Fibronectin Splice Isoforms in Wound Healing and Vascular Development

by

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ABSTRACT

Fibronectins are a large family of extracellular matrix proteins that regulate cell adhesion, migration, differentiation and survival. Different isoforms of fibronectin arise via alternative splicing of the transcript of a single gene. Although many of the general functions of fibronectins have been elucidated, few specific functions of the alternatively spliced isoforms have been described. Studies of the expression patterns of the splice isoforms show that these proteins are present at high levels during embryonic development. In adult animals, these isoforms are expressed around the perimeter of blood vessels and are induced in response to injury. We have developed an in vitro system using a set of bacterially expressed fibronectins and fibronectin-null cells to look at the functions of the alternatively spliced isoforms in wound healing and vascular development. Our results indicate that the bacterially expressed fibronectins are fully functional and promote a number of well-established fibronectin functions. Therefore, these fusion proteins should be useful for investigations of splice isoform specific functions. Our experiments with fibronectin-null cells show that fibronectin negatively regulates the expression of the differentiation marker α-smooth muscle actin which is expressed by myofibroblasts during wound healing and by peri-endothelial cells during blood vessel development.

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LITERATURE CITED
Chapter 1

An Introduction to Fibronectin Forms and Functions
One of the major goals of cell biological research is to understand how cell adhesion affects cellular behavior. During development adhesive interactions between cells and their surroundings provide cues that instruct cells to proliferate, migrate, differentiate, and organize into specialized structures. Once development is complete cell adhesion helps to maintain the organization and stability of tissues. In addition, cell adhesion is important during tissue repair and remodeling.

These adhesive interactions fall into two general classes, cell-cell adhesion and cell-matrix adhesion. Cell-cell adhesion refers to the binding of cell surface receptors such as cadherins and immunoglobulin superfamily molecules on one cell to coreceptors on adjacent cells. Cell-matrix adhesion refers to the interaction of cells with a network of proteins and carbohydrates that are synthesized intracellularly and then secreted into the extracellular space. This network is commonly referred to as the extracellular matrix (ECM).

The work described in this thesis is an investigation of the functions of a closely related set of ECM proteins collectively referred to as fibronectins. The different fibronectins arise via alternative splicing of the mRNA transcript from a single gene. Although many general functions of fibronectins have been elucidated (e.g. adhesion, migration, differentiation, cell survival) few isoform specific functions have been described. This chapter provides a general introduction to fibronectin biology. The introduction begins with a review of well-established aspects of fibronectin biology. This first section will be followed by a brief description of the discovery of the splice isoforms and of their expression patterns. Studies that have reported functions specific to particular fibronectin isoforms will then be described. The final section is a review of literature that supports the hypothesis that some fibronectin isoforms regulate the differentiation of particular cell types (e.g. fibroblasts, smooth muscle cells, and pericytes) during wound healing and blood vessel development.

Overview

Fibronectins are good model proteins for studies of cell-matrix interactions because many of the details of their structures and functions have been elucidated. Fibronectins are also interesting because they influence a wide range of cellular processes (e.g. adhesion, migration, proliferation, cytoskeletal organization, intracellular signaling,
differentiation, and cell survival). The importance of fibronectin in development has been demonstrated by the generation of mutant mice that lack fibronectins. Fibronectin-null embryos die around embryonic day 9 and exhibit defects in mesodermal development (George et al. 1993). Fibronectins are also implicated in a number of important physiological and pathological processes in adult animals including hemostasis, wound healing, fibrosis, tumorigenesis, and metastasis (Hynes, 1990).

**Fibronectin Structural Features**

Figure 1-1 provides a schematic representation of fibronectin structure. Fibronectins are modular proteins made up of three different types of repeating homologous motifs termed fibronectin repeats Type I, Type II, and Type III. They are secreted as high molecular weight (420-450kD) disulfide-bonded dimers. Cysteine residues found in the carboxy-terminus of each monomer mediate dimerization. The amino-terminal region of fibronectin which contains the Type I and Type II repeats exhibits extensive intrachain disulfide bonding (Balian, 1979a; Wagner and Hynes, 1979). Fibronectins are glycoproteins which contain 5-7% carbohydrate in which complex oligosaccaride is linked to asparaginy1 residues (Duskin, 1978; Graham et al., 1978; Hynes et al., 1978; Hynes and Destree, 1978; Olden, 1978). Fibronectin monomers vary in molecular weight (220-250kD) due to alternative splicing of the mRNA transcript from a single gene (reviewed in Hynes 1990). Alternative splicing occurs at three positions within the fibronectin transcript (Figure 1-1). The resulting alternatively spliced regions are termed extra Type III repeat A (EIIIA), extra Type III repeat B (EIIIB), and variable region (V) respectively. In various situations, the alternatively spliced segments are included or excluded from the molecule (see below).

Like many ECM proteins, fibronectins consist of a series of independently folded globular domains. These domains are separated by flexible extendable stretches of protein, which contribute to fibronectins’ elasticity (Ohashi et al., 1999; Ohashi et al., 2002). The globular domains can be separated by proteolysis and many of them have been shown to independently mediate one or another of the functions of the full-length molecule. For example, some isolated domains of fibronectins bind to the ECM molecules collagen, gelatin, heparin, and fibrin (reviewed in Hynes 1990) (Figure 1-1).
In addition to binding to a number of different ECM molecules, fibronectins can also bind to cells. The cell-binding activity of fibronectin has likewise been mapped to smaller domains of the protein. For example, a central 120kD fragment of fibronectins binds to cells via the sequence Arg-Gly-Asp (RGD) found in the tenth fibronectin Type III repeat (Type III10) (Figure 1-1). Smaller pieces of the molecule and even RGD peptides show cell-binding activity (Pierschbacher et al., 1983; Pierschbacher et al., 1981; Pierschbacher and Ruoslahti, 1984a; Pierschbacher and Ruoslahti, 1984b).

**Fibronectin Receptors**

Cells bind to fibronectins via cell surface receptors called integrins. Integrins are a large family of heterodimeric transmembrane proteins that are composed of an α subunit and a β subunit (Pytel et al., 1985a; Pytel et al., 1985b). Each subunit is composed of a large extracellular domain, a transmembrane domain, and an intracellular cytoplasmic tail. The extracellular domain binds to ECM ligands such as fibronectin, laminin, vitronectin, and collagen or to cell-surface counter-receptors of the immunoglobulin superfamily. There are approximately 24 different integrin heterodimers formed from pairings between 8 different beta subunits and 18 different alpha subunits.

Integrin pairs exhibit heterodimer specificity such that only certain α and β combinations are observed. For example, α4 can pair with β1 or β7 but pairings between α4 and β2 or β4 are not observed. Integrin heterodimers exhibit ligand-binding specificity such that no single integrin can bind to all of the known integrin ligands. However, there is considerable overlap in ligand specificity since many integrins can bind to more than one ligand. Furthermore, many ligands are bound by several different integrins (Hynes, 1992). For example, a subset of at least seven integrins is capable of binding to fibronectins. These include α4β1, α5β1, α8β1, αvβ1, αvβ3, αvβ6, and αIIibβ3. As mentioned above some of these integrins, including the major fibronectin receptor α5β1 and the αv integrins bind to the RGD sequence found within the tenth Type III repeat of fibronectin (Figure 1-1).

**Integrin Cytoplasmic Interactions**

The cytoplasmic domains of integrins can bind to a number of intracellular proteins. These interactions establish a link between ECM molecules such as fibronectin
and intracellular components of the cytoskeleton. The cytoskeleton was first described as a network of membrane-associated proteins that was insoluble in nonionic detergents such as Triton-X 100. After treatment with Triton-X, the cytoskeleton could be pelleted as a Triton-insoluble structure with dimensions similar to those of the cell prior to solubilization (Yu et al., 1973). The relationship between fibronectin and the actin cytoskeleton was demonstrated by immunofluorescence studies showing that actin filament bundles and extracellular fibronectin fibrils exhibit largely coincident staining patterns suggesting that these proteins are often found in close proximity to one another (Hynes and Destree, 1978).

Physical links between actin, integrins, and fibronectin have been studied extensively (reviewed in Juliano, 2002). It is well established that ligand binding induces clustering of integrins into structures termed focal contacts or focal adhesions. Focal contacts are regions of the plasma membrane that are close (10-15nm) to the substratum. These dense cytoplasmic plaques are associated with bundles of actin filaments called stress fibers (Geiger et al., 1984a; Geiger et al., 1984b). Immunofluorescence staining for integrins or other focal contact proteins appears as a pattern of bright dots of staining on the cell surface.

It is now recognized that focal contacts play a crucial role in many cellular processes including adhesion, spreading and migration. This is due in part to the high concentration of structural, regulatory, and signaling molecules present in focal contacts. In addition to containing integrins, focal contacts contain structural proteins such as talin, α-actinin, and vinculin, which link to the actin cytoskeleton. They also contain a large number of signaling molecules such as focal adhesion kinase (FAK), paxillin, Src family kinases, phosphatidylinositol-3'-kinase (PI3K), and phospholipase C (PLC)-γ (reviewed in Clark and Brugge, 1995).

The molecules present in focal contacts interact in a complex web of protein-protein binding. Some of the interactions between integrins and other focal contact molecules have been described. These interactions form the molecular basis for the effects of integrins and fibronectin on cytoskeletal rearrangements and intracellular signaling. For example, the βι integrin cytoplasmic tail binds to several proteins associated with the cytoskeleton (e.g. talin, α-actinin, and FAK) (Horwitz et al., 1986;
Otey et al., 1993; Schaller et al., 1995). Integrin binding to α-actinin and talin provides a connection between integrins and actin filaments since both talin and α-actinin bind directly to actin (reviewed in Jockusch et al., 1995). While connections to structural components link integrins to the cytoskeleton, association with components of signaling pathways enables integrins to feed into intracellular signaling cascades. For example, integrin clustering induces phosphorylation of protein tyrosine kinases (e.g. FAK, Src, Csk, and Syc), serine threonine kinases (mitogen-activated protein (MAP) kinase/ERK and protein kinase C (PKC), and some phospholipid kinases (phosphatidylinositol (PI)-3 kinase) (reviewed in Clark and Brugge, 1995). These molecules in turn activate downstream targets leading to changes in cellular behavior. Integrin activation of these molecules is probably indirect since integrin cytoplasmic tails are short and lack enzymatic activity. Instead, integrin clustering is believed to bring kinases and their substrates into close enough proximity to interact (Guan et al., 1991; Kornberg et al., 1991).

Of the various kinases activated in response to integrin clustering, FAK is believed to play a central role in regulating assembly and activation of other molecules in the signaling complexes. This is due in part to the large number of associations that FAK can make with other molecules including Src, Csk, p130Cas, the Ras pathway adapter proteins Grb2, PI-3kinase and paxillin (reviewed in (Hanks and Polte, 1997). Ultimately, signaling through intergrins can lead to changes in gene expression and consequently in cellular behavior. For example, integrin-induced activation of ERK/MAPK is important for integrin-mediated regulation of cell proliferation. ERK in turn is involved in induction of cyclin D1 a molecule that is necessary for entry into the G1 phase of the cell cycle (Danen and Yamada, 2001).

The effects of integrins on signaling and on the cytoskeleton can also modulate cellular behaviors such as adhesion and migration. Several intracellular signaling molecules that are regulated by integrins have been implicated in such processes. For example, cells from FAK mutant embryos show migration defects (Illic et al., 1995). The effects of fibronectin and integrins on adhesion and migration also depend on a family of small GTPases that includes Rac, Rho, and cdc42 (Clark et al., 1998). These molecules regulate membrane extensions and cytoskeletal rearrangements (e.g. stress fibers, focal
contacts, lamellipodia, and filopodia) that are necessary for cell adhesion and migration (Small et al., 1999).

**The Discovery of Fibronectin Alternative Splicing**

While a great deal is known about the structure and function of fibronectins less is known about the specific functions of fibronectin splice isoforms. Early on in the study of fibronectins, it was observed that fibronectins differed in their solubility, with plasma fibronectins (pFNs) being highly soluble while the fibronectins associated with cells, cellular fibronectins (cFNs), were insoluble. Fibronectin isoforms also differed in their electrophoretic mobility suggesting that structural modifications might be responsible for this diversity. Furthermore, while polyclonal antibodies recognized many of the different isoforms of FN, some monoclonal antibodies recognized only some isoforms and not others (Hynes, 1990).

The mystery of this diversity began to be unraveled in the early 1980s when the gene for fibronectin was cloned in several different species. During the initial cloning of rat fibronectin three cDNA clones were identified that were identical over large stretches of sequence. Interestingly, large deletions were identified in some of these otherwise identical clones. This raised the question of whether or not these clones had arisen via cloning artifacts or if they were the products of three similar but distinct mRNAs. Using S1 nuclease mapping, researchers were able to confirm that the different clones arose from three mRNAs present in rat cells (Schwarzbauer et al., 1983). The fact that the nucleotide sequences in overlapping regions of the clones were identical strongly suggested that all three mRNAs arose from the same gene. This hypothesis was later confirmed by sequencing and Southern blotting of genomic clones of rat fibronectin. In addition, canonical 5’ and 3’ splice donor and acceptor sites preceding and following the missing exon were identified. This showed that at least some of the different fibronectin isoforms were generated via alternative splicing of fibronectin transcripts (Tamkun et al., 1984). The story became even more interesting when another possible 3’ splice site was identified within this alternatively spliced exon. This site allowed the first 25 amino acids to be spliced independently of the last 95.

The region identified in these studies was named the variable (V) region because it lacked homology to any of the previously identified fibronectin Type I, Type II, and
Type III domains. In the literature, the V region is also sometimes referred to as the Type III connecting segment (IIICS) because it connects the 14th and 15th type three repeats. Since these early reports, alternative splicing of the V region has been examined in many different species. The splicing of this region is complex and varies from species to species. In rats and mice, there are three possible splicing patterns. First, all 120 amino acids of the V region can be spliced in (V120) or spliced out (V0). Alternatively, the first 25 amino acids can be spliced out leaving a 95 amino acid region (V95).

In humans, the story is even more complicated since an additional site of alternative splicing can give rise to five forms. The human V region can be spliced in (V120), spliced out (V0), or the first 25 amino acids can be spliced out (V95). In addition, the last 31 carboxy-terminal amino acids can be spliced out (V89). Exclusion of the first 25 amino acids and the final 31 amino acids in the same molecule generates a 5th form (V64) (ffrench-Constant, 1995).

Soon after the discovery of V region alternative splicing, further analysis of fibronectin genomic clones revealed alternatively splicing of another carboxy-terminal exon (Odermatt et al., 1985; Vibe-Pedersen et al., 1984). The domain encoded by this exon was homologous to fibronectin Type III repeats and was accordingly named extra Type III repeat A (EIIIA). Interestingly, EIIIA was excluded in cDNA clones from rat liver but was included in cDNAs generated from human cell lines and in mRNAs taken from rat cell lines. Subsequently, a third region of alternative splicing was discovered. This region encoded a Type III repeat which was named extra Type III repeat B (EIIIB). The alternative splicing of both EIIIA and EIIIB was confirmed using RNase protection of RNA from several different cell types (Schwarzbauer et al., 1987). Like EIIIA, EIIIB was excluded by hepatocytes but included in mRNAs produced by many other cell lines.

The discovery of alternative splicing within fibronectin immediately gave rise to more questions. What was the significance of the alternative splicing? It was clear from the work described above that some cell types such as hepatocytes included the V region but excluded EIIIA and EIIIB. Consequently, much of the early research on the alternatively spliced regions focused on determining the cell type specificity and temporal regulation of their expression. It had been shown previously that isolated domains or fragments of fibronectin could perform many of the functions of intact
fibronectin (e.g. ECM molecule binding; cell adhesion) independently of the larger molecule. This, along with the observation that EIIIA and EIIIB showed a high degree of conservation between species, raised the question of whether the alternatively spliced domains perform specific functions.

**Expression of Fibronectin Splice Isoforms**

Analysis of the expression of fibronectin splice isoforms during development has been performed in chicken, Xenopus, rat, and mouse embryos. These studies show that fibronectin splice isoforms are expressed at high levels during embryonic development. RNAase protection analysis of total RNA from chicken embryos revealed that EIIIA+, EIIIB+, and V+ isoforms were abundant at all of the stages examined (E2.5 through E11) (Norton and Hynes, 1987). A follow-up study in chicken embryos used *in situ* hybridization in addition to RNAase protection. This study showed that staining for the alternatively spliced isoforms colocalized with staining for total fibronectin.

RNAase protection studies of early Xenopus embryos revealed that 90% of fibronectins present during cleavage, the mid-blastula transition, and gastrulation contain EIIIA and EIIIB. RT-PCR analysis of the V region confirmed that early stage Xenopus fibronectins are also V+ (DeSimone et al., 1992). Studies using RNAase protection on tissues from E17 rat embryos showed high levels of EIIIA+ and EIIIB+ fibronectin in many tissues. In mouse embryos expression of fibronectin splice isoforms has been examined by indirect immunofluorescence microscopy. In mice, the fibronectins present during gastrulation and early morphogenesis are largely EIIIA+, EIIIB+, and V120+.

Interestingly, later in development the inclusion of the EIIIA and EIIIB regions decreases. In chicken embryos (E16), the levels of EIIIA and EIIIB drop and these splice isoforms are excluded from many tissues (ffrench-Constant and Hynes, 1989). As in the chicken, the levels of EIIIA and EIIIB drop in late stage rat embryos. These levels remain low in 5, 12, and 17-month-old adult rats (Pagani et al., 1991). In mice, the levels of the alternatively spliced isoforms decreases as development progresses and, in some tissues, these isoforms disappear at later stages of development (Peters and Hynes, 1996a). Adult mice show much lower expression of EIIIA and EIIIB than did embryos. Furthermore, splice isoform expression in adult mice is restricted to a few specific structures such as the walls of blood vessels (Peters et al., 1996b). Taken together the
expression studies suggest that EIIIA, EIIIB, and V120 might mediate cellular behaviors that are important for development such as cell migration, proliferation, or differentiation. This hypothesis is further supported by the observation that expression of these segments is reduced in adult animals where tissues are more stable.

Additional support for the idea that the alternatively spliced isoforms promote migration comes from expression studies of migrations that occur during the development of chicken embryos. During development, neural crest cells originate at the dorsal region of the neural tube and migrate dorsally, eventually giving rise to melanocytes, or ventrally giving rise to certain neurons, Schwann cells, and adrenomedullary cells (see Gilbert, 1994). In chicken embryos, the pathways along which the neural crest cells migrate contain EIIIA⁺, EIIIB⁺, V120⁺ fibronectin. In addition, EIIIB⁺ fibronectin is found in the chick chorioallantoic membrane during the time when endothelial cells are migrating into this tissue (E4-5). When endothelial cell migration ceases the fibronectin in this region becomes EIIIB⁻. Another study looked specifically at two additional migrations that take place in early chicken embryos, namely mesenchymal cell migration into the area vasculosa and endocardial cushion cell migration in the heart. In both cases, migration pathways and the migrating cells showed positive staining for all three alternatively spliced domains (ffrench-Constant and Hynes, 1988). These experiments were consistent with a role for fibronectin isoforms in cell migration.

Given the general hypothesis that the alternatively spliced isoforms of fibronectin might play a role in cell migration, proliferation, and differentiation, researchers predicted that these isoforms might also be expressed during tissue repair or remodeling in adult animals. These situations require extensive cell migration, proliferation and differentiation. As predicted, the alternatively spliced isoforms of fibronectin were upregulated after injury in many model systems including corneal repair, liver regeneration, peripheral nerve regeneration, and cutaneous wound healing (reviewed in ffrench-Constant, 1995).

**Functional Studies of Fibronectin Isoforms**

Although expression studies provided some clues as to the function of the alternatively spliced segments, more direct methods were necessary for testing
hypotheses about isoform function. Researchers were also eager to identify proteins that interact with the alternatively spliced segments. They hoped this would allow them to identify EIII-A, EIIIB, or V cell surface receptors along with receptor associated intracellular molecules. Experiments attempting to identify alternatively spliced isoform functions have been performed using in vitro systems. In these experiments, recombinant fibronectin splice isoforms purified either from mammalian cells or from E. coli are used as substrates. Cells are plated on these recombinant proteins and various responses measured. Several studies have looked at whether or not these segments modulate well-established fibronectin functions such as adhesion, spreading, migration, focal contact formation, and actin assembly.

**V Region Functions and Receptors**

In vitro approaches aimed at elucidating the functions of the V region of fibronectin have yielded some successes. Early studies using five non-overlapping peptide fragments spanning the V region revealed that the addition of two of these soluble fragments inhibited adhesion of B16-F10 melanoma cells. One of these peptides mapped to the amino-terminal region of V (CS1 peptide) and the other to the carboxy-terminal region (CS5 peptide). CS1 and CS5 were then used as substrates in melanoma adhesion assays. Both peptides supported spreading of melanoma cells. The ability of these sites to mediate adhesion was 2.4-fold (CS1) or 320-fold (CS5) lower than that of intact fibronectin. The cell-binding activity of these peptides was specific to the melanoma cells since they did not promote the spreading of baby hamster kidney cells (Humphries et al., 1987). Subsequently, (Dufour et al., 1988) showed that CS1 promoted neural crest cell adhesion. Furthermore, they showed that this site worked synergistically with the RGD site in Type III10 to promote migration of neural crest cells.

Using a retroviral expression system to produce full-length recombinant fibronectin splice isoforms, Guan et al. (1990) were able to identify the CS1 binding site and its receptor. They plated various cell lines (Nil8, NRK, Rat1, NIH3T3, and B16F10) on different fibronectin splice isoforms and then measured cell adhesion, spreading, focal contact formation, stress fiber assembly, and migration. They found that fibronectins containing the V region support the adhesion and spreading of the lymphoid cell line WEHI231 while fibronectin isoforms lacking V do not. This suggested that WEHI231
cell adhesion and spreading is specifically mediated by V and raised the question of whether these cells might express a cell surface receptor capable of binding to the V region.

In order to identify the V region cell surface receptor, Guan et al. (1990) surface-labeled proteins from WEHI cells and incubated labeled proteins with a Sepharose column coupled to V region peptides that were known to block WEHI binding to V-fibronectin. Bound material was eluted using V region peptides. The bound proteins were recognized by antibodies raised against α4 and β1 integrins. These anti-α4 and anti-β1 antibodies were able to block WEHI adhesion to the V-fibronectin. The exact binding site for α4β1 was mapped using smaller peptides within the V region. A 10 amino acid peptide containing a highly conserved EILDV sequence was found to block binding of α4β1 to V peptide-Sepharose columns. Interestingly, this binding site falls within the first 25 amino acids of the V region which as mentioned above can be selectively removed by alternative splicing. Thus, loss of V25 would result in loss of this binding site and consequently would alter the ability of certain cells (e.g. WEHI231) to bind to fibronectin. The binding site in the CS5 region had previously been identified as a four amino acid sequence (REDV in humans or RGDV in rats) (Humphries et al., 1986). Antibody blocking experiments later demonstrated this REDV sequence is also a binding site for integrin α4β1 (Mould and Humphries, 1991).

Additional functions for the V region continue to be elucidated and the field surrounding the V region is rapidly expanding. Furthermore, the function of the V region receptor α4β1 has been extensively studied. Some experiments related to the function of the V region will be described in this thesis (Chapter 2), however, most of our investigations will focus on the functional significance of EIIIA.

**EIIIA Functional Studies**

Several *in vitro* studies have focused on the EIIIA region. In the study described above using retrovirally-expressed fibronectins, inclusion of EIIIA did not affect the adhesion, spreading, focal contact formation or migration of several established cell lines (Guan et al., 1990). Other studies have reported effects of EIIIA, but in general these reports have been contradictory perhaps because different researchers have used different cell lines and different configurations of fibronectin substrates.
Some of these studies have used bacterially expressed fibronectins to study EIIIA function. (Xia and Culp, 1995) reported that the isolated EIIIA domain alone could support attachment and spreading of NIH 3T3 fibroblasts. Furthermore, in this study, the presence of different oncogenes within the cells could abrogate (ras) or enhance (src) cell binding to EIIIA. The fact that EIIIA alone could mediate adhesion of these cells suggests the presence of a specific cell surface receptor for EIIIA on these cells. The authors did not investigate the presence of integrins or other putative receptors on the surface of these cells. However, it would be interesting to determine which integrins are present on these cells as well as whether or not the levels of such putative receptors change in the cells expressing different oncogenes.

As mentioned above, results of such studies of EIIIA function are conflicting. For example, another group has reported that inclusion of EIIIA into bacterially expressed fibronectin Type III repeats 7-12 decreased adhesion, focal contact formation, and actin assembly in Nil8 cells (Hashimoto-Uoshima et al., 1997). The difference between their findings and those of Xia and Culp (1995) might be due to the fact that the EIIIA domain alone was used in the first study while in the second study EIIIA was presented in the context of a larger portion of fibronectin.

(Manabe et al., 1997) transfected full-length fibronectin constructs into CHO cells in order to obtain full-length fibronectin isoforms containing or lacking EIIIA. In contrast with the findings of Guan et al. (1990) who saw no differences in the adhesion of Nil8, NRK, Rat1, NIH3T3, or B16F10 cells to EIIIA fibronectin, they found that inclusion of EIIIA produced a 2-fold enhancement of the attachment of several cell lines, including HT1080 fibrosarcoma cells, CHO cells, rat NRK cells, and mouse L cells. They also showed that HT1080 cells migrated farther on fibronectins containing EIIIA than on those lacking this segment. However, in contrast to the findings of Xia and Culp (1995), Manabe et al. (1997) reported that the isolated EIIIA domain did not promote attachment of the cells they were studying. This suggested either that EIIIA did not exert its effects via direct interaction with a cell surface receptor or alternatively that such a receptor could not recognize EIIIA outside of the context of the larger fibronectin molecule.
It is well established that sequences within the Type III9 region of fibronectin affect cell adhesion by enhancing the ability of the neighboring Type III10 RGD site to bind α5β1 (Obara et al., 1988). Therefore, it seemed possible that EIIIA might affect cell adhesion in a similar manner. In order to investigate this hypothesis, the authors first showed that α5β1 antibodies blocked the increases in adhesion and migration observed on EIIIA-fibronectin. Second they showed that α5β1-containing liposomes also showed enhanced binding to EIIIA-fibronectin. Finally, the authors demonstrated that antibodies to α5β1 could block the enhancement in liposome binding. These experiments suggest a model in which inclusion of the EIIIA segment induces conformational changes in fibronectin that alter the position of the RGD binding site and thereby modulate its interaction with integrin α5β1. This model and the notion that there is a cell surface receptor for EIIIA are not mutually exclusive, but both ideas should be kept in mind when designing and interpreting experiments related to the alternatively spliced isoforms.

Proteolysis and Regulation of EIIIA Activity

An additional consideration related to the mechanism of action of the alternatively spliced domains is the potential role of extracellular proteases in regulating their activity. There are a number of different classes of secreted proteases that are capable of degrading extracellular matrix molecules. These include matrix metalloproteinases, adamalysin-related membrane proteinases, and tissue serine proteinases (Werb, 1997). These proteins play an important role in regulating the assembly and remodeling of the extracellular matrix. In addition, several studies implicate these proteases in the generation of bioactive fragments from extracellular matrix molecules. These bioactive fragments exhibit biological activities that are not promoted by the intact molecule. This phenomenon has perhaps been studied most extensively in relation to blood vessel growth (angiogenesis). Proteolytic fragments of several extracellular matrix molecules including plasminogen, collagen, prolactin, and osteopontin have been shown to modulate endothelial cell adhesion or cell cycle progression (Sage, 1997). As a result, many of these fragments can inhibit new blood vessel growth while the intact molecule from which they are released cannot. A study of the ability of fragments of fibronectin to regulate gene expression showed that fragments of fibronectin could display activities that were not promoted by intact fibronectin (Huhtala et al., 1995). In this study, plating
rabbit synovial fibroblasts on a central 120kD fragment of fibronectin which included the TypeIII10 RGD cell-binding site induced an increase in metalloproteinase expression. However, metalloproteinase expression was much lower on intact fibronectin. In this case, it was shown that a region of the alternatively spliced V domain that binds to α4β1 inhibited the 120kD fragment induced expression of metalloproteinase. Clearly, in this case, release of the central fragment from the rest of the fibronectin molecule allows this fragment to function in a way it could not when presented in the context of the intact molecule.

It is possible that release of the alternatively spliced domains from intact fibronectin might be required for them to exert some of their effects. Two different studies suggest that, at least in some instances, EIII A can function as an isolated domain in situations where its has no effect when presented in the context of the intact fibronectin. In one study, the isolated recombinant EIII A domain induced matrix metalloproteinase expression in chondrocytes and synovial fibroblasts (Saito et al., 1999). However, this activity was abolished when the adjacent Type III repeats were added to each side of EIII A or when EIII A was presented within the intact fibronectin molecule. Interestingly, a proteolytic fragment isolated from human placenta and containing a portion of fibronectin from C-terminus of EIII A through the NH₂ terminus of the intact molecule induced metalloproteinase expression. A second study showed that the isolated EIIIA domain but not EIIIA within the intact fibronectin molecule serves as a ligand for the Toll-like receptor 4 (TLR4) which is also a receptor for lipopolysaccharide (Okamura et al., 2001). In this case, HEK293 cells that normally do not express TLR4 were transfected with TLR4 and plated on recombinant EIIIA. The isolated EIIIA domain, but not EIIIB, FN TypeIII11, or intact fibronectin containing EIIIA, induced expression of metalloproteinases in the transfected cells. The authors of this report concluded that endogenously generated EIIIA fragments are bioactive signaling molecules. Furthermore, they suggested that other regions of intact fibronectin might suppress the ability of EIIIA to perform at least some of its functions. These results suggest yet another method by which the activity of the alternatively spliced domains might be regulated. This might also explain why some previous studies using full-length fibronectin isoforms did not reveal functions for the alternatively spliced domains.
The Search for EIIIA Cell Surface Receptors

Clearly, the TLR4 is one candidate receptor for the isolated EIIIA domain. However, in another recent study, (Liao et al., 2002) suggested that integrins α4β1 and α9β1 might also serve as cell surface receptors for EIIIA. They collected anti-EIIIA antibodies that had been shown to block some of the putative functions of EIIIA (see below) including promoting condensation during chondrogenesis (Gehris et al., 1997) and induction of fibroblast differentiation (Serini et al., 1998). The binding site of these inhibitory antibodies was then mapped to a sequence in the EIIIA domain. The sequence of the binding site was observed to be very similar to a previously described motif in the ECM molecule tenascin (Liao et al., 1999). Since this region of tenascin is a binding site for the integrin α9β1, the authors tested the ability of α9β1 to bind to EIIIA. The integrin α9 subunit is 39% identical to the α4 subunit and many α9β1 ligands also bind to α4β1 (Palmer et al., 1993; Takahashi et al., 2000). Therefore, binding of EIIIA to the integrin α4β1 was also tested.

Several lines of evidence were used to suggest that EIIIA does indeed mediate binding to α9β1 and α4β1. First, SW480 cells transfected with α9, but not mock-transfected cells, adhered to the isolated EIIIA domain but not to the invariant Type III4 fibronectin domain. This binding was dose-dependent and could be blocked with antibodies to α9 but not to α5 or αVβ3. Second, Molt4 cells, which express α4β1, could also bind to the isolated EIIIA segment and anti-α4 antibodies, anti-β1 antibodies, or peptides from the V region, which as described above had been shown to bind to α4β1, blocked binding. Third, an EIIIA function-blocking antibody that mapped to the putative α9 binding site also blocked binding of α9 transfected cells and Molt4 cells to EIIIA. EIIIA deletion mutants lacking the putative EIIIA binding site showed decreased ability to bind the cells. While these results suggest that α4 and α9 are receptors for EIIIA, the absence of evidence for direct binding of these receptors to EIIIA leaves open the possibility that EIIIA acts indirectly by increasing the ability of some other extracellular molecule produced by the cells to bind to these receptors. This could explain why Guan et al. (1990) did not observe binding of α4β1 expressing WEHI cells to EIIIA-fibronectins. The use of EIIIA-Sepharose columns analogous to those used to identify α4β1 as a V region receptor might help to resolve this issue.
Signaling Pathways Regulated by EIIIA

Given the hypotheses that EIIIA might promote differentiation or proliferation there have been some reports that investigate whether or not EIIIA can regulate the intracellular signaling molecules that are involved in these processes. One such report investigated cell cycle progression and mitogenic signal transduction in cells plated on EIIIA (Manabe et al., 1999). In this study the authors first examined the ability of different fibronectin isoforms to promote cell cycle progression. They found that isoforms containing EIIIA promoted entry into S phase of the cell cycle to a greater extent than isoforms that lacked EIIIA. In order, to investigate the molecular basis for this effect, they then examined several signaling molecules that had previously been shown to be expressed or activated in response to fibronectin (see discussion of integrin signaling above). These included two molecules involved in cell cycle progression, cyclin D1 and pRb, and three other signaling molecules, extracellular regulated kinase ERK2, p130Cas, and FAK. While fibronectin lacking EIIIA stimulated expression of cyclin D1, phosphorylation of pRb, ERK2, p130Cas and FAK, EIIIA-fibronectin increased all of these responses except FAK phosphorylation. In this study, the authors postulated that EIIIA exerted its effects by augmenting the ability of the RGD site to interact with integrin α5β1.

However, other studies that have reported on EIIIA receptors have also suggested that EIIIA might signal directly through these receptors. For example, Liao et al. (2002), the authors who suggested that α4β1 and α9β1 are receptors for EIIIA, also examined activation of MAP kinase. They assayed MAP kinase phosphorylation in MOLT-3 α4β1- expressing cells plated on EIIIA and showed that MAP kinase phosphorylation was induced by EIIIA. In the study that examined the interaction of EIIIA with Toll-like receptor 4 (Okamura et al, 2001), EIIIA was found to stimulate expression and activity of the transcription factor NF-κB. Since NF-κB is known to induce MMPs (Sato and Seiki, 1993) the ability of EIIIA to regulate it seems to account for the effects of EIIIA on MMP expression.

The EIIIB Region

EIIIB remains the most elusive of the three alternatively spliced segments in that there are very few reports that relate to the function of this isoform. Its inclusion did not
affect the adhesion, migration, or spreading of a number cell lines (Guan et al., 1990). However, EIIIB shows a strikingly high degree of sequence conservation. Its amino acid sequence is completely identical between humans, rats, and mice and it is 96% identical between humans and chickens (Norton and Hynes, 1987). Furthermore, EIIIB appears to be the most tightly regulated, both spatially and temporally, of the three isoforms (Peters et al. 1996b). All of these observations suggest that it has some functional significance.

**Project Overview**

In this thesis three hypotheses related to the function of the fibronectin splice isoforms are investigated. The first is that the alternatively spliced isoforms of fibronectin promote the migration of keratinocytes and dermal fibroblasts. The second is that the EIIIA splice isoform of fibronectin promotes fibroblast differentiation during wound healing. The third is that the alternatively spliced isoforms of fibronectin promote the differentiation of mural cell precursors during blood vessel development. All three of these hypotheses relate to processes that take place during wound healing (although blood vessel development plays an important role during embryonic development as well). The second two hypotheses also relate to the effects of the fibronectin isoforms on the differentiation of mesodermally derived cells. The next few sections of the introduction will provide an overview of wound healing and mesodermal development. They also will provide evidence supporting each of the three hypotheses.

**Cutaneous Wound Healing and Fibronectin Splice Isoforms**

Wound healing requires the coordinated action of many different cell types (Clark, 1993; Gailit and Clark, 1994). After tissue damage extravasation of blood components activates cells in the bloodstream including platelets, which are involved in blood clotting, and neutrophils and monocytes, which are involved in the inflammatory response. Activated platelets bind to the wounded site. Platelet aggregation and degranulation help to reestablish hemostasis and prevent excessive blood loss. During this process platelets release fibronectins that contain EIIIA* and EIIIB*.

Monocytes are attracted to the wound by growth factors. The monocytes then differentiate into phagocytic macrophages that clear bacteria and debris. The expression of the alternatively spliced isoforms in macrophages has been examined in a rat model of cutaneous wound healing. In this study, probes for the different fibronectin isoforms,
along with probes for the macrophage marker lysozyme, were used to show that macrophages produce EIIIA+, EIIIB+, and V+ fibronectins during the early stages of wound healing (Brown et al., 1993).

The inflammatory phase is followed by reepithelialization and revascularization of the wound. This period is also characterized by the formation of granulation tissue. Granulation tissue refers to the disrupted tissue that lies just below the blood clot. This tissue is so named because an influx of cells and newly forming vessels gives it a granular appearance. Granulation tissue contains a provisional matrix that is rich in fibronectin crosslinked to the ECM protein fibrin. This provisional matrix provides a substrate over which cells migrate.

Reepithelialization begins soon after wounding with the migration of keratinocytes at the edges of the wound into the granulation tissue. Keratinocyte proliferation within the wound bed is required for the establishment of a new epithelial layer. Interestingly, an in situ hybridization study of fibronectin in healing wounds (see above) showed that EIIIA+, EIIIB+, and V+ forms of fibronectin are highly expressed in the pathway over which the advancing keratinocytes migrate. Interestingly, keratinocytes, which express several integrin receptors, upregulate the expression of the fibronectin receptor α5β1 in response to wounding and levels of this integrin remain high during their migration (Grinnell et al., 1987).

Revascularization is another important process that occurs during wound healing. This process involves the sprouting of capillary cells from preexisting vessels in response to angiogenic growth factors (see below) present in the wound bed. These sprouts invade the granulation tissue and become remodeled into a dense network of small vessels that provide nutrients and oxygen to the cells in the wounded site. As wound healing progresses this capillary network becomes less dense and the endothelial cells within it become quiescent. This phase of vessel development is marked by the recruitment of smooth muscle cells and pericytes which are characteristic of quiescent vessels (Tonnesen et al., 2000).

Finally, the dermal layer of the skin is reestablished in part by dermal fibroblasts that migrate into the wound from sites in the surrounding dermis. During their migration and early on in the process of wound healing, these cells produce fibronectin isoforms
that are EIIIA\(^+\), EIIIB\(^+\), and V\(^+\). Later these cells begin producing collagen and laminin matrix molecules that will form a new epidermal basement membrane. As the repair process comes to an end, the dermal fibroblasts differentiate into myofibroblasts that supply the force needed to contract the wound edges.

**Fibronectin in Mesodermal Development**

In mammals, the formation of mesodermal tissues begins during gastrulation with the migration of a population of mesenchymal cells into the embryo to positions that will eventually give rise to mesodermal structures within the embryo and the extraembryonic membranes. Interestingly the pathways over which these cells migrate is rich in the alternatively spliced fibronectins (Peters and Hynes, 1996a). Gastrulation generates an embryo comprised of three layers with the mesodermal layer lying between the outer layer of ectoderm and the innermost endodermal layer. Subsequently, the mesoderm becomes divided into different regions and each region gives rise to different structures. Chordamesoderm forms a transient structure called the notochord. This tissue also directs the formation of the neural tube and plays a role in the formation of the anterior-posterior body axis. Some of the presumptive mesodermal cells migrate laterally from the notochord and line up along the notochord and neural tube to form the paraxial mesoderm. Paraxial mesoderm separates into blocks of tissue called somites that give rise to the dorsal connective tissue of the bones, muscles, cartilage, and dermis. In another region of the embryo, lateral plate mesoderm forms and later gives rise to the heart, blood vessels, and blood cells. In the extraembryonic yolk sac, mesodermal cells give rise to yolk sac vasculature (see Gilbert, 1994).

Fibronectin is expressed in and/or around many mesodermally derived structures during their formation (e.g. head mesenchyme, heart, notochord, somites, and dorsal aorta). Mouse embryos lacking fibronectin exhibit extensive mesodermal defects. They have a shortened anterior to posterior axis and have deformed neural tubes. These mice also lack the notochord and somites. Furthermore, they exhibit severe defects in blood vessel development in the embryo and in the yolk sac (see below) (George et al., 1993). Further studies revealed that cells expressing markers characteristic of the notochord and somite lineages arose in the correct location at the appropriate time in the null embryos. This suggested fibronectin is required for the morphogenesis of these structures rather
than for the differentiation or migration of cells of these lineages (Georges-Labouesse et al., 1996).

There is a great deal of evidence that fibronectin plays a central role in directing the differentiation of a number of cell types many, but not all, of which are mesodermally derived (reviewed in Hynes 1990). Recent work suggests that some of these differentiation events might also involve fibronectin splice isoforms. One of the most interesting examples of this involves the acquisition of smooth muscle cell characteristics by fibroblasts and smooth muscle cell/pericyte (mural cell) precursors. In addition to sharing some similar differentiation markers, these three cell types share a common lineage in that all three are derivatives of pluripotent mesenchymal cells. The induction of smooth muscle cell markers in these cells imparts them with the ability to exert contractile force. This ability is important for their functions in wound healing (fibroblasts) and blood vessel maturation (pericytes and smooth muscle cells). Evidence implicating fibronectin isoforms in the differentiation of fibroblasts during wound healing and mural cell precursors during blood vessel development is discussed in the following sections.

**EIIIA and Myofibroblast Differentiation**

Towards the end of the wound healing process, wound fibroblasts differentiate into a contractile cell type. These contractile cells exhibit morphological and biological features that are intermediate between fibroblasts and smooth muscle cells and are therefore called myofibroblasts. Myofibroblasts are most often characterized by their expression of the $\alpha$-smooth muscle actin protein. Mammals have six different actin genes and thus produce six distinct actin isoforms (Vandekerckhove and Weber, 1978). Although the amino-acid sequence of actin isoforms is generally highly conserved, heterogeneity in the sequence of 17-amino-terminal amino acids allows them to be differentiated based on their isoelectric points. Each isoform has one of three isoelectric points termed $\alpha$, $\beta$, or $\gamma$. One of the $\alpha$ isoforms, $\alpha$-smooth muscle actin, is most commonly expressed by smooth muscle cells. The discovery of myofibroblasts demonstrated that under certain conditions other cell types could express this marker as well. Myofibroblasts were thought to be derived from fibroblasts because they express
fibroblast markers such as vimentin but lack many other smooth muscle cell markers such as desmin and smooth muscle myosin (Darby et al., 1990; Eddy et al., 1988).

Myofibroblasts were first described as a population of cells that appeared in the granulation tissue of healing wounds and which aided in wound contraction (Gabbiani et al., 1971). Subsequent studies have shown that myofibroblasts can also be found in various organs under normal conditions. Interestingly, myofibroblasts are present in and around structures that require mechanical forces for stability, morphogenesis, or remodeling both during development and in adult animals. For example, these cells are found around the developing capillaries, glomerulus, and lung alveolar sac in embryos and in contractile interstitial cells of alveolar septa, uterine submucosa and adrenal gland capsule in adult animals. These observations support the hypothesis that myofibroblasts perform a contractile function (Desmouliere and Gabbiani, 1994; Lindahl and Betsholtz, 1998).

Myofibroblasts also appear during a variety of pathological conditions in which tissue remodeling and retraction occur. Some of these include liver cirrhosis, hypertropic scarring, pulmonary fibrosis, burn contracture, and hepatic fibrosis. Many of these maladies involve tissue injuries that fail to heal. In such cases, the wound healing process is not resolved normally, but rather it continues unchecked and leads to tissue damage. In other cases, myofibroblasts arise as part of pathological prineoplastic proliferations. They are also characteristic of fibroblastic proliferations that accompany the growth and invasion of some epithelial tumors (Sappino et al., 1990).

Clearly, myofibroblasts play an important physiological role during wound healing. Their contractile properties help to close the wound and their synthesis of collagen matrix aids in reestablishing a normal basement membrane. However, it is also clear that loss of the normal regulation of these beneficial wound-healing functions can have dire consequences. In the pathological situations described above, deposition of matrix leads to fibrosis and contraction leads to tissue damage. Because of the prominent role of myofibroblasts in repair and pathology, there is great interest in identifying factors that regulate their differentiation.

EIIIA-fibronectin has been shown to be upregulated during wound healing, during tissue injury, and in tumors. Thus, it is clearly present in many of the situations where
myofibroblasts arise. This correlation lead to the hypothesis that EIIIA-fibronectin might be involved in myofibroblast differentiation. The hypothesis that EIIIA fibronectin induces myofibroblast differentiation has been tested directly in a rat hepatic fibrosis model (Jarnagin et al., 1994). In this model, liver injury induced by bile duct ligation is characterized by the conversion of lipocytes (like fibroblasts, lipocytes are mesodermally derived) to α-smooth muscle actin-expressing myofibroblasts. Using this system, the authors showed that normal lipocytes plated on EIIIA-fibronectin increased their expression of α-smooth-muscle actin mRNA. This increase could be blocked by an anti-EIIIA antibody or by soluble EIIIA-peptides.

More recent studies have elaborated this model by suggesting a link between EIIIA-fibronectin, myofibroblast differentiation, and the growth factor TGF-β1. TGF-β1 is a heterodimeric molecule that belongs to a large family of cytokines that regulate cell proliferation, differentiation, and motility (Roberts et al., 1995; Shull et al., 1992; Staehling-Hampton et al., 1994). TGF-β is secreted into the extracellular space as a latent precursor protein that is biologically inactive (Zhu and Burgess, 2001). In the extracellular space, TGF-β activation occurs via a number of mechanisms (e.g. proteolysis, thrombospondin dependent mechanism) and the active form of TGF-β binds to its cell-surface receptors and mediates biological effects. For example, this growth factor plays an important role in pattern formation and differentiation during embryogenesis. In adult animals, TGF-β1 regulates inflammation, tissue repair, and transformation (reviewed in Kingsley, 1994; Roberts and Sporn, 1993).

TGF-β1 has long been considered a regulator of myofibroblast differentiation. It induces the expression of characteristic myofibroblastic markers including α-smooth muscle actin and Type I collagen in fibroblasts both in vivo and in vitro (Desmouliere et al., 1993; Kurkinen et al., 1980; Ronnov-Jessen and Petersen, 1993; Zhang et al., 1994). It also upregulates other smooth muscle markers such as SM22α (Hautmann et al., 1999). In fibroblasts, TGF-β addition directly regulates the activity of the α-smooth muscle actin promoter (Hautmann et al., 1999; Roy et al., 2001). This activation relies on the presence of cis elements within the promoter and in particular on a region termed the transforming growth factor-β control element (TCE) (Roy et al., 2001).
TGF-β1 also regulates the expression of fibronectin and increases the inclusion of EIIIA into the fibronectin molecule (Balza et al., 1988; Borsi et al., 1990; Kocher et al., 1990). Like EIIIA, TGF-β1 is expressed in a wide variety of pathological fibrotic lesions and in tumors (Branton and Kopp, 1999; Pasche, 2001; Reiss, 1999). Based on these studies, it was proposed that upregulation of EIIIA by TGF-β1 might also be required for the regulation of myofibroblast differentiation.

The relationship between EIIIA-fibronectin, TGF-β1, and myofibroblast differentiation was examined by Serini et al. (1998). First, the authors determined the temporal relationship between expression of EIIIA-fibronectin and α-smooth muscle actin in vivo in a rat exisional model of wound repair. Immunostaining of sections of healing wounds showed that deposition of EIIIA fibronectin precedes α-smooth muscle actin expression by fibroblasts during granulation tissue formation. In vitro stimulation of rat dermal fibroblasts with TGF-β1 also induced an increase in the levels of EIIIA-fibronectin. This increase in EIIIA-fibronectin preceded increases in the expression of α-smooth muscle actin. In this study, anti-EIIIA antibodies blocked the TGF-β1 mediated increase in the expression of both α-smooth muscle actin and Type I collagen. This result suggests a model in which TGF-β1 induction of α-smooth muscle actin occurs via an EIIIA-dependent mechanism. In contrast with findings from the study of EIIIA in hepatic fibrosis that showed that EIIIA was sufficient to induce α-smooth muscle actin expression (see above), in this study, EIIIA was not sufficient for upregulation of α-smooth muscle actin. In this case, plating dermal fibroblasts on the isolated EIIIA domain did not lead to induction of α-smooth muscle actin.

**Fibronectin Isoforms and Blood Vessel Development**

During development, the cardiovascular system is the earliest organ system formed. Vascular development begins in the yolk sac where endothelial and hematopoietic cell precursors differentiate in situ from mesoderm (vasculogenesis). In the yolk sac, the endothelial and hematopoietic precursors form aggregates called blood islands that are progressively organized into a network of capillaries. Vasculogenesis subsequently generates a network of vessels within the embryo proper and aids in formation of vessels supplying the heart and dorsal aorta. Subsequent vascularization of the yolk sac and of embryonic organs occurs via a process called angiogenesis. During
angiogenesis, endothelial precursors migrate as individual cells or as sprouts from preexisting vessels into unvascularized areas (reviewed in Pepper, 1997).

In adult organisms, the vasculature is, for the most part, quiescent. However, in some situations formation of new blood vessels occurs in adult organisms. In most cases, this occurs during physiological tissue remodeling (e.g. vascularization during the ovarian cycle) or in tissue repair (e.g. wound healing). However, angiogenesis also occurs in a variety of pathological situations. For example, tumor size is highly dependent upon angiogenesis. If tumors do not become vascularized, they commonly undergo necrosis or apoptosis (reviewed in Carmeliet and Jain, 2000 and; Folkman, 1995).

Molecules such as vascular endothelial growth factor (VEGF), and its receptor Flk-1, are necessary for endothelial cell differentiation (Risau, 1997). VEGF is also important during angiogenesis in adult animals since it promotes endothelial cell proliferation, migration, and vascular permeability. Newly formed endothelial vessels are fragile and prone to regression. However, these vessel are gradually stabilized by virtue of their association with an outer layer of peri-endothelial smooth muscle cells/pericytes (mural cells) (Conway et al., 2001). Endothelial cells recruit mural cell precursors and these cells in turn differentiate into contractile cells. Although this association blocks endothelial cell proliferation and migration it protects the vessels from apoptosis and leads and to the formation of a stable quiescent vasculature.

Several molecules involved in the association between endothelial cells and mural cells have been identified (Carmeliet and Collen, 2000). Endothelial cells recruit mural cells via production of the cytokine PDGF-BB. PDGF-BB binds to PDGFβ receptors on the mural cells and induces their migration. Angiopoietin-1 (Ang-1), a ligand for the endothelial cell specific receptor Tie2, is produced by mural precursor cells. Ang-1 signaling via Tie2 inhibits vascular permeability by strengthening the associations between endothelial cells. Ang-1/Tie2 signaling also appears to be important for endothelial associations with peri-endothelial cells (Vikkula et al., 1996).

In a model reminiscent of the one proposed for myofibroblast differentiation (see above), it has been suggested that TGF-β plays a role in inducing the differentiation of mural cell precursors into α-smooth muscle actin-expressing smooth muscle cells and
pericytes. The importance of α-smooth muscle actin expression in these cells has been demonstrated by the generation of α-smooth muscle actin-null mice. Although such mice are viable and fertile, they exhibit compromised vascular contractility, tone, and blood flow (Schildmeyer et al., 2000).

In smooth muscle cells, TGF-β directly induces expression of the α-smooth muscle actin promoter (Hautmann et al., 1997). This induction depends on the presence of two CArG elements in the promoter as well as the TCE that was found to be important for myofibroblast differentiation. Regulation of the expression of smooth muscle cell markers has also been studied in 10T1/2 mesenchymal cells which can serve as smooth muscle cell progenitors (Hirschi et al., 1998). In these cells, γ-smooth muscle actin expression was assayed because this marker is upregulated late in smooth muscle cell development and typically is not expressed by myofibroblasts and other non-smooth muscle cell types. Uppregulation of γ-smooth muscle actin was also shown to depend on CArG/ serum response element (SRE) motifs (Hirschi et al., 2002).

Further evidence for a role for TGF-β in this process comes from co-culture experiments of 10T1/2 cells and endothelial cells. These studies have shown that TGF-β is produced and activated in cultures that contain both endothelial cells and mural cells. Although, TGF-β is also produced in cultures containing endothelial cells or mural cells alone, it is not activated in these cultures. More importantly, co-culturing 10T1/2 with endothelial cells induced α-smooth muscle actin expression in the 10T1/2 cells and this induction was blocked by antibodies that inhibit TGF-β function (Hirschi et al., 1998). These results suggest that the heterotypic cell interactions of mural cells and endothelial cells can activate TGF-β and thereby induce mesenchymal cell differentiation.

In the myofibroblast differentiation model (see above), it has been suggested that fibronectin plays a role in TGF-β mediated α-smooth muscle actin induction. There is some evidence that fibronectin might mediate TGF-β’s effects on mural cell precursors as well. First, there is good evidence that fibronectin plays a role in the development of blood vessels. Furthermore, fibronectin seems specifically to be necessary for mesenchymal cell interactions with the endothelium. Finally, some evidence indicates that fibronectin may be important for TGF-β’s effects on vessel development.
As mentioned previously, ablation of the fibronectin gene in the mouse has revealed that fibronectins are necessary for blood vessel formation during development (George et al., 1993). Within the fibronectin-null embryo proper, the blood vessels are dilated and abnormal in appearance. In the yolk sac, blood vessels fail to form at all. A similar defect in vasculogenesis is observed in mice mutant for the fibronectin integrin receptor subunit α5 (Yang et al., 1993).

Fibronectin-null embryos have been examined for the presence of endothelial cells (George et al., 1997). This analysis revealed that PECAM (platelet endothelial cell adhesion molecule) and Flk-1/VEGFR-2 positive endothelial cells are present in the null embryos. Furthermore, these endothelial cells are, for the most part, present in the correct locations at the appropriate times. Therefore, fibronectin appears to be required for the morphogenesis or maintenance of blood vessel structure rather than for endothelial cell differentiation or migration.

Interestingly, in fibronectin-null embryos the dorsal aorta does not form properly. In these embryos the endothelial layer of cells fails to associate with the surrounding mesenchymal layer. These data suggest that fibronectins are required for endothelial cell associations with the peri-endothelial support cells. Interestingly, Ang-1 knockout mice show similar defects in endothelial/mesenchymal interactions (Suri et al., 1996).

Genetic evidence links fibronectin and TGF-β in pathways that regulate blood vessel development. Fifty percent of TGF-β-null mice (Kulkarni et al., 1993; Shull et al., 1992) and all TGF-β receptor I and TGF-β receptor II null mice (Larsson et al., 2001; Oshima et al., 1996) show defects in yolk sac vasculogenesis. These defects resemble those seen in the fibronectin-null mice in that endothelial cell association with the surrounding mesenchyme is disrupted.

A role for fibronectin in the TGF-β null phenotype is suggested by studies using TGF-β somatic chimeras (Goumans et al., 1999). In these experiments, researchers generated ES cells that overexpressed either the wild-type TGF-βRII or a truncated dominant negative form of the receptor. They then aggregated the mutant or wildtype ES cells and attached the aggregates to morula stage wildtype ROSA blastulas. The resulting embryos were then implanted into host mothers. LacZ staining confirmed that the endodermal layer of the yolk sac was host derived while the mesodermal component that
gives rise to the vessels was derived from the mutant ES cells. In this model, severe
defects in vasculogenesis arose in the yolk sacs derived from the TGF-βR mutant ES
cells. The vessels that formed were collapsed and disorganized and some areas of the
mutant yolk sacs lacked vessels altogether. The authors also observed defects in the
mesodermal interactions with the endoderm.

Staining of sections from mutant yolk sacs showed a drastic reduction in
fibronectin staining. Furthermore, the small amount of fibronectin that was present was
disorganized when compared with fibronectin staining in wild-type animals. Embryoid
bodies formed from the mutant ES cells also showed a drastic reduction in fibronectin by
immunofluorescence staining and western blotting of whole cell extracts. These results
suggest that TGF-β’s effects on yolk sac vasculogenesis are dependent on its ability to
upregulate fibronectin.

There is no direct evidence that any particular splice isoform of fibronectin plays
a role in mural cell differentiation, however, the temporal and spatial expression patterns
of EIIIA and EIIIB fibronectins correlate with the formation of blood vessels. These
isoforms are expressed around the developing vasculature during mouse embryogenesis
(Peters et al. 1996b) and, in the adult mouse, prominent staining for EIIIA staining is
observed around all blood vessels and appreciable EIIIB staining is present around small
vessels and capillaries. Furthermore, as mentioned earlier these isoforms are upregulated
in situations which require new blood vessel growth (e.g. in healing wounds and during
tumorigenesis) (see above).

**An In Vitro System for the Analysis of Fibronectin Splice Isoforms**

This introduction provides an overview of what was known about fibronectin
isoforms at the point at which I began my research. At that time several interesting
observations had been made which required further study. For example, it was unclear
whether or not the use of primary cell types that interact with or produce fibronectin
splice isoforms in vivo might yield previously overlooked information about the function
of the splice isoforms. Furthermore, a growing body of evidence suggested that
fibronectin isoforms might play a role in blood vessel development and more generally in
the acquisition of smooth muscle cell features in a variety of situations. However, more
direct evidence in support of this hypothesis was needed. The goal of the work described
in this dissertation was to develop an *in vitro* system to address these questions. I have made considerable progress towards this goal in that I have produced recombinant fibronectin splice isoforms and assayed the responses of two primary cell types, keratinocytes and dermal fibroblasts, to these isoforms (Chapter 2). I have also isolated and characterized fibronectin-null fibroblasts and begun isolating fibronectin-null endothelial cells to address the role of fibronectin splice isoforms in myofibroblast differentiation and blood vessel development (Chapter 3; Appendix). This research thus establishes a useful framework for those interested in testing hypothesis related to the function of fibronectin isoforms. Furthermore, it provides novel and interesting insights into the function of fibronectins.
Figures and Legends
Figure 1-1: Schematic drawing of FN (adapted from Hynes, 1990). Small rectangles represent FN Type I repeats. Open circles represent FN Type II repeats. Open squares represent FN Type III repeats. The two closed squares show the position of the alternatively spliced Type III repeats EIIIA and EIIIB. The hatched rectangle represents the V region and is subdivided to reflect the complex splicing pattern of this region. The black dot shows the position of the RGD cell-binding domain in Type III10 repeat.
Chapter 2

Production and Characterization of Recombinant Fibronectin Splice Isoforms
INTRODUCTION

Both keratinocytes and dermal fibroblasts are recruited to sites of tissue injury where keratinocytes mediate reepithelialization and dermal fibroblasts function in wound contraction. During wound healing keratinocytes migrate from an area containing EIII A<sup>+</sup>, EIII B<sup>+</sup>, V<sup>+</sup> fibronectins into granulation tissue rich in EIII A<sup>+</sup>, EIII B<sup>+</sup>, V<sup>+</sup> fibronectins. Although the migrating keratinocytes do not make fibronectin, other cells in the wound bed deposit alternatively-spliced isoforms of fibronectin along keratinocyte migration pathways. This fibronectin helps to direct keratinocyte movement. Unlike keratinocytes, dermal fibroblasts produce EIII A<sup>+</sup>, EIII B<sup>+</sup>, V<sup>+</sup> fibronectins while migrating into the wound. Given this expression pattern, it seemed possible that the alternatively spliced isoforms might facilitate keratinocyte and dermal fibroblast migration. Cell migration is an integrated process that involves changes in the strength of adhesion to the extracellular matrix, in the rearrangements of actin cytoskeleton, and in the formation of focal contacts (Lauffenburger and Horwitz, 1996). Therefore, it seemed possible that the alternatively spliced fibronectins might also affect these processes.

In order test the effects of the fibronectin isoforms on cell migration and related processes, I used a bacterial expression system to produce fibronectin fragments that differed in their inclusion of the various alternatively spliced regions. The bacterial expression system has several advantages over mammalian expression systems. First, this system allows for relatively rapid purification of protein. One can prepare milligram quantities of protein within a week whereas this process requires months when using a mammalian system. Second, since bacterial cells do not make fibronectin one can avoid issues of contamination often encountered when using mammalian cells. Despite these advantages, the bacterial system does have some limitations. There are protein solubility issues and post-translational modifications that occur in mammalian cells do not take place in bacteria.

Once I obtained the recombinant fibronectin fragments, I examined their effects on the adhesion, spreading, stress fiber formation, focal contact assembly, and migration of keratinocytes and dermal fibroblasts. I found that the recombinant fragments were able to promote all of these responses in both keratinocytes and dermal fibroblasts. I also found that more keratinocytes were able to migrate on fibronectins containing the
alternatively spliced regions than on isoforms lacking these segments. No differences were observed in the ability of isoforms containing the alternatively spliced regions and isoforms lacking these regions to promote the other functions that I examined.
MATERIALS and METHODS

Recombinant Fibronectin Fragments

Dr. Laird Bloom and Jane Trevithick designed constructs used for the production of recombinant fibronectin fragments. Clones were constructed by standard molecular biological methods (Ausubel, 1991; Sambrook, 1989). Expression vectors for the production of the GST-and His₅-tagged fibronectin fusion proteins are derivatives of the pGH expression vector and have been described (Bloom et al., 1999). Seven additional splice isoform fusion proteins containing rat fibronectin Type III repeats 7-15 and the alternatively spliced exons were generated by cloning HincII fragments from a set of seven different rat fibronectin cDNAs (Guan et al., 1990) into pGH. These cDNAs contained EIIIA, EIIIB, or the V region singly or in all possible combinations of the three (EIIIA and EIIIB or EIIIA and V or EIIIB and V or EIIIA, EIIIB, and V). The RGDS deletion mutation in the fibronectin Type III₁₀ domain was derived from a baculovirus vector containing full-length rat FN lacking RGDS (a gift from Jean Schwarzbauer, Princeton University, Princeton, NJ). A DraIII fragment containing the RGDS mutation was then used to replace a DraIII fragment containing the wildtype RGDS site in pGH.F7-15 (pGH.F7-15 RGDS'). The other seven alternatively splice constructs were then used to generate seven additional alternatively spliced isoforms that lacked the RGD cell-binding site.

Production of Fusion Proteins

Expression of fusion proteins in Escherichia coli strain DH5α grown to late log phase in Luria-Bertani medium and 100μg/ml ampicillin was induced with 1mM isopropyl-β-D-thiogalactopyranoside (Boehringer Mannheim, Indianapolis, IN) for 4h. Cells were harvested by centrifugation and stored at −80°C in PBS containing 2mM PMSF (Sigma, St. Louis, MO). Bacterial pellets were thawed at 4°-8°C. The buffer was then adjusted to 50mM sodium phosphate, pH8, 10mM imidazole, 300mM NaCl, 2mM PMSF, 1:100 aprotinin stock (Sigma), and 12.5 μg/ml leupeptin (Sigma). Pellets were incubated with lysozyme (2mg/ml, Sigma) for 30 min at 4°C, lysed by sonication, brought to 1% Triton X-100 (Sigma), and centrifuged at 28,000g for 20 min. Lysates were incubated with Co²⁺ chelated resin (TALON; Clontech, Palo Alto, CA), for 3 hours at room temperature. The resin was washed three times in 10 volumes of wash buffer (50 mM sodium phosphate,
pH 8.0, 300mM NaCl, and 10mM imidazole). Proteins were eluted with 6 volumes of elution buffer (wash buffer adjusted to 150mM imidazole). The first 5 fractions eluted from the TALON resin were then incubated with glutathione-agarose (Sigma) overnight at 4°C. The glutathione-agarose was washed three times in 10 volumes of PBS, 2mM PMSF. Proteins were eluted in 4 column volumes of CAPS/glutathione elution buffer (20mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH11, 150mM NaCl, 2 mM EDTA, 2mM PMSF, and 10mM glutathione). Elution was performed at pH 11 because at high pH fibronectin assumes an extended conformation that decreases nonspecific protein aggregation. Protein purity and size were verified by SDS-PAGE electrophoresis on a 7.5% polyacrylamide gel followed by Coomassie Blue staining.

**Western Blotting**

Purified recombinant fibronectin fusion proteins V0, EIIB⁺, EIIMA⁺, V⁺, or EIIB⁺EIIMA⁺V⁺ were electrophoresed on 7.5% SDS-PAGE gels and transferred for 1 hour and 15 minutes to Protran nitrocellulose (Schleicher and Schuell, Keene, NH). Filters were blocked overnight at 4°C in blocking buffer (5% Shaw's non-fat dry milk reconstituted in PBS/0.1% Tween-20). Blots were incubated overnight at 4°C or for 1 hour at room temperature with an appropriate dilution of antibody in blocking buffer. The rabbit polyclonal antibody 28 STU that recognizes all of the recombinant FNs was used at 1:2000. The affinity-purified rabbit polyclonal antibody R264 that recognizes EIIB (Peters et al., 1995) was used at 1:10,000. The rabbit polyclonal antibody 73N that recognizes an epitope in V95 was used at 1:5000 (Peters et al. 1996a). The murine monoclonal antibody 3E2 ("anti-cellular FN") that recognizes EIIMA was obtained as ascites (Sigma, St.Louis, MO) and was used at 1:4000. Blots were washed with PBS/0.01% Tween-20 for at least 1 hour. Filters were then incubated with appropriate HRP-conjugated secondary antibodies (Jackson Immunoresearch Labs, Inc., West Grove PA) diluted 1:2000-1:5000 in blocking buffer for 1 hour at room temperature and then washed as above. Bands were visualized using the Renaissance chemiluminescence kit (DuPont-NEN, Boston, MA) and autoradiography.

**Quantitation of Fusion Protein Concentration**

Fusion proteins along with a BSA standards (Pierce) were run on a SDS-PAGE gel (7.5%) and protein concentrations of fusion proteins were determined by densitometry.
using the Alpha Imager 2200 system and AlphaEase Image Processing analysis Software (Alpha Innotech Corporation, San Leandro, CA). Protein concentrations were verified using a protein assay kit based on the Bradford method (Bio-Rad Laboratories, Hercules CA) followed by measurement of the absorbance at 595nm.

**Keratinocyte Cell Culture**

Human epidermal keratinocytes from neonatal foreskins were a gift of Dr. K. Hodivala-Dilke. Keratinocytes were cultured as described (Rheinwald, 1989). Briefly, cells were cultured in low calcium FAD medium to prevent keratinocyte differentiation (0.5mM Ca²⁺). FAD medium consisted of 3 parts DME, 1 part Ham’s F-12, and adenine at a final concentration of 1.8 x 10⁻⁴ M. Medium contained 10% fetal bovine serum (Hyclone), 100U/ml penicillin, 100μg/ml streptomycin, and 2mM L-glutamine. Several additional supplements including 5μg/mL insulin (Sigma), 2x10⁻⁶ M T3 (Sigma), 0.4μg/mL hydrocortisone (Calbiochem), 10⁻¹⁰ M cholera toxin (ICN), and 10ng/ml EGF (UBI) were also added. Keratinocytes were cultured on a layer of J2 (Swiss 3T3 clone) feeder cells that had been mitotically inactivated by pretreatment with 4μg/ml of mitomycin-C (Sigma). Before keratinocytes were harvested for experiments, feeder cells were selectively removed using EDTA.

**Isolation of Dermal Fibroblasts**

Newborn pups were sacrificed by CO₂ narcosis and washed in 0.01 N iodine in PBS for 10 minutes. Pups were then washed in 70% ethanol followed by a wash in a solution of gentamycin (diluted 1:50)(Gibco Laboratories). The skins of the mice were removed and floated on 0.25% trypsin solution (Gibco Laboratories) overnight at 4°C with the epidermis facing upwards. Skins were transferred to 60mm dishes and the epidermis was removed from the dermis using sterile Pasteur pipettes. Dermises were then incubated with 3mls of 212U/ml collagenase (Worthington Biomedical Corp) for 10 minutes at 37°C. They were then filtered through a 70-μm sterile nylon filter (Becton Dickinson, Franklin Lakes, NJ) to separate the cells from collagenous material. Cells were spun down and resuspended in E4HG medium containing 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, and 2mM L-glutamine. Cells were then plated on tissue culture plates that had been precoated with 30μg/ml of denatured rat tail collagen (Collagen Corp., Palo Alto, CA). Unless otherwise indicated, all cells were used at passage 6.
Keratinocyte Adhesion Assays

Wells of 96-well dishes were coated overnight with recombinant fibronectin fragments or full length rat or human fibronectin (BD Biosciences) as a positive control. Negative control wells were coated with 2mg/ml of heat-inactivated bovine serum albumin (BSA). All wells were blocked with BSA and washed prior to plating the cells. Passage six human keratinocytes were pretreated with 2.5μg/ml of cycloheximide (CHX; Sigma) for one hour to inhibit protein synthesis. After cycloheximide treatment, cells were trypsinized and 1x10⁵ cells were plated in each well in 500μL of serum-free medium with cycloheximide. After one hour, wells were washed and fixed with 4% paraformaldehyde. Cells were stained with 0.1% crystal violet for 5 minutes. The dye was then released using 10% acetic acid and the amount of dye present in samples from the different wells was quantitated by measuring the absorbance at 560nm. In a follow-up experiment, 2x10⁵ cells were used and cell adhesion was measured after 2 hours. To quantitate cell adhesion cells were stained with the green fluorescent dye 5-octadecanoylaminofluorescein (Molecular Probes, Eugene OR). Cells in four fields per sample were counted manually. Results are the average of three replicates.

Dermal Fibroblast Adhesion Assays

Shallow rings in the covers of 96-well plates (Falcon, Becton Dickinson) were coated with 80μl drops of recombinant FN substrates. Substrates were incubated either overnight at 4°C or for 2 hours at 37°C. Wells were coated with either 2mg/ml BSA or 50μg/ml poly-D-lysine as negative and positive controls. All wells were then blocked with 2mg/ml heat-inactivated BSA for 1h at 37°C. Passage 4 dermal fibroblasts were pretreated with 2.5μg/mL of cycloheximide. Cell adhesion was then measured by plating 1x10⁴ cells per well in serum-free medium containing cycloheximide for 2 hrs at 37°C. Nonadherent cells were washed off by gently submerging the plate in PBS then draining excess liquid. Cells were fixed for 10 minutes with 4% paraformaldehyde, washed 3x with PBS, stained with 0.1% crystal violet for 5 minutes, washed 3x in water and air-dried. Stained cells were counted in video images of 5 fields for each sample by Macintosh computer using Scion Image, a derivative of the public domain NIH Image program (developed at the National Institutes of Health, Bethesda, MD). Three experiments with six replicates of each condition were performed. Cell adhesion values
from the three experiments were normalized to the poly-D-lysine (PDL) positive control and then averaged.

**Spreading, Actin Assembly, and Focal Contact Formation**

Glass coverslips were coated with 30 μL drops of recombinant fibronectin substrates. Substrates were incubated for 2hrs at 37°C or overnight at 4°C. Substrates were then aspirated and the coverslips were washed 3x with PBS containing Ca^{2+} and Mg^{2+}. Coverslips were blocked for 1h with 2mg/ml heat-inactivated BSA. For short term spreading assays cells were pretreated for 30 minutes with 2.5μg/ml of cycloheximide (CHX; Sigma) to inhibit protein synthesis. The cells were then trypsinized and resuspended in serum-free medium. 5x10^3 cells were added to each coverslip. Cells were incubated 37°C and different timepoints were assayed depending upon the experiment. (10min-20 hours). The medium was aspirated and the coverslips were washed 3x with PBS containing Ca^{2+} Mg^{2+}. Cells were fixed in 3.7% PF containing Ca^{2+} Mg^{2+} at room temperature for 10 minutes, washed 3x with Ca^{2+} Mg^{2+} PBS and permeabilized in 0.5% of the nonionic non-denatured detergent (octylphenoxy)polyethoxyethanol (IGEPAL) (Sigma, St.Louis MO) for 10 minutes at room temperature. Coverslips were blocked in 10% NGS in PBS Ca^{2+} Mg^{2+} for 30 minutes at 37°C. For actin assembly assays, cells were then stained with rhodamine–conjugated phalloidin (Molecular Probes, Eugene OR or Sigma) for 30 minutes. For focal contact assays, cells were stained for 30 minutes at 37°C with either a mouse monoclonal anti-paxillin antibody at a 1:1000 dilution (Transduction Labs), a mouse monoclonal anti-vinculin antibody, VIN-11-5, at 1:50 (Sigma), or a mouse monoclonal anti-phosphotyrosine antibody PY99 at 1:1000 (Santa Cruz). Coverslips were washed 3x by dunking into PBS. Cells were then stained with rhodamine or fluorescein-conjugated anti-mouse secondary antibody (1:200) (TAGO) for 30 minutes at 37°C. Coverslips stained for actin or focal contact proteins were then washed 3x by dunking into beakers of PBS, washed once more by dunking into dH2O, blotted dry, and mounted face down on a drop of prewarmed Gelvatol. Stained cells were visualized using the 63x objective on the Axiophot (Zeiss) fluorescence microscope.
Migration Assays

Migration assays were performed as described in (Albrecht-Buehler, 1977). Briefly, 8-chamber wells of chambers affixed to a glass slide (Nunc or Falcon, Becton Dickinson) were blocked with 1% BSA in deionized, distilled water, washed with ethanol, air dried and baked for 10 minutes at 80°C. Wells were then coated with colloidal gold solution for 45 minutes at room temperature. Excess gold was aspirated from the wells, and wells were then coated with 100μl of substrate for 2 hours at 37°C. Substrate solutions were then aspirated and wells were blocked with 100μL of heat inactivated BSA (2mg/ml) for 2 hrs at 37°C. After aspirating the blocking solution, 100μl of serum-free medium were added to each well. Cells (1.5x10³) in 150μL of medium containing 10% FN-depleted serum were then added to each well. Fibronectin-depleted serum, a gift of Dr. K. Hodivala-Dilke and Jane Trevithick, was produced by passing serum over a gelatin-Sepharose column as described (Hynes, 1990). Cells were allowed to migrate for 14 hrs at 37°C. At the end of the incubation, cells were fixed with 4% paraformaldehyde for 10 minutes. Slides were dipped into distilled water and then air-dried. The area cleared by migratory cells was scored using a CCD camera. Analysis was performed on a Power Macintosh G3 computer using CG-7 RGB Color Frame Grabber (Scion corporation, Frederick, MD) and Scion Image Version 1.62a. Migration areas of single cells were traced and areas were assigned values based on computer screen pixels (arbitrary units).
RESULTS

Generation of recombinant fibronectin fusion proteins

To investigate the response of dermal fibroblasts and keratinocytes to the alternatively spliced segments of fibronectin, I produced alternatively spliced recombinant fibronectins as fusion proteins in bacteria (see Materials and Methods). The recombinant fibronectins include Type III repeats 7-15 of rat fibronectin preceded by an amino-terminal GST tag and followed by a carboxy-terminal His$_6$ tag (See Figure 2-1). These constructs encode proteins containing one (EIIIIB, EIIIA, V, or V95), two (EIIIIB and EIIIA together, EIIIIB and V together, EIIIA and V together), or all three (EIIIIB and EIIIA and V together) of the alternatively spliced regions, or lacking all three alternatively spliced regions (V0). In addition, these proteins contain either a wild-type RGD cell-binding site or lack the RGD binding site found in the fibronectin Type III10 repeat (See Figure 2-1). This site binds to several integrins including $\alpha5\beta1$ and $\alpha\nu$ containing-integrins. The constructs lacking RGD may allow for the identification of RGD-independent functions of the alternatively spliced segments. The V95 constructs produce a V region isoform that lacks the first 25 amino acids of the V region (V25). V25 contains the EILDV cell-binding site which is important for binding to integrin $\alpha4\beta1$. In all cases, the use of the single letter V without an associated number refers to the complete 120 amino acid V region (also referred to asV120).

I purified the fusion proteins by two successive rounds of affinity chromatography on Co$_{2+}$ resin followed by glutathione-Sepharose (See Materials and Methods). Figure 2-2 shows a representative purification of one of the fusion proteins. Although some protein fails to bind to the Co$_{2+}$ column (Figure 2-2, lane3) a large fraction bound to the column and could be eluted by competition with imidazole (Figure 2-2, lanes 4-9). Generally, we were able to recover from 20 to 40 percent of the protein in the original cell lysate in the glutathione eluates (Figure 2-2, lanes 11-14). Although this yield is relatively low, the glutathione fractions obtained were sufficiently concentrated (100-200$\mu$g/ml) for use as substrates in cell biological experiments. In order to verify that the correct complements of alternatively spliced segments were present in the recombinant proteins, I performed Western blotting using splice isoform-specific antibodies and a polyclonal antibody that recognized all of the fusion proteins (Figure 2-3). The proteins
appeared to be the correct size (120kD-150kD), with the rFN7-15 (V0) isoform being smaller than isoforms containing one alternatively spliced insert and with the isoform containing all three inserts being the largest. Antibodies specific for EIIIIB, EIIIA, or V recognized only the isoforms containing the appropriate splice segment. All of the antibodies recognized the isoform that contains all three splice segments.

**Ability of fibronectin isoforms to support cell adhesion**

I wanted to test the validity of using these recombinant proteins as substitutes for full-length mammalian fibronectin and to dissect the requirements for various fibronectin splice isoforms in adhesion. In order to determine whether or not the recombinant splice isoforms promote keratinocyte and dermal fibroblast adhesion, I measured the ability of these cells to adhere to wells coated with different recombinant splice isoforms of fibronectin. Keratinocyte adhesion was assayed first by a crystal violet dye-binding assay (Figure 2-4). Cells did not bind to wells coated with bovine serum albumin (BSA) and little crystal violet dye was retained in these wells. Cells adhered readily to wells coated with the recombinant fibronectin isoforms and these wells showed about 10-fold higher levels of crystal violet retention. These results suggested that the recombinant fibronectins were as effective as the full-length rat plasma fibronectin (rFN) control at promoting adhesion. However, the alternatively spliced isoforms promoted cell adhesion to the same extent as did the V0 isoform, which lacks all of the alternatively spliced segments. This suggests that the alternatively spliced segments do not modulate keratinocyte adhesion. The adhesion of keratinocytes to the fragments was dependent on the presence of the RGD cell-binding site since we noted that these cells did not adhere to fusion proteins lacking RGD (see cytoskeleton section).

To further explore the function of these fragments, I examined whether adhesion to the fragments was dose dependent. I selected a subset of the alternatively spliced fragments and tested their ability to promote keratinocyte adhesion at different doses of fibronectin (Figure 2-5). In this second experiment, I counted the number of cells that adhered to bacterial dishes coated with different concentrations of fibronectins. This experiment confirmed that keratinocytes adhere readily to the recombinant isoforms and showed that adhesion is dose-dependent (data not shown). It also confirmed that there is no difference in adhesion to proteins containing and lacking the alternatively spliced
isoforms. As with the keratinocytes, I found that dermal fibroblasts adhered to all of the recombinant splice isoforms of fibronectin and to the human fibronectin control in a dose-dependent fashion (Figure 2-6). As expected, dermal fibroblasts failed to adhere to control wells coated with BSA. There was no difference in the ability of the alternatively spliced isoforms to promote dermal fibroblast adhesion compared with V0.

**Effect of fibronectin isoforms on the cytoskeleton**

Cytoskeletal rearrangements play an important role in the molecular mechanisms responsible for cell migration. Therefore, I wanted to know if different fibronectin isoforms affect the cytoskeleton and its associated proteins. In order to test this, I used immunofluorescence microscopy to visualize actin stress fiber assembly and focal contact formation in keratinocytes and dermal fibroblasts plated on the recombinant fibronectin isoforms. Keratinocytes formed robust stress fibers on all of the fibronectin substrates (Figure 2-7). Furthermore, no difference in the rate of stress fiber formation was observed between cells plated on the alternatively spliced isoforms and those plated on V0 (data not shown). Keratinocytes were also able to form focal contacts on all of the substrates tested (Figure 2-7). The ability of V0 and the alternatively spliced isoforms to promote focal contact formation were similar over a range of FN concentrations (20µg/ml, 10µg/ml, or 5µg/ml) and at several different time points (1 hour, 2 hours, 3 hours or 4 hours) (data not shown).

I also examined focal contact formation and cytoskeletal organization using recombinant proteins lacking the RGD site that is important for cell adhesion. In general, coverslips coated with these proteins looked similar to BSA-coated negative controls (data not shown). On BSA-coated coverslips, adherent cells are rare and the few cells that are present on BSA-coated coverslips do not spread. The failure of the RGD− fragments to promote cell adhesion suggests that the alternatively spliced segments do not themselves promote keratinocyte adhesion. This result also implicates keratinocyte integrins that bind to RGD, such as α5β1, αvβ5, and αvβ6, in keratinocyte adhesion to RGD+ fusion proteins.

Interestingly, although very few keratinocytes could be observed on RGD− fragments, a few cells plated on the RGD+ constructs containing the V splice isoform were able to spread and form focal contacts. Spread cells were never observed on RGD−
fragments that lacked the V segment. In order to determine if spreading on V was dependent on the EILDV binding site found in the first 25 amino acids of the V region (see Chapter 1), I tested the ability of cells to spread on V95 RGD1 constructs which lack this binding site. I found that spread cells were observed on the recombinant V95 RGD1 as well as on V120 RGD1. This suggests that cell spreading is mediated by the RGDV site which has been mapped to the carboxy-terminal portion of the V region (see Chapter 1) rather than, or in addition to, the EILDV binding site (Figure 2-8).

In an experiment comparing the behavior of dermal fibroblasts on the different recombinant fragments, I initially observed a difference in the ability of V-containing fragments to promote focal contact formation (data not shown). However, upon repetition of these experiments I found that there was no difference in the rate of focal contact formation on V as compared to V0 (Figure 2-9).

**Migration of keratinocytes and dermal fibroblasts on fibronectin splice isoforms**

I wanted to know whether or not the alternatively spliced isoforms modulate keratinocyte and dermal fibroblast migration. Therefore, I assayed the affect of the splice isoforms on migration by measuring the area cleared by keratinocytes and dermal fibroblasts plated on colloidal gold coated with the different splice isoforms. As the cells migrate they phagocytose the gold particles leaving a cleared area. This area can be used as a measure of distance migrated by the cells. In this assay, I defined migratory cells as those cells clearing an area greater than 500 units. Although individual cells were only about 100 units large, I set the cut off higher in an attempt to distinguish true cell migration from the increases in cleared area that resulted from cell spreading.

Keratinocytes plated on gold particles coated with the recombinant fusion proteins migrated creating areas devoid of gold particles. No clearing was observed when cells were plated on gold particles coated with BSA. Interestingly, the cells appeared to migrate more robustly on fragments containing alternatively spliced segments than on V0 (Figure 2-10). Only about 25% of keratinocytes were migratory on V0 while about 60% of cells were migratory when any of the splice isoforms were present (Figure 2-11a). However, the same amount of clearing was seen in the area that each migratory cell was able to clear on V0 as compared to the splice isoforms (Figure 2-11b). This suggests that
the alternatively spliced isoforms affect the ability of keratinocytes to initiate migration but do not affect the rate of cell migration.

Dermal fibroblasts plated on BSA rarely cleared any of the gold particles. Roughly 60% of dermal fibroblasts were migratory on all the different substrates that were tested (Figure 2-12a). No difference in the percentage of migratory cells was seen in wells coated with V0 versus the other substrates. Among the migratory population, there was also no difference in the area that cells were able to clear on the different substrates (Figure 2-12b). Cells appeared to migrate slightly further on fragments coated at 10µg/ml than on fragments coated at 3µg/ml suggesting that fibroblast migration was dose dependent (data not shown).
DISCUSSION

Expression studies suggest a role for alternatively spliced fibronectin isoforms in wound healing process. In the experiments described in this chapter, recombinant splice isoforms of fibronectin were used to investigate the role of the various isoforms in the wound healing. More specifically, the effect of different isoforms on the adhesion, spreading, focal contact formation, and migration of keratinocytes and dermal fibroblasts was examined. All of the RGD+ recombinant proteins promoted all of these functions in both keratinocytes and dermal fibroblasts. However, the there was no difference in adhesion, focal contact formation, or stress fiber assembly by keratinocytes, or adhesion, focal contact formation, and migration by dermal fibroblasts plated on isoforms containing the alternatively spliced segments compared with isoforms lacking these segments. When the RGD site was deleted from the isoforms, it was possible to observe V-region mediated spreading of keratinocytes. Finally, more keratinocytes were migratory on fibronectins containing alternatively spliced segments than on those lacking these segments.

Fibronectin Splice Isoforms Promote Keratinocyte Migration

The finding that a higher percentage of keratinocytes are migratory on isoforms containing alternatively spliced regions fits well with expression data showing that these isoforms are present at high levels in keratinocyte migration pathways during wound healing. This is an interesting preliminary result; however, more work is needed to confirm it. Experiments comparing migration on isoforms containing the splice segments and on V0 should be repeated. If the fragments do indeed stimulate keratinocyte migration then several follow up experiments could be performed (see Chapter 4). The significance of the fact that all of the alternatively spliced segments stimulate keratinocyte migration is unclear. It seems unlikely however that there is some defect in the V0 isoform used in these experiments since this protein was as effective as the other fragments in promoting keratinocyte adhesion and cytoskeletal rearrangements. Furthermore, V0 supported dermal fibroblast migration to a similar extent as the other fragments suggesting that the effects of the splice isoforms on migration are keratinocyte specific.
V-region mediated cell spreading

We also observed that a small number of cells in the keratinocyte cultures were able to spread on V120 RGD and on V95 RGD. This suggests that a subset of these cells is able to recognize the RGDV site present in the C-terminal portion of the V region. However, it is also possible that the cells that adhere and spread in these experiments represent a small subpopulation of contaminating cells, most probably fibroblasts. Visualization of intermediate filament markers for keratinocytes (keratins) and fibroblasts (vimentin) could help to resolve this issue. Integrin expression in these cells could also be examined. In humans, α4β1 binds to the binding site REDV in the C-terminal portion of V, however, because there is an amino acid change in this binding site in rats, (RGDV rather than REDV) α5β1 might be responsible the effects observed in these experiments. In any case, analysis of the expression of both integrins could be informative.

The response of primary cells to fibronectin splice isoforms

Aside from the finding that more keratinocytes migrate on the alternatively spliced fragments, no other effects of the alternatively spliced isoforms were observed. There are several explanations for this result. It is possible that we did not see differences because we used bacterially expressed proteins. Our fusion proteins differ from intact fibronectins in several important ways. First, these proteins are not full-length fibronectins. They contain only the TypeIII7-TypeIII15 C-terminal portion of fibronectin. It was necessary to exclude the N-terminal portion of the protein because this region contains a large number of intramolecular disulfide bonds that cannot be produced in bacteria. It is possible that sequences in the N-terminus of fibronectin facilitate the functioning of the alternatively spliced isoforms. This seems unlikely given that protease digestion studies have shown the C-terminus of fibronectin to be sufficient for many fibronectin functions. Second, these fusion proteins lack glycosylation again because they are expressed in bacteria. However, it is also unlikely that these differences explain the results. Full-length fibronectins that have been produced in the presence of tunicamycin are synthesized and secreted normally. These proteins are however less stable than glycosylated forms suggesting that glycosylation might have a protective function (Olden, 1978). In any case, deglycosylated fibronectins are functional in adhesion assays (Atherton and Hynes, 1981; Olden, 1978; Paul and Hynes, 1984).
Furthermore, this system has been used with great success in this laboratory to demonstrate a role for the fibronectin Type III13 repeat in stress fiber formation (Bloom et al., 1999). Therefore, this approach in general and this system in particular have yielded interesting information in the past.

The results reported here are in agreement with those of Guan et al. (1990) who assayed the spreading, adhesion, migration, and focal contact formation of cell lines plated on full-length fibronectin splice isoforms. The authors suggested that one reason no differences were observed in their experiments might be that they had used cell lines rather than primary cells. Primary cells that interact with the alternatively spliced isoforms in vivo might be expected to express receptors and specific intracellular proteins appropriate for recognition of and response to the alternatively spliced isoforms. The use of primary cells represents the main difference between the studies described in this thesis and those reported previously. When the work described here was begun, little had been done to address this issue. The results of this work suggest that the use of established cell lines may not account for the failure to observe differences in previous studies. These results are perhaps not so surprising given that plasma fibronectin that lacks EIIIA and EIIIB supports all of the functions that were tested. While looking at these well-established functions was useful for demonstrating the efficacy of the recombinant proteins it may be necessary to look at functions that are not supported by plasma fibronectin in order to see effects of the alternatively spliced isoforms. Finally, it may be necessary to look at the effect of the isolated alternatively spliced domains, since, as discussed in Chapter 1, their activity may be suppressed when they are presented in the context of the intact fibronectin molecule.
Figures and Legends
Figure 2-1: Schematic representation of recombinant fibronectin-fusion proteins.
Open squares represent fibronectin Type III repeats. Black squares represent the alternatively spliced Type III repeats EIIIA and EIIIB. The black rectangle represents V95. The additional 25 amino terminal amino acids of V (V25) are represented by a small gray rectangle. The black dot represents the RGD cell-binding site in Type III10. Note that although only 5 of the recombinant FN proteins are shown, a total of twenty-one proteins were produced. Eight proteins represented all possible combinations of EIIIB, EIIIA, and V and contained a wild-type RGD site. Eight additional proteins were produced that were identical to the first eight but which lacked RGD (RGD'). Four additional proteins included the wild-type RGD but lacked the V25 region. These proteins represented all possible combinations of EIIIB, EIIIA, and V95. V95 RGD protein was also produced.
Figure 2-2: SDS-PAGE analysis of fusion protein purification. Aliquots (30μl) of protein were taken at various stages during the purification process and analyzed on 7.5% SDS-PAGE gels in order to monitor protein purity and yield. Each lane contains the following: molecular weight markers Lane 1) crude lysate Lane 2) material that did not bind to the cobalt column Lane 3) aliquots of successive elutions from the cobalt column Lanes 4-9) material recovered after boiling cobalt beads Lane 10) aliquots of elutions from the glutathione column Lane 11-14). Typically one milligram of fusion protein could be produced by this method. In most cases, both the first and second glutathione fractions had concentrations (100 μg/ml-200 μg/ml) sufficient for use in cell biological assays.
Figure 2-3: Western blot analysis of recombinant fibronectins. Purified fibronectins (0.1μg/lane) immunoblotted with antibodies against total fibronectin, anti-EIIIB, anti-EIIIA, or anti-V region. These antibodies recognize bands ranging from 120-150 kD depending upon the number and type of extra splice regions included.
Figure 2-4: Keratinocytes adhere to recombinant fibronectin splice isoforms.

Human keratinocytes were plated in 96 well dishes coated with 2μg/ml of recombinant fibronectin, 2μg/ml of full-length rat fibronectin, or 2mg/ml of BSA as a negative control. After 1 hour, cells were fixed and stained with crystal violet. Results are expressed as the O.D. 560 of the crystal violet solution released from the stained cells. Values are the average of triplicate wells for each condition. Error bars represent the standard deviation of the three points taken for each condition. This result indicates that the recombinant proteins can promote cell adhesion. However, the alternatively spliced isoforms are no better than V0 at promoting keratinocyte adhesion.
Figure 2-5: Keratinocyte adhesion to recombinant fibronectin isoforms is dose dependent. Keratinocytes were plated on 10, 2, or 1μg/ml of recombinant EIIIb*EIIIA*V* (BAV) fibronectin or recombinant V0. As controls, cells were plated on human fibronectin (hFN) or 2mg/ml of BSA.
Figure 2-6: Dermal fibroblast adhesion to recombinant fibronectin splice isoforms is dose dependent. Dermal fibroblasts were plated on 4,2,1.5, or 1 μg/ml of the different recombinant fibronectins. Cells were plated on 50μg/ml of poly-D-lysine or 2mg/ml of BSA as controls. Five fields per well were counted and these counts were then averaged to obtain the number of adherent cells per field. Values are the average of three experiments with six replicates. Results are expressed as a percentage of the number of cells that adhered in the poly-D-lysine control. The error bars represent the standard deviation between the results for the three experiments. Although adhesion to the fragments is dose dependent, there is no difference in the amount of adhesion of the fibroblasts to the different isoforms. The alternatively spliced isoforms are no better than V0 at promoting adhesion under these conditions.
Figure 2-7: Keratinocytes assemble stress fibers and form focal contacts when plated on recombinant fibronectin splice isoforms. Keratinocytes were plated on 5μg/ml of recombinant fibronectin and allowed to adhere and spread for 3 hours. At the end of the incubation, cells were fixed with paraformaldehyde and stained with rhodamine phalloidin to visualize actin assembly into stress fibers (left panel). Alternatively, cells were stained with an anti-paxillin antibody followed by a rhodamine-conjugated secondary antibody to visualize focal contacts (right panel). V0 and the alternatively spliced isoforms showed no difference in their ability to promote stress fiber assembly or focal contact formation.
Figure 2-8: Analysis of recombinant fibronectin splice isoforms lacking the RGD cell-binding motif. Keratinocytes were plated on coverslips coated with 20μg/ml of recombinant fibronectin lacking the RGD site. Cells were incubated for 4 hours. At the end of the incubation, cells were fixed and stained with an anti-paxillin antibody followed by a rhodamine-conjugated secondary antibody. Very few cells could be found on coverslips coated with BSA or with EIIIB RGD\(^\prime\), EIIIA RGD\(^\prime\), or V0 RGD\(^\prime\). When cells were observed on these coverslips they were unspread. There were also very few cells observed on coverslips coated with RGD\(^\prime\) fragments containing the V region. However, occasionally spread cells were observed on these fragments. A few cells could also spread on V95 RGD\(^\prime\). This suggests that while the majority of the keratinocytes require the RGD site to spread and form focal contacts on the recombinant fibronectins, some cells may be able to adhere and spread using a binding site in the V95 region.
Figure 2-9: Rate of dermal fibroblast focal contact formation on recombinant fibronectin splice isoforms containing or lacking V. Dermal fibroblasts were plated on 10μg/ml of recombinant V or V0 and allowed to adhere and spread for different amounts of time (1 hour, 3 hours, 5 hours, or 20 hours). At the end of each timepoint, cells were fixed and stained with an anti-paxillin antibody followed by a rhodamine-conjugated secondary antibody. Two hundred cells were scored for spreading and focal contact formation and the percentage of spread cells with focal contacts was then calculated. Triplicate coverslips were scored and values are the average of the three counts. Error bars represent the standard deviation between the three values. There was no difference in the rate of focal contact formation on V and V0.
Figure 2-10: Recombinant fibronectin fusion proteins promote keratinocyte migration. Keratinocytes were plated on colloidal particles were precoated with 2mg/ml BSA (left), 3μg/ml of V0 fibronectin (center), or 3μg/ml of EIIIB 'EIIIA+V' (BAV) fibronectin (right). Keratinocytes cleared more area on BAV fibronectin that on V0 suggesting that one or more of the alternatively spliced isoforms enhance keratinocyte migration.
Figure 2-11: The alternatively spliced regions of fibronectin enhance keratinocyte migration. Keratinocytes were plated on colloidal gold coated with 10μg/ml of recombinant fibronectins. As a control, cells were plated on gold coated with 2mg/ml of BSA. Cells were allowed to migrate for 14hrs and the area cleared by each cell was measured. The percentage of cells, which migrated a) and the area cleared by each migratory cell b) were then, calculated for each substrate. Values are the results of one experiment in which triplicate wells were scored. These results are in agreement with one preliminary experiment that shows an increase in keratinocyte migration on BAV versus V0 and with two preliminary experiments that show an increase in keratinocyte migration on V versus V0. Error bars represent the standard deviation between the three values. The percentage of cells that migrated on isoforms containing EIIIB, EIIIA, V, or any combination of these was higher than the percentage of cells which migrated on V0. However, no difference in the area cleared was observed between V0 and the other isoforms. This suggests that the alternatively spliced segments of fibronectin increase the number of keratinocytes that migrate on fibronectin but not the area cleared by each migratory cell.
Figure 2-12: Recombinant fibronectin fusion proteins promote dermal fibroblast migration. Dermal fibroblasts were plated on colloidal gold coated with 10μg/ml of recombinant fibronectins or with 2mg/ml of BSA as a control. Cells were allowed to migrate for 14hrs and the area cleared by each cell was measured. The percentage of migratory cells a) and the distance cleared by each cell b) was then calculated for each substrate. Results are the average of triplicate counts and error bars are the standard deviation between these values. There was no difference in the percentage of migratory cells or the area cleared between the different fragments that were tested.
Chapter 3

The Role of Fibronectin in Myofibroblast Differentiation
INTRODUCTION

As the wound healing process progresses, dermal fibroblasts differentiate into myofibroblasts. Myofibroblasts are contractile cells that are characterized by the expression of $\alpha$-smooth muscle actin. A great deal of evidence shows that the growth factor TGF-$\beta$1 acts to promote the differentiation of myofibroblasts during wound healing (see Chapter 1). There is also some evidence that EIIIA-fibronectin may be required for TGF-$\beta$1 mediated induction of the myofibroblastic phenotype. Experiments examining the differentiation of rat dermal fibroblasts into myofibroblasts revealed that TGF-$\beta$1 induced smooth muscle actin expression in myofibroblasts. Antibodies that recognize the EIIIA splice segment blocked this induction (Serini et al., 1998). In these experiments, plating cells on the isolated EIIIA domain did not induce smooth muscle actin expression. Based on these results, the authors concluded that EIIIA is necessary but not sufficient for TGF-$\beta$1 mediated myofibroblast differentiation.

In order to repeat and extend the results of Serini et al. (1998), I used dermal fibroblasts and the recombinant fibronectin fusion proteins generated in Chapter 2 to assay $\alpha$-smooth muscle actin expression by fibroblasts in response to EIIIA. In order to minimize the effects of endogenous fibronectin on these experiments, I obtained fibronectin-null fibroblasts either from fibronectin chimeras or from ES cells that had been differentiated in vitro. Surprisingly, I found that the regulation of $\alpha$-smooth muscle actin expression in vivo differentiated fibronectin-null fibroblasts was different from the regulation in heterozygous fibroblasts. Specifically, the null cells showed higher basal levels of smooth muscle actin expression than did heterozygous cells suggesting that fibronectin is a negative regulator of smooth muscle actin expression. Furthermore, TGF-$\beta$1 induced an increase in $\alpha$-smooth muscle actin expression in heterozygous but not in fibronectin-null fibroblasts.
MATERIALS and METHODS

Smooth Muscle Actin Immunofluorescence Staining
Dermal fibroblasts (1 x 10^3) were plated on coverslips that had been coated overnight with 5μg/ml of V0 or EIIIA containing recombinant fibronectin fragments. Cells were plated in 2% serum, 0.1% serum, or under serum free conditions and incubated at 37°C. In a fourth experiment, cells were plated in serum free medium on higher concentrations (25mg/ml) of the fibronectin fragments. At various timepoints cells were fixed in 3.7% PF containing Ca^{2+}Mg^{2+} at room temperature for 10min, washed 3x with Ca^{2+} Mg^{2+} PBS and permeabilized in 0.5% IGEPAL (Sigma) at room temperature for 10min. Coverslips were blocked in 10% NGS in PBS Ca^{2+} Mg^{2+} for 30 minutes at 37°C. Cells were stained with a mouse monoclonal antibody against smooth muscle actin 1:1000 (Sigma) for 30 minutes at 37°C. Coverslips were washed 3x by dunking into beakers of PBS and then stained with a rhodamine-conjugated anti-mouse secondary antibody (TAGO) for 30 minutes at 37°C. Coverslips were then dipped in into PBS. After a rinse in dH_{2}O coverslips were blotted dry and mounted face down on a drop of prewarmed Gelvatol. Stained cells were visualized using the 63x objective on the Axiophot fluorescence microscope. In each experiment, one hundred cells were scored per coverslip. Cells with strong α-smooth muscle actin staining were scored as positive.

Fibronectin Immunofluorescence Staining
Dermal fibroblasts (1 x 10^4) were plated on coverslips in 10% fibronectin-depleted serum and incubated at 37°C. After 72hrs cells were fixed in 3.7% paraformaldehyde at room temperature for 10 minutes, washed 3x with Ca^{2+} Mg^{2+} PBS and permeabilized in 0.5% IGEPAL (Sigma) for at room temperature for 10 minutes. Coverslips were blocked in 2mg/ml BSA in PBS Ca^{2+} Mg^{2+} for 30 minutes at 37°C. Cells were stained either with the rabbit polyclonal anti-fibronectin antibody 61.2 at a dilution of 1:200 or with the mouse monoclonal anti-EIIIA antibody 3E2 (Sigma) at a dilution of 1:200 for 30 minutes at 37°C. Coverslips were washed 3x by dunking into beakers of PBS and then stained with a rhodamine-conjugated goat anti-rabbit or fluorescein-conjugated anti-mouse secondary antibody (1:1000) (TAGO) for 30 minutes at 37°C. Coverslips were then dipped into PBS and dH_{2}O, blotted dry, and mounted on a drop of prewarmed Gelvatol. Stained cells were visualized using a 40x objective on the Axiophot fluorescence microscope (Zeiss).
Generation and Characterization of FN-/- chimeras

Fibronectin-null ES cells were generated previously by Dr. Stephen Robinson (see Saoncella et al. 1999) by harvesting blastocysts from matings of FN heterozygous mice (George et al., 1993). The inner cell masses of individual blastocysts were removed and plated in order to obtain ES cells. ES cell clones were screened by polymerase chain reaction (PCR). Two independent null ES cell clones were generated (see Saoncella et al., 1999). To generate chimeric mice, ES cells were cultured and prepared as described by George et al. (1993). ES cells were injected into C57BL6 blastocysts by John Mkandawire in the MIT-CCR transgenic facility and Helen Rayburn (Program of Excellence). Chimeras used for preparation of neonatal dermal fibroblasts were identified by PCR using tail DNA from pups. Briefly, tails (5-10mm pieces) were digested overnight at 55°C in 400µl of Glenn's buffer (50mM KCl, 10mM Tris-Cl, pH 8.3, 2.5 mM MgCl₂, 0.1 mg/ml gelatin 0.45% wt/volume Nonidet P-40, 0.45% wt/volume Tween 20) and 20µg/ml proteinase K. 2.5µl of DNA were mixed with 10x buffer, dNTPs, H₂O, Taq polymerase, and 3 primers (George et al., 1993). Thirty or forty cycles (94°C for 1 minute, 65°C for 2 minutes, 72°C for 3 minutes) were run. The percentage chimerism was determined using a glucose-6-phosphate isomerase assay (see below). Alternatively, the percentage chimerism was estimated from coat color.

Glucose phosphate isomerase assay

The percentage of chimerism was estimated by glucose-6-phosphate isomerase (GPI) assay. Extracts of neonatal lung, liver, and paw were ground up using a mini-pestle (Kontes Co., Vineland,NJ) then lysed by repeated freezing and thawing. After addition of ddH₂O, samples were loaded onto cellulose acetate plates that had been presoaked with 1xTG buffer (10xTG buffer: 30g Tris base, 144g glycine, in 1l ddH₂O). The plates were then loaded into a GPI electrophoresis apparatus (Zip Zone chamber, 1283; Helena Laboratories) and run for 1-2hrs at 150V in order to separate the different strain-specific isozymes of GPI. GPI substrate mix (2mls of 0.2M Tris-HCl, pH 8.0, 0.1ml of 54.1 g/liter magnesium acetate, 0.1ml of 10 mg/ml NADP (Sigma), 0.1ml of 10mg/ml methylthiazolium tetrazolium (MTT) (Sigma), 0.05ml of 2.5 mg/ml phenazine methosulphate (PMS) (Sigma) ) was prepared. Just prior to staining, 8µl of glucose-6-phosphate dehydrogenase in 1.5% melted agarose were added to the substrate mix. The
agarose substrate mixture was then poured onto the plates. Plates were incubated in the dark. After approximately one hour stained bands corresponding to the different isozymes developed on the plates. Plates were then photographed and band intensities on the photographs were determined by densitometric analysis using the IS-1000 digital Imaging System (Alpha Innotech, San Leandro, CA). The percentage of ES cell-derived (129/Sv) isoforms of GPI was calculated by dividing the densitometry units of the upper band (129/Sv) by the total densitometry units of the upper and lower bands (C57BL6) and multiplying by 100%.

**Isolation of Fibronectin-null Dermal Fibroblasts from Fibronectin chimeras**

Dermal fibroblasts were isolated from newborn chimeras and from age-matched wild-type and heterozygous 129Sv/Jae strain mice. Isolation of dermal fibroblasts is described in Chapter 2. Cultures of FN-null dermal fibroblasts were obtained by growing dermal fibroblasts from chimeric mice in selective media containing 600µg/ml of G418. The purity of the cultures was assessed by immunofluorescence staining for FN (see below). Cultures were estimated to be 60-90% pure by this method.

**In vitro differentiation of Fibronectin-null Fibroblasts**

Dr. Steven Robinson generated FN-null fibroblasts. Their production has been described (Saoncella et al., 1999). Briefly, cells were differentiated from two independent clones of FN null ES cells (4D and 5F) and from one clone of FN heterozygous ES cells as a control. Fibronectin-null ES cells were cultured in 1% dimethylsulfoxide and enriched for fibroblastic morphology by passaging the cells several times. Cells were immortalized using a retrovirus to transduce simian virus 40 large T antigen and cloned by limiting dilution. In vitro differentiated fibroblasts were cultured in E4HG with 10% fetal bovine serum supplemented with 2mM glutamine, 100U/ml of penicillin, and 100µg/ml of streptomycin.

**Smooth Muscle Actin Detection by Western Blotting**

Dermal fibroblasts were plated in medium containing serum depleted of fibronectin. After 24 hours the cells were washed 1x with medium and fresh medium was added. For positive controls, 10ng/ml of TGF-β1 (Calbiochem) were added. For some experiments, different serum conditions were tested. For low serum conditions, medium was supplemented with a mix of insulin-transferrin-selenium-X (ISTX) 1:100 (GibcoBRL), 2-
mercaptoethanol 1:1000, and lipid rich bovine serum albumin (AlbuMAX-II) 1:100 (GibcoBRL). After 72 hours, cells were washed 1x with PBS and then lysed on ice in 100\(\mu\)L of lysis buffer (0.2% SDS, 150mM NaCl, 10mM Tris pH 7.4, 2mM EDTA). Lysates were centrifuged for 10 minutes at 13,000. Protein concentrations were determined using the Pierce Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Equal amounts of protein were run on 7.5% SDS–PAGE gels. Proteins were blotted on nitrocellulose. Blots were blocked in 5% BSA, incubated with a mouse monoclonal IgG2a anti-\(\alpha\)-smooth muscle actin antibodies as ascites 1A4 (Sigma) at 1:500, and washed in 0.1% Tween 20 in PBS. Blots were then incubated 1:2000 with sheep anti-mouse IgG conjugated to HRP (JacksonImunoResearch). After a second wash, bound antibodies were visualized using chemiluminescence reagents from New England Nuclear. For some experiments, cells were lysed in 0.1% SDS, 1mM PMSF, 1% DTT, 0.4M Tris-HCl pH 6.8 and 1mM Na-tosyl-L-arginine methyl ester. Equal amounts of lysate were loaded on SDS-PAGE gels. In these cases, vimentin was used as a loading control. Briefly, blots were stripped by placing them in a container filled with stripping buffer (62.5mM Tris (pH 6.8), 2%SDS, 100mM beta mercaptoethanol) and shaken for 30 minutes at 60\(^\circ\). They were then rebotted using a mouse monoclonal IgM anti-vimentin antibody VIM 13.2 (Sigma) to detect vimentin.

In vitro differentiated cell lines were plated at 1x10^4 cells/well in 1% serum depleted of FN. After 24 hours the cells were washed 1x with medium and the medium was replaced with medium containing 1% fibronectin-free serum. As a control, some wells received this same medium with the addition of 10ng/ml TGF-\(\beta\)1 (Calbiochem). After 72h cells were washed 1x with PBS and then lysed on ice in 100\(\mu\)L of lysis buffer (0.2% SDS, 150mM NaCl, 10mM Tris pH 7.4, 2mM EDTA). Lysates were centrifuged for 10 minutes at 13,000. Protein concentrations were determined using the Pierce Micro BCA Protein Assay Reagent Kit (Pierce, Rockford,IL). Equal amounts of protein were run on 7.5% SDS–PAGE gels. Proteins were blotted to nitrocellulose. Blots were blocked in 5% BSA, incubated with a mouse monoclonal IgG2a anti-\(\alpha\)-smooth muscle actin antibody as ascites1A4 (Sigma) at 1:500, washed in 0.1% Tween 20 in PBS, and then incubated with sheep anti-mouse IgG (1:2000) conjugated to HRP (JacksonImunoResearch). After a second wash, proteins were visualized using
chemiluminescence reagents (NEN). As an additional loading control, some blots were stripped by placing them in a container filled with stripping buffer (62.5mM Tris-HCl (pH 6.8), 2% SDS, 100mM beta mercaptoethanol) and shaken for 30 minutes at 60°. Blots were then washed five times with PBS and then rebotted using a mouse monoclonal anti-vimentin IgM antibody VIM 13.2 (Sigma) or a mouse monoclonal IgG1 ascites fluid (clone SAP.4G5) recognizing β-tubulin (Sigma).
RESULTS

The Role of EIIIA Fibronectin in Dermal Fibroblast Differentiation

I wanted to determine whether or not EIIIA could induce the differentiation of dermal fibroblasts into myofibroblasts. In order to address this question, I used immunofluorescence microscopy to examine the expression of a myofibroblast differentiation marker, α-smooth muscle actin, in dermal fibroblasts plated on fibronectins containing or lacking EIIIA. Dermal fibroblasts were plated on 5μg/ml of EIIIA-FN or V0, allowed to adhere and spread for 1, 3, or 9 hours and then stained for α-smooth muscle actin. This experiment was performed under different serum conditions (2% serum, 0.1% serum, or serum free). I chose the serum free and 0.1% serum concentrations because I was concerned that growth factors in the serum might induce α-smooth muscle actin expression and mask any effects of the recombinant fibronectins. However, I was also concerned about the viability of the cells. Therefore, I also plated the cells in 2% serum. The intensity of α-smooth muscle actin staining appeared to be similar on EIIIA and V0. Furthermore, there was little difference in percentage of smooth muscle actin positive cells on the two fragments although there was a slight trend towards higher expression of α-smooth muscle actin on the EIIIA-containing isoform (Figure 3-1 a-c). Previous reports examining α-smooth muscle actin expression in dermal fibroblasts examined later timepoints (e.g. 48hrs). In order, to determine if α-smooth muscle actin expression was different at later time points I performed a second set of experiments under the different serum conditions at 6hrs, 26hrs, and 48hrs. Approximately 50-60% of cells plated on EIIIA-fibronectin, V0, or the ECM protein collagen were strongly positive for α-smooth muscle actin at all of the later timepoints (data not shown). These results suggest that that EIIIA does not affect the expression of α-smooth muscle actin in these cells perhaps because they express high basal levels of this protein.

Some studies have used higher concentrations of EIIIA fusion proteins (20-80μg/ml) to demonstrate effects on cell adhesion (Xia and Culp, 1994; Manabe et al., 1997). In order to determine whether or not a higher concentration of EIIIA was required for induction of α-smooth muscle actin expression, I repeated these experiments using 25μg/ml of the fragments, a five-fold higher concentration than the concentration that I
had used previously (Figure 3-1 d). At the one hour timepoint, the number of α-smooth muscle actin-positive cells on EIIIA-fibronectin was 2-fold greater than on V0. However, by six hours roughly equal percentages of cells on EIIIA or V0 showed intense α-smooth muscle actin staining. By 12 hours almost all of the cells in both conditions showed high levels of α-smooth muscle actin staining. Taken together these results suggest that while EIIIA may increase the rate at which α-smooth muscle actin is expressed by these cells, the cells are able to express high levels of α-smooth muscle actin even in the absence of exogenously added EIIIA.

One possible reason for the similar results on EIIIA and V0 is that endogenous fibronectin deposited by the dermal fibroblasts induces smooth muscle actin expression in these cells. In order to determine whether or not the dermal fibroblasts deposit endogenous fibronectin that contains EIIIA, I stained these cells with antibodies recognizing the EIIIA segment of fibronectin (Figure 3-2). Within 48 hours after plating EIIIA-containing fibronectin fibrils were present on the surface of some of the dermal fibroblasts. Given this result, it seemed possible that the presence of endogenous EIIIA fibronectin in these experiments might account for the expression of smooth muscle actin in the cells.

**Generation and Characterization of Fibronectin Chimeric Mice**

The high basal levels of smooth muscle actin expression in the dermal fibroblasts revealed two difficulties with the design of the experiments described in the previous section. The results of that section may have been uninterpretable because A) a small difference in the amount of smooth muscle actin expressed on EIIIA and on V0 might not be detectable using an immunofluorescence assay or B) endogenous EIIIA fibronectin produced by these cells prior to or during these experiments might affect smooth muscle actin levels. To overcome both of these potential problems, I generated fibronectin-null dermal fibroblasts and used quantitative western blots to examine α-smooth muscle actin levels.

Fibronectin null embryos die during development. Consequently, it is not possible to obtain differentiated adult cells from fibronectin null animals. Therefore, I obtained fibronectin-null cells by generating fibronectin-chimeric mice. Fibronectin null embryonic stem cells (ES) cells were transferred into wild-type (WT) blastocysts (see
Materials and Methods). The resulting chimeric mice were comprised of a mixture of fibronectin genotypes. Some of these cells would be expected to arise from the fibronectin-null ES cells and would therefore be genetically null for fibronectin. Because the fibronectin-null ES cells carry the gene for neomycin-resistance, it is possible to select these cells from a mixture of null and wild-type cells.

In order to identify chimeric pups, I performed diagnostic PCR on a 5mm sample of the tail from each pup (Figure 3-3). In order to obtain an estimate of the percent chimerism of the animals, I isolated various organs or tissues from the pups and prepared them for GPI analysis (See Materials and Methods). This analysis showed that fibronectin-null cells were able to contribute to the liver, lung, and paw (Figure 3-4). The percentage chimerism estimated by this method ranged from 25% to 70%.

**Isolation of Fibronectin-Null Dermal Fibroblasts**

Skin was isolated from newborn pups and used for the preparation of dermal fibroblasts. In order to obtain pure populations of fibronectin-null dermal fibroblasts we selected the cells with the drug G418 (see Materials and Methods). Diagnostic PCRs revealed that G418 selection enriched the neo band that is present only in the fibronectin-null cells (Figure 3-5). In order to estimate purity of the selected cultures, I used immunofluorescence to visualize fibronectin on the surface of the cells after selection (Figure 3-6). Although results varied between cultures, I estimated that the purity of the cultures ranged from 60-90% fibronectin-negative cells. The amount of contamination varied between animals, but not from preparation to preparation from the same animal. Different vials of cells from the same pup routinely gave 90% pure cultures while the cultures from another pup always gave cultures that were 60% pure. This is probably due to differences in the percentage of chimerism of the animals. Although this level of contamination was less than ideal (see Discussion), these cultures contained a very low level of fibronectin compared with wild-type cultures.

**Differentiation Assays with Dermal Fibroblasts Lacking Fibronectin**

With these cells in hand, I performed a preliminary experiment to test the hypothesis that interaction with EIIIA fibronectin induces the upregulation of α-smooth muscle actin in these cells. I grew wild-type or fibronectin-null dermal fibroblasts on three different substrates, tissue culture plastic, EIIIA-fibronectin, or V0 for 72 hours.
Each condition was repeated in the presence of TGF-β as a positive control for α-smooth muscle actin induction. In this experiment we found that, as expected, TGF-β upregulated α-smooth muscle actin in wild-type dermal fibroblasts on all three substrates tested. The fibronectin-null cells yielded unexpected results. These cells expressed high levels of smooth muscle actin in the presence or absence of TGF-β, suggesting that, in the absence of fibronectin, α-smooth muscle actin is induced in these cells. This was true on all of the different substrates tested (Figure 3-7). Unfortunately, I was not able to repeat these results in an independent experiment. In the second round, null cells still expressed high levels of α-smooth muscle actin in a TGF-β independent manner, but the wild-type control cells also produced high levels of α-smooth muscle actin expression in the presence and absence of TGF-β (Figure 3-8). Consequently, the result with the null cells was uninterpretable. In an attempt to reproduce the initial result, I performed an experiment to compare different plating densities and serum concentrations for the ability to restore normal regulation of α-smooth-muscle actin expression in the wild-type cells. However, under the conditions examined in this experiment, wild-type dermal fibroblasts failed to show the expected regulation of α-smooth muscle actin by TGF-β (Figure 3-9).

**Differentiation Assays with in vitro differentiated fibronectin-null cells**

Because of contamination issues and the failure of wild-type control dermal fibroblasts to behave as expected, I decided to begin using fibronectin-null fibroblasts that had been differentiated in vitro for these experiments (see Materials and Methods). Although the dermal fibroblasts are primary cells and therefore may be more representative of the in vivo situation, there were some advantages to using the in vitro differentiated cells. Isolation of null dermal fibroblasts required selection in order to get rid of the contaminating wild-type cells. In contrast, the in vitro differentiated cells were prepared from FN-null ES cells. Immunofluorescence staining showed that even without selection, these cells lacked fibronectin (Figure 3-10). This eliminated the need for selection and therefore, the concern that selection in G418 would affect the health of the cultures or influence the induction of α-smooth muscle actin.

In order to investigate the expression of α-smooth muscle actin in the fibronectin-null and heterozygous cells, we obtained two fibronectin-null cell lines which were derived from two independent fibronectin-null ES cell clones and one heterozygous cell
line derived from heterozygous ES cells. I used immunofluorescence to visualize α-smooth muscle actin in these cells (Figure 3-11). Heterozygous cells showed little staining for α-smooth muscle actin. However, the two independent fibronectin-null cell lines appeared to express α-smooth muscle actin. This difference in basal expression levels seemed interesting given that I had obtained a similar result in my initial experiments using the dermal fibroblasts. Therefore, in order to extend these results, I used western blotting to compare the levels of smooth muscle actin expressed by the null and heterozygous in vitro differentiated fibroblasts (Figure 3-12). I found that fibronectin-null fibroblasts expressed higher levels of smooth muscle actin than did fibronectin heterozygous cells. Furthermore, while heterozygous cells could be induced to increase their expression of α-smooth muscle actin when stimulated with TGF-β, fibronectin-null cells expressed similar levels of α-smooth muscle actin in the presence or absence of TGF-β (Figure 3-12).
DISCUSSION

Myofibroblasts play a critical and beneficial role in wound closure after injury. However, these cells also arise in various pathological situations and this leads to tissue damage caused by excessive tissue contraction. An understanding of the molecular mechanism governing the differentiation of these cells may eventually give rise to strategies to improve wound healing and to prevent pathological proliferations of myofibroblasts. Various studies have implicated TGF-β1 and EIIIA-fibronectin in myofibroblast differentiation. In order to test the hypothesis that EIIIA promotes the differentiation of dermal fibroblasts into myofibroblasts, the expression of α-smooth muscle actin by fibronectin-null and wild-type fibroblasts plated on EIIIA and V0 was investigated by immunofluorescence and western blotting.

These studies yielded several observations and results. First, it was observed that fibronectin-null cells are able to contribute to many different tissues in the fibronectin chimeric animals. Second, high percentage chimeras could be obtained and these animals appeared to be normal. Third, no differences in the expression of α-smooth muscle actin by dermal fibroblasts was observed when these cells were plated on EIIIA and V0 and analyzed by immunofluorescence. Finally, fibronectin-null fibroblasts differentiated in vitro exhibited phenotypic differences when compared with fibronectin-heterozygous control cells. The null cells express higher basal levels of α-smooth muscle actin than do heterozygous control cells. Furthermore, while TGF-β increased the expression of α-smooth muscle actin in the heterozygous cells addition of TGF-β to the null cells did not further increase the high levels of TGF-β expressed by these cells.

Fibronectin Chimeras Appear Normal

The observation that fibronectin-null chimeras appear normal was somewhat surprising given the fact that fibronectin-null embryos die during development. A likely explanation for the observation that high-percentage fibronectin-null chimeras are viable is that fibronectin is a secreted molecule. Therefore, fibronectin produced by wild-type cells is available to the null cells. A knockout of plasma fibronectin has been created by crossing floxed fibronectin mice with mice expressing Cre under the control of the interferon and polyinosinic-polycytidic acid (pI-pC) inducible Mx promoter (Mx-Cre). Injection of pI-pC into progeny from these crosses resulted in Mx-Cre mediated deletion.
of fibronectin in the liver. Three days after injection, these mice lacked detectable levels of plasma fibronectin. The mice appear normal and are fertile (Sakai et al., 2001). Given these results it is clear that plasma fibronectin is dispensable in adult animals. It is also possible that upregulation of fibronectin by wild-type cells occurs in the chimeras and that this accounts for the viability of high-percentage chimeras.

Coat color provides an estimate of percent chimerism, but this method may overestimate the contribution of null cells in tissues whose development or maintenance require fibronectin. We did not look closely at the contribution of null cells in different regions of the body. However, such analysis is possible and has been performed in alpha5 integrin chimeras using immunohistochemistry (Taverna et al., 1998a; Taverna et al., 1998b). The expression of fibronectin in the fibronectin chimeras could be analyzed by in situ hybridization. Alternatively, one could track the cells arising from the FN-null ES cells by transfecting the ES cells with DNA encoding markers such as lacZ or GFP.

Finally, it is possible that we have overlooked some subtle effects of the loss of fibronectin. Closer analysis of the mice described above which lack plasma fibronectin showed subtle defects including an increase in neuronal apoptosis and larger infarction areas after transient focal cerebral ischemia. In addition, analysis of the muscles of α5 integrin chimeric mice revealed signs of muscular dystrophy (Taverna, et al. 1998a). One might expect to see similar defects in the fibronectin chimeras. It was observed that the fibronectin chimeras seemed generally larger than wild-type mice and that they had noticeable deposits of fat. However, a careful comparison of the size and fat content of the chimeras and wild-type mice has not been performed. Given that fibronectin inhibits adipocyte differentiation (Kamiya et al., 2002; Spiegelman and Ginty, 1983), it might be worthwhile to perform such a comparison.

**Differentiation of Dermal Fibroblasts**

Studies using wild-type dermal fibroblasts suggested that there was no difference in the amount of α-smooth muscle actin expressed by these cells when grown on EIIIA as compared with V0. Interpretation of these results was complicated by the fact that the wild-type dermal fibroblasts produce EIIIA fibronectin. To address these problems fibronectin-null dermal fibroblasts were isolated and their expression of α-smooth muscle actin was analyzed by western blotting. In most experiments, cells from both chimeric
and wild-type cultures expressed high levels of α-smooth muscle actin in the presence or absence of TGF-β. Thus both the immunofluorescence assays and the western blotting assay suggest that there are high background levels of smooth muscle actin present in the dermal fibroblasts and this probably complicates analysis of α-smooth muscle actin induction in these cells.

Serini et al. (1998) used human dermal fibroblasts from explants of adult tissue to show that upregulation of α-smooth muscle actin in these cells is dependent on EIIIA. The experiments described here used cells from neonatal mice. Neonatal mice were used because these animals do not yet have coats. Coat hairs hinder the isolation of the dermal cells. Since fetal wound healing is faster than wound healing in adults and does not lead to scarring, it is conceivable that the stage at which the cells were taken (neonatal versus adult) accounts for the differences observed in these assays. The neonatal cells may be more similar to fetal cells than to adult cells in the expression of molecules involved in wound healing. Mice develop hair 2-3 days after birth. Therefore, it might be interesting to compare cells taken from neonates with those taken from animals that are a 1-2 days older. It might also be worthwhile to isolate fibroblasts from older mice and assay their responses to TGF-β. It is also possible that species-specific differences between humans and mice might explain the differences in the results.

Induction of α-smooth muscle actin in dermal fibroblasts was observed in an initial experiment. This suggests that, under some conditions, it is possible to see the expected response to TGF-β using these cells. (Masur et al., 1996) found that plating density regulates the myofibroblast phenotype and that cultures plated at low densities produce more myofibroblasts than do those plated at high densities. Furthermore, they found that addition of TGF-β to subconfluent cultures, but not to confluent cultures induces the myofibroblast transition. In addition, low cell density was shown to correlate with increased functional expression of TGF-β receptors and receptor signaling (Petridou et al., 2000). Although plating density was investigated as a possible factor in the conflicting observations reported here it might be worthwhile to test a wide range of densities at which to maintain the cells.

The selection of FN-null cells from chimeric cultures proved more difficult than expected as a number of contaminating fibronectin-positive cells persisted even after
selection in G418. It may be possible to obtain pure cultures by altering the dose of G418 or by using a cell-sorting system to remove the fibronectin-positive contaminants. We have used such a system to selectively isolate endothelial cells using anti-ICAM-2-coated magnetic beads (See Appendix 2).

**Novel Phenotype of Fibronectin-Null Fibroblast Lines**

Experiments using the *in vitro* differentiated fibroblasts yielded unexpected results. Both clones of the fibronectin-null fibroblasts express higher basal levels of $\alpha$-smooth muscle actin than did heterozygous cells. Furthermore, while TGF-$\beta 1$ induces an increase in smooth muscle actin expression in heterozygous cells TGF-$\beta$ had no effect on the levels of $\alpha$-smooth muscle actin produced by the null cells. There are at least three models that would explain these results. First, fibronectin might act as a repressor of smooth muscle actin expression. Second, loss of fibronectin might radically alter the type of cells that are isolated during the *in vitro* differentiation of the ES cells. This could occur as a result of selection for a subpopulation of cells that survive in the absence of fibronectin. This could also occur if fibronectin normally blocks the differentiation of ES cells into myofibroblastic or smooth muscle cells. Third, loss of fibronectin might cause the induction of factors that induce expression of $\alpha$-smooth muscle actin (e.g. TGF-$\beta$). There is some evidence for the first two models since fibronectin protects cells against apoptosis (Frisch et al., 1996; Hungerford et al., 1996; Ilic et al., 1998; Xu et al., 1996) and has also been shown to block the differentiation of a number of different cell types including myoblasts, keratinocytes, chondrocytes, and adipocytes (reviewed in Adams and Watt, 1993). Clearly, it will be important to determine whether the reintroduction of fibronectin into this system can rescue this phenotype. Such studies may help to provide support for one or another of these models. Preliminary experiments show that plating the null cells on exogenous fibronectin does not restore the wild-type phenotype suggesting that the null cells are fundamentally different than the heterozygous cells (see Appendix 1). However, more experiments related to rescue of this phenotype are needed.

A more thorough characterization of the markers expressed by the null and wild-type cells might also help sort out whether or not the null cells have differentiated into a different cell type. Additional smooth muscle markers such as smooth muscle-myosin,
calponin, and SM22α (Duband et al., 1993; Gimona et al., 1990) and myofibroblast markers such as collagen I and SM22α (reviewed in Hautmann et al., 1999; Scrinia and Gabbiani, 1999) could be used to determine whether or not the fibronectin-null cells showed additional characteristics of these cell types.

**Altered response of fibronectin null cells to TGF-β1**

One of the most intriguing findings of this study was that fibronectin-null and wild-type cells differ in their responses to exogenously added TGF-β. While the heterozygous fibroblasts were induced to express α-smooth muscle actin in response to TGF-β, expression of α-smooth muscle actin by the null cells was unaffected by the addition of TGF-β. This raises the interesting possibility that fibronectin-null cells upregulate TGF-β. If this were the case, addition of exogenous TGF-β might fail to induce an increase in α-smooth muscle actin in the null cells because a maximal response to TGF-β has already been reached. Indeed, the level of α-smooth muscle actin expression in null cells is similar to that in the wild-type cells stimulated with exogenous TGF-β. Since one of the functions of TGF-β is to increase deposition of fibronectin it is plausible that cells might respond to reduced fibronectin levels by upregulating TGF-β. Consequently, fibronectin-null cells, which obviously cannot respond to a demand for increased fibronectin production, might be expected to constitutively produce TGF-β.

This is a particularly attractive hypothesis given two observations concerning fibronectin, TGF-β, and tumorigenesis. First, loss of fibronectin from the surface of cells correlates with oncogenic transformation (reviewed in Plantefaber and Hynes, 1989) and overexpression of fibronectin in some cell lines can suppress their motility and growth in vivo (Akamatsu et al., 1996). Second, many tumors show an increase in the production of TGF-β that is postulated to enhance tumorigenesis and metastasis by increasing angiogenesis, suppressing host immunosurveillance, and promoting epithelial to mesenchymal transition (reviewed in Derynck et al., 2001). Upregulation of TGF-β by cells that have lost the ability to produce fibronectin might provide a link between these two observations. In one study, reintroduction of the von Hippel-Lindau (pVHL) tumor suppressor into a renal carcinoma cell line that overproduced TGF-β and lacked pVHL led to a decrease in TGF-β1 synthesis (Ananth et al., 1999). Interestingly, fibronectin has been shown to bind intracellularly to pVHL and disruption of this interaction leads to loss
of fibronectin from the cell surface (Ohh et al. 1999). Therefore, it is possible that in this system pVHL regulates TGF-β synthesis indirectly via its effects on fibronectin assembly. Clearly, more experiments will be needed to address the role of TGF-β signaling in fibronectin null cells (see Chapter 4).

The experiments reported here have uncovered an interesting and novel role for fibronectin, namely negative regulation of α-smooth muscle actin expression. The implications of this result allow us to make testable models about the effect of loss of fibronectin on cell differentiation and gene expression (e.g. of TGF-β). Finally, these results have important implications for a preexisting model that EIIIA-fibronectin induces myofibroblast differentiation. Although these results and that model are not mutually exclusive, reconciling the two will require some variation of this original hypothesis (see Chapter 4).
Figures and Legends
Figure 3-1: Dermal fibroblast differentiation on EIII A and V0 fibronectin. Dermal fibroblasts were plated on coverslips coated with 5μg/ml of EIII A or V0 fibronectin fragments in 2% serum a) 0.1% serum b) serum-free medium c) or on 25μg/ml of EIII A or V0 in serum-free medium d). Cells were incubated for different amounts of time (1 hour, 3 hours (a-d) and 6 hours (d) or 9 hours (a-c)). Cells were then fixed, stained with a mouse monoclonal antibody to α-smooth muscle actin followed by a rhodamine-conjugated secondary antibody, and scored for the presence of α-smooth muscle actin. Each graph represents the result of one experiment. In each experiment, duplicate coverslips were scored and results are the average of these counts. At the lower concentration, there was no difference in α-smooth muscle actin staining on EIII A and V0. At the higher concentration, the rate at which α-smooth muscle actin staining appeared was increased relative to V0 suggesting that EIII A upregulates smooth muscle actin.
Figure 3-2: Dermal fibroblasts produce EIII A fibronectin. Dermal fibroblasts were grown in culture for 72 hours and then fixed and stained for with a primary anti-EIII A antibody followed by a fluorescein -conjugated secondary antibody. In the left panel, no 1st antibody was used. Cells in the center and right panels are stained with anti-EIII A.
Figure 3-3: Identification of chimeric pups by PCR. Tail DNA was taken from pups born to the host mothers of embryos that had been injected with FN-null ES cells. A band of 1060bp indicates the presence of a targeted allele containing the neomycin (neo) resistance gene. The 900bp band results from the presence of the wild-type allele of fibronectin. White arrows indicate that neo bands appeared in two of the PCR reactions from animals in this litter.
Figure 3-4: Glucose phosphate isomerase assay of tissues from fibronectin chimeras. Tissues from chimeras were prepared and samples were run on cellulose acetate plates to separate glucose phosphate isozymes. Chimeric animals (Sv/Jae129/C57BL6 background) were compared with wild-type Sv/Jae129 controls to assess the percentage chimerism in different tissues.
Figure 3-5: PCR analysis of dermal fibroblast cultures. DNA was isolated from dermal fibroblasts of heterozygous, wild-type, or chimeric animals grown in the presence (selected) or absence (unselected) of G418. A band of 1060bp indicates the presence of a targeted allele containing a neomycin resistance cassette. The 900bp band arises from the presence of the wild-type fibronectin allele. This result indicates that G418 enriched the cultures for fibronectin-null cells.
Figure 3-6: Fibronectin staining of chimeric dermal fibroblast cultures after selection. Heterozygous (left) or fibronectin-chimeric (right) cultures were grown for 72 hours in medium containing 600μg/ml of G418 and 10% serum from which fibronectin had been depleted. Cells were then stained with an anti-fibronectin antibody followed by a rhodamine-conjugated secondary to visualize fibronectin matrix. G418 selection enriches these cultures for fibronectin-null fibroblasts.
Figure 3-7: Expression of smooth muscle actin in dermal fibroblasts on different fibronectin substrates. Wild-type or fibronectin-null dermal fibroblasts were plated in 0.1% serum on tissue culture plastic (-), recombinant EIII A fibronectin (EIII A), or recombinant fibronectin lacking EIII A (V0). One day after plating, fresh serum-free medium containing TGFβ (10 ng/ml) or lacking TGFβ was added to the cells. After 72 hours, cells were lysed and lysates were run on a SDS-PAGE gel. Proteins were transferred to nitrocellulose filters. Filters were blotted for α-smooth muscle actin or vimentin as a loading control. While wild-type cultures upregulated α-smooth muscle actin in response to TGFβ, chimeric cultures (selected inG418) expressed high levels of α-smooth muscle actin in the presence and absence of TGFβ.
Figure 3-8: Expression of $\alpha$-smooth muscle actin in wild-type and chimeric (G418 selected) dermal fibroblasts. $3 \times 10^4$ wild-type or fibronectin-null dermal fibroblasts were plated on tissue culture plastic. One day after plating, fresh medium containing 1% fibronectin-free serum and containing or lacking TGF-\(\beta\) (10ng/ml) was added to the cells. After 72 hours, cells were lysed and equal amounts of protein were run on a SDS-PAGE gel. Proteins were transferred to nitrocellulose and blotted with an antibody to $\alpha$-smooth muscle actin. $\alpha$-smooth muscle actin levels were determined by densitometry and the data are expressed in densitometry units. Black bars represent $\alpha$-smooth muscle actin levels in cells treated with TGF-\(\beta\) while striped bars represent the levels in untreated cells. Values are the average of four independent experiments (wild-type cells) or three independent experiments (null cells). Error bars represent the standard deviation between values obtained in the experiments. These data show that neither wild-type, nor fibronectin-null dermal fibroblasts upregulate $\alpha$-smooth actin in response to TGF-\(\beta\). Furthermore, fibronectin-null dermal fibroblast express high levels of $\alpha$-smooth muscle actin suggesting that fibronectins are not required for $\alpha$-smooth muscle actin expression in these cells.
Figure 3-9: α-Smooth muscle actin expression in wild-type dermal fibroblasts plated at different densities and under different serum conditions. Cells were grown in different serum conditions (0.1% or 1% serum) and at different densities (10^5 cells/well in a 24 well dish or 3x10^4 cells/well in a 24 well dish) for 72 hours with or without 10ng/ml of TGF-β. α-Smooth muscle actin expression was measured by Western blotting. α-smooth muscle actin levels were determined by densitometry of bands on the Western blots. Black bars represent α-smooth muscle actin levels in cells treated with TGF-β while striped bars represent the levels in untreated cells. These values represent the results of one experiment performed in triplicate and error bars are the standard deviation between the three values. No differences in the levels of α-smooth muscle actin were observed between the different conditions tested.
Figure 3-10: Fibronectin staining of in vitro differentiated fibroblasts. Heterozygous (left) or fibronectin-null (right) cells were grown for 72 hours in medium containing 10% serum from which fibronectin had been depleted. Cells were then stained with anti-fibronectin antibody followed by a fluorescein-conjugated secondary antibody to visualize the fibronectin matrix.
Figure 3-11: α-smooth muscle actin staining of *in vitro* differentiated fibroblasts.

Heterozygous (left) or fibronectin-null (center and right) cells were grown for 72 hours in medium containing 10% serum from which fibronectin had been depleted. Cells were then stained with an anti-α-smooth muscle actin antibody followed by a rhodamine-conjugated secondary antibody to visualize α-smooth muscle actin.
**Figure 3-12: Smooth muscle actin levels in fibronectin-null and heterozygous fibroblasts.** Null and heterozygous cells were plated in 1% serum. After 24 hours, some cells were lysed for the T=0 timepoint (gray bars) while others received fresh medium with or without 10ng/ml of TGFβ. After 72 hours, cells were lysed and lysates were electrophoresed on SDS-PAGE gels. Proteins were transferred to nitrocellulose and Western blotting for α-smooth muscle actin was performed. α-Smooth muscle actin levels were quantitated using densitometry and values are expressed in densitometry units. Black bars represent α- smooth muscle actin levels in samples treated with TGF-β1 for 72 hours and striped bars represent untreated cells. Values given for the heterozygous clone and the null clone 5F represent the average of 4 independent experiments. For the null clone 4D the data are the average of three independent experiments. Error bars are the standard deviation of the values obtained in the three experiments. Null fibroblasts showed higher levels of smooth muscle actin staining than their heterozygous counterparts. While heterozygous cells upregulated α-smooth muscle actin in response to TGFβ, fibronectin-null cells did not appear to respond to TGFβ.
Chapter 4

Conclusions and Future Directions
Fibronectins play an important role in many fundamental processes. Although there is a wealth of information about the functions of fibronectins in general, very few functions have been attributed to specific alternatively spliced isoforms of fibronectin. Despite this fact, research on the splice isoforms suggests some intriguing possibilities about their functions. The expression of these isoforms is spatially and temporally regulated. While fibronectins lacking the alternatively spliced isoforms are present at high levels in the connective tissues of many parts of the body in adult organisms, the alternatively spliced isoforms make their appearance during dynamic processes such as development and wound healing during which tissues are rapidly changing and growing. Indeed one of the most fascinating aspects of fibronectin biology is the ability of these molecules to present themselves in different forms for different situations. Furthermore, this observation immediately raises questions about the functional significance of the alternatively spliced domains. Clearly, the functions necessary for stability and maintenance of structures, which is desirable in adult animals, are different from the functions that would be desirable in developing tissues. This suggests that alternative splicing of fibronectin is a sort of switch that allows one gene to produce different proteins depending on whether stability or growth is required.

The work described in this thesis addresses some of the hypotheses about the function of fibronectin splice isoforms. Thematically, the different sections of this work are related in that they dwell on the potential functions of the alternatively spliced isoforms in wound healing and development. Specifically, three different hypotheses were investigated. First, the role of the alternatively spliced isoforms in the migration of keratinocytes and dermal fibroblasts was examined. Second, the ability of EIIIA-fibronectin to promote the differentiation of fibroblasts into α-smooth muscle actin expressing myofibroblasts was tested. Finally, a third hypothesis, namely that the EIIIA splice segment is important for the differentiation of mural cells during blood vessel development, was proposed. These studies therefore have implications not only for the role of fibronectin during wound healing but also during embryonic development.

This work has produced several interesting results. First, the alternatively spliced isoforms of fibronectin were found to promote keratinocyte migration. Second, fibronectin null cells were found to express constitutively high levels of α-smooth muscle
actin. Furthermore, these cells did not show normal responses to TGF-β. The following sections contain a discussion of experiments that could be done in order to extend these results. First, experiments to probe the mechanism by which fibronectin isoforms modulate keratinocyte migration are proposed. Second, experiments to test the hypothesis that loss of fibronectin induces production of TGF-β are described. Third, the general consequences of loss of fibronectin are also discussed. Fourth, the implications of these results will be examined in the context of an established model of myofibroblast differentiation. Finally, the implications of the recent generation of EIIIA and EIIIB null mice by two different groups (Dr. E. George and Dr. K. Sekiguchi personal communication) will be discussed.

Models to explain the effects of fibronectin splice isoforms on keratinocyte migration

The percentage of keratinocytes that migrated on fragments containing the alternatively spliced segments was greater than the percentage of cells that migrated on the V0 isoform which lacks these segments. Furthermore, inclusion of any one of the recombinant splice isoforms could promote migration. This raises questions regarding the mechanism by which these fragments exert their effects. In general there are two models used to describe the mechanism by which the alternatively spliced isoforms of fibronectin might modulate fibronectin function. The first model is that receptors on the cell surface specifically recognize the alternatively spliced segments and that receptor binding leads to changes in cell signaling and behavior. The second model is that inclusion of an alternatively spliced segment alters the conformation of fibronectin such that another site within the protein (e.g. RGD) can interact with its receptor in a manner that favors the initiation of a particular cellular response. In order to differentiate between these two models in the keratinocyte migration system, one could repeat these experiments using mixed substrates of V0 which would provide a wildtype RGD binding site and either EIIIA RGD, EIIIB RGD, or V RGD. If specific receptors on the surface of keratinocytes recognize the splice segments, one would expect to observe a higher percentage of migratory cells on the mixed substrates than on V0 alone. However, if the segments exert their effects by inducing a structural change and, more specifically, a change that affects binding to another site within the molecule, one would expect
migration on V0 and on the mixed substrates to be similar. This general approach might prove useful when attempting to determine whether or not the effect of inclusion of a particular splice isoform is direct or indirect.

**Experiments with fibronectin-null cells, chimeras, and embryos**

Our finding that fibronectin-null cells show constitutive expression of α-smooth muscle actin suggests that fibronectin-null cells may exhibit fundamentally different phenotypes than do wild-type cells. This is perhaps not surprising given the diversity of functions regulated by fibronectins, the defects seen in fibronectin-null mice, and the loss of fibronectin matrix from the surface of transformed cells. If fibronectin-null cells are to be used in future investigations of the functions of fibronectin splice isoforms it will be important to carefully characterize them with respect to normal cells. Our observations do suggest however, that fibronectin-null cells might be useful in investigating the consequences of loss of fibronectin at the single cell level. Studies of tumor models have suggested that loss of fibronectin enhances tumor growth and motility. Therefore, the growth patterns and motility of the fibronectin-null and wild-type cells could be compared in order to address some of these observations. Other assays for tumorigenesis including the ability to grow in suspension or the ability to form colonies in soft agar could also be tested. Finally, it might be interesting to perform expression-profiling studies on fibronectin-null and wild-type cells. The results described here would predict that α-smooth muscle actin would be upregulated in the null cells. Furthermore, other differences identified by this method might shed light on the mechanism by which upregulation of α-smooth muscle actin occurs. It should also be possible to isolate other fibronectin-null cell types such as endothelial cells and look at the effect of loss of fibronectin on gene expression in these cells.

One possible explanation for the observation that fibronectin-null cells express high levels of α-smooth muscle actin is that fibronectin commonly inhibits their terminal differentiation. If this is the case, one might expect null embryos to show increased expression, altered localization, or aberrant temporal regulation of α-smooth muscle actin expression. α-Smooth muscle actin expression is first observed during early vascular development and is upregulated after the formation of the heart tube (McHugh et al., 1991). Since fibronectin null embryos reach the stage of heart tube formation, it should
be possible to examine smooth muscle actin expression in the null embryos by immunostaining of embryos or by western blotting of extracts from whole embryos.

If expression of α-smooth muscle actin in mural cell precursors is similarly inhibited by forms of fibronectin lacking EIIIA, this might suggest that the expression of EIIIA around blood vessels serves to stabilize the expression of α-smooth muscle actin in these peri-vascular cells. This in turn might be important for maintaining the stability of these vessels. Therefore, one might expect EIIIA- fibronectin to stabilize blood vessels while other forms of fibronectin might be more conducive to destabilization and angiogenesis. It would be interesting to test this hypothesis and we have begun setting up a system to do so (see Appendix 2).

The TGF-β Pathway in fibronectin-null cells

One model proposed to explain the constitutive expression of α-smooth muscle actin by fibronectin-null cells is that loss of fibronectin causes an increase in TGF-β synthesis or signaling. As described in Chapter 3, such a model would have important implications for the process of tumorigenesis. In order to investigate this model one might examine the levels and activation state of TGF-β and components of its signaling pathways in the null and heterozygous fibroblasts. TGF-β1 exerts its effects via binding to cell surface receptors. TGF-β binding induces receptor phosphorylation and phosphorylated receptors in turn bind to and phosphorylate intracellular molecules known as SMADS (reviewed in Heldin et al., 1997). Once phosphorylated, the receptor binding SMADS (R-SMADS) form complexes with SMAD4. These complexes can bind DNA and activate transcription of TGF-β target genes. TGF-β has also been shown to signal via at least one other signaling pathway, the MAP kinase pathway. Specifically, TGF-β activates ERK, JNK, and p38 MAP kinases (Atfi et al., 1997; Frey and Mulder, 1997; Hannigan et al., 1998; Hocqvar et al., 1999; Mucsi et al., 1996). The levels of TGF-β1 synthesis, TGF-β receptor molecules, effector SMAD phosphorylation, and MAP kinase phosphorylation in null and heterozygous cells could be determined by Western blotting. The addition of TGF-β neutralizing antibodies to cultures of null cells might also be useful in testing this hypothesis. It would also be interesting to see if conditioned medium from the fibronectin-null cells affects the heterozygous cells and vice versa. If TGF-β is expressed and activated in the fibronectin-null cell cultures one would expect
null conditioned medium to induce differentiation of the heterozygous cells while heterozygous cell medium would be expected to have no effect on the null phenotype. Finally, one could extend these results with in vivo experiments. For example, one might expect the fibronectin chimeras to have higher levels of TGF-β than do wild-type mice. It should be fairly straightforward to measure TGF-β levels in the blood of high percentage fibronectin-null chimeras and in whole cell extracts of fibronectin null embryos by Western blotting.

**Models of Myofibroblast differentiation**

Finally the role of EIIIA fibronectin in dermal fibroblast differentiation remains to be addressed. Although the use of fibronectin null fibroblasts is problematic, it is probably worthwhile to revisit this question using the in vitro differentiated heterozygous fibroblasts. Unlike the wild-type dermal fibroblasts, these cells showed the expected induction of α-smooth muscle actin in response to TGF-β. Clearly, the presence of endogenous fibronectin in this system must be taken into account when interpreting results, but these cells do at least demonstrate the ability to modulate α-smooth muscle actin expression in response to a physiological signal.

The results presented in this thesis suggest that EIIIA fibronectin can promote a process (e.g. differentiation) while other forms of fibronectin actively inhibit the same process. This observation raises the following interesting questions: How do cells regulate the opposing activities of fibronectins containing and lacking EIIIA and how can inclusion of EIIIA into fibronectin change it from an inhibitor of differentiation into a factor that promotes differentiation. Clearly, one mechanism by which these activities could be regulated would be via the regulation of EIIIA expression. As discussed previously, expression of EIIIA is highly regulated both spatially and temporally. Support for the idea that spatial regulation of EIIIA plays a role specifically during differentiation processes comes from studies of condensation during chondrogenesis in the developing chicken. During this process mesenchymal cells condense and then differentiate to form cartilage. In both chicken and mouse embryos, EIIIA is included in the fibronectin that is expressed during condensation. Immediately following condensation, there is a drop in EIIIA to undetectable levels while expression of fibronectins lacking EIIIA persists (Gehris et al., 1996). This suggests that in this
situation the activity of EIIIA is controlled by spatial and temporal regulation of expression. However, studies of fibronectin alternative splicing during wound healing suggest that the ability of EIIIA to promote differentiation may be dominant to the ability of fibronectin which lacks EIIIA to block differentiation since both forms of fibronectin are present in wounds. Alternatively, it may be that the relative levels of EIIIA-containing and EIIIA-lacking isoforms in determine whether or not differentiation will be favored in a particular situation.

As discussed previously, various models can be proposed to explain the mechanism by which EIIIA could alter the function of fibronectin. First, EIIIA might interact with receptors on the surface of cells and thereby induce changes in the cytoskeleton or in signaling. Second, EIIIA might shift the conformation of the fibronectin molecule thus disrupting interactions needed for inhibitory activity. There is some support for the second model. Based on their finding that EIIIA appears to enhance RGD binding to α5β1, Manabe et al. (1997) have proposed that inclusion of EIIIA can affect fibronectin functions by altering its conformation. Furthermore, conformational changes in fibronectin have been shown to relieve the inhibitory effect of fibronectin on myoblast differentiation (Garcia et al., 1999). Addition of a soluble peptide containing the TypeIII14 repeat of fibronectin which inhibits FN-fibril formation, (Kamiya et al., 2002) can relieve the inhibitory effects of fibronectin on differentiation of adipocytes. Given these two observations one can postulate a model in which conformational changes in fibronectin structure uncover previously sequestered regions of the molecule and thereby modulate its function.

**EIIIA and EIIIB Null mice**

One of the major advances that has taken place in the study of fibronectin alternative splicing since this project was begun is the generation of mutant mice which lack the EIIIA or the EIIIB region of fibronectin (Dr. E. George and Dr. K. Sekiguchi personal communication). These mice are viable and appear normal. Wound closure and appears to be normal in both the EIIIA and EIIIB-null mice. Vascular development also occurs normally in both EIIIA and EIIIB null mice. In addition, several studies of angiogenesis in the EIIIA mutant mice have been performed (personal communication Dr. S. Snitkovsky). Specifically two models of physiological angiogenesis (retina
vascularization and bone growth plate vascularization) and one model of pathological angiogenesis (tumor angiogenesis) have been examined. Vascular density in the retinas of EIIIA-null and wild-type animals appears to be similar. Angiogenesis was also examined indirectly by measuring the length of the growth plates that give rise to bones. Growth plate length is an indirect measure of vascularization. In the absence of angiogenesis, ossification of the growth plates is delayed and growth plated length is increased. Growth plate length appeared normal in the EIIIA-null mice. Thus no defects in angiogenesis in the EIIIA mice were observed in two different physiological models of angiogenesis. Angiogenesis was also studied in the Rip-Tag tumor model of pancreatic cancer (Dr. S. Snitovsky personal communication) which has been described (Hager and Hanahan, 1999). In this model, vessel density was similar in EIIIA-null and wild-type mice. In this case, expression of α-smooth muscle actin and the pericyte marker NG2 was also examined. These markers appeared to be expressed at similar levels and with similar patterns of localization in the null and wild-type animals.

These results show that vasculogenesis and angiogenesis can proceed normally in mice lacking EIIIA or EIIIB. This suggests that neither EIIIA nor EIIIB is required for these processes. However, given the expression patterns of these isoforms and the high degree of conservation of the splice segments between species is seems likely that they do play some role in this or other important cellular processes. Failure to observe defects in these mice may be due to compensation by other molecules. It is possible that these isoforms usually function in blood vessel morphogenesis but that in the absence of one of these the other splice form can perform the same function. Our results regarding keratinocyte migration indicate that any one of the alternatively spliced segments was sufficient to enhance keratinocyte migration suggesting that in at least some situations the presence of only one of these segments can mediate a given function. Generation of mutant mice, which lack both EIIIA and EIIIB, will be necessary to resolve this issue. Clearly, it is also possible that other factors can compensate for the loss of EIIIA or EIIIB in these mice.

**Conclusions**

This dissertation describes the development of an *in vitro* system for investigating the function of the alternatively spliced isoforms of fibronectin. This work has verified
the efficacy of a set of bacterially expressed fibronectin fusion proteins in a number of different biological assays. Furthermore, we describe the isolation and generation of fibronectin-null cells. This work has revealed some of the limitations of the use of these fibronectin-null cells in elucidating the functions of the alternatively spliced isoforms. However, these results suggest that further characterization of these cells may produce interesting insights into the function of fibronectins. Finally, the finding that the fibronectin seems to inhibit at least one marker of myofibroblast differentiation raises a number of questions about the mechanism by which this occurs. It will be particularly important to examine the role of TGF-β in fibronectin-null cells.
Appendix 1

Rescuing the Phenotype of the Fibronectin-Null Fibroblasts
INTRODUCTION

Experiments in Chapter 3 of this thesis show that fibronectin-null fibroblasts express higher levels of \( \alpha \)-smooth muscle actin than do cells that produce fibronectin. Given my initial hypothesis that EIII A fibronectin induces \( \alpha \)-smooth muscle actin expression, I had expected to observe lower levels of \( \alpha \)-smooth muscle actin in the null cells since these cells cannot produce any fibronectin isoforms. One interpretation of this result is that fibronectin normally acts as a negative regulator of smooth muscle actin expression and myofibroblast differentiation. Negative regulation of \( \alpha \)-smooth muscle actin by fibronectin has not been reported previously and this finding represents a new fibronectin function.

These results do not fit neatly with previous reports suggesting that EIII A fibronectin upregulates \( \alpha \)-smooth muscle actin. There are two possible models to explain this. First, it is possible that fibronectin which lacks EIII A usually represses \( \alpha \)-smooth muscle actin expression but that this repression is relieved by the inclusion of the EIII A. Second, ablation of fibronectin may cause selection for different subpopulations of cells or force the differentiation of null cells into a smooth muscle-actin expressing cell type. Such cells might exhibit a fundamentally different regulation of \( \alpha \)-smooth muscle actin than do the heterozygous fibroblasts.

It should be possible to distinguish between these two models by restoring fibronectin to the cells. If these cells are identical to heterozygous cells except for the fact that they do not make fibronectin, addition of fibronectin should result in the restoration of normal \( \alpha \)-smooth muscle actin regulation. If however, these cells are different than the heterozygous cells, addition of fibronectin would not be expected to alter smooth muscle actin expression. I have attempted to restore the wild-type phenotype of these cells by two different methods, plating the cells on fibronectin and transfecting cells with fibronectin. I found that plating the cells on fibronectin did not restore the wild-type phenotype. Experiments attempting to transfecct cells with fibronectin were unsuccessful due to technical problems.
MATERIALS and METHODS

Smooth Muscle Actin Western Blotting

Western blots were performed as described (see Chapter 4).

Immunofluorescence Assays

Cells were grown on glass coverslips in medium containing 10% serum that had been depleted of fibronectin. After 72 hours, cells were washed with PBS Ca\(^{2+}\)Mg\(^{2+}\) and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells were then washed with PBS Ca\(^{2+}\)Mg\(^{2+}\), and permeabilized in 0.5% IGPAL for 10 minutes at room temperature. After washing, cells were incubated with either the rabbit polyclonal anti-fibronectin antibody 61.1(1:200) or an anti-α-smooth muscle actin antibody 1A4(1:1000) (Sigma) in 2mg/ml BSA/PBS for 30 minutes at 37°C. The coverslips were washed and then incubated with fluorescein-conjugated anti-rabbit or rhodamine-conjugated anti-mouse secondary antibodies in 10% normal goat serum/PBS for 30 minutes at 37°C. After washing, the coverslips were mounted in Gelvatol and photographed using an inverted fluorescence microscope.

Transfections

2×10^6 Phoenix retroviral packaging cells were plated in 5mls of DMEM 10% fetal bovine serum, 2mM glutamine, 100μg/ml streptomycin, 100U/ml penicillin in 6 cm dishes. After 24 hours cells were transfected. A pMIG vector containing a full length FN cDNA derived from pAIPFN and the green fluorescent protein gene (GFP) (Akamatsu et al., 1996) was a gift from Dr. Kaan Certel. Five minutes before transfection the medium was replaced with 3mls of fresh medium containing chloroquinone (25μM). 40μg of DNA was prepared in 61μl of 2M CaCl\(_2\) and 417 μl of H\(_2\)O. 0.5mls of 2xHBS (8.0g NaCl 6.5g HEPES, 10 Na\(_2\)HPO\(_4\)) was then added to the DNA solution. The HBS/DNA solution was vortexed and then added dropwise to the medium. Cells were then incubated at 37°C for 24 hours at which time the medium was replaced with fresh medium. After 24 hours, supernatant from the transfected Phoenix cells was removed and centrifuged at 1500 rpm for 5 minutes and centrifuged through a 70 μM filter to remove cells. FN-null cells 4D and 5F were plated at 2×10^6 cells in 6-cm dishes. One ml of medium was removed from the target cells and 3μl of 5mg/ml polybrene were added. Then 1ml of viral supernatant was added to the target cells and incubated for 24 hours.
After incubation the supernatant was removed and cells were washed and fed with fresh medium.

**Metabolic Labeling**

Fibronectin heterozygous cells, fibronectin null cells, and null cells that had been transfected with the pMIG-FN construct were plated (3x10^5 cells/ml) in six-well dishes. After 24 hours in culture, normal medium was removed and replaced with labeling medium (90% E4HG without methionine, 10% normal medium, and 100μCi/ml of Express Label [35S] methionine (New England Nuclear). After 24 hours at 37°C, 1.5mls of culture medium was removed from each well and protease inhibitors were added (2mM PMSF; 2mM EDTA). Samples were spun for 10 minutes. 30μl were then added to buffer (0.1M Tris (pH8), 0.2%SDS, 1% NP-40, 1% DOC, 2mM EDTA, 2mM PMSF) in tubes coated with 10% ovalbumin. The rabbit polyclonal anti-fibronectin antibody 61.1 (10μl) or control preimmune serum 61A (10μl) was added to each tube. The sample buffer mixture was incubated for 1 hour at 37°C. After the incubation, a secondary goat anti-rabbit antibody (100μl) was then added and incubated for 2 hours at 37°C. Samples were then spun and washed 3 times in wash buffer (0.1% SDS, 0.5%NP-40, 0.5% DOC, 0.1M Tris (pH 8), 2mM PMSF, 2mM EDTA). Precipitates were boiled in 2% SDS and 0.1M dithiothreitol and electrophoresed on a 7% SDS-PAGE gel. Gels were dried and bands were developed onto Scientific Imaging Film (Kodak).
RESULTS

I wanted to determine whether plating the null cells on fibronectin or transfecting these cells with fibronectin would restore the wild-type phenotype. I found that plating cells on full length human plasma fibronectin or recombinant fibronectin containing different splice isoforms did not restore the wild-type regulation of α-smooth muscle actin expression (Figure A1-1). This suggests that these cells are indeed a different cell type than are the wild-type cells. Next, I attempted to transfect these cells using a retroviral pMIG vector which contain a full-length fibronectin gene as well as the gene for GFP. After transfection, cells were sorted into high and low GFP-expressing populations (Figure A1-2). Cells were then checked for expression of fibronectin. However, the transfected cells did not stain positively for fibronectin (Figure A1-3) suggesting that the transfection had not worked. It was possible that the transfected cells produced fibronectin, but were unable to bind it to the cell surface. Therefore, I assayed the ability of these cells to assemble a fibronectin matrix when exogenous human fibronectin was added to the culture (Figure A1-4). The null cells were able to assemble this fibronectin into a pericellular matrix. As an additional test of the ability of the transfected cells to produce fibronectin, I metabolically labeled the cells with 35S methionine (Figure A1-5). While the heterozygous cells produced fibronectin, neither the null cell line 5F nor the transfected derivative of 5F showed a fibronectin band. The 4D line and its transfected derivative both showed faint bands that were also present in the preimmune serum. These bands might represent background however they might be the result of a contaminating heterozygous or wildtype ES cells present in cell cultures used to generate these lines. These results indicate that our attempts to restore fibronectin to these cells were unsuccessful.
DISCUSSION

To address the question of whether or not exogenous fibronectin can rescue the wild-type phenotype null and heterozygous cells were grown on different isoforms of full-length fibronectin. Exogenous fibronectin did not revert the phenotype of the null cells. It is possible that the dose of fibronectin provided in the rescue experiments or the length of time the cells were allowed to grow on fibronectin was insufficient to revert the phenotype. However, the most straightforward interpretation of this result is that regulation of α-smooth muscle actin expression in fibroblasts derived from fibronectin-null ES cells is fundamentally different than expression in cells derived from heterozygous ES cells.

It might also be the case that, in order to rescue the wild-type phenotype, cells must produce their own fibronectin. One possible explanation for the requirement for endogenous fibronectin might be that intracellular interactions of fibronectin with other proteins. This interaction might affect fibronectin’s ability to repress α-smooth muscle actin expression. Fibronectin has been shown to bind intracellularly to the von Hippel-Lindau tumor suppressor protein (pVHL) (Ohh et al., 1998). In the absence of pVHL, fibronectin matrix fails to assemble on the cell surface. While many of the implications of this interaction remain to be explored, it is possible that alterations in fibronectin binding to pVHL are sensed intracellularly. In this case loss of binding of pVHL to fibronectin might cause changes in cellular phenotype.

In order to address this possibility, a retroviral vector containing fibronectin and a GFP marker was used to transfect the null fibroblasts. The transfectants that were obtained expressed GFP, but failed to express fibronectin. The percentage of GFP-positive cells in the initial sort was less than 1%. This suggests low transfection efficiency. The rare positive clones probably represent events in which GFP could be inserted independently of fibronectin. Northern blotting to look for expression of fibronectin RNA in these cells might provide information about whether or not they have acquired the fibronectin gene. If this technique were attempted again it would be worthwhile to try and clone Phoenix cell lines that express high levels of GFP and then to check the insertion before proceeding with the transfections. It should also be possible to use alternative methods such as liposome-mediated transfection to transfer fibronectin
into the target cells. This approach gets around the packaging problems that arise when retroviruses are used to package large inserts such as fibronectin. Cotransfection of vectors containing an appropriate selectable marker should allow the generation of stable fibronectin transfectants using the lipofection method. In any case, the isolation of transfectants should shed light on the role of fibronectin in these processes.
Figures and Legends
Figure A1-1: Expression of smooth muscle actin in fibronectin-heterozygous and fibronectin-null fibroblasts cultured on different substrates. Fibroblasts were plated on tissue culture plastic, full length hFN, or full length BAV in 1% serum from which fibronectin had been depleted. One day after plating fresh medium containing or lacking TGF-β (10ng/ml) was added to the cells. 72 hours later, cells were lysed and lysates were run on an SDS-PAGE gel. Proteins were transferred to nitrocellulose filters. Filters were blotted for smooth muscle actin or vimentin as a loading control.
Figure A1-2: Transfection experiments. Fibronectin-null cell lines (4D and 5F) were transfected with a pMIG vector containing pMIG FN-GFP. Transfected and untransfected cells were then observed using a fluorescence microscope to visualize GFP.
Figure A1-3: Fibronectin staining of FN-null cells transfected with GFP. Cells transfected with fibronectin-GFP or untransfected cells were grown for 72 hours in medium containing 10% serum from which fibronectin had been depleted. Cells were then stained with an anti-fibronectin antibody followed by a rhodamine-conjugated secondary antibody to visualize the fibronectin matrix. The transfected cells did not show fibronectin staining.
Figure A1-4: Fibronectin-null cells can assemble exogenously added fibronectin.

Fibronectin-null cell lines and null cell lines transfected with fibronectin-GFP were grown for 72 hours in medium containing 10% serum from which fibronectin had been depleted. 10μg/ml of exogenous human fibronectin were added to the culture medium. Cells were then stained with anti-fibronectin antibody followed by a rhodamine-conjugated secondary antibody to visualize the fibronectin matrix. Both the transfected and untransfected cells were able to assemble exogenous fibronectin into a cell associated matrix.
Figure A1-5: Metabolic labeling of transfected cells. Fibronectin-null cell lines or transfected cell lines were grown for 24 hours in medium containing $^{35}$S methionine. Conditioned medium was then removed from the cells and fibronectin was immunoprecipitated by incubating samples with an anti-fibronectin antibody followed by incubation with a goat anti-rabbit antibody. Immunoprecipitates were run on an SDS-PAGE gel. Gels were dried and used to expose films. A band corresponding to fibronectin was immunoprecipitated from heterozygous cells. Neither the null cell lines nor the transfected cells contained strong fibronectin bands. Light bands were visible in the 4D samples probably due to wild-type cells carried over during the preparation of these cells.
Transfected FN Null cells do not Produce FN Protein
Appendix 2

A Model System for Investigation of the Role of
Alternatively Spliced Fibronectin Isoforms in Angiogenesis
INTRODUCTION

The field of blood vessel development has expanded rapidly in recent years. This is due in part to the demonstration that changes in angiogenesis play a central role in tumorigenesis. Much of this research has focused on factors that regulate the early stages of blood vessel development especially those that induce or inhibit endothelial cell proliferation and migration. While endothelial cells were the focus of many early studies of angiogenesis, recent studies illustrate that peri-endothelial cells also play an important role in blood vessel maturation and function. The differentiation of these mural precursors is postulated to involve TGF-β. The fact that TGF-β is known to induce expression of fibronectin splice isoforms and that the splice isoforms are expressed around blood vessels suggests that alternatively spliced isoforms of fibronectin may also be involved in the differentiation of mural cells.

We wanted to test the hypothesis that the alternatively spliced isoforms of fibronectin are involved in endothelial cell associations with mural cells in an *in vitro* system. *In vitro* experimental systems that serve as a three dimensional model of vessel formation have been used to examine the effects of co-culture of endothelial cells with other cell types. In one such system, endothelial cells embedded in collagen gels undergo morphogenesis into tubular structures. In this system, tube formation is endothelial cell-specific. Furthermore, it is also dependent on new RNA and protein synthesis and on endothelial cell adhesion molecules such as platelet endothelial cell adhesion molecule (PECAM) and vascular endothelial cadherin (VE-cadherin) (Yang et al., 1999). Specific angiogenic growth factors such as VEGF induce endothelial cell morphogenesis into tubes that display proper apical/basal polarity and form lumens (Davis and Camarillo, 1996). Given the similarities between the tubes formed in this system and those formed in vivo, the *in vitro* system seems like a promising model for studying early and late steps of blood vessel formation such as lumen formation and mural cell associations.

In order to investigate whether fibronectin splice isoforms are involved in endothelial and mural cell differentiation, we hoped to incorporate the reagents and cells generated in Chapters 2 and 3 into the culture system. To set up the culture system we obtained endothelial cells from the fibronectin-chimeras described in Chapter 3. Below, I
describe some preliminary efforts to isolate these cells and to establish the co-culture system.
MATERIALS and METHODS

Endothelial Cell Tube Forming Assays

Endothelial tube forming assays were performed according to the protocol of Yang et al. 1999. Human umbilical vein endothelial cells (HUVECS) cells were trypsinized and resuspended in basal medium (100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, and 50µg/ml of ascorbic acid in 1x M199 medium). One part cells was mixed with 4 parts of ice-cold gelation solution (10xM199, H2O, 0.53mol/L NaHCO3, 200mM/liter L-glutamine, type 1 collagen (Upstate Biotechnology Lake Placid NY), 0.1mol/L NaOH; 100:27.7:50:10:750:62.5 by volume) to give a final concentration of 3x10^6cells/ml. Gels were allowed to solidify by placing them in a CO2-free incubator at 37°C for 1 hour. The gels were then overlaid with 1x basal medium containing M199 medium supplemented with 26.5mM NaHCO3, 40ng/ml bFGF (PeproTech), 40ng/ml VEGF (PeproTech), 80nM PMA (Sigma), ISTX (GibcoBRL), and 1% serum. Cells were incubated at 37°C and 5% CO2, tubes were photographed, and images were prepared using a DC290 ZOOM digital camera and Aquire Software (KODAK).

Isolation of Mouse Lung Endothelial Cells

Chimeric mice FN+/+;−/− (generated as described in Chapter 3) were sacrificed by cervical dislocation at 6-20 weeks. Age-matched heterozygous FN or wild-type 129Sv/Jae strain mice were sacrificed for use as controls. Animals were perfused with 1U/ml heparin in PBS (Sigma). Lungs were dissected and placed into 20mls of ice-cold Ham’s F12 medium (BioWhittaker, Walkersville,MD). Lungs were dipped into 70% ethanol and quickly transferred to a dish containing Ham’s F-12 medium. Lungs were then placed into the top of a petri dish and minced into fine tissue for 1-2 minutes using surgical dissecting scissors. The minced tissue was treated with 0.1% collagenase (Worthington Type I) for one hour at 37°C. Tissue was then homogenized by passing it 5-10 times through a blunt-ended 14G cannula (Becton Dickinson, Franklin Lakes,NJ) until large pieces were eliminated. The homogenized tissue was filtered through a 70µm nylon cell strainer (Falcon, Becton Dickinson #2350), centrifuged at 4°C for 5 minutes, and pelleted cells were plated into T75 flasks that had been precoated with 0.1% gelatin and 10mg/ml of human plasma fibronectin (BD Biosciences). Endothelial cells were maintained in endothelial cell medium consisting of 40% Ham’s F12, 40% DME low
glucose, 20% heat-inactivated FBS, 50 μg/ml endothelial mitogen (Biomedical Technologies Inc., BT-203 Stoughton, MA), 0.1 mg/ml heparin (Sigma H3393), 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine. Cells were sorted using antibody-coated magnetic beads (Sheep anti-rat IgG coated Dynabeads, Dynal A.S. Oslo, Norway) using a protocol provided by Dr. Julie Lively (MIT). Non-endothelial cells were removed when the cultures reached 60% confluence (approximately 24 hours after plating the cultures). For negative sorts, 50μl of Dynabeads were loaded overnight at 4°C with 10μl of rat anti-mouse Fcγ receptorII/III (Pharmingen-01241D) in 500μl of PBS/2%FBS. Medium was removed from cells and 3 mls of fresh medium were added. Cells were cooled by placing them at 4°C for one hour. At the end of the incubation, coated beads were added to the cells in 3 mls of medium. Beads and cells were incubated for 1 hour at 4°C with occasional shaking to redistribute the beads. At the end of the incubation, cells were trypsinized and collected in to a magnetic particle concentrator (Dynal MPC-1, Dynal). The supernatant containing cells that did not bind to the magnet was replated in endothelial cell medium. After 2-3 days, positive sorts for endothelial cells were performed. Beads were coated overnight with 10μl of rat anti-mouse ICAM-2 (Pharmingen-01800D). Sorts were performed using essentially the same procedure as described for negative sorts. For positive sorts, supernatant containing cells not bound to the magnetic beads were discarded and endothelial cells trapped by the magnet were replated. If contamination with non-endothelial cells was observed, a second positive sort was performed 2-3 days later.

**Endothelial Cell PCR Assays**

PCR assays using DNA taken from chimeric, heterozygous and wild-type endothelial cell cultures were performed as described for dermal fibroblasts in Chapter 3.
RESULTS

We were interested in determining whether or not the alternatively spliced isoforms of fibronectin play a role in the morphogenesis and maturation of blood vessels. Therefore, we decided to use a collagen gel assay to monitor endothelial cell tube formation (Figure A2-1). Using this assay system, I observed that in the presence of the growth factors VEGF and bFGF, human umbilical vein endothelial cells spread within the gel and neighboring cells formed associations (Figure A2-2). In the absence of growth factors, cells failed to spread or associate (Figure A2-2). This preliminary result suggested that the collagen assay worked as expected and that in this model association was regulated by factors that had previously been shown to be important for endothelial cell tube formation.

We wanted to test the role of fibronectin isoforms in vessel morphogenesis. Therefore, we decided to isolate endothelial cells that were unable to make their own fibronectin in order to compare tube formation by these cells with tube formation by wild-type cells. We would expect to see a defect in the ability of the null cells to form tubes. If this were the case, we could then compare the ability of the different splice isoforms to rescue the null phenotype. In order to obtain fibronectin null endothelial cells, we used the fibronectin-null chimeras as a source of these cells (Figure A2-3). Our strategy involved using magnetic beads coated with antibodies to either the Fcγ receptor to sort out contaminating macrophages or the endothelial specific marker ICAM-2 to selectively sort out endothelial cells from mouse lung cultures (Figure A2-4). Figure A2-5 shows a culture of wild-type mouse lung endothelial cells that have undergone a negative sort to remove macrophages and a positive sort to purify endothelial cells. I also prepared cultures of endothelial cells from FN chimeras or control wild-type or heterozygous animals. After a positive and negative sort, I verified the genotypes of the different cultures using PCR. Figure A2-6 shows the results of these PCR reactions. As expected chimeric and heterozygous cultures showed a band corresponding to the allele containing the neo cassette while wild-type cultures gave rise only to a wild-type band. Although the cultures have not yet been selected, the presence of the targeted allele indicates that at least some of the cells in the culture are null for fibronectin. Preliminary
experiments illustrate that this approach can successfully be used to isolate fibronectin-null endothelial cells.
DISCUSSION

The project described in this chapter is in its preliminary stages and much work remains to be done to further purify and characterize the fibronectin-null endothelial cells and to establish the co-culture assay. Dr. Julie Lively (personal communication) has extensively characterized wild-type mouse lung endothelial cells prepared using this method. These cells have been shown to express endothelial markers such a PECAM, von Willebrand's factor, and endothelial cell integrins (αvβ3). However, fibronectin-null endothelial cells will need to be selected from the mixed population of null and wild-type cells. They must also be analyzed by immunofluorescence and Western blotting to confirm the absence of fibronectin expression. Null cells should also be analyzed for the expression of endothelial cell markers given results in Chapter 3 that suggest that loss of fibronectin might lead to unexpected phenotypic changes.

Obtaining healthy endothelial cell cultures proved to be difficult. This was partly because these cells appear to grow poorly and lose endothelial morphology when grown at low cell densities. My initial cell preparations yielded sparse cultures. This was detrimental to the health of the cells. The problem of cell yield can probably be fixed by using more mice or by changing other details of the preparation protocol. However, the need for high cell densities may present a problem during selection of fibronectin-null cells with G418. The death of large numbers of wild-type cells in the chimeric cultures would be expected to drastically decrease cell density in these cultures and consequently affect the quality of the remaining cells. One might be able to avoid this problem by using magnetic beads coated with an anti-fibronectin antibody to remove wild-type cells prior to selection. These relatively pure cultures could then be grown up to high densities and selected. Since these cultures would already be enriched for null cells the density should remain relatively high even after selection and this should result in less stress on the cells.

If selection of pure cultures of fibronectin-null cells using the methods described above is unsuccessful, an alternative approach would be to perform in vitro differentiation of fibronectin-null ES cells into endothelial cell. In vitro differentiation of murine ES cells into structures termed embryoid bodies has been described (Wang et al., 1992). Vascular channels that develop within these embryoid bodies have been
extensively characterized (Vittet et al., 1996). Immunofluorescence and RT-PCR show that the endothelial cells within embryoid bodies are positive for endothelial cell markers including PECAM, VE-cadherin, Flk-1 (a receptor for vascular endothelial cell growth factor), and the Tie-2 angioptin receptors. Furthermore, in embryoid bodies these markers are turned on in the same order as they appear during in vivo vasculogenesis. (Yamashita et al., 2000) have reported an efficient system for obtaining pure cultures of either endothelial cells or smooth muscle actin expressing mural cell precursors from ES cell cultures. In this system, ES cells are cultured on a collagen substrate and then Flk-1 positive cells are sorted from the cultures. These cells can then be induced to express endothelial cell markers by addition of VEGF. In the absence of VEGF, 95% of the Flk1+ cells express α-smooth muscle actin. Therefore, it should be possible to use this method to obtain fibronectin-null mural cell precursors as well.

Although generation of fibronectin-null endothelial cells using the method developed by Yamashita et al. (2000) has not been reported, fibronectin-null embryoid bodies have been described (Francis et al., 2002). These embryoid bodies show a defect in capillary plexus formation and maturation, however these structures do contain some cells that stain positive for the endothelial cell marker PECAM. The defect in capillary plexus formation can be rescued by adding exogenous fibronectin to the culture system. This report also indicates that the number of PECAM positive cells can be increased by the addition of fibronectin. This suggests that including fibronectin in the culture system might increase the yield and quality of FN-null endothelial cells obtained from embryoid bodies.

In addition, various aspects of the collagen assays remain to be worked out. For example, we have tested the use of cell surface markers to mark the endothelial cells and fibroblasts so that they can be easily distinguished in co-culture. The use of different markers should also aid in assessment of the ability of fibroblasts and endothelial cells to associate. Eventually, we would like to co-culture fibronectin-null fibroblasts and endothelial cells. However, we must first establish that one or both sets of fibroblasts we generated in Chapter 3 will associate with endothelial cells. Some studies have suggested that only certain undifferentiated mesenchymal cell types can function as peri-vascular cell precursors since 3T3 fibroblast lines do not associate with endothelial tubes in vitro.
(Hirschi et al., 1998). However, association of human neonatal dermal fibroblasts with endothelial cells in co-culture assays has been reported (Xin et al., 2001).
Figures and Legends
Figure A2-1: Schematic representation of an assay for blood vessel formation.
During blood vessel formation endothelial cells line up and form hollow tubes. During vessel maturation endothelial cells recruit smooth muscle cells which contribute to vessel stability. In this assay endothelial cells are embedded in collagen gels along with growth factors. The rate of tube formation of fibronectin null and wild-type endothelial cells can then be determined. Co-culture of endothelial cells and fibroblasts can be used to assay the ability of endothelial cells to recruit and associate with these cells. In the co-culture assays, fluorescent labels are used to mark the different cell types.
3D Endothelial Fibroblast Cocultures

Fluorescently labeled endothelial cells

Fluorescently labeled dermal fibroblasts

Collagen gel
**Figure A2-2: Endothelial cell tube-forming assays.** Human umbilical vein endothelial cells (HUVECS) embedded in a collagen gel either with or without 40ng/ml of VEGF and 40ng/ml of bFGF. Arrows show the position of endothelial cells within the collagen gel. In the absence of growth factor, HUVECs remained round and unspread and appeared fragmented. In the presence of growth factors, the cells spread and associated with neighboring cells.
**Figure A2-3:** Scheme for isolating endothelial cells from fibronectin chimeras. Lungs of chimeric mice were used as a source of fibronectin-null endothelial cells.
Generation of FN-/- Endothelial Cells

Harvest lungs from tissues of FN chimeras

+ mince and add collagenase

○ ○ ○ ○ ○ ○ ○ ○ ○
Figure A2-4: Endothelial cell sorting. Protocol for sorting endothelial cells from other cell types obtained from mouse lung. Magnetic beads coated with an anti-ICAM-2 antibody were incubated with cultures obtained from mouse lung. Endothelial cells, but not fibroblasts and macrophages, express ICAM-2 and bind to the beads. After one hour, cells are trypsinized, cells are collected into a 15 ml Falcon tube which can be placed inside of a clamp attached to a small magnet. Supernatant containing contaminating cells is removed. The tube is then removed from the magnet and the endothelial cells are replated in fresh medium.
**Figure A2-5:** Wild-type endothelial cells grown in culture exhibit a typical cobblestone morphology.
Mouse Lung Endothelial Cells +/+
Figure A2-6: Analysis of endothelial cells by PCR. Small flasks of mouse lung endothelial cells obtained from FN-wild-type, FN-heterozygous, or FN-null mice were trypsinized and placed into Glenn’s buffer with proteinase K. Cells were then analyzed using PCR. A band of 1060bp indicates the presence of a targeted allele containing a neomycin resistance cassette. The 900bp band indicated the presence of the wild-type fibronectin allele.
LITERATURE CITED


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