with an array of 84 full and 6 half EGFL repeats, a fibronectin-type III array and a terminal fibrinogen globe (Jones and Jones, 2000a). Recently, we demonstrated that select EGFL repeats of human tenascin C (e.g. the 14^{th} repeat, Ten14), when presented in soluble form, can signal through EGFR in a receptor-dependent fashion (Swindle et al., 2001). However, micromolar concentrations of Ten14 were required to elicit responses comparable to those observed with EGF in the nanomolar range. A similar function has been reported for EGFL repeats from laminin V (Schenk et al., 2003).

The finding that EGFL repeats can signal through a classical receptor such as EGFR invited attention on a new class of receptor ligands, matrikines (Schenk and Quaranta, 2003; Tran et al., 2004), that are encoded as part of larger matrix components. The significantly lower affinities of these ligands would reflect the matrix-constrained situation of their physiological environment, in which limited diffusion and multimeric presentation would result in avidities approximately three orders of magnitude greater than individual soluble affinity constants. However, how this low affinity binding would be accomplished at the submolecular level has evaded explanation.

We hypothesized that the low affinity of Ten14 for EGFR is driven by weak inter-residue contacts with the receptor due to deletions and substitutions of key residues in the EGFL – binding domain of Ten14 required for tight binding, resulting in a more flexible mode of interaction that could accommodate a constraining environment. EGFR binding of ligands is

usually accompanied by conformational changes in the complex that accommodate/optimize the interactions in the bound form (De Crescenzo et al., 2000). Our postulate assumes an enhancement in this type of conformational flexibility and its persistence even after binding.

Structural analysis of the complex showed that though Ten14 lacks the C-terminal loop present in EGF and TGFα found to be important for high affinity interaction with EGFR (Kramer et al., 1994), other structural contacts are established that may be sufficient for activation of receptor. Accordingly, a much weaker K_D and increased mobility for the Ten14-EGFR interaction is observed as compared to EGF. As a result, Ten14 is neither internalized nor degraded over short and long term signaling via EGFR, and leads to compartmentalization of EGFR at the cell surface. This may lead to altered biochemical and biophysical signaling responses downstream of the receptor. An effort into characterizing the interaction of EGFL repeats with cell surface receptors has not been undertaken before, and elucidation of mechanistics of EGFL repeat-mediated signaling will allow for a more complete understanding of this new class of low-affinity growth factors embedded within the ECM.

2.3 MATERIALS AND METHODS

2.3.1 Structure Prediction for Ten14

Homology modeling and *ab initio* techniques were used to predict the 3D structure of Ten14. The chains corresponding to the active conformations of TGFα (PDB code IMOX-chain C (Ogiso et al., 2002)) and EGF (PDB code 1IVO-chainC (Garrett et al., 2002)) were chosen as templates. Sequence analyses for Ten14, TGFα and EGF were first performed using

CLUSTALW (Pearson and Lipman, 1988). Model structures were obtained using three servers – Robetta (Kim et al., 2004), ESyPred3D (Lambert et al., 2002)and Swiss-Model (Schwede et al., 2003). Four queries were submitted to Robetta: Ten14 without a template (*ab initio*), Ten14 with TGFα as the template, Ten14 with EGF as the template and Ten14 with TGFα-EGFR as the template. In all, 25 models were obtained – ten for Ten14 without template and five each for the other three prediction queries with templates. For predictions with ESyPred3D and Swiss-Model, one model was obtained from each server with TGFα as template, resulting in a total of 27 models for Ten14. Distance root-mean-square deviations (dRMSD) between each model and the known TGFα and EGF structures were then calculated The Ten14 model with the lowest dRMSD was chosen for docking with EGFR.

2.3.2 Circular Dichroism Measurements

CD spectra for Ten14 were recorded in 10mM phosphate buffer, pH 7.4, using an AVIV 202 series CD spectrophotometer (Lakewood, NJ) held at 25^{o}C with a thermostatically controlled cell holder in a fused quartz cell with a path-length of 0.1cm. For protein concentration of 0.18mg/ml, ten spectra measured every 1nm in the far UV-region (185 – 280nm) were averaged. CD spectra were subjected to subtraction from buffer blank, normalization and smoothing, using the AVIV data manipulation software. Analysis of the data was carried out with the program CDSSTR, which used seven reference datasets, and is available through the DICHROWEB web server at www.cryst.bbk.ac.uk/cdweb/html/home.html (Lobley et al., 2002; Whitmore and Wallace, 2004).

2.3.3 Docking of Ten14 with EGFR

Ten14 was docked onto EGFR in two binding conformations. EGF-EGFR and TGFα-EGFR crystal structures were used as reference. For Structure I, we used the results from 'Consensus' server (Prasad et al., 2003; Prasad et al., 2004), which indicated that residues 21 through 31 in Ten14 had good overlap with active EGF and TGFα. Hence, we superimposed the co-ordinates of residues numbered 21 though 31 from Ten14 (the model with lowest dRMSD was chosen) onto corresponding residues in TGFα, in addition to matching the corresponding cysteines in Ten14 and TGFα. The remaining residues were transposed so as to maintain the overall structure of Ten14. For Structure II, Ten14 was docked to EGFR in a manner so as to allow for the anchoring interaction of Leu12 of Ten14 with the hydrophobic pocket in domain I of EGFR, determined by Leu14, Leu69 and Leu98 of receptor. This transposition of the entire Ten14 structure also led to a favorable interaction between Arg19 of Ten14 and Asp354 of EGFR (a residue forming a key salt bridge with EGF and TGFα). Finally, with only the backbone atoms of Ten14 and all atoms of EGFR fixed, the docked structure was minimized using CHARMM. Structures I and II for Ten14-EGFR were then analyzed using the Gaussian network model (GNM) and 'FastContact'.

2.3.4 Structure Evaluation

The binding dynamics of the ligands complexed with EGFR were analyzed using the *iGNM* database and server (http://ignm.ccbb.pitt.edu/). *iGNM* generates residue mobilities in different modes of motion accessible near native conditions. We examined the most cooperative (lowest frequency) modes for EGFR structure alone (chain A from 1MOX), and for the complexes of

EGFR with EGF (chains A and B from 1IVO), with TGFα (chains A and B from 1MOX) and with Ten14 (Structures I and II). The slow mode fluctuations for receptor alone and with ligand were visualized using both color-coded ribbon diagrams and mobility distribution curves (eigenvectors) as a function of residue index.

The key residues in ligand and receptor that contribute towards favorable and unfavorable interactions were identified using the software 'FastContact' (Camacho and Zhang, 2005). This software identifies interactions between ligand and receptor residues that contribute maximally towards overall electrostatic and desolvation energies, and total binding energy. The resulting high affinity interactions between both structures of Ten14-EGFR were compared against those occurring in the EGF-EGFR and TGFα-EGFR complexes.

2.3.5 Surface Plasmon Resonance (SPR) Analysis

Ten14 binding kinetics was examined by SPR using the BIAcore 3000 system. Expression and purification of Ten14 was performed as described previously (Swindle et al., 2001). Recombinant human EGF (hEGF) (BD Biosciences, Bedford, MA) was used as the control analyte. The Ten14 and hEGF runs were performed on separate chips; each experimental series was repeated. For both runs, 25μg/ml of recombinant EC domain of EGFR (EGFR-ED) (Research Diagnostic Inc., Flanders, NJ) in 10mM sodium acetate (pH 5.0) was cross-linked to a CM5 sensor chip surface using the EDC/NHS coupling method (Amine coupling kit, BIAcore Inc., Uppsala, Sweden), resulting in immobilizations of ~7000 resonance units (RUs) of EGFR-ED for Ten14 studies and ~2500 RUs for hEGF studies. Separate flow cells from each chip derivatized without EGFR-ED were used as control. For Ten14, sterile 0.22μm filtered phosphate buffered saline (PBS) was used as the running buffer and 10mM Glycine-HCl, pH 3.0

(Regeneration kit, BIAcore Inc., Uppsala, Sweden) was used for regeneration of the chip surface. Increasing concentrations of Ten14 (1.88, 3.75, 7.5, 15 and 30μM) or hEGF (0.03, 0.06, 0.125, 0.25, 0.5, 1, 3, 10μM) were then injected into their respective flow cells. For each concentration of Ten14, 30min of association, dissociation and regeneration cycles were used, all at a flow rate of 5μl/min. Increasing concentrations of human EGF was prepared in sterile HBS-EP buffer (BIAcore Inc., Uppasala, Sweden), and injected over the surfaces at flow rate of 30μl/min, with a 10min association pulse and 15min of dissociation, without the need for a regeneration step. The sensograms obtained for each ligand concentration for all runs were graphed and analyzed for steady state binding using the BIAEvaluation software (BIAcore Inc., Uppsala, Sweden).

2.3.6 Internalization and Depletion Assays

For all *in vitro* experiments, NR6WT fibroblasts expressing ~100,000 human EGFR/cell were cultured and maintained as described previously, and quiesced in medium containing 0.5% dialyzed serum (Chen et al., 1994a; Wells et al., 1990). Depletion assays were performed by incubating quiesced NR6WT cells in medium containing various concentrations of murine EGF (mEGF) or Ten14. Cells were lysed and growth factor concentrations determined for time 0 and 48h via immunoblotting using anti-Xpress™ antibody (Invitrogen, Carlsbad, CA). To measure ligand internalization, quiesced NR6WT cells were incubated in binding buffer at 37°C. I^{125}-EGF (0.6nM) and I^{125}-Ten14 (100nM) were introduced for varying time points at 37°C. After washing, cell-surface associated ligand was removed with stripping buffer (HCl, pH 2.0). Cells were then lysed with 1N NaOH. Internalization was measured as counts per minute (CPM) on a Packard 5005 Cobra Gamma Counter. To measure EGF receptor internalization and degradation, mEGF (1nM) and Ten14 (2μM) were introduced into the media at various time points at 37°C.

Samples were collected and total EGFR levels were determined using immunoblotting utilizing the monoclonal EGFR antibody (BD Transduction Labs, San Jose, CA). Equal loading was assured using the anti-GAPDH antibody (Abcam Inc. Cambridge, MA). Relative densitometric values were derived with NIH image shareware v1.63 and Adobe Photoshop. Each experiment was repeated at least twice.

2.3.7 Immunofluorescence Assays

To assess localization of active EGFR, 10,000 NR6WT cells quiesced on glass coverslips and treated with increasing concentrations of mEGF (10nM, 1nM) or Ten14 (1μM, 0.1μM, 0.01μM) for 30min. After washing with cold PBS, cells were fixed in 3% paraformaldehyde for 30min and lysed for 30min with buffer containing 1% triton X-100, 1mM PMSF and 1μg/ml aprotinin, followed by blocking in 5% BSA. In order to assess the localization of total versus phosphorylated EGFR, cells were incubated for 3h at room temperature in a mixture of rabbit polyclonal anti-EGFR antibody (Santa Cruz Biotech., Santa Crux, CA) and mouse monoclonal phospho-EGFR antibody (Upstate, Chicago, IL), both at a final concentration of 5μg/ml. After a brief wash in PBS containing 0.5% BSA, coverslips were incubated in a mixture of Alexa Fluor 647 anti-mouse secondary antibody, Alexa Fluor 488 anti-rabbit secondary antibody (both at 1μg/ml) and 25μg/ml propidium iodide at room temperature for 30min. In order to assess the co-localization of ligand and receptor, slips treated with 1μM Ten14 or 10nM mEGF were incubated with a mixture of mouse monoclonal anti-Xpress™ antibody(Invitrogen, Carlsbad, CA) and rabbit polyclonal anti-EGFR antibody, followed by appropriate secondary antibodies. After a last wash, the slips were washed and mounted onto glass slides using gelvatol. After overnight drying, the slides were imaged using a Zeiss Axioplan confocal laser-scanning microscope, with

each one imaged simultaneously for all channels. Each image was scanned along the Z-axis in 7-10 sectional planes with 0.43μm steps (512x512 pixels per sectional plane). Images were collected and analyzed using Adobe Photoshop ver. 6.0. All RGB images were first pasted onto a canvas and RGB levels were adjusted from 0 to 128 bits achieve an optimal signal to noise ratio. Individual channel images were then separated and pasted separately onto another canvas to display green, red, and composite images.

2.4 RESULTS

2.4.1 Predicted Structure for Ten14 Conforms to Other EGFR Ligands

Ten14 shares low but adequate sequence homology with EGF (25%) and TGFα (32%) for homology modeling techniques (Figure 6A). Of the 27 models generated, the best model was selected from dRMSD calculations based on inter-residue distances between C^{α} atoms of 11 conserved residues: Cys5, Pro6, Cys9, Gly13, Cys15, Cys20, Cys22, Gly25, Tyr26, Gly28 and Cys31 (numbers refer to residue number in Ten14 sequence; corresponding residues were selected from EGF and TGFα as shown in Figure 6A). The dRMSD value for the selected model was less than 1Å when compared to EGF and TGFα (Table 1).

Table 1. dRMSD Values Between Templates and the 27 Predicted Models for Ten14

We generated 27 models using three protein structure prediction servers – Robetta, Swiss-Model and EasyPred3D. In all, 27 models were obtained using either no template (ten models using Robetta), or TGFα (five models using Robetta and one each from Swiss-Model and EasyPred3D), or EGF (five models using Robetta), or crystal structure

of EGFR with bound TGFα as templates (five models using Robetta). ClustalW was first used to identify residues in Ten14, TGFα and EGF that were conserved based on sequence (Figure 6A). The distance Root Mean Square Deviation (dRMSD) analysis was performed on each model for Ten14 against both TGFα and EGF for the 11 conserved residues. The structures were rank ordered starting with the structure that had the lowest dRMSD. The table shows that the Ten14 models obtained with Robetta using TGFα as template yielded the best scores, including four of the top five structures predicted. The EasyPred3D prediction was also consistent with this set of results, scoring second best overall (rank two – in italics).

Ten14 Model Number	**Template For Prediction**	**RMSD Vs. TGFα**		**Ten14 Model Number**	**Template For Prediction**	**RMSD Vs. EGF**
1	**TGFα**	**0.804**		**1**	**TGFα**	**0.831**
2	***TGFα***	***1.091***		***2***	***TGFα***	***1.176***
3	**TGFα**	**1.103**		**3**	**TGFα**	**1.230**
4	**TGFα**	**1.576**		**4**	**TGFα**	**1.753**
5	**TGFα**	**1.643**		**5**	**TGFα**	**1.833**
6	**EGF**	**2.180**		**6**	**EGF**	**2.188**
7	**EGF**	**3.012**		**7**	**EGF**	**3.125**
8	**EGF**	**3.330**		**8**	**EGF**	**3.538**
9	**EGF**	**3.479**		**9**	**EGF**	**3.589**
10	**TGFα-EGFR**	**3.676**		**10**	**TGFα-EGFR**	**3.800**
11	**EGF**	**3.927**		**11**	**EGF**	**3.923**
12	**NONE**	**4.026**		**12**	**NONE**	**4.215**
13	**NONE**	**4.036**		**13**	**NONE**	**4.230**
14	**TGFα-EGFR**	**4.233**		**14**	**TGFα-EGFR**	**4.372**
15	**NONE**	**4.880**		**15**	**NONE**	**4.983**
16	**NONE**	**4.904**		**16**	**NONE**	**4.989**
17	**NONE**	**5.018**		**17**	**NONE**	**5.119**
18	**NONE**	**5.357**		**18**	**NONE**	**5.461**
19	**NONE**	**5.385**		**19**	**NONE**	**5.506**
20	**NONE**	**5.570**		**20**	**NONE**	**5.706**
21	**NONE**	**5.821**		**22**	**TGFα-EGFR**	**5.818**
22	**TGFα-EGFR**	**5.832**		**24**	**TGFα-EGFR**	**5.878**
23	**NONE**	**5.860**		**21**	**NONE**	**5.909**
24	**TGFα-EGFR**	**5.897**		**23**	**NONE**	**5.993**
25	**TGFα-EGFR**	**6.045**		**25**	**TGFα-EGFR**	**6.026**
26	***TGFα***	***8.149***		***26***	***TGFα***	***8.112***
27	**TGFα**	**8.155**		**27**	**TGFα**	**8.122**

Ten14 is predicted to be composed of an anti-parallel β-hairpin encompassing the six backbone cysteines and an N- and C-terminal loop (Figure 6B). This agrees well with the experimentally determined secondary structure for soluble Ten14 in CD studies, which indicates that soluble Ten14 is composed of 27% β-sheet and only 1% α-helix (Figure 6C). As CD is particularly powerful for the prediction of α-helices (Johnson, 1990), our structural predictions for Ten14 are very compelling for lack of helical content. Also, binding geometry of the six cysteines of Ten14 agrees well with EGF and TGFα, in addition to residues in the beta-sheet region (Fig. 6B). As this binding is crucial for activity of EGFL repeats of tenascin C, we believe that the predicted Ten14 structure represents the functional form of the native soluble monomer (Zanuttin et al., 2004).

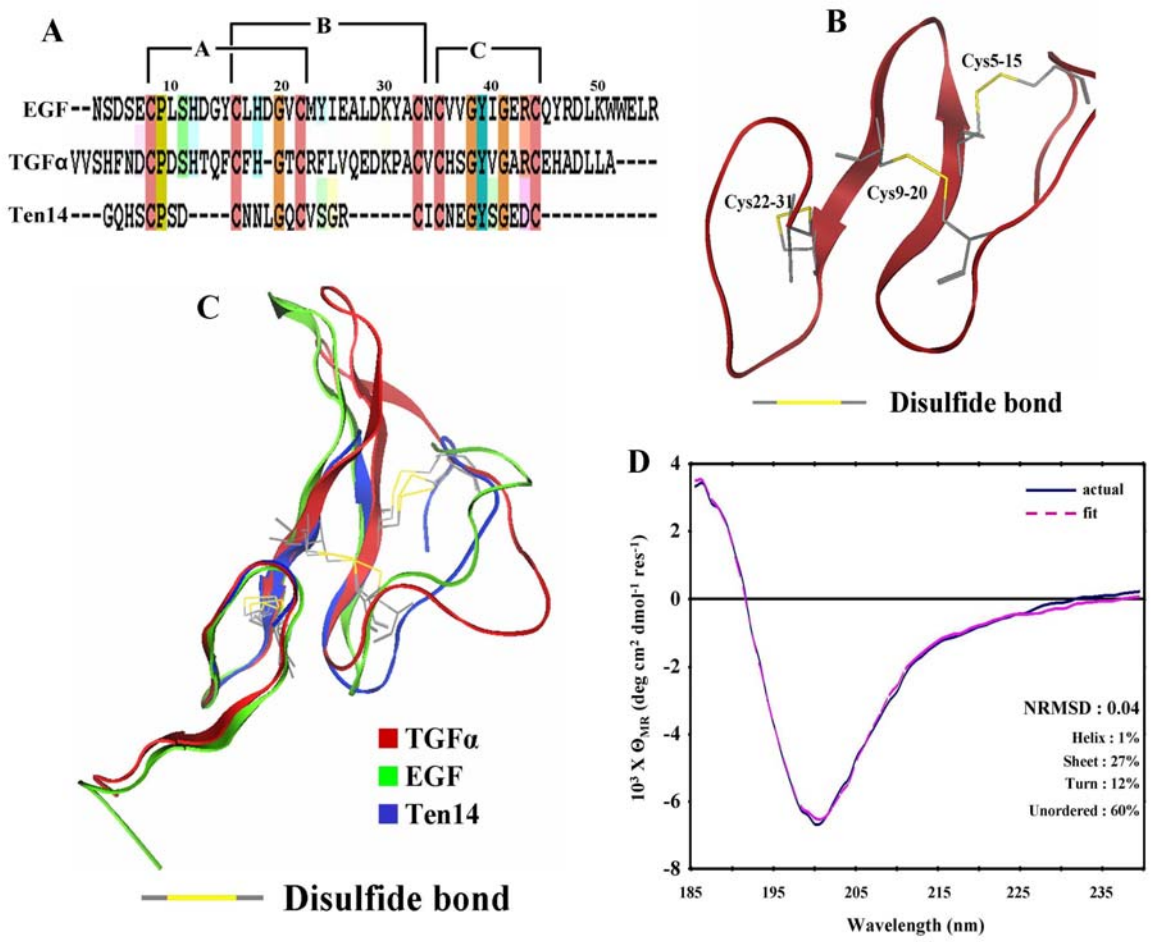

Figure 6. Structural Modeling of Ten14

A. Multiple sequence alignment indicates that Ten14 has 25% sequence homology with EGF and 32% with TGFα; EGF and TGFα have 38% sequence homology. The six cysteines of Ten14 align with those of EGF and TGFα in addition to Pro6, Gly13, Gly25, Tyr26 and Gly28 of Ten14. Note however that the conserved arginine (Arg42 in TGFα) corresponds to an oppositely charged (aspartate) residue in Ten14.

B. Ten14 is composed of an anti-parallel β-hairpin, with six cysteines forming the disulphide bridges in 1-3 (Cys5-Cys15), 2-4 (Cys9-Cys20), and 5-6 (Cys22-Cys31) orientation.

C. Deletions at both N- and C-terminal regions make Ten14 significantly shorter compared to EGF and TGFα. Nevertheless, there is close overlap in the position of the six cysteines for all three structures.

D. Analysis using CDSSTR yielded a best fit (pink dotted) for averaged Ten14 CD experimental spectra (blue) with a NRMSD of 0.04. Ten14 is composed mainly of β-sheet and β-turn, with negligible α-helix content, in excellent agreement with the modeled Ten14 structure.

2.4.2 Ten14 May Dock Onto EGFR in Alternative Structural Conformations

Ten14 binds to EGFR in or near the same region as EGF since Ten14 binding was competed by EGF, and an antibody that blocked EGF binding also blocked Ten14 (Swindle et al., 2001). As such, we modeled Ten14 binding to EGF/TGFα-binding pocket of EGFR (Figure 7A). Rigid-body docking of Ten14 was performed using two methodologies based on distinct underlying principles, yielding two complexes (Structures I and II, Figure 7). Structure I was generated on the intuitive assumption that if an unknown ligand shares sequence and structure homology with a known ligand in regions that directly interact with its receptor, the unknown ligand will most likely bind the receptor in a fashion similar to the known ligand. Hence, we first identified structurally conserved regions between Ten14, EGF and TGFα using the Consensus server (http://structure.bu.edu/cgi-bin/consensus/consensus.cgi). Consensus yields high-quality alignments for comparative modeling and identifies the alignment regions reliable for copying from a given template, even under low target-template identity. With maximum confidence on a scale of 0-9 (Table 2), the C^{α} atoms of residues 20-31 of Ten14 were superimposed onto the corresponding TGFα residues in the ligand-binding pocket of TGFα-EGFR structure, with transposition of the other residues (Figure 7B).

Table 2. Consensus Results for Direct Overlap of Ten14 with EGF and TGFα

The Consensus server was used to identify the residues in Ten14 that were conserved both in sequence and structure as compared to EGF and TGFα. The 3-dimenisional coordinates for the selected Ten14 structure, TGFα and EGF (chains C of 1MOX and 1IVO, respectively).were submitted to the Consensus server. A consensus is generated for each residue of the query (Ten14) with the template (TGFα and EGF), and the degree of consensus both structurally and sequentially is graded on a scale of 0 though 9, with 0 for minimum consensus and 9 for maximum. The results for Ten14 are summarized in the table. As shown, residues 20 through 31 (indicated in italics with confidence level of 9) rank highest, and could be transposed to structurally align with the corresponding residues in TGFα and EGF. This allowed us to effectively dock Ten14 to EGFR in the first conformation (Structure I). Residues 17-19 in Ten14 also had a maximum confidence level of 9 when compared to TGFα, but were not chosen because they did not rank as well when compared with EGF.

Ten14	TGFα Overlap	Confidence Level (0-9)		Ten14	EGF Overlap	Confidence Level (0-9)
G1	H4	1		D8	E5	3
Q2	F5	1		C9	C6	3
H3	N6	1		N10	S9	3
S4	D7	1		N11	H10	3
C5	C8	6		L12	D11	3
P6	P9	6		G13	G12	3
S7	D10	6		Q14	Y13	3
D8	S11	6		C15	C14	3
C9	C21	6		V16	L15	3
N10	R22	6		S17	H16	3
N11	F23	6		G18	D17	3
L12	L24	6		R19	A30	6
G13	V25	6		*C20*	*C31*	*9*
Q14	Q26	6		*I21*	*N32*	*9*
C15	E27	6		*C22*	*C33*	*9*
V16	D28	6		*N23*	*V34*	*9*
S17	K29	9		*E24*	*V35*	*9*
G18	P30	9		*G25*	*G36*	*9*
R19	A31	9		*Y26*	*Y37*	*9*
C20	*C32*	*9*		*S27*	*I38*	*9*
I21	*V33*	*9*		*G28*	*G39*	*9*
C22	*C34*	*9*		*E29*	*E40*	*9*
N23	*H35*	*9*		*D30*	*R41*	*9*
E24	*S36*	*9*		*C31*	*C42*	*9*
G25	*G37*	*9*				
Y26	*Y38*	*9*				
S27	*V39*	*9*				
G28	*G40*	*9*				
E29	*A41*	*9*				
D30	*R42*	*9*				
C31	*C43*	*9*				

We modeled Structure II based on two observations: that the leading interaction responsible for molecular recognition (Rajamani et al., 2004) for both EGF and TGFα ligands corresponds to two ligand leucines binding to the same two hydrophobic pockets in EGFR, and that the leading salt bridge for both ligands involves Asp354 (Figure 2). These interactions were missing in Structure I. In Structure II, we not only were able to accommodate the single leucine of Ten14 (Leu12) in one of the hydrophobic pockets in EGFR, but the model complex also

formed a salt bridge between Arg19 and Asp354 (Figure 7C). Hence, the binding modes of EGF, TGFα, and Ten14 are quite homologous. We note that Structure II does not fulfill a second salt bridge present in EGF and TGFα (see Table 3), thus missing at least a third of the binding energy relative to these complexes.

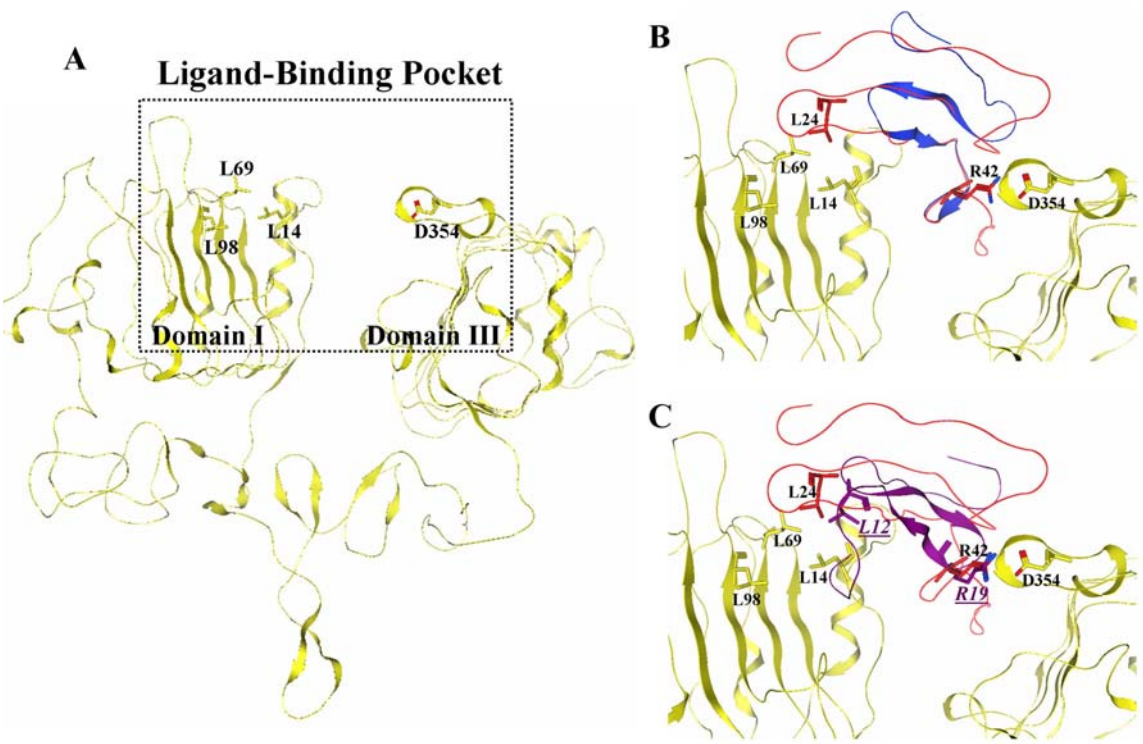

Figure 7. Docking of Ten14 with EGFR

A. Chain A of 1MOX shows the ligand binding pocket of active EGFR. Leucines 14, 69 and 98 form an important ligand-dock site in domain I and Asp354 is involved in a crucial interaction with ligand in domain III. Both EGF and TGFα bind the receptor in this pocket. An additional interaction between Leu47 of TGFα with receptor has been shown in Figure 2.

B. Structure I of Ten14 (blue) docks to EGFR in the binding pocket. This docking was performed by overlap of the C-terminal region of Ten14 with the corresponding motif in TGFα (red).

C. Structure II represents an alternative conformation, where Leu12 and Arg19 (underlined italicized) of Ten14 interact in a similar fashion as TGFα, forming contacts with the leucine pocket and Asp354 of EGFR respectively.

2.4.3 GNM Analysis Suggests High Mobility of the EGFR-Ten14 Complexes

GNM calculations were performed to assess the collective dynamics of the ligand-receptor complexes. With GNM, the complex is modeled as an elastic network, the nodes of which are the α-carbons and the connectors (between all pairs of residues located within a cutoff distance of 8Å) account for the equilibrium interactions that stabilize the native fold (Bahar, 1999; Bahar et al., 1997; Bahar and Jernigan, 1998; Demirel et al., 1998; Yang and Bahar, 2005). We used the *iGNM* web server for global mode analysis EGFR with or without ligand (Yang et al., 2005).

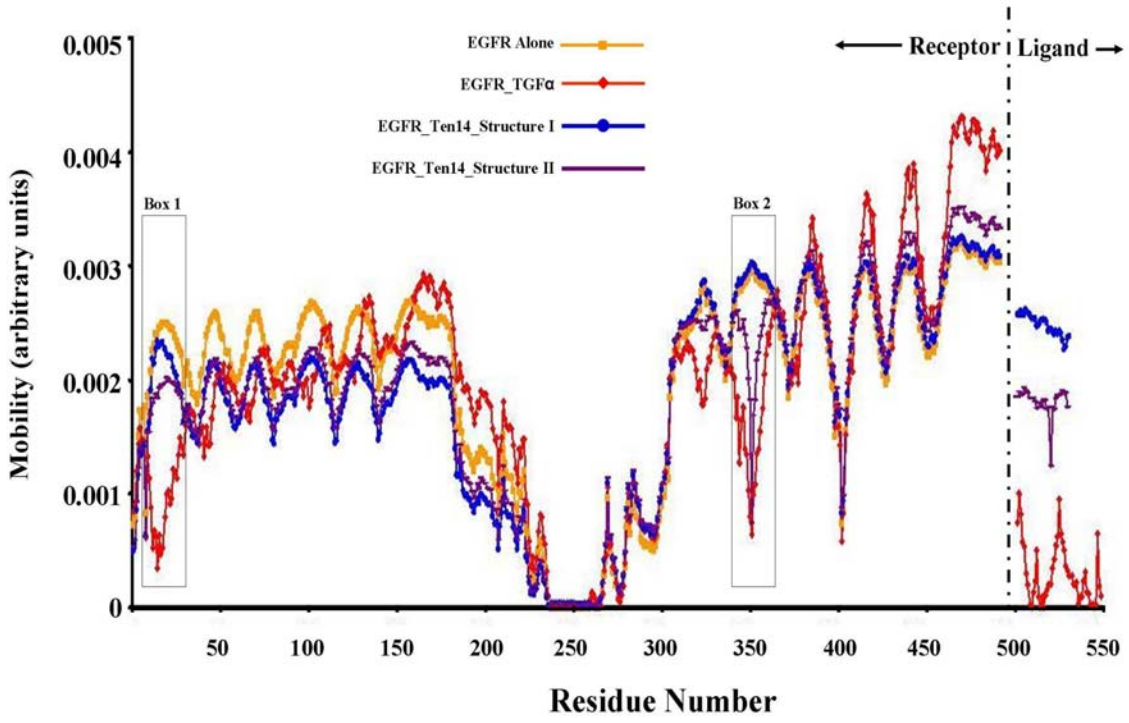

Figure 8. GNM Analysis for Ten14 Bound to EGFR Suggests Weak Binding

The figure shows the distribution of fluctuations in the slowest modes for EGFR structure alone, and of the complexes TGFα-EGFR, Ten14-Structure I EGFR and Ten14-Structure II EGFR. The curves represent the normalized mobility of each residue in the complex (Y-axis) as a function of residue index (X-axis). At the ligand-binding pocket entrance encompassing residues 10-40 (Box 1) and 350-370 (Box 2), EGFR alone (yellow) has considerably higher mobility than EGFR bound to TGFα (red), while the mobility in the Ten14-bound forms (blue and purple) is comparable to the unbound forms. This comparison confirms that these regions involve stable and strong contacts with EGFR in the case of TGFα binding, which are either lost or weaker in the Ten14-bound form. Also, Ten14 ligand in both conformations is much more mobile than TGFα (right terminal portion of the curves).

Our analysis shows that the ligand binding pocket of EGFR between domains I and III is highly mobile in absence of ligand, allowing easy access and local rearrangements to

accommodate ligand binding, as expected from experiments (De Crescenzo et al., 2000) (Figure 8, Figure 9). The ability of receptors to undergo conformational movements that facilitate substrate-binding is consistent with our recent examination of intrinsic mobilities of proteins near their substrate binding sites (Tobi and Bahar, 2005).GNM analysis of collective dynamic for the TGFα-EGFR and EGF-EGFR confirmed that a very stable interaction is established for both EGF and TGFα with EGFR, i.e. the mobilities of both ligands are significantly suppressed, indicating that both ligands bind with high affinity to the receptor as expected (Figure 8, Figures 9B and 9C). However, in both Ten14-EGFR structures, Ten14 is found to be much more mobile in the bound form than classical EGFR ligands (Figure 8, Figures 9D and 9E). As evidenced by the mobility plots for EGFR complexed with the ligands, differences exist in the mobility of residues near the ligand-binding pocket (Figure 8) (Also see Movies 1 (EGFR alone), 2 (EGF-EGFR), 3 (TGFα-EGFR), 4 (Ten14_Structure I-EGFR) and 5 (Ten14_Structure II-EGFR)).The curves represent the normalized distribution of mobilities in the most cooperative (slowest) modes of motions. Regions near the ligand-binding pocket, (Boxes 1 and 2) are much more mobile in the Ten14-EGFR complexes (blue and purple) compared to the TGFα- (red) bound forms. Likewise, the terminal portions of the curves, detailing the mobility of the ligand residues, clearly indicate the significantly higher flexibility of Ten14 compared to TGFα for both structures, albeit the binding of Structure II to EGFR is more stable than Structure I.

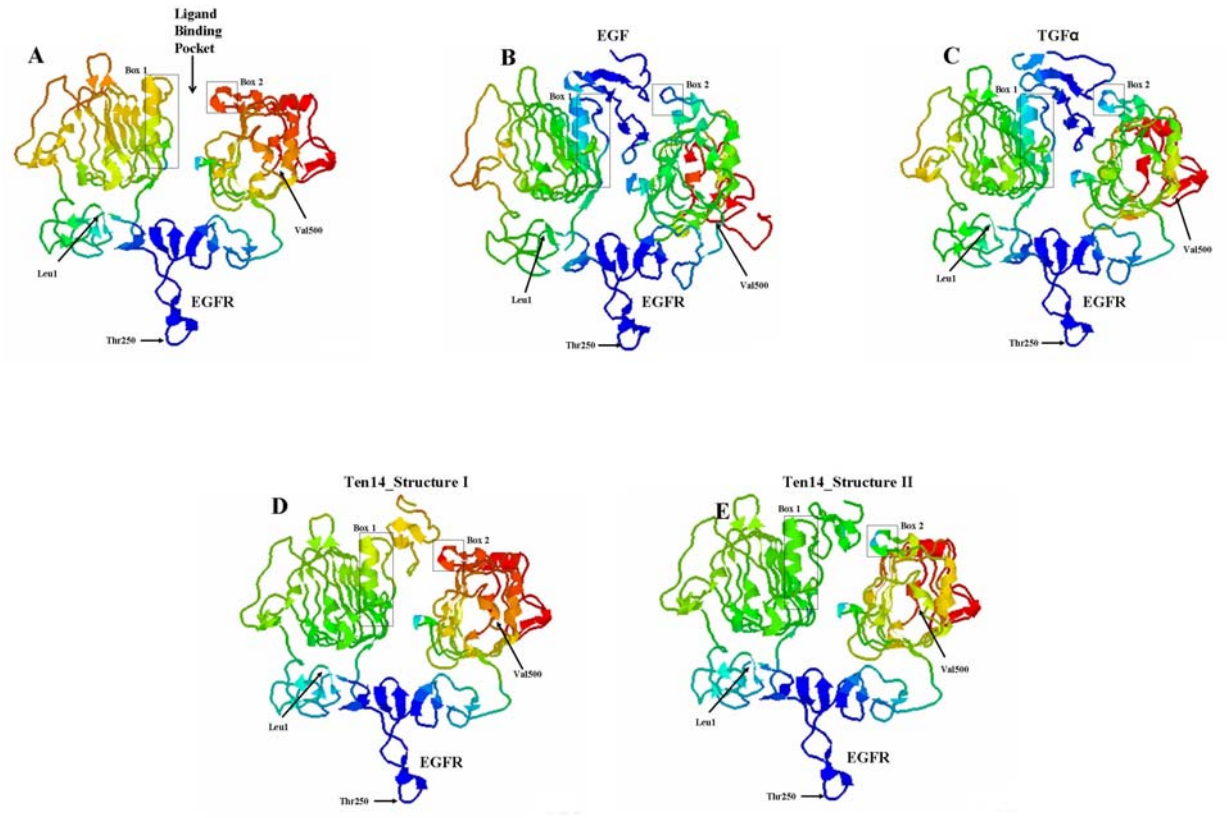

Figure 9. GNM Ribbons for Ligands Bound to EGFR

Color-coded ribbon representations of the most cooperative (lowest frequency) modes of equilibrium fluctuations for EGFR in the unbound form **(A)**, for EGF-EGFR complex **(B)**, TGFα-EGFR complex **(C)**, and for the predicted Ten14-EGFR complexes in both conformations **(D&E)** were generated using the *i*nternet based Gaussian Network Model (*iGNM*) server. The ribbon structures are colored coded from blue to red, with blue for regions with maximum stability, red for maximum mobility, and green as intermediary. EGFR alone without ligand is very mobile near the ligand binding pocket between domains I and III (A) , but is stabilized by a high affinity interaction with TGFα (B) and EGF (C) – this can be inferred by the shift from red in the ligand binding pocket of EGFR alone (A) to mostly blue in the bound receptor structure (B & C). Ten14 binds with a relatively lower affinity in both structural conformations I and II, resulting in higher mobility of the complex in the binding pocket of EGFR (green in D and E). Though Arg19 in Structure II seems to favorably interact with Asp354 (Box II in E), the overall structure is still fairly unstable, indicated by green in the structure.

2.4.4 Analysis of EGFR-Ten14 Interactions

We analyzed the interface between Ten14 and EGFR using 'FastContact', a program that computes the relative free energy of receptor and ligand residue contacts by summing electrostatic interactions and desolvation potentials that encapsulate hydrophobic interactions, the self-energy change upon desolvation and side chain entropy loss. Stronger interactions between ligand and receptor are manifested by lower (more negative) electrostatic and desolvation potentials. However, FastContact only accounts for inter-molecular contacts, and does not estimate differences in configurational entropy. Analysis of EGF-EGFR, TGFα-EGFR and Structures I and II of Ten14-EGFR complex yielded total electrostatic (ΔE) and desolvation energy potentials of -27.68 and -7.1 kcal/mol respectively for EGF-EGFR; -34.72 and -9.35 kcal/mol for TGFα-EGFR; -6.72 and 1.51 kcal/mol for Structure I, and -23.58 and -1.51 kcal/mol for Structure II of Ten14-EGFR. We emphasize that these are only relative energies - figuring in the error contributions arising from conformational entropy of the ligands and errors due to inherent shortcomings in the computation of binding energies using FastContact would significantly reduce the differences we observe with in silico binding energies, especially with EGF and TGFα. However, these estimates strongly suggest that Structure I, the model based on the same binding mode as EGF-EGFR, is not a good candidate for the complex. On the other hand, even after accounting for the aforementioned errors, Structure II recovers only a part (maybe about two thirds) of the affinity observed for EGF and EGFR, which may result in about a significant reduction in Ten14 affinity for EGFR with respect to EGF.

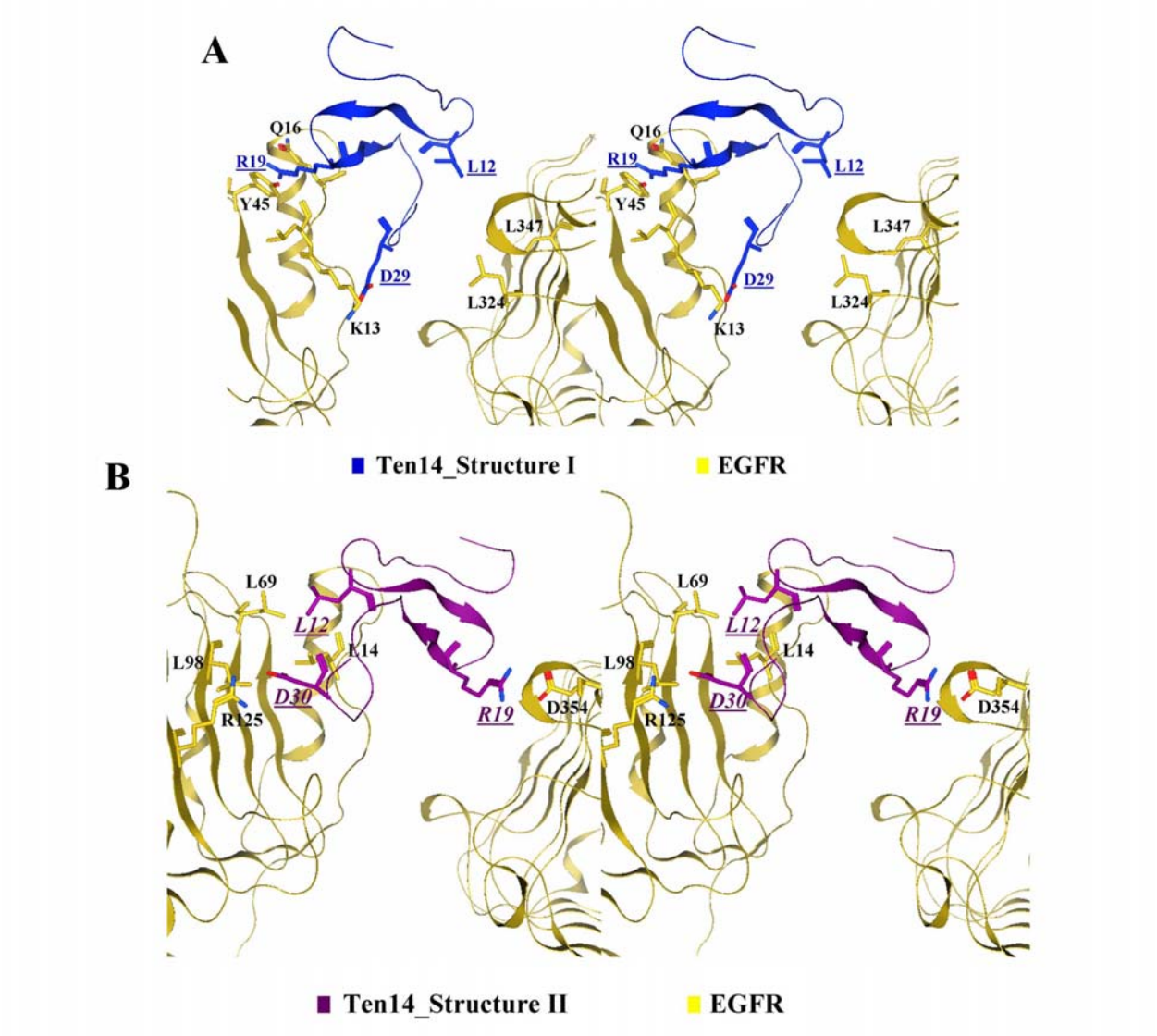

Figure 10. Interactions between Ten14 and EGFR

Stereo views of two important sets of interactions for both conformations of Ten14 (blue on top and purple on bottom) with EGFR (yellow ribbon) were generated using MOE.

A. Interactions between Ten14-Structure I EGFR include a high-affinity salt-bridge between Lys13 of EGFR and Glu29 of Ten14. The side chains of the two residues are only 3 Å apart. This interaction is energetically much more favorable than the corresponding interaction between EGF and TGFα (See Table 3). Arg19 of Ten14 and Tyr45 and Gln16 of EGFR stabilize the binding of Ten14 to EGFR. This interaction buries Leu14 of EGFR and restricts it from

solvent accessibility. Also, a potential interaction may be possible between Leu12 of Ten14 and a pocket formed on the receptor by Leu324 and Leu347.

B. For Structure II of EGFR-bound Ten14, Leu12 of Ten14 sits in the hydrophobic pocket formed by Leu14, Leu69 and Leu98 of EGFR. This coupling leads to interaction of Arg19 with Ten14 with Asp354 of EGFR. Note also that Asp30 of Ten14 interacts with Arg125, a key residue for the TGFα-EGFR interaction (See Table 3).

Detailed examination of the structures reveals that the highly attractive interactions between Asp345-Arg42 (Arg41 in EGF), Glu90-Lys29 (Lys28 in EGF), Lys464-Ala50 (Trp49 in EGF) and Arg125-Glu27 (Asp27 in EGF) between EGFR and the ligands EGF or TGFα are absent in the case of Ten14-Structure I (See Table 3). However, for Structure I, an important salt bridge between Lys13 of the receptor and Glu29 of Ten14 is formed (Figure 10A). This interaction is energetically more favorable for Ten14 as compared to those for EGF and TGFα (Table 3). Also, favorable electrostatic interactions take place between Arg19 of Ten14 and Gln16 and Tyr45 of EGFR, shielding the hydrophobic Leu14 of EGFR and stabilizing the binding of Ten14 to EGFR (Figure 10A). For Structure II, a number interactions are recapitulated in Ten14 that exist in EGF and TGFα, with Arg19 forming a highly favorable interaction with Asp354, and Glu29 interacting with Arg125, both of which lead to a tighter binding of Ten14 with EGFR in this conformation than Structure I (Figure 10B). However, these interactions are not sufficiently stable to allow for tight binding of Ten14 to EGFR, evident from GNM analysis of Structure II (see Figure 9E).

Table 3. FastContact Results for Ten14 Show Favorable Interactions with EGFR

We used FastContact to analyze the energetics of the predicted Ten14-EGFR bound structures (Ten I and Ten II). The program ranks the 20 most important inter-residue interactions that contribute to overall electrostatic and binding free energies between two proteins in a complex. The results were generated for the Ten14-EGFR structures, tabulated and then compared with results for EGF-EGFR and TGFα-EGFR structures.

(A) The most important residue pairs in Ten14-EGFR structures (Ten-I and Ten-II), TGFα-EGFR and EGF-EGFR that contribute most towards free energy are tabulated. As we can see, nearly all the important interactions in Ten14-EGFR structures are electrostatic in nature.

(B) This table identifies the residue interactions from the Ten14-EGFR structures, EGF-EGFR and TGFα-EGFR structures that contribute most towards the electrostatic energies of the complex. Lys13 interacts very strongly with Glu29 of Ten14-I (see Figure10A), forming a salt bridge and this interaction may play a key role in maintaining Ten14 bound to EGFR. For Ten14-II, The important interaction is between Asp354 and Arg19 of Ten14, and Leu12 of Ten14 with the hydrophobic pocket in domain I of EGFR (see Figure 10B).

EGF-EGFR RESIDUE INTERACTION			TGFα-EGFR RESIDUE INTERACTION			Ten14-EGFR RESIDUE INTERACTION					
EGFR	EGF	FREE ENERGY (kcal/mol)	EGFR	TGFα	FREE ENERGY (kcal/mol)	EGFR	Ten14-I	FREE ENERGY (kcal/mol)	EGFR	Ten14-II	FREE ENERGY (kcal/mol)
Asp354	Arg41	-10.010	Arg125	Glu27	-9.261	Lys13	Glu29	-4.908	Asp354	Arg19	-9.88
Glu90	Lys28	-7.939	Asp354	Arg42	-9.086	Leu17	Cys22	-1.236	Arg125	Asp30	-9.281
Lys13	Glu40	-1.802	Glu90	Lys29	-3.738	Gln16	Cys22	-0.965	Lys13	Glu29	-6.658
Arg29	Asp46	-1.746	Lys464	Ala50	-2.457	Gln16	Ile21	-0.958	Phe356	Ile21	-1.43
Phe257	Tyr13	-1.503	Gln16	Arg22	-1.765	Thr15	Glu29	-0.809	Leu14	Leu12	-1.1
Lys464	Trp49	-1.342	Phe356	Phe15	-1.488	Leu14	Arg19	-0.723	Glu90	Glu29	-1.07
Lys464	Leu47	-1.262	Phe411	Leu48	-1.446	Thr15	Cys22	-0.574	Thr15	Cys20	-0.85
Phe411	Leu47	-1.111	Thr15	Cys32	-1.274	Asn12	Gly28	-0.533	Thr15	Cys9	-0.672
Thr15	Cys31	-1.079	Leu17	Cys34	-1.143	Thr15	Cys20	-0.517	Gln15	Cys9	-0.527
Leu98	Leu26	-1.074	Ile437	Leu47	-1.118	Gly18	Cys22	-0.406	Gly18	Pro6	-0.476
Leu17	Cys33	-1.072	Ser355	Thr13	-1.086	Lys13	Asp30	-0.400	Leu14	Cys31	-0.467
Ile437	Leu47	-1.071	Arg29	Asp47	-1.064	Arg29	Glu24	-0.399	Lys13	Asp30	-0.448
Lys464	Asp46	-1.000	Gln16	Cys34	-1.059	Thr15	Cys31	-0.384	Gly18	Ser7	-0.441
Leu69	Leu26	-0.922	Val349	Phe17	-0.868	Arg352	Asp8	-0.376	Arg125	Cys31	-0.439
Gly18	Cys33	-0.898	Gln16	Val33	-0.842	Leu17	Ser27	-0.354	Leu69	Leu12	-0.393
Leu17	Ile38	-0.862	Leu17	Val33	-0.814	Glu42	Arg19	-0.335	Leu14	Cys20	-0.382
Gln16	Cys33	-0.834	Thr15	Ala41	-0.756	Thr15	Gly28	-0.310	Thr15	Cys15	-0.37
Gln383	Gln43	-0.775	Leu14	Leu24	-0.696	Leu17	Tyr26	-0.298	Gln16	Ser7	-0.366
Leu69	Ile23	-0.764	Asn12	Gly40	-0.669	Thr15	Asp30	-0.283	Glu90	Gly28	-0.352
Val349	Leu15	-0.763	Phe411	Leu49	-0.656	Gly18	Ile21	-0.283	Tyr89	Asp30	-0.343

EGF-EGFR RESIDUE INTERACTION			TGFα-EGFR RESIDUE INTERACTION			TEN14-EGFR RESIDUE INTERACTION					
EGFR	EGF	ELEC. ENERGY (kcal/mol)	EGFR	TGFα	ELEC. ENERGY (kcal/mol)	EGFR	Ten14-I	ELEC. ENERGY (kcal/mol)	EGFR	Ten14-II	ELEC. ENERGY (kcal/mol)
Asp354	Arg41	-10.540	Arg125	Glu27	-9.835	Lys13	Glu29	-5.957	Asp354	Arg19	-10.394
Glu90	Lys28	-8.726	Asp354	Arg42	-9.554	Leu14	Arg19	-0.808	Arg125	Asp30	-9.874
Lys13	Glu40	-2.958	Glu90	Lys29	-4.275	Gln16	Arg19	-0.797	Lys13	Glu29	-7.696
Lys464	Trp49	-2.052	Lys464	Ala50	-2.852	Asn12	Gly28	-0.595	Glu90	Glu29	-1.57
Arg29	Asp46	-1.838	Gln16	Arg22	-2.582	Tyr45	Arg19	-0.466	Asn91	Glu29	-0.604
Lys464	Leu47	-1.629	Arg29	Asp47	-1.071	Asn12	Ser27	-0.447	Tyr89	Asp30	-0.458
Gln410	Lys48	-1.099	Ser355	Thr13	-0.843	Gln16	Cys22	-0.432	Lys13	Asp30	-0.448
Gln16	Asn32	-1.054	Gln383	Glu44	-0.821	Thr15	Glu29	-0.412	Arg125	Cys31	-0.439
Lys464	Asp46	-1.029	Asn128	Gln26	-0.767	Gln16	Ile21	-0.403	Tyr89	Glu29	-0.419
His408	Lys48	-1.017	Asn12	Gly40	-0.722	Lys13	Asp30	-0.400	Glu90	Gly28	-0.403
Gln383	Gln43	-0.952	Thr15	Cys32	-0.627	Arg29	Glu24	-0.399	Asp322	Arg19	-0.32
Thr15	Cys31	-0.649	Gln16	Cys34	-0.615	Arg352	Asp8	-0.376	Gln16	Ser7	-0.319
Ser467	Glu51	-0.648	Tyr45	Arg22	-0.602	Leu69	Arg19	-0.337	Gly18	Ser7	-0.267
Gly18	Cys33	-0.615	Leu14	Arg22	-0.578	Glu42	Arg19	-0.335	Lys321	Glu29	-0.266
Asp435	Lys48	-0.542	Arg29	Ala50	-0.546	Tyr89	Glu29	-0.289	Arg125	Glu29	-0.189
Tyr89	Lys28	-0.504	Lys462	Ala50	-0.537	Gly18	Arg19	-0.266	Lys13	Cys31	-0.173
Gln383	Arg45	-0.416	Gln16	Cys32	-0.459	Asn40	Glu29	-0.249	Gly18	Pro6	-0.168
Arg125	Asp27	-0.408	Asp322	Arg42	-0.443	Leu14	Glu29	-0.199	Ser127	Asp30	-0.164
Gln16	Cys31	-0.382	Thr15	Ala41	-0.422	Ser11	Gly28	-0.175	Thr15	Asp8	-0.146
Asp322	Arg41	-0.355	Thr15	Gly40	-0.413	Asp102	Arg19	-0.173	Gly353	Arg19	-0.105

2.4.5 Ten14 Exhibits Ultra-Low Affinity for EGFR as Compared to EGF

Previous studies show that much higher concentrations of Ten14 are required to have biological and biochemical effects equivalent to EGF. This, coupled with the structural data

indicating very weak binding, suggests that Ten14 may activate receptor in a manner distinct from other classical soluble ligands. A low affinity ligand such as Ten14 would follow a staccato mode of signaling, whereby it binds EGFR for a period sufficient to elicit signaling, but dissociates from the receptor before internalization. In order to confirm this, SPR analyses were performed with increasing concentrations of Ten14 and human EGF on sensor surfaces derivatized with the EC ligand binding domain of EGFR (Figure 11). We obtained a K_D of 74 μM for Ten14, nearly a thousand-fold higher than that of EGF (~110nM), which was similar to published values for EGF-EGFR interactions (Brown et al., 1994; Domagala et al., 2000; Zhou et al., 1993). Though we could not directly determine the association/dissociation rates for the interaction due to technical limitations pertaining to low fidelity of Ten14 to changes in buffer conditions, the K_D values agree well with our observations in vitro and similar experiments performed with EGFR-ED (see discussion below). This unprecedented high K_D for an EGFR-ligand interaction is in accordance with predictions of high mobility of Ten14 in the ligand binding pocket of EGFR from structural modeling of the complex (Figure 8).

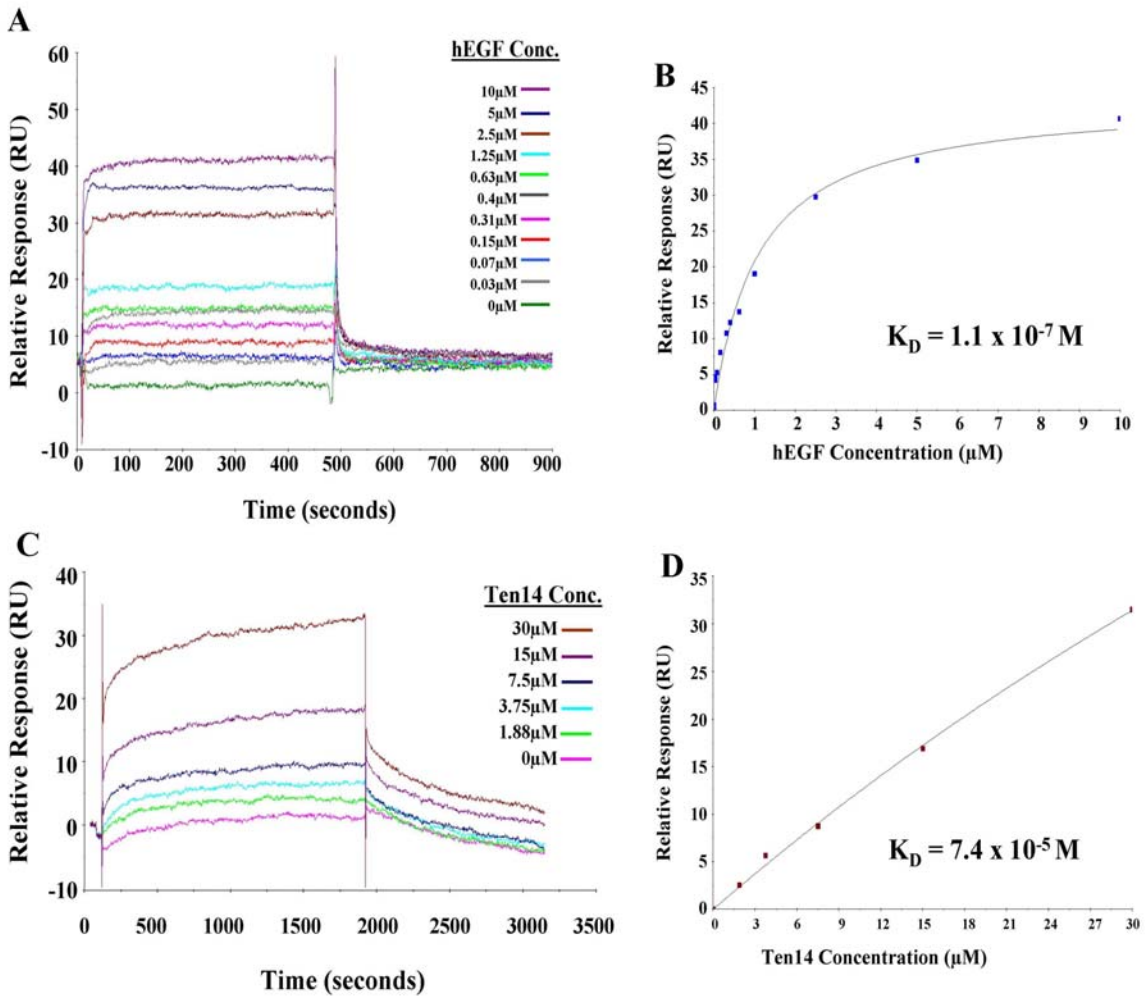

Figure 11. Surface Plasmon Resonance Analysis of EGF/Ten14 Binding to EGFR

A. Increasing concentrations of EGF (0.039, 0.078, 0.153, 0.31, 0.625, 1.25, 2.5, 5 and 10μM) were run over CM5 surfaces derivatized without or with ~2500 RUs of extracellular domain (ED) of EGFR, using HBS-EP as running buffer. Sensograms were plotted against time using the BIAEvaluate software package after subtraction from blank.

B. Steady state RUs were plotted against concentration for each EGF level, and curves were fit for calculating the equilibrium dissociation constant K_D using the 'steady state' module in the BIAEvaluation software. We obtained a K_D of 110nM for EGF.

C. Increasing concentrations of Ten14 (1.88, 3.75, 7.5, 15 and 30μM) were run over surfaces derivatized without or with ~7000 RUs of EGFR-ED, using PBS as running buffer. Sensograms were plotted after blank subtraction.

D. Sensograms were analyzed and a K_D of 74μM was obtained for Ten14, nearly a thousand-fold higher than EGF.

2.4.6 Ten14 Does Not Undergo Internalization and Depletion

The much higher ligand mobility and K_D for Ten14-EGFR interaction predicts an unstable binding unlikely to result in either ligand or receptor internalization/degradation. Specifically, we determined whether Ten14 was internalized and/or depleted in a manner similar to EGF when presented in soluble form to NR6WT murine fibroblasts over-expressing EGFR. Over a 48h period, EGF concentrations of 10nM and 1nM were depleted from the medium; however, we saw no significant depletions of Ten14 at similar concentrations (Figure 12A). In order to distinguish between the possibilities of Ten14 being recycled to cell surface as in the case of TGFα (French et al., 1995) versus rapid uncoupling from the receptor before internalization, we assessed internalization of radio-iodinated EGF and Ten14 ligand using a commonly derived protocol over a short time period that precludes recycling (Wiley et al., 1991). Although EGF was internalized dramatically, Ten14 was not (Figure 12B). We also measured EGFR internalization under high levels of EGF and Ten14 to ensure that Ten14-EGFR binding did not persist to engage internalization and subsequent degradation. As expected, no significant Ten14-mediated degradation of EGFR was observed over extended time periods (Figure 12C). Also, this in vivo data corroborated by the results we obtain for the Ten14-EGFR interaction in vitro (Figure 11), where the high K_D would drive rapid dissociation of Ten14. We conclude that Ten14 does not undergo receptor-mediated internalization, implying a rapid dissociation from the receptor leading to surface restricted activation of EGFR. Accordingly, immunofluorescent detection of active EGFR showed that upon treatment with EGF, significant co-localization of total and active EGFR was observed in internal compartments of the cell in the form of punctate dots (Figure 12D). However, Ten14 treatment resulted in surface staining alone, with no active receptors in internal compartments.

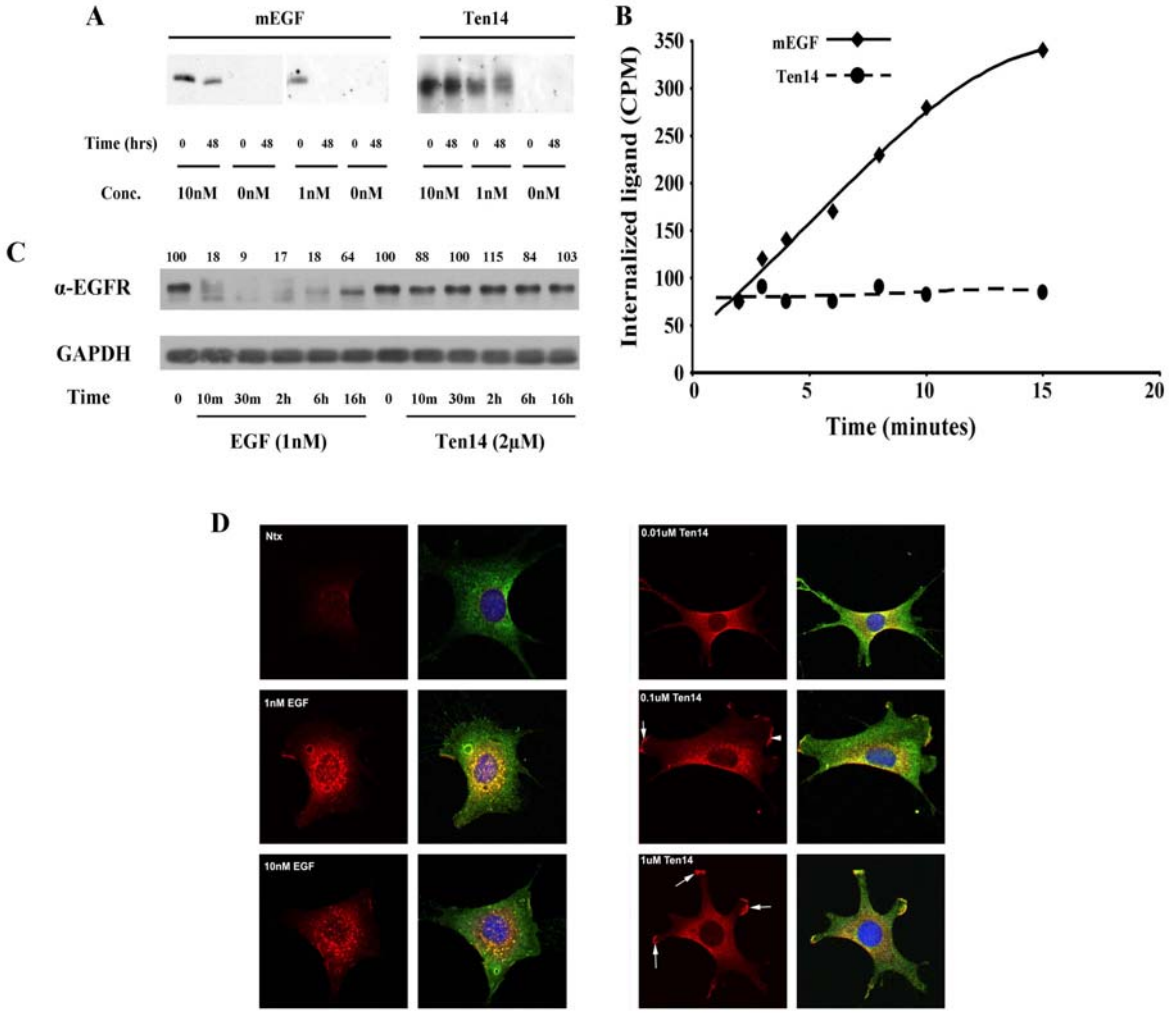

Figure 12. Ten14 Activates EGFR on the Cell Surface without Internalization

A. Following treatment of NR6WT cells for 48 hr with Ten14 and mEGF, concentrations of growth factors in the supernatant were determined using the antibodies against ligands. Ten14 is not depleted from the medium over 48 hr, compared to murine EGF.

B. Over 20 min, minimal internalization of ^{125}I-Ten14 is observed as compared to ^{125}I-EGF.

C. As opposed to EGF, Ten14 does not lead to degradation of EGFR over a 16 hr time period.

D. With 1nM and 10nM EGF treatment, active EGFR (phospho-tyrosyl EGFR, red) is internalized into endosomal compartments, appearing as punctate blobs. Staining for total EGFR (green) shows this is the fate of the majority of cellular receptors. Internalization of active EGFR is accompanied by EGF internalization (data not shown). On the

other hand, Ten14 causes localization of active EGFR solely at the cell surface with no internalization into intracellular compartments. All concentrations of Ten14 lead to activation and localization of EGFR into lammelipods (arrows). Also, Ten14 co-localizes with active receptor at the cell surface without internalization (data not shown). Blue stains for the nucleus.

2.5 DISCUSSION

EGF-like repeats are found in many ECM proteins and have been implicated in signaling through EGFR (Schenk et al., 2003; Swindle et al., 2001). This creates a conundrum in that high affinity binding of a concactemerized tethered ligand to a growth factor receptor, resulting in ultra-high avidity, would not allow for internalization-mediated attenuation that is critical to prevent excess signaling and aberrant cell responses (Masui et al., 1991; Wells et al., 1990). Even dephosphorylation attenuation would be limited as the physical constraints coupled to the high affinity would results in highly persistent ligandation. As an answer to this confounding aspect, our data indicates that Ten14 may be a low affinity ligand with altered binding dynamics as compared to other soluble prototypical ligands such as EGF and TGFα. Thus, signaling from the tethered EGFL would be attenuated by loss of ligandation and subsequent dephosphorylation. Although we evaluate the binding (both *in silico* and *in vitro*) of the 14th EGFL repeat of tenascin C, it is to be noted that at least three other repeats in the EGFL domain of tenascin C can potentiate signaling through EGFR (Swindle et al., 2001). We focus on Ten14 because we have previously optimized the purification and refolding process for Ten14 such that high yields of active ligand could be obtained for effective *in vitro* studies over a broad range of ligand

concentrations. Also, the folding of the EGFL repeat 14 has been previously studied (Zanuttin et al., 2004).

Structural modeling of Ten14-EGFR complex was the first step towards understanding the basis of the low affinity of Ten14 for EGFR. Modeling was chosen over classical techniques such as X-ray crystallography due to limitations in the purification process of Ten14; Ten14 undergoes non-physiological aggregation at very high concentrations that are required for successful protein crystallization. Ten14 is also extremely sensitive to changes in pH and buffer conditions. Lastly, enzymatic de-glycosylation of EGFR, a required step for co-crystallization of EGFR with its ligands (Ogiso et al., 2002), may drive down the affinity of Ten14 further, as seen previously with EGFR (Soderquist and Carpenter, 1984; Wang et al., 2001). Alternative purification techniques are being explored, but these efforts lie beyond the scope of this communication. Modeling of Ten14-EGFR complex structure offers a reasonable alternative to crystallography. Structural models have been generated for a number of receptor-ligand complexes, with good correlation between the predicted model and the actual crystal structure (Paas et al., 2000). Also, templates for the Ten14-EGFR complex exist in the form of EGF-EGFR and TGFα-EGFR crystal structures (Garrett et al., 2002; Ogiso et al., 2002), an important consideration for structural modeling. These facts provided us with sufficient impetus to simulate the bound structure of Ten14 and EGFR and analyze the complex to identify important interactions between them. An important consideration for studying Ten14-EGFR interactions using structural modeling was the identification of the ligand binding region, as this would directly impact inferences drawn from detailed analysis of the resulting complex. We employed the following approaches to elucidate interactions between Ten14 and EGFR. The first approach relies on the fact that given sufficient sequence and structural homology, similar domains in

distinct ligands may bind substrate in an identical manner (Rajamani et al., 2004). This is clearly evident with the EGF-EGFR and TGFα-EGFR complexes, where, despite their structural differences, significant overlap exists in the key ligand-motifs that interact with the receptor. Independently, we generated homology models of Ten14 based on the EGF and TGFα structures, and found that the top ranked models had a striking structural homology with the previously identified binding motifs. Based on this homology, we modeled a bound structure in a manner so as to satisfy the key molecular interactions, achieved by first fulfilling the hydrophobic requirements, followed by coupling of hydrophilic interactions resulting from the initial dock.

Both GNM and 'FastContact' analysis of the models suggests that conformation of Structure II of Ten14 is much more stable in the EGFR binding pocket compared to Structure I (lower mobility of Structure II in GNM analysis of the complex (Figure 8) and the lower ΔE of Structure II as compared to Structure I). Interestingly, Structure I provided us with a direct experimental test as it juxtaposed a non-conserved amino acid at the site of a salt bridge in EGF-EGFR and TGF□-EGFR complexes. We replaced Asp30 of Ten14 with a positively charged arginine corresponding with Arg41 in EGF (Arg42 in TGFα), and assessed its affinity for EGFR. If Structure I represents the true binding conformation of Ten14, the restoration of an important salt bridge between Ten14 and EGFR (by interaction of Arg30 in a D30R mutant of Ten14 with Asp354 of EGFR) should result in increased affinity of D30R for EGFR. This would also be reflected in the 'FastContact' analysis of the D30R mutant complexed with EGFR, resulting in much lower ΔE as compared to Structure I of native Ten14, and much tighter binding. However, SPR measurements for the D30R mutant with EGFR-ED and 'FastContact' analysis of the modeled D30R-EGFR complex yielded results showing no increase in affinity of the mutated form (data not shown). 'FastContact' analysis suggests that Structure II loses a significant part of

its affinity, which could result in a K_D that is well within the range observed for Ten14 *in vitro* using SPR (Figure 11B). Therefore, even though there is a lack of mutations predicted to increase affinity empirically, we believe Structure II most likely represents the true binding conformation of Ten14 for EGFR. However, mutational analysis of Structure II (in a manner similar to that for Structure I) with substitution of the key Arg19 to decrease affinity for EGFR further would not be technically feasible with any level of certainty. Even with native Ten14, we barely register binding affinities by SPR and not at all by standard Scatchard binding assays. Nevertheless, additional experiments are underway to validate the binding conformation of Structure II of Ten14 by assessing binding of Arg19 of Ten14 with Asp354 of EGFR using bi-functional crosslinking followed by receptor and ligand fragmentation and affinity purification. These technically daunting experiments lie beyond the scope of the present communication.

In order to assess if the low affinity is a direct result of altered binding dynamics of Ten14 to EGFR as compared to other soluble ligands, we performed SPR analysis using the ED domain of EGFR. SPR can be effectively used to predict kinetic binding parameters even in the micromolar levels (van der Merwe et al., 1994) as opposed to other biochemical techniques that are optimal only for studying high-affinity binding interactions. Our results indicate a K_D of 110nM for EGF, and though this is nearly two logs higher relative to measurements using live cells, it is in excellent agreement with similar experiments performed previously with EGFR-ED monomers (Brown et al., 1994; Domagala et al., 2000; Zhou et al., 1993). Also, the predicted K_D for Ten14 is nearly thousand-fold higher than EGF which agrees well with concentrations of ligand required to stimulate equivalent levels of activation of EGFR *in vitro.* As we could not directly measure the on-off rates of binding due to limitations arising from buffer considerations

for Ten14, we attempted to verify our data with other independent techniques such as dynamic light scattering, but technical limitations hindered effective analysis of binding.

Receptor compartmentalization and trafficking are important aspects of regulation of EGFR-mediated cellular responses. Proliferation and differentiation are initiated by signaling cascades triggered at the cell surface and are maintained by signaling cascades that are functional in intracellular compartments (Haugh and Meyer, 2002). Cell migration though, seems to be a mainly cell surface signaling mediated phenomenon, and active EGFR in endosomal compartments contribute minimally to triggering PLCγ1 required cell migration (Chen et al., 1994b; Glading et al., 2001; Haugh et al., 1999a). In vitro experiments with EGF presented as a tethered ligand by coupling to a polymer matrix showed that EGF can promote cell migration as effectively as soluble EGF (Griffith, Wells, et al. personal communication). We also observe Ten14-mediated restriction of active EGFR at the cell surface over a range of ligand concentrations. We contend that Ten14, and possibly other select EGFL repeats of tenascin C, may play a similar role in physiological conditions, presenting itself as a two-dimensional matrikine ligand with low affinity for EGFR, leading to compartmentalization of receptor and steady activation of migratory cascades at the cell surface.

Based on this and previous studies, we posit that multiple EGFL repeats can potentially bind numerous EGFR as part of a signaling complex. In this context, EGFR signaling could be mediated by EGFL repeats being part of an intact tenascin C, or released as aggregates containing multiple EGFL-repeat subunits. In fact, we previously reported that simple dimerization of Ten14 stabilized the Ten14 EGFL-repeat interactions with EGFR (Swindle et al., JCB 2001). The release of these subunits can potentially be mediated by the action of matrix metalloproteinases (MMPs) on tenascin C (Siri et al., 1995), and it has also been demonstrated *in*

vivo for EGFL domains of laminin (Schenk et al., 2003). Thus, while the individual affinity would be low, the matrix constraints would increase the avidity; a similar situation is found with integrin binding sites (Carman and Springer, 2003). However, overall avidity for interactions with EGFR via multimeric ligand domains may increase or decrease, and would be a function of both the intrinsic affinity of individual EGFR-EGFL repeat bonds, and the number of such bonds. Interestingly, we prefer Structure II binding precisely because it better allows for an integral EGFL repeat as part of intact tenascin C or an EGFL repeat domain to fit in the EGFR binding pocket.

Receptor binding to such matrix-constrained ligands would be enabled by the greater motility of the receptor-ligand interaction as we note, resulting in the lessened binding affinity and more transient occupation and activation profile. Such signaling may be relevant from the ECM standpoint, where a number of proteins, particularly tenascin C are found to be up-regulated only during wound healing or tumor progression, both of which require potent activation of migratory signaling cascades (Ilunga et al., 2004; Juuti et al., 2004; Zagzag et al., 2002). Interestingly, an up-regulation of MMPs is also observed concurrently with expression of tenascin C during numerous patho-physiological scenarios, characterized especially by instances involving potent cell migration (Cai et al., 2002; Jian et al., 2001; Kalembeyi et al., 2003). EGFL repeats of tenascin C may thus temporally and spatially activate select pathways downstream of EGFR, driven primarily by the low affinity of Ten14 for the receptor.

2.6 ACKNOWLEDGEMENTS

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3.0 CELL SURFACE RESTRICTION OF EGFR BY A TENASCIN CYTOTACTIN-ENCODED EGF-LIKE REPEAT IS PREFERENTIAL FOR MOTILITY-RELATED SIGNALING

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3.1 ABSTRACT

The 14th EGFL-repeat (Ten14) of human tenascin cytotactin activates the EGF receptor (EGFR) with micromolar affinity; however, unlike EGF, Ten14-mediated activation of EGFR does not lead to receptor internalization. As different signaling pathways downstream of EGFR have been shown to be triggered from plasma membrane and cytosolic locales, we investigated whether Ten14-mediated surface restriction of EGFR resulted in altered biochemical and cellular responses as compared to EGF. Molecules associated with migratory cascades were activated to a relatively greater extent in response to Ten14, with very weak activation of proliferation-associated cascades. Activation of phospholipase C γ (PLCγ) and m-calpain, associated with protrusion and tail retraction respectively, were noted at even at sub-saturating doses of Ten14. However, activation of ERK/MAPK, p90RSK and Elk1, factors affecting proliferation, remained low even at high Ten14 concentrations. Similar activation profiles were observed for EGF-treated cells at 4^{o}C, a maneuver that limits receptor internalization. We demonstrate a concurrent effect of such altered signaling on biophysical responses - sustained migration was observed at levels of Ten14 that activated PLCγ, but did not stimulate proliferation to significant levels. Here, we present a novel class of EGFR ligands that can potentially signal as a part of the extracellular matrix, triggering specific signaling cascades leading to a directed cellular response from an otherwise pleiotropic receptor.

3.2 INTRODUCTION

Many cell surface receptors elicit pleiotropic cellular responses when activated, although some of these responses might be mutually exclusive in any given or at a given time point. One prime example involves the epidermal growth factor receptor (EGFR), which, upon ligandation, triggers cell migration and proliferation, two responses that do not occur simultaneously (Wells, 1999). How a cell distinguishes between these two outcomes likely involves differential activation of the myriad of intracellular signaling pathways that are activated by this receptor (Bhalla and Iyengar, 1999).

We have demonstrated previously that EGFR-mediated migration and proliferation are distinct cell responses that negatively impact each other; i.e. when cells are driven to migrate, the fraction of the cell population undergoing proliferating diminishes (Chen et al., 1994a; Chen et al., 1996b). EGFR-induced motility requires the activation of phospholipase C-γ (PLCγ) (Chen et al., 1996a; Chen et al., 1996b; Polk, 1998), whose activation negatively impacts EGFR-mediated cell proliferation. Interestingly, both proliferation and migration are downstream of extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK); however, motility requires ERK to be activated at the plasma membrane (Glading et al., 2001), while proliferation can be triggered by ERK at either the plasma membrane or from intracellular sites (Chen et al., 1994a; Wang et al., 2002). Additionally, cell proliferation requires ERK translocation into the nucleus along with other transcription factors such as Ets-related protein ELK1 and the 90-kDa ribosome S6 protein kinase (p90RSK) that are activated by intracellular ERK (Ebisuya et al., 2005; Rocks et al., 2006). Thus, cellular responses mediated by EGFR signaling appear to be triggered independently by the spatial separation of these key molecules.

This mode of signal control adds an important dimension to controlling biophysical responses downstream of EGFR (Burke et al., 2001; Di Fiore and De Camilli, 2001; Ebisuya et al., 2005; Rocks et al., 2006).

EGFR is activated by soluble growth factors such as EGF, TGFα, amphiregulin, heparin-binding EGF, betacellulin and a few virally encoded factors (Citri and Yarden, 2006). These peptides bind with very high affinity and lead to internalization of EGFR, activating both motogenic and mitogenic cascades in the process (Wells, 2000). Ligand binding is key to internalization of EGFR – unliganded EGFR fail to internalize and activate downstream molecules from such locales (Haugh et al., 1999b).

This led us to speculate that a ligand that limits EGFR activity in a manner so as to trigger signaling selectively from the cell surface might elicit a different spectrum of responses than the classical soluble EGFR ligands such as EGF and TGFα. We and others recently demonstrated that a new class of ligands for EGFR – EGF-like repeats (EGFL) – can activate EGFR with binding modes qualitatively distinct from the classical soluble peptide ligands for EGFR (Schenk et al., 2003; Swindle et al., 2001). EGF-like repeats of tenascin cytotactin, an extracellular matrix protein, bind with ultra-low affinity and a fast off-rate, leading to compartmentalization of active receptors at the cell surface without internalization of either receptor or ligand (Iyer et al., 2007). This may be characteristic of binding of EGF-like repeats in general (Schenk and Quaranta, 2003; Tran et al., 2004; Tran et al., 2005), allowing for a novel signaling mechanism distinct from classical growth factors.

We hypothesized that compartmentalization of liganded EGFR at the cell surface would lead to selective activation of intracellular cascades and that this would influence the overall cell response. Specifically, we postulated that in response to the transient nature of binding of EGFL

repeats, EGFR would activate PLCγ and m-calpain at the cell surface, leading to enhanced migration but lacking the tonic intracellular activation of ERK that drives the cells toward proliferation. Our results indicate relatively robust activation of molecules associated with the migratory cascade downstream of EGFR in response to Ten14, leading to preferential activation of cell migration at concentrations of Ten14 that failed to stimulate proliferation. This work presents a novel mechanism by which ECM proteins containing EGFL signal EGFR, leading to a more selective and directed cell response from a potentially pleiotropic receptor.

3.3 MATERIALS AND METHODS

3.3.1 Expression and Purification of Ten14

Mid-log phase cultures of *Escherichia coli* strain BL21/DE3/pLys-S (Stratagene, La Jolla, CA) transformed with the individual expression plasmids were induced for recombinant protein expression (Ten14 or mEGF) with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3h at 37°C. Bacteria were harvested by centrifugation for 10min at 5,000 *g* at 4°C, and bacterial lysates were prepared by extraction with 0.02 culture volumes of B-PER detergent (Pierce, Rockford, IL). Recombinant proteins were purified from bacterial lysates by nickel-agarose chromatography with imidazole elution as stated previously (Swindle et al., 2001). Purified protein was dialyzed against PBS, 0.25mM β-mercaptoethanol for 24h at room temperature. Each experiment was repeated at least twice.

3.3.2 Mitogenesis Assays

Cells were quiesced for 24h in quiescence medium (serum-free growth medium supplemented with 0.5 % dialyzed fetal calf serum) (Swindle et al., 2001). The ligand-induced ^{3}[H]-thymidine incorporation assay has been described previously (Chen et al., 1994a). In brief, after quiescence cells were exposed to EGF or various concentrations of EGF-like repeat proteins for 24h. ^{3}[H]-thymidine was added to the cells for the last 10h to determine stimulation of proliferation. Mitogenesis assays were performed in tandem with *in vitro* wound healing assays with common stocks of ligand.

3.3.3 *In Vitro* Wound Assay

Basal and EGF-induced migration was assessed by the ability of the cells to move into an acellular area (Chen et al., 1994a). Cells were plated on a 24-well plastic dish and grown to confluence in DME with 7.5% FBS. After quiescence for 24h in medium with 0.5% dialyzed FBS, an area was denuded by a rubber policeman. The cells were then treated with or without ligand at 37°C. Photographs were taken at 0h and 24h, and the distance traveled by the cells at the acellular front was determined. Motility assays were performed in tandem with mitogenesis assays with common stocks of ligand.

3.3.4 Cell-Based Calpain Assay

NR6WT fibroblasts were plated with equal density and quiesced at 50% confluence in a Labtek II glass chamber (Nunc, Rochester, NY) and loaded for 20min at 37°C with 50μM Boc-LM-CMAC (Invitrogen, Carlsbad, CA), a synthetic calpain substrate (Glading et al., 2001). After loading, the cells were treated with growth factor for 5min and then mounted on glass slides, and images of the Boc-LM-CMAC fluorescence were obtained using pre-fixed exposures to enable comparisons between specimens. Equal density of cells per field was obtained via inspection under light microscopy. The BOC substrate is designed so that calpain cleavage results in fluorescence. Following imaging, representative images were quantified for blue fluorescence (with at least 15 cells per field) and graphed using Microsoft Excel. For cells that were incubated at 4°C, cells were loaded with BOC-LM-CMAC, then preincubated at 4°C and ligand added at 4°C for 5min. repeated at least twice.

3.3.5 Immunofluorescence Assays

To assess localization of p90RSK, 10,000 NR6WT cells quiesced on glass coverslips and treated with increasing concentrations of EGF or Ten14 for 30min. After washing with cold PBS, cells were fixed in 4% paraformaldehyde for 30min and lysed for 30min with buffer containing 1% triton X-100, 1mM PMSF and 1µg/ml aprotinin, followed by blocking in 5% BSA. In order to assess the localization of total versus phosphorylated EGFR, cells were incubated overnight at 4°C in rabbit polyclonal phospho-p90RSK1 antibody (Cell Signaling Tech. Danvers, MA). After a brief wash in PBS containing 0.5% BSA, coverslips were incubated in Alexa Fluor 488 anti-rabbit secondary antibody (1µg/ml) and 25µg/ml propidium iodide at room temperature for 30min. After a last wash, the slips were washed and mounted onto glass slides using gelvatol. After overnight drying, the slides were imaged for total fluorescence using a Zeiss Axioplan confocal laser-scanning microscope. Each image was scanned along the Z-axis in 7-10 sectional planes with 0.43µm steps (512x512 pixels per sectional plane). Following imaging, representative images were quantified for green fluorescence (with at least 15 cells per field) using Adobe Photoshop ver. 6.0 and then graphed using Microsoft Excel. 4°C for 5min. repeated at least twice.

3.3.6 In Vivo MAP Kinase Luciferase Assay

ERK/MAPK phosphorylation and activation of downstream substrate ELK1 in NR6WT murine fibroblasts was quantified using an MAPK *in vivo* kinase assay kit (Clontech, Mountain View, CA) according to the manufacturer's protocol. Briefly, a pTet-ELK vector (50ng/rxn) expressing a fusion protein with the functional domain of ELK and Tet repressor (TetR) domain and a

luciferase reporter vector, pTRE-Luc (0.5μg/rxn), containing a tet-responsive element (TRE) upstream of the luciferase gene, were transiently co-transfected into NR6WT cells seeded into 6-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as the transfecting agent. pTet-Neg (50ng/rxn) and pTet-Off (50ng/rxn) vectors, negative and positive control plasmids respectively co-transfected with pTRE-Luc, were also used in parallel. At 4h post transfection, cells were washed in PBS and incubated with quiescent medium containing 0.25% dialyzed FBS for 3h. Increasing concentrations of either EGF (10, 1 and 0.1nM) or Ten14 (1, 0.1 and 0.01μM) were incubated for 15min, 60min and 4h. Following incubation in serum free medium, cells were harvested on ice and assayed for luciferase expression using Luciferase™ Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Light emission was measured for 20sec with LB Lumat 9505 luminometer (Berthold Tech., Oakridge, TN, USA).

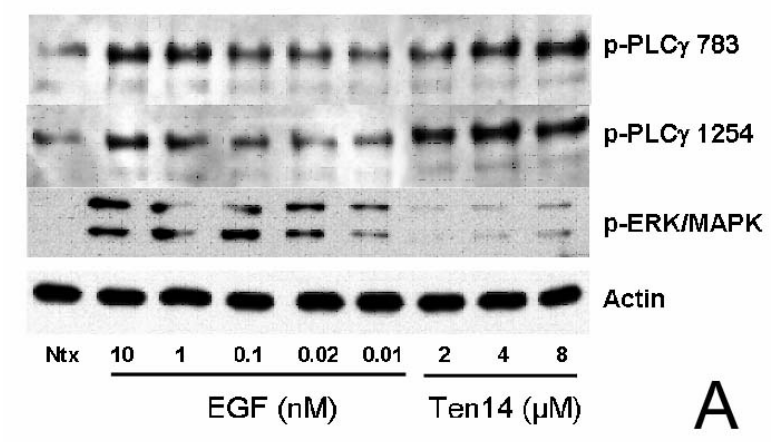
3.3.7 Phosphorylation Assay

NR6WT cells grown to 80% confluence, quiesced for 24h and were stimulated with indicated doses of EGF or Ten14 alone for 5min at 37°C or to cells (preincubated at 4°C) at 4°C. Cells were then lysed with sample buffer and separated on 7.5%-10% SDS-PAGE and western blotted for the indicated proteins - anti-phosphorylated PLCγ1 at tyrosine 783 and 1253 (Santa Cruz, CA, USA), anti-phosphorylated ERK/MAPK (Upstate Biotech., Boston, MA), anti-∝-actin (Sigma, St. Louis, MO).

3.4 RESULTS

3.4.1 Ten14 Exhibits Differential Activation of Signaling Cascades Downstream of EGFR Due to Surface Restriction of Receptor

We have previously demonstrated that at least some of the EGFR-mediated signaling pathways stimulating cell migration and proliferation are separable (Chen et al., 1994a). These diverge at the immediate and intermediate post-receptor stage, with motility requiring direct phosphorylation and activation of PLCγ (Chen et al., 1994b) and indirect activation of m-calpain secondary to plasma membrane-associated ERK (Glading et al., 2000a). As the low affinity and rapid off-rate of Ten14 detaches it prior to internalization of EGFR and restricts active EGFR to the cell surface (Iyer et al., 2007), we investigated whether such binding results in a biochemical activation profile different from that of classical soluble ligands such as EGF. Both PLCγ and ERK/MAPK were robustly activated and modulated in a dose dependent manner in response to EGF, as expected (Figure 13). For Ten 14, we observed strong activation of PLCγ as determined by phosphorylation at both the activation site, Y783, and the IP3 formation site, Y1254 (Kim et al., 1991; Nishibe et al., 1990). However, the phosphorylated, active ERK remained at near basal levels. Phosphorylation of the cytosolic target of ERK, p90RSK, was similarly absent. As the majority of active ERK derives from endosome-associated ERK (Haugh et al., 1999a), this dichotomy in signaling suggested that Ten14 binding leads to parsing of the signaling cascade downstream of EGFR.



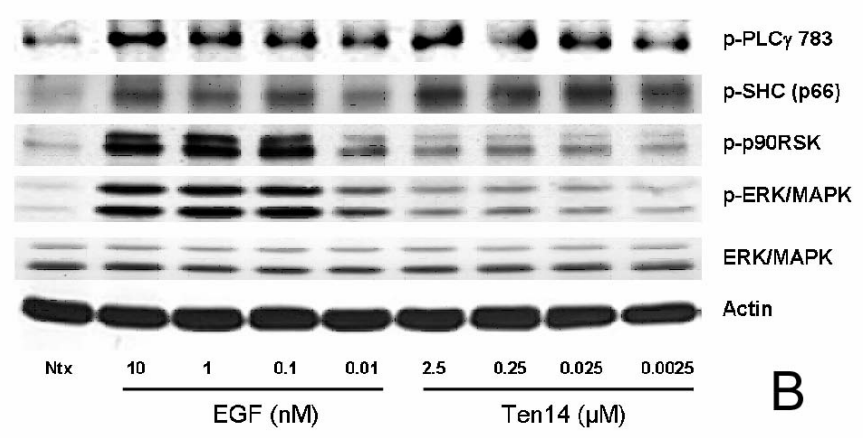


Figure 13. Ten14 Stimulates PLCγ1 preferentially over ERK/MAPK

Quiesced NR6WT cells were stimulated with indicated doses of EGF or Ten14 for 5min at 37°C. Cells were then lysed, proteins separated through SDS-PAGE gels, and immunoblotted for the indicated proteins. Shown are representative blots of at least four for each analysis.

A. Both Ten14 and EGF simulate PLCγ1 to equivalent levels at comparable concentrations of ligand. However, ERK/MAPK is weakly phosphorylated even at the high concentrations of Ten14.

B. PLCγ1 and SHC are robustly activated even by nanomolar levels of Ten14. However, unlike EGF, the phosphorylation of ERK/MAPK and p90RSK remains weak even at micromolar concentrations of Ten14.

We had previously demonstrated that Ten14 fails to drive internalization of EGFR even at high ligand concentrations (Iyer et al., 2007). Therefore, we proposed that this differential activation of molecules downstream of EGFR was due to pools of active EGFR compartmentalized at the cell surface in response to Ten14. The biochemical profile observed in response to Ten14 models similar ERK signaling as seen after inhibition of EGFR internalization via mutation of the endocytic pathway or artificial obstruction (Daaka et al., 1998; Di Fiore and De Camilli, 2001; Pierce et al., 2000; Tong et al., 2000; Vieira et al., 1996). Therefore, we assessed the signaling activation profiles in response to EGF under physical conditions that limit EGFR internalization (Figure 14). At 4^{o}C, the biochemical signaling profile of EGF was strikingly similar to Ten14 at 37^{o}C, with weak activation of ERK even at saturating levels of EGF (10nM). This difference was not a result of impaired signal transduction through EGFR as evinced by the phosphorylation of PLCγ and Src homologous and collagen like (SHC) protein which is the initial, immediate post-receptor adaptor leading to ERK activation. Thus, the differential signaling profile is driven primarily by the restriction of active EGFR at the cell surface. It is important to note that the phosphorylated form of PLCγ has been shown to associate mainly with surface associated EGFR (Haugh et al., 1999b; Matsuda et al., 2001). Also, we infer that the ERK being activated by Ten14, albeit at low levels, is part of pool of membrane-proximal ERK, and this could result in activation of signaling cascades qualitatively distinct from endosomal ERK/MAPK signaling (Glading et al., 2001).

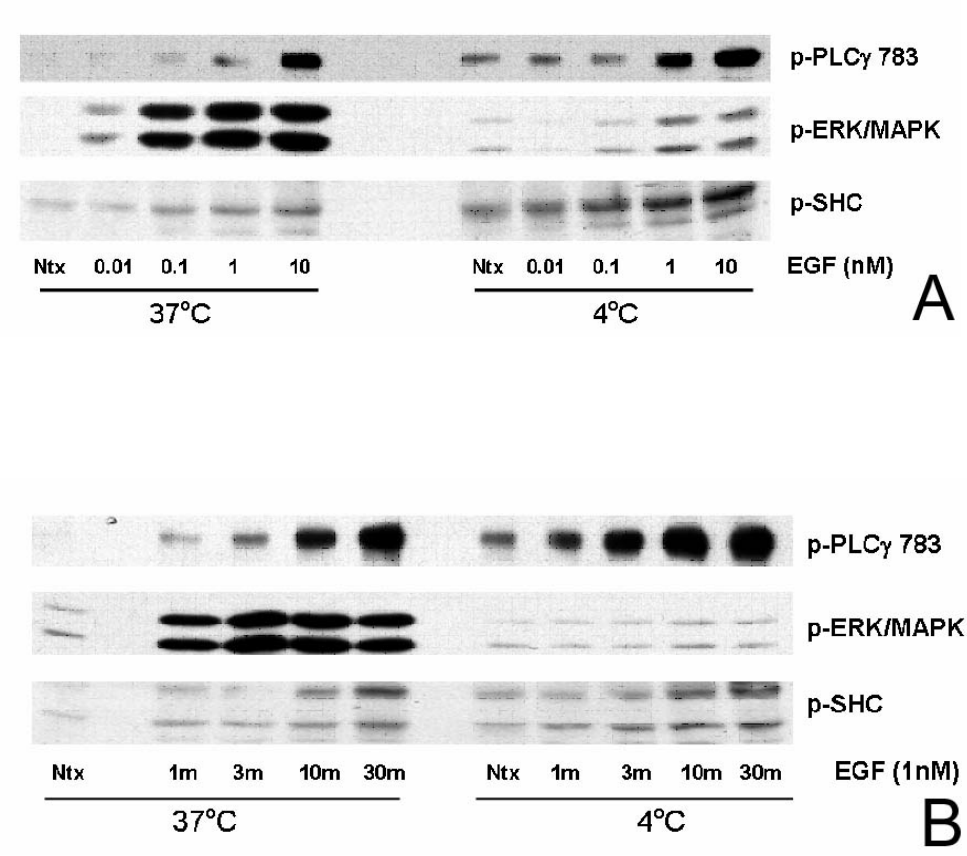


Figure 14. Activation Profile of Proteins Stimulated by Ten14 at 37°C is Similar to the Profile Observed with EGF at 4°C

NR6WT cells were quiesced, and then stimulated with indicated doses of EGF for the set time points or 5min at 37°C and 4°C. Cells were then lysed, proteins separated through SDS-PAGE gels, and immunoblotted for the indicated proteins. Shown are representative blots of at least four for each analysis.

A. Over a 5min period, EGF leads to robust dose-dependent activation of ERK/MAPK at 37°C, accompanied by phosphorylation of PLCγ1 and SHC. However, at 4°C, the activation profile for EGF-treated cells changes, with increased phosphorylation of PLCγ1 and SHC and very low levels of ERK/MAPK activation. This profile is strikingly is similar to that observed with Ten14 at 37°C (see Figure 13B).

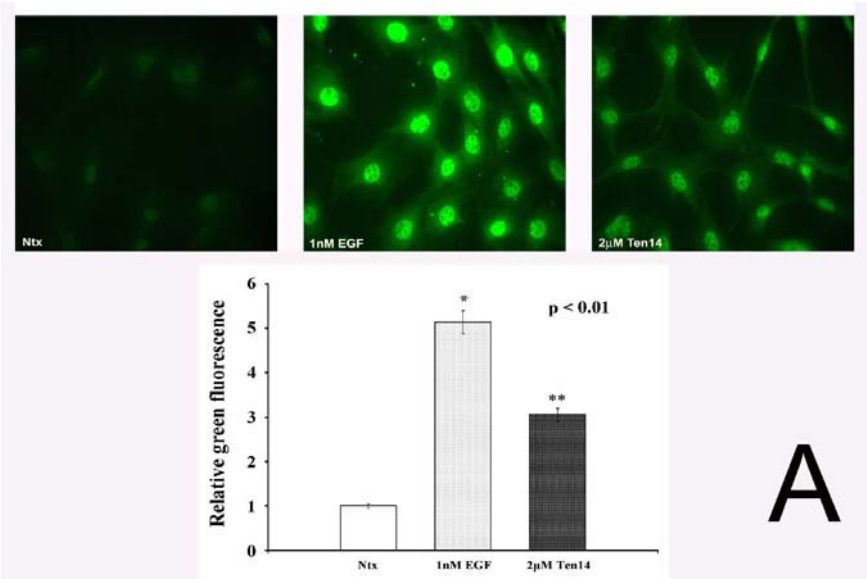
B. A differential activation profile is observed for EGF treated cells over a period of 30min, where the robust activation of ERK/MAPK seen at 37°C is lost at 4°C, but increased phosphorylation of PLCγ1 is observed.

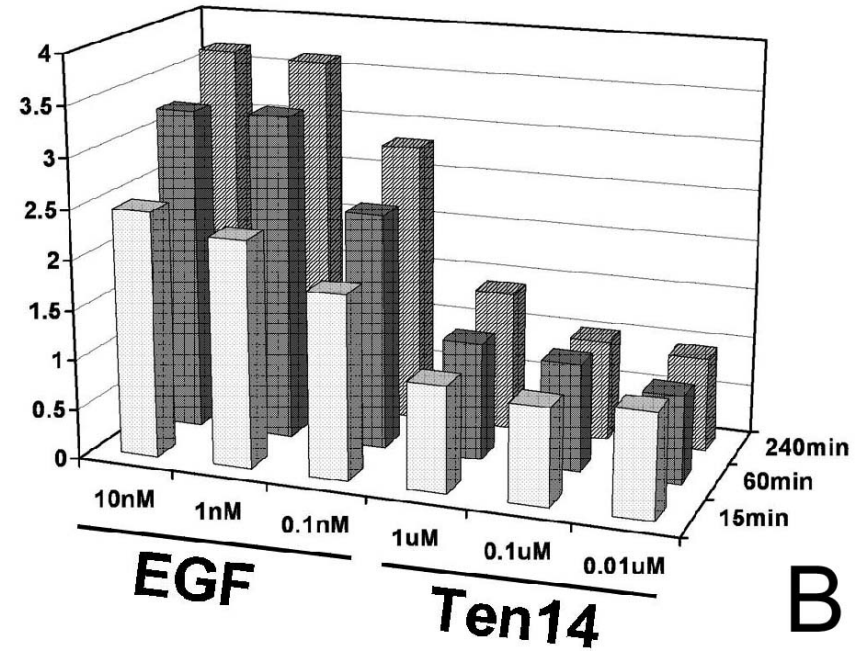
3.4.2 Ten14 Differentially Activates Divergent Signaling From ERK Downstream of EGFR

In order to delineate the effects of such divergent EGFR-mediated signaling at the cell surface in response to Ten14, we selected key downstream molecules that would transduce the differential signaling effects of ERK depending on signaling locale. Signaling downstream from ERK bifurcates with plasma membrane-associated ERK leading to activation of m-calpain (Glading et al., 2001) and cytosolic ERK activating p90RSK and ELK1 (Brunet et al., 1999; Gille et al., 1995), with the latter transiting to the nucleus (Brunet et al., 1999; Hochholdinger et al., 1999).

We assessed the activation of p90RSK and ELK1, both transcription factors that signal to promote cell proliferation in response to robust activation of ERK, particularly that of endosomal ERK (Wang et al., 2002) (Figure 15A,B). EGF resulted in strong activation of ELK1 as expected, as measured by the expression of the luciferase reporter that was placed under the action of ELK1. However, we observed only near basal levels of ELK1 activity for all concentrations of Ten14 over a 4 hour time period. This was also reflected in the weak activation of p90RSK for Ten14 as assessed by immunofluorescence and immunoblotting (see Figure 13). Interestingly, the motility-associated pathway downstream from ERK, m-calpain, was robustly activated in response to Ten14 (Figure 15C, D). That this m-calpain activity was inhibited by PD98059, a specific ERK inhibitor, and CI IV, a specific m-calpain inhibitor, confirms the signaling pathway involving EGFR-triggered ERK (data not shown). Also, increased m-calpain activation was observed for EGF-treated cells at 4^{o}C (data not shown), resulting from increased pool of active ERK at the cell surface due to restriction of EGFR (see Figure 14). These data support a model of surface-restricted signaling of EGFR in response to Ten14 leading to

preferential activation of molecules along the motility cascade as opposed to the mitogenic pathways.





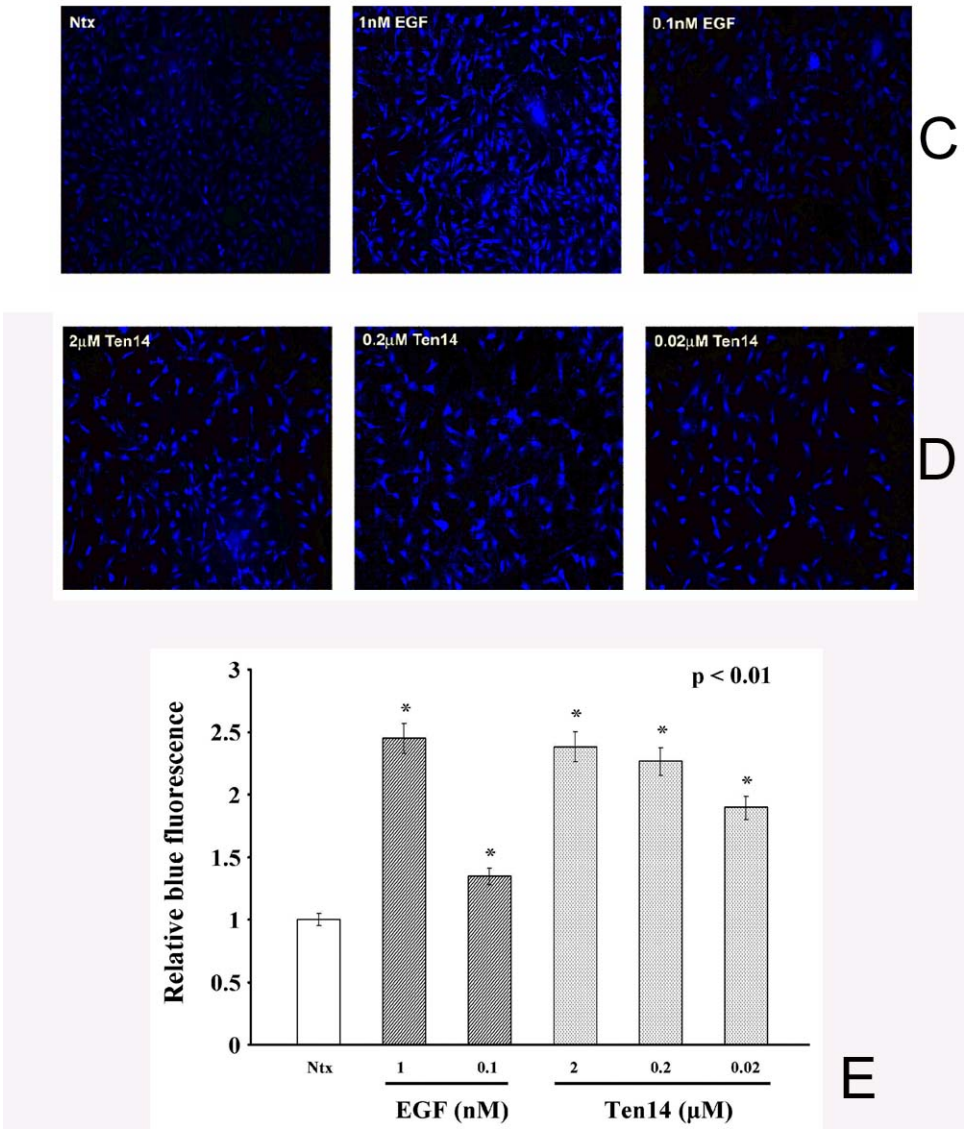


Figure 15. Differential Activation of Signaling Effectors by Ten14 Suggests a Lack of Potent Mitogenic Response and a Strong Motogenic Response Downstream of EGFR

A. Quiesced NR6WT cells plated on cover-slips were assayed for phosphorylated p90RSK (green). We observe much lower levels of active p90RSK with Ten14 as compared to EGF. Shown are representative immunofluorescent images of three separate experiments. EGF increased phosphorylated p90RSK staining 5.14 ± 0.25 fold versus 3.04 ± 0.09 fold for Ten14 ($P < 0.05$) as determined by quantification of fluorescent intensity of 15 randomly selected cells in one randomly selected experiment.

B. .NR6WT cells transiently co-transfected with the pTet-ELK and pTRE-LUC reporter vectors were incubated with increasing concentrations of either EGF (10, 1, 0.1nM) or Ten14 (1, 0.1, 0.01μM) over increasing periods of time, with control plates receiving no ligand. Cells were harvested and assayed for luciferase expression, and data plotted as a value relative to basal levels. A dose response was observed over time for EGF, with even the lowest levels activating ELK robustly. However, Ten14 did not stimulate activation of ELK over basal levels. Shown is mean, normalized to luciferase activity in serum-free media for each time point, of experiments at least four time for each stimulation/timepoint (each in triplicate); s.e.m. is < 10% of value and are not shown to limit visual clutter. At all time points Ten 14 is statistically different from EGF and only at the highest concentration (1μM Ten14) is Ten14 statistically different from background ($P < 0.05$).

C & D. Cells were incubated with 50uM BOC-LM-CMAC (Molecular Probes) for 15min at 37°C. Various concentrations of EGF and Ten14 were added and cells were incubated at 37°C for 15min. Calpain activity (blue) was assessed by fluorescence microscopy. We observe robust activation of Calpain even at 0.02μM, whereas calpain activity for 0.1nM is lower, suggesting sustained activation of Calpain with Ten14. Shown are representative immunofluorescent images of five separate experiments. (C) EGF at its highest concentration (1nM) increased BOC fluorescence 2.45 ± 0.09 fold which is similar to the 2.38 ± 0.08 fold for Ten14 (P is not significantly different from each other though $P < 0.01$ compared to control for both ligands) as determined by quantification of fluorescent intensity of 15 randomly selected cells in one randomly selected experiment. (D) However, at lower concentrations of each ligand, Ten14 stimulation led to greater BOC fluorescence than EGF ($P < 0.01$ comparing the two ligands).

3.4.3 Ten14 Preferentially Activates Cell Migration over Proliferation

The foregoing data would suggest that cell responses would be different upon EGFR activation by EGF or Ten14 (Figure 16). This should be most pronounced at limiting concentrations of ligand. As we titered out EGF, both motility and proliferation decreased in parallel. However, for Ten 14, motility was effected at concentrations that failed to stimulate proliferation over basal

levels (Figure 16B). Though ERK-mediated cell proliferation can be driven by strong endosomal ERK/MAPK signaling, the signals required to generate mitogenesis need only be above a threshold and exigent over a long period of time (Jones and Kazlauskas, 2001; Reddy et al., 1998), and can be achieved by surface-restricted EGFR (Ebisuya et al., 2005). This would explain the increase in cell proliferation at higher concentrations of Ten14, where the threshold required for proliferation is being met more effectively than at lower levels of Ten14.

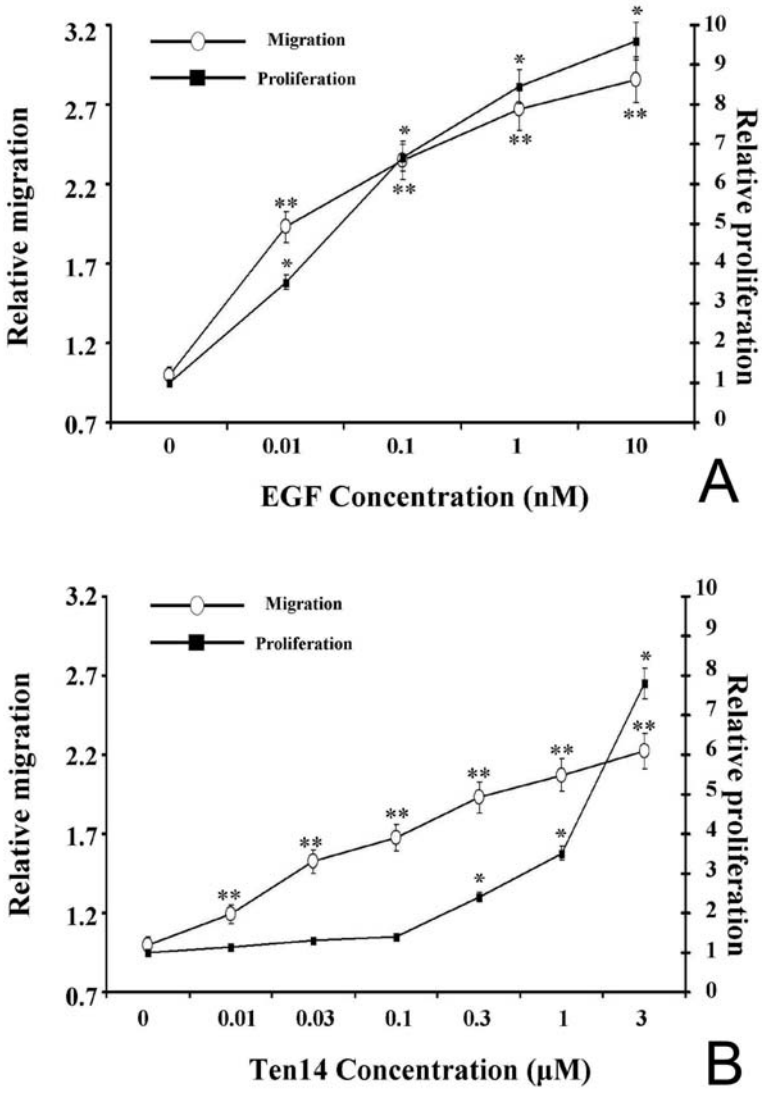


Figure 16. Ten14 Activates Migration at Levels That Fail to Stimulate Proliferation

Quiesced NR6WT cells were exposed to various concentrations of EGF (A) or Ten14 (B). Proliferation (filled squares) and motility (open circles) were assessed as described, and values plotted. Shown are mean ± s.e.m., normalized to treatment with diluent alone, of experiments performed at least 5 times (in triplicate). * for proliferation and ** for migration denotes $P < 0.05$ compared to diluent alone.

3.5 DISCUSSION

Herein, we presented data that activation of EGFR by an ultralow affinity matrikine signals to induce fibroblast motility preferentially over mitogenesis. This separation of cellular signaling is noted not only in the cell response but also in the biochemical switches that are key for these responses. What distinguishes the ultralow affinity matrikines, the EGFL in tenascin-C and laminin (Schenk et al., 2003; Swindle et al., 2001), from the classic high affinity soluble peptide ligands, EGF, TGFα and related ligands, is the mode of binding. These matrikines bind only transiently to the receptor, with the result that neither receptor nor ligand undergoes ligand-induced internalization (Iyer et al., 2007). Thus, essentially all of the EGFR signaling occurs from the plasma membrane locale. Based on the parsing of the signaling, we propose that plasma membrane-associated signaling of EGFR is preferential for motility. This finding provides a new mechanism by which a cell can select responses from otherwise pleiotropic signals.

The mode of binding of EGFL repeats to EGFR is akin to the interaction observed with other low affinity receptor classes such as the integrin family of receptors binding to fibronectin. Although the biophysical response elicited is adhesion (as opposed to migration with EGFL repeats), avidity of multiple ligand-receptor pairs rather than individual affinity is the critical property that drives the interaction between fibronectin and integrins (Carman and Springer, 2003).

It goes without saying that many other molecules such as the GTPases Rho, Rac & Cdc42 and the protein focal adhesion kinase (FAK) may also be involved as part of the post receptor signal transduction pathways that contribute towards the overall response that we observe with EGFL repeats. The fact that we are able to account for the observed responses (Figure 16) using

only PLCγ and ERK qualifies these molecules as critical rate-limiting factors in EGFL repeat signaling via EGFR, as expected from literature (Hautaniemi et al., 2005; Kharait et al., 2006; Ridley et al., 2003). In support of this, pharmacological inhibitors against ERK and PLCγ were able to negate the effects on migration and proliferation observed in response to Ten14 (data not shown). However, assaying for the other aforementioned molecules may further the understanding of the effect of EGFL repeats, particularly in situations such as wound-healing and metastasis, where multiple factors influence the eventual outcome.

While we support the postulate that it is the plasma membrane locale that is preferential for motility, we must note that other differences may account for this outcome preference. Non-internalizing EGFR, due to elimination of AP-2 binding sites (Chen et al., 1989) or abrogation of dynamin-mediated internalization (Vieira et al., 1996), are fully capable of driving proliferation (Vieira et al., 1996; Wells et al., 1990), though motility could not be determined in these cells due to lack of activation of key molecular switches such as PLCγ (Chen et al., 1994b). Further, not only does the transient ligandation result in predominantly plasma membrane-associated signaling, but matrikines also produce a highly staccato mode of signaling with the receptor avoiding activation-related downregulation (Iyer et al., 2007). These latter properties may also channel the signaling towards specific pathways. However, we cannot distinguish between these properties and the plasma membrane locales at present, as ligand or receptor engineering are not capable of separating these ligand-receptor binding properties. Physically tethering ligand may address this issue and is being actively pursued.

The mode of matrikine signaling is physiologically relevant, as the EGFL-containing tenascin-C displays a very discrete and transient pattern of expression during embryogenesis, wound healing, and tumor progression (Jones and Jones, 2000a). This pattern coincides with

situations of increased motility of adherent cells. In addition, EGFL-repeats of laminin are released as biologically relevant matrikines *in vivo*, where they stimulate the release of matrix metalloproteinases during mammary gland involution, and potently trigger EGFR-mediated cell migration without significant activation of ERK/MAPK (Schenk et al., 2003). Thus, the appearance of tenascin-C would imply enhanced migration, whether physiological during (neo)organogenesis or pathological during tumor invasion and dissemination (Wells, 2000). Our finding not only sheds light on a basic biological question of how cells respond differently via the same receptor but also holds promise for tissue engineering approaches to alter pathological wound repair and tumor invasion.

3.6 ACKNOWLEDGEMENTS

We thank Samantha L. Hess, Ali Jiwani, Christelle Akati, and Caitlin Q. Marlow for insightful discussions and protein purification assistance. We acknowledge Jean-Francois Hamel and BPEC for advice and biotechnology assistance. This work was supported by grants from the National Institute of General Medical Sciences (NIH).

4.0 SUMMARY AND FUTURE PROSPECTS

4.1 OVERALL CONCLUSIONS

Tenascin C is and ECM protein that has gained considerable attention in the past few years, mainly due to its emerging status as a regulatory molecule in numerous physiological processes including cutaneous wound healing, angiogenesis and development of brain, bone and other organs, and as a prognostic factor for several conditions including tumor progression and metastasis (Orend and Chiquet-Ehrismann, 2006). Different functional domains of TN-C have been implicated in eliciting and directing select responses, either by directly triggering signal transduction pathways downstream of select receptor classes, or by regulating the binding of other soluble and insoluble ligands that bind to their respective receptors (Jones and Jones, 2000a).

The functional aspects of the various domains of TN-C have been well documented. The EGFL domain was involved in neuronal outgrowth and counteradhesion of fibroblasts (Fischer et al., 1997; Gotz et al., 1996; Prieto et al., 1992; Spring et al., 1989). The fibronectin like domain play multiple roles in different tissues and cells. Among its many functions includes the adhesion and spreading of different cell types mediated by interactions with fibronectin and integrin receptors, induction of various matrix metalloproteinases and in tissue and ECM remodeling (Bell et al., 1999; Chung and Erickson, 1997; Krushel et al., 1994; Probstmeier and Pesheva,

1999; Tremble et al., 1994; Yokosaki et al., 1996). The terminal fibrinogen globe is also involved in cell elongation and spreading, mediated by interactions with heparin sulphate-containing proteins (Chiquet-Ehrismann et al., 1988; Joshi et al., 1993; Schenk et al., 1999). In addition, TN-C can regulate the function of other binding partners such as annexin, syndecans and actin (Chung and Erickson, 1994; Jones and Rabinovitch, 1996) (Chiquet-Ehrismann and Chiquet, 2003). However, since the major role of TN-C in vivo has been attributed to mediating cell deadhesion (which is thought to be regulated mainly by the various interactions of FNIII domain of TN-C with integrins), focus on EGFL repeats in this context has been limited. The EGFL repeat array has been viewed as more of an auxiliary domain that merely augments the effects of the FNIII domain in select cell types.

As mentioned earlier, the temporal and spatial pattern of TN-C expression sets it apart from most other ECM proteins which are found to be more ubiquitously expressed. In almost all situations, both physiological and pathological, TN-C expression correlates strongly with cell migration. Therefore, in light of our recent work with the EGFL repeats of TN-C, and work done by a few others with similar domains in other ECM proteins; we believe strongly that a paradigm shift is in order that highlights and strengthens the role of EGFL repeats in regulating cell responses.

We demonstrated that select EGFL repeats of tenascin C in soluble form could bind and activate EGFR. Using structural bioinformatics and selecting the 14^{th} repeat (Ten14) as a representative, we characterized the interaction of EGFL repeats with EGFR, and identified binding domains that stabilized the interaction of ligand and receptor. The ultralow affinity of Ten14 predicted by structural modeling was verified using surface plasmon resonance. An affinity in the micromolar range is not surprising; given that the presentation of these repeats as

part of the ECM restricts their binding, and that multiple subunits of the array can potentially bind a cluster of receptors, thus increasing the effective affinity in vivo. Due to their ultra-low affinity, EGFL repeats were unable to drive ligand-mediated internalization of EGFR; restricting active EGFR complexes to the cell surface, where select signal transduction pathways downstream of EGFR were triggered.

An examination of the signal transduction pathway of EGFR indicated that migratory signals arising PLCγ and m-calpain activation were stimulated strongly, with weak activation of mitogenic signals mediated by active pools of intracellular ERK, p90RSK and ELK1. As a result, robust migration was observed for subsaturating concentrations of Ten14, levels at which proliferation remained basal. Mitogenesis was observed only at very high concentrations of Ten14, whereas motility was signaled positively in a dose dependent manner for all levels of ligand.

These observations shed new light on the possible role of EGFL repeats in vivo as part of the ECM. It is interesting to note that for almost all physiological and pathological situations that tenascin C is found to be upregulated in, EGFR is also seen to be expressed. In all situations involving tenascin C signaling, its EGFL repeats may trigger signaling cascades similar to what we observe in vitro, leading to directed cell responses. As mentioned previously, tenascin C expression is observed under conditions that involve cell migration. EGFL repeats of tenascin C potently stimulate cell migration, as we have reported, and this effect must have obvious implications for tenascin C function. This aspect of tenascin C function has not been investigated so far, and in light of our recent work, sheds new light on the role of EGFL repeats from ECM proteins in vivo. Such signaling must have broad implications in various physiological and pathological settings that involve tenascin C, and an understanding of the associated pathways

could help identify possible targets that can potentially be exploited to generate better therapeutic solutions to disease.

Here, we briefly overview of how EGFL repeats of tenascin C and other ECM proteins may be processed and signal in vivo, thereby regulating EGFR-mediated cell responses in pathophysiological scenarios.

4.2 EGF-LIKE REPEATS - PROCESSING AND SIGNALING *IN VIVO*

In order to fully appreciate the functional aspects of EGFL repeat signaling in vivo, let us consider wound healing as a case in point (Figure 17). As mentioned earlier, wound healing involves the temporal and spatial upregulation of tenascin C during phases requiring intense cell migration, particularly in the inflammatory phase and granulation phase, after which TN-C expression decreases and dissipates towards the start of the resolution phase. Interestingly, the granulation phase also witnesses the upregulation of matrix metalloproteinases (MMPs) such as gelatinases, collagenases, matrilysins, etc (Mignatti P, 1996). These MMPs typically cleave ECM proteins at specific sequence recognition sites, leading to either degradation of the protein or its processing such that an active cleavage product is obtained (Birkedal-Hansen et al., 1993). Finally, this phase involves active expression of EGFR by fibroblasts, epithelial and endothelial cells (Hudson and McCawley, 1998; McCawley et al., 1998; Tran et al., 2004).

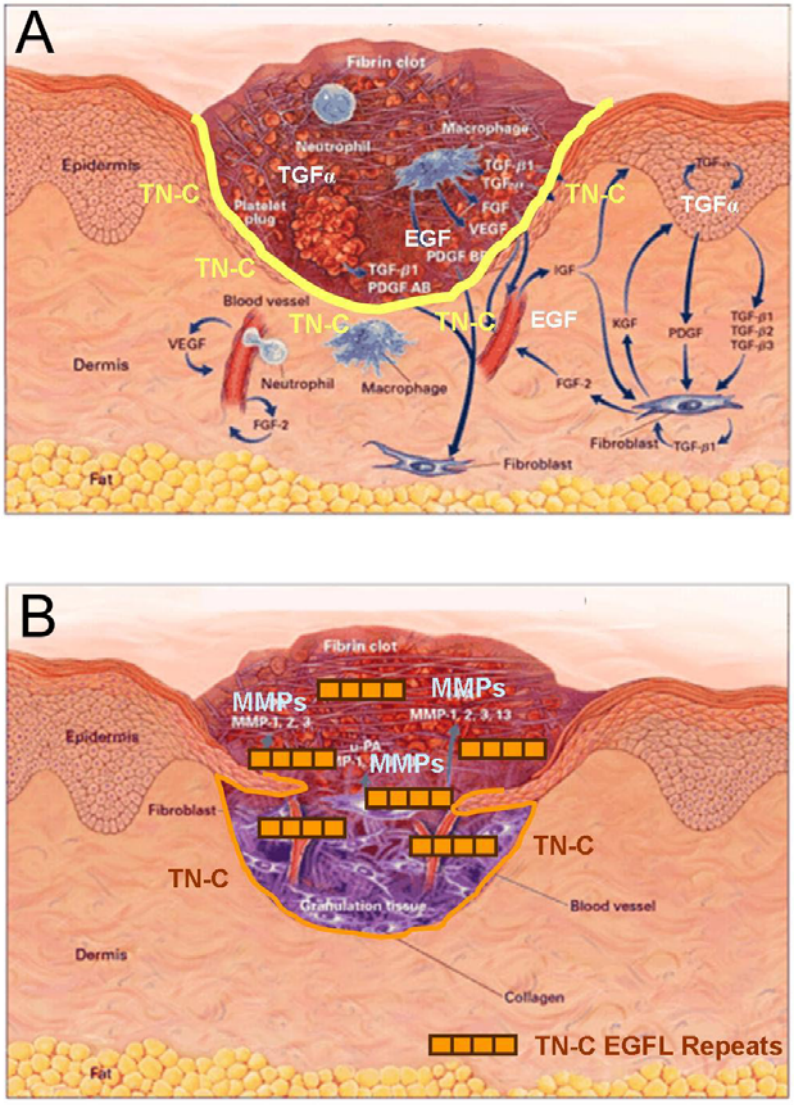


Figure 17. Tenascin C Expression Patterns during Wound Healing

A. During the inflammatory phase, TN-C levels increase, and peak at the start of the reepithelialization phase. TN-C is expressed along the leading edge of the wound. EGF and TGFα are also found to be expressed.

B. Towards the end of the reepithelialization phase, and towards the start of remodeling phase, TN-C expression starts to decrease. During this period, an increase in MMPs is observed. EGFL repeats of TN-C may be potentially cleaved by the action of these MMPs, thereby releasing them to facilitate local interactions with EGFR. Additionally, the action of these MMPs on TN-C may degrade it and thus downregulate its expression.

Figures 17A and **17B** were adapted from Singer and Clark, 1999. *NEJM.* 341:738-746.

The proposed model for the action of EGFL repeats in vivo is as follows. During the initial part of inflammatory phase of wound healing, TN-C is expressed at the leading extracellular margins of keratinocytes and invading fibroblasts. EGFL repeats of TN-C and other matrix proteins such as laminins may bind EGFR with ultralow affinity, activating those receptors being expressed by the cells at their leading edge and triggering the signaling cascade downstream of EGFR. Alternatively, throughout the granulation phase, a number of MMPs, particularly MMP2, MMP3 and MMP9, are found to be overexpressed in the stroma. Previous studies have shown that MMP2 can cleave EGFL repeats from laminin in vivo, releasing active EGFL domains in soluble form (Schenk et al., 2003). Sequence analysis indicates that EGFL repeats of tenascin C also contain the recognition site for MMPs, and can be potentially cleaved so that they can be released in soluble form.

Such activation may also lead to transient aggregation of EGFR on the cell surface into clathrin coated pits, increasing the effective avidity of EGFL repeats due to simultaneous binding of multiple EGFL domains to receptor. Upon activation, EGFR undergoes autophosphorylation at critical tyrosine residues in the intracellular domain that can then activate and phosphorylate a number of downstream substrate proteins, including PLCγ1 and the Shc/Grb2 adaptor complex. Cell migration is robustly triggered, influenced by the generation of DAG and IP3 through PLCγ1-mediated hydrolysis of membrane-bound PIP2 and release of several actin binding proteins such as profiling and gelsolin, whereas RAS activation though the Shc/Grb2 complex yields active pools of ERK at the cell surface. Pools of active ERK at the cell surface contribute towards cell migration via disruption of focal adhesions at the rear end of the cell via activated m-calpain (See Figure 18 and Movie 6).

Presentation of EGFL repeats as an integrated part of an ECM protein such as tenascin C should restrict its internalization simply on the basis of its mode of presentation. However, we have demonstrated that even in soluble form, EGFL repeats fail to internalize due to their weak affinity for EGFR; therefore, independent of their mode of presentation (i.e. both when bound to TN-C or when released into the stroma possibly by the action of MMPs), EGFL repeats will tend to undergo rapid decoupling from the receptor. Active EGFR that are now left unoccupied at the cell surface are quickly dephosphorylated by intracellular phosphatases, allowing for another round of EGFR activation (Hernandez-Sotomayor et al., 1993).

Receptor occupation is critical for EGFR internalization, and is a key step for potent ERK-driven mitogenesis through endosomal EGFR signaling. Since this is lacking with EGFL repeat signaling, we postulate that a strong motogenic cue might be selectively established over a proliferative response in vivo by EGFL repeats due to constant triggering of 'migration arm' of the EGFR signal transduction machinery, propelling cells in the direction of the healing wound. The significance of such parsed signaling is highlighted by the fact that initial stages of wound healing are characterized mainly by the involution of EGFR-expressing fibroblasts and keratinocytes into the wound bed from the leading margin of the wound, where tenascin C is selectively overexpressed. Interestingly, low levels of proliferation are observed in this leading edge. Under these situations, EGFL repeats can selectively signal migration as we observe in vitro, and play an important regulatory role in mediating migration in concert with the FNIII domain and other such domains in ECM proteins.

The selective activation of cell motility by EGFL repeats has broad implications in other pathophysiological conditions that involve EGFR signaling and migration. In order to highlight the potential role of EGFL repeats, a few processes have been discussed below in further detail.

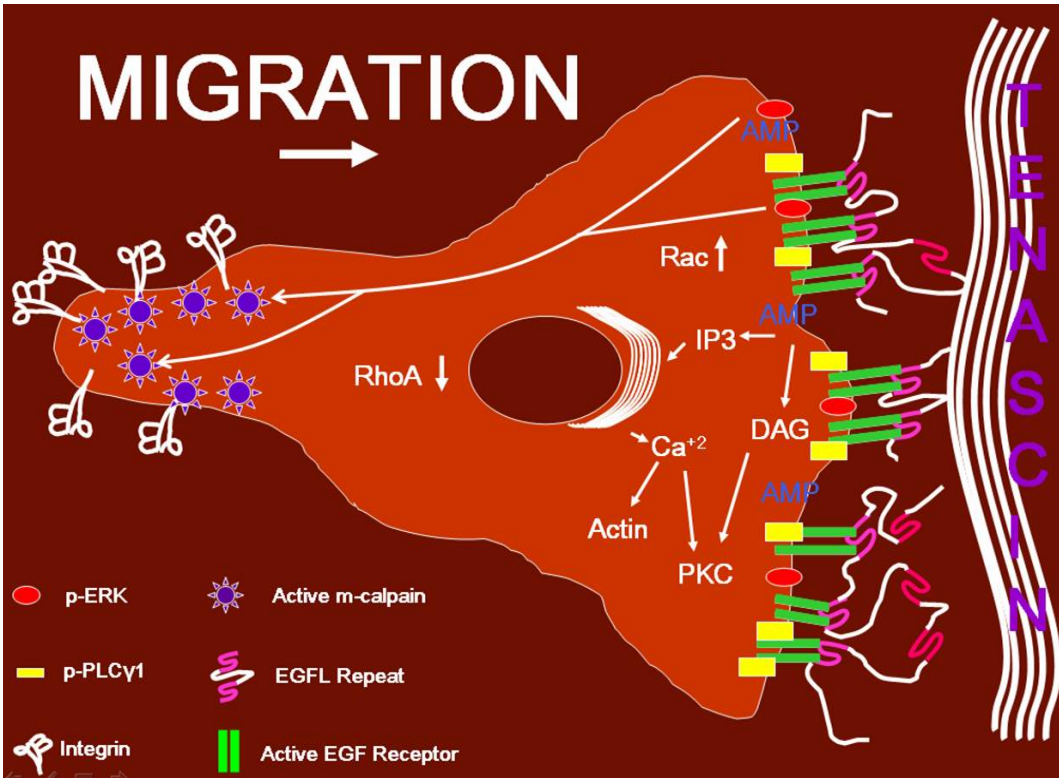


Figure 18. Potential Signaling for Migration by EGFL Repeats of TN-C In Vivo

When cells presenting EGFR (such as fibroblasts and keratinocytes) encounter tenascin-C during wither physiological or pathological conditions, EGFL repeats may bind receptor either as part of the intact proteins, or when released as subdomains (as shown here). These subdomains can simultaneously engage multiple receptors, but dissociate from the receptor due to their low affinity, thus circumventing ligand-mediated internalization of receptor. Active receptors at the cell surface can trigger potent activation of PLCγ, leading to hydrolysis of PIP2 and release of actin-modifying proteins (AMP) such as profiling and gelsolin, which can lead to cytoskeletal reorganization. Hydrolysis of PIP2 yields two secondary messengers, diacyl glycerol (DAG) which can activate PKCδ that causes cell contractility, and inositol-triphosphate that releases intracellular stores of calcium leading to actin reorganization. On the other hand, active ERK at the cell surface triggers m-calpain, which may then cleave focal adhesion complexes at the rear end of the cell. Additional factors such as RhoA, Rac and Cdc42 may also play a role, leading to migration of the cell towards the motogenic cue that is tenascin-C.

4.3 PROSPECTIVES

4.3.1 Implications for Embryogenesis, Morphogenesis and Development

As an organism develops from just a fertilized egg to transform into a fully developed entity, numerous morphological changes occur that involve tissue growth, differentiation and maturation of distal organs. Tenascin C is found to be intricately involved in all these processes, particularly during phases that require the migration and involution of cells. It has been found to regulate development in numerous organ systems including brain, bone, skin, digestive system, lung, etc (Orend and Chiquet-Ehrismann, 2006). Tenascin C is also expressed during CNS development in embryogenic stages, with a differential pattern of expression. The radial glial fibers of purkinje cells in the developing cerebellum express tenascin oriented according to the migratory direction of these cells (Porcionatto, 2006). In the avian embryo, TN-C is found to be present along neural crest cell migration pathways prior to the onset of migration (Riou et al., 1992). TN-C is found to be overexpressed at neural stem cell margins in the sub-ventricular zone of the developing brain, where neural stem cells involute and differentiate into neurons and glial cells. Interestingly, expression of EGFR is also observed in this region of the brain during development. Tenascin C is essential for the timely expression of the EGFR in neural stem cells - TN-C knockouts show a significant delay in the onset of EGFR, which results in delayed neuronal and glial development (Garcion et al., 2004). EGFL repeats of TN-C may have a potential role here in signaling through EGFR and facilitating the maturation process of neuronal cells in the developing brain. In developing cartilage and bone, TN-C is expressed selectively in the chondrogenic mesenchyme, especially by migrating perichondrial cells and chondrocytes that participate in appositional growth of the cartilage (Mackie and Murphy, 1998). EGFL motifs of

versican contribute to the inhibitory effect of the protein on mesenchymal chondrogenesis (Zhang et al., 1998), suggesting that EGFL repeats may play a role in migration as well as differentiation of chondrocytes, possibly via signaling through EGFR. In the human gastric mucosa of the developing stomach, TN-C is found to be uniformly distributed throughout the entire mesenchymal layer at 10 weeks of gestation and increases by 20 weeks. However, in the adult, TN-C is expressed only at the epithelial-stromal interface, without any detectable levels in the connective tissue (Tremblay and Menard, 1996). Such spatiotemporal expression of TN-C is also found in the mesenchymal core of the developing small intestine. In both scenarios, EGFL repeats may act as migratory effectors, allowing for the movement of epithelial cells in regions undergoing morphogenesis (Beaulieu et al., 1993).

4.3.2 Implications for EGF-Like Repeats in Tumor Growth and Metastasis

Tenascin C was first identified in embryonic chick brain, with widespread tissue distribution, and shown to mediate glia-neuron adhesion in vitro (Grumet et al., 1985). Although initial studies with TN-C were focused on its role in brain development and morphogenesis, there were studies in adult mammary tumors that showed an upregulation of TN-C in the surrounding mesenchyme (Chiquet-Ehrismann et al., 1986). Since then, TN-C has been implicated in the progression of tumors in various tissues including brain, breast, prostate, liver, lung, colon, pancreas, etc. (Orend and Chiquet-Ehrismann, 2006). The metastatic potential of TN-C is realized by the select activation of signaling cascades, including the promotion of deadhesion, increase in cell proliferation, increase in matrix remodeling proteins and induction of epithelial-mesenchymal transition which then promotes tumor progression and metastasis (Chiquet-Ehrismann and Chiquet, 2003; Chiquet-Ehrismann et al., 1986; Huang et al., 2001; Kalembeyi et al., 2003;

Martin et al., 2003; Tremble et al., 1994). Most of these reports have been directed mainly towards unraveling the role of fibronectin repeats in promoting metastasis. However, given that EGFR is upregulated in almost all tumorigenic pathologies that involve TN-C, and with the recent discovery that EGFL repeats can signal EGFR to selectively trigger migration, a new dimension has been added to the mechanisms the lead to cancer progression via EGFR. Mechanistically, epithelial-mesenchymal transition (EMT) appears to be a critical step during metastatic progression of tumor cells in all tissue, and involves the downregulation of adhesion molecules such as E-cadherin and an upregulation of EGFR (Lee et al., 2006). Tenascin C was found to be expressed selectively at the invasion border of colorectal carcinoma cells that also expressed nuclear b-catenin, which is a marker for cells that have undergone EMT (Beiter et al., 2005). Also, tenascin-C was found to be present breast cancer cells together with vimentin, another marker for mesenchymal cells which may produce a more aggressive and invasive phenotype (Dandachi et al., 2001). These and other studies confirm that TN-C is spatiotemporally regulated during EMT, and plays an important role in establishing and maintaining the mesenchymal phenotype of stromal cells during EMT in both normal and pathological settings (Orend and Chiquet-Ehrismann, 2006). Under such conditions, EGFL repeats of TN-C could have a pivotal role in regulating invasiveness of cancer cells. In a recent study where colon cancer cells were co-cultured with myofibroblasts from colon cancer tissue, EGFL repeats of TN-C were found to play an important regulatory role for cancer cell invasion in vitro. They found that neutralizing antibodies against the EGFL domain of TN-C could completely block the proinvasive activity of colon cancer cells that were treated with conditioned medium of myofibroblasts (De Wever et al., 2004). They also found that signaling of EGFL repeats through EGFR lead to priming of cancer cells for the proinvasive activity of hepatocyte

growth factor through c-Met. Such a role for EGFL repeats could be envisioned in similar scenarios for other tissues, and could thus be important for EGFR-mediated metastasis.

In addition to EMT, angiogenesis is another important aspect of cancer metastasis that leads to the formation of new blood vessels in order to provide oxygen and nutrients to the growing tumor. Tenascin C is expressed during early angiogenesis, exerting its effects on endothelial cells through VEGF and inducing migration possibly through regulation of focal adhesion kinase phosphorylation (Zagzag et al., 2002) (Tanaka et al., 2004). Tenascin C expression has also been shown to be correlated with the degree of tumor neovascularization in human gliomas (Herold-Mende et al., 2002). EGFL repeats could play an important role by signaling EGFR, thereby regulating endothelial cell migration, as well as mediating signaling of other proangiogenic factors such as VEGF.

4.3.3 Implications for EGF-Like Repeats in Other Pathologies

Numerous other pathologies are associated with a spatiotemporal overexpression of TN-C. EGFL repeats could play an important role in quite a few, especially those that involve an upregulation of EGFR concomitant with levels of TN-C expression.

Keloids result from abnormal wound healing of skin, and are characterized by the formation of dense fibrotic tissue in the wound, and they extend beyond the confines of the wound, recurring even after excision (Datubo-Brown, 1990). Although the cause for keloids is not known, TN-C is a strong in vivo marker for keloids, and stimulated keloidal fibroblasts continue to produce TN-C throughout the stroma even without circulating factors (Dalkowski et al., 1999). Interestingly, human keloid fibroblasts also show an increased basal level of activation of EGFR, although EGFR numbers were comparable with adult human fibroblasts

(Cheng et al., 2002; Chin et al., 2000). However, in vitro, keloid fibroblasts show diminished EGF-mediated motility due to rapid loss of EGFR driven by EGF binding. The acuity of EGFL repeat signaling though EGFR comes to light under such situations, given that EGFL repeats bind with ultra-low affinity to the receptor, and do not lead to ligand mediated internalization or degradation of receptor. The fact that keloids continue to extend beyond wound margins suggests a dysregulation of migratory response of these fibroblasts. Given the increased phosphorylation potential of EGFR and the overexpression of TN-C in keloids, it is fair to postulate that the increased migration is caused due to hyperstimulation of EGFR by EGFL repeats without receptor degradation, leading to excessive migration of fibroblasts and keratinocytes across the wound and an extension of the wound margin as a result. Additionally, matrix metalloproteinases which are overexpressed in keloids could also play a role by liberating EGFL repeat domains from TN-C, thereby providing easier access for EGFR to EGFL repeats and increasing the potentiation of receptor signaling. In the heart, Its expression correlated with inflammation and cardiac injuries such as myocarditis and myocardial infarction (Imanaka-Yoshida et al., 2001; Willems et al., 1996). After myocardial infarction, TN-C appeared at the borderzone between the infarcted area and the intact myocardium where extensive remodeling occurred. In addition, Tenascin C also upregulates the production of MMP2 and MMP9, mediated by transformed myofibroblasts that are recruited by TN-C (Imanaka-Yoshida et al., 2001). Interestingly, although EGFR has a global expression pattern in the heart, it is upregulated during pathological situations in mesenchymal cells that closely interact with myofibroblasts(Erickson et al., 1997) (Jackson et al., 2003). Under such situations, in addition to stimulating migration directly via EGFR, EGFL repeats could also play a role in enhancing the migratory potential of other EGFR ligands such as HB-EGF (Nishi et al., 2001).

4.4 FINAL WORD

This thesis presents not only our research but also the efforts by various investigators that have sought to elucidate the role of tenascin C and other such ECM proteins that express EGFL repeats. Our work has led to the discovery of a new mode of signaling by EGFL repeats that until now, although ubiquitously expressed, seemed to play more of a tertiary role as compared to the FNIII arrays and other such domains in ECM proteins. This work certainly adds another dimension to the already multifarious functions of ECM proteins, not to mention the unique mode of signaling of such spatially restricted peptides through receptor tyrosine kinases that were traditionally thought to elicit responses only to soluble cues. It is interesting to note that EGFL repeats can potentially exploit the effects that receptor compartmentalization has on cellular responses, and suggests an evolutionary selection and retention of such a mode of signaling so as to elicit select responses from an otherwise multifaceted receptor system.

This work has broad implications, not only for the development of better techniques to address physiological outcomes such as wound healing, but also to design better therapies to target cancer metastasis and other such serious pathologies that involve EGFR signaling.

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