CHAPTER 1

MOLECULAR INSIGHTS INTO ANGIOGENESIS
– ROLE OF FIBRONECTIN:
A BRIEF REVIEW
1. MOLECULAR INSIGHTS INTO ANGIOGENESIS – ROLE OF FIBRONECTIN: A BRIEF REVIEW

The vascular system that ensures an adequate blood flow is required to provide the cells with sufficient supply of nutrients and oxygen. Angiogenesis, the formation of new vessels from preexisting ones, is regulated by a delicate balance of pro- and anti-angiogenic factors. Physiologically, angiostatic mediators outweigh the angiogenic molecules and angiogenesis does not occur. Under certain conditions such as tumor formation or wound healing, the positive regulators of angiogenesis predominate and the endothelium becomes activated. Angiogenesis is initiated by vasodilatation and increased vessel permeability. After destabilization of the vessel wall, endothelial cells proliferate, migrate and form a tube, which is finally stabilized by pericytes and smooth muscle cells. Numerous soluble angiogenic growth factors, angiostatic factors, cytokines and proteases as well as extracellular matrix proteins and adhesion molecules strictly control this multi-step process. Understanding the molecular mechanisms involved in modulation of endothelial cell function by fibronectin and its relevance in the angiogenic process form the subject matter of this thesis. A brief review of the angiogenic process, its importance in physiology and pathology, the properties and interactions of modulators of angiogenesis and the molecular basis of cell-fibronectin interactions with relevance to angiogenesis is given below.

1.1. Angiogenesis

Growth of the vasculature is primarily a developmental process, which occurs during embryogenesis and only to a limited extent in postnatal life. During early embryogenesis, blood islands composed of progenitor blood cells (hematopoietic cells) and endothelial cells (angioblasts) differentiate from the mesoderm. The formation of blood vessels de novo from differentiating angioblasts and their organization into a primordial vascular network known as primary capillary plexus, consisting of the major blood vessels of the embryo, is called vasculogenesis (Flamme et al., 1997). While the primitive capillary plexus is still emerging, modifications occur to remodel it into a more complex and differentiated secondary network (Distler et al., 2003). The endothelial cell lattice thus created by vasculogenesis then serves as a scaffold for angiogenesis, which further extends the vasculature by sprouting and branching of new vessels from the pre-existing ones in a multi-step process (Carmeliet,
Angiogenesis is a complex process involving extensive interplay between cells, soluble factors, membrane bound factors and extracellular matrix (ECM) components (Liekens et al., 2001). The formation of a vascular network involves different sequential steps including: (i) the release of proteases from activated endothelial cells (ECs), (ii) degradation of the basement membrane (BM) underlying the existing vessel endothelium, (iii) migration of the endothelial cells into the interstitial space, (iv) endothelial cell proliferation, (v) lumen formation, (vi) generation of new basement membrane with the recruitment of pericytes, (vii) fusion of the newly formed vessels and (viii) initiation of blood flow.

Angiogenesis is initiated by vasodilatation and an increased permeability (Carmeliet, 2000; Distler et al., 2003). Upon activation of the ECs, mural cells are detached, and the EC basement membrane is degraded so that the ECs invade the stroma of the neighboring tissue (Mignatti and Rifkin, 1996; Moses, 1997). After destabilization of the vessel wall, ECs proliferate and migrate (Distler et al., 2003) and form a tube, which is finally stabilized by recruiting pericytes and smooth muscle cells (Carmeliet, 2000; Kurz, 2000). When sufficient neovascularisation has occurred, angiogenic factors are down regulated or the local concentration of inhibitors increases. As a result, the ECs become quiescent and the vessels remain or regress if no longer needed (Liekens et al., 2001). Thus, angiogenesis requires multiple interactions that must be tightly regulated in a spatial and temporal manner.

Despite the abundance of angiogenic factors present in different tissues, EC turnover in a healthy adult organism is remarkably low with a turnover rate in the order of thousands of days. The maintenance of endothelial quiescence is thought to be due to the presence of endogenous negative regulators (Iruela-Arispe and Dvorak, 1997) (Table: 1.1). But under certain conditions such as tumor formation or wound healing, the positive regulators of angiogenesis predominate and the endothelium becomes activated. Physiologically, angiostatic mediators outweigh the angiogenic molecules and angiogenesis does not occur. Besides during embryogenesis, physiological angiogenesis is also activated during the development of follicles, corpus luteum formation and embryo implantation (Folkman, 1995; Modlich et al., 1996).
Table: 1.1. — Major stimulators and inhibitors of angiogenesis and their role in the formation of new vessels

<table>
<thead>
<tr>
<th>ANGIOGENIC FACTORS</th>
<th>Biological Functions</th>
<th>References</th>
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<tbody>
<tr>
<td>VEGF-family</td>
<td>↑ Permeability, Plasminogen activators and Interstitial collagenase EC proliferation, migration and angiogenesis in vivo, ↓EC apoptosis</td>
<td>Veikkola et al., 1999; Ferrara, 1999</td>
</tr>
<tr>
<td>FGF-family</td>
<td>↑ Plasminogen activators, αβ₁ integrin and other adhesion molecules EC proliferation, migration and angiogenesis in vivo</td>
<td>Bussolino et al., 1996</td>
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<tr>
<td>Angiopoietin-1</td>
<td>EC sprouting and vessel stabilization</td>
<td>Holash et al., 1999; Hayes et al., 1999; Koblizek et al., 1998</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>EC proliferation, EC migration and EC sprouting only in the presence of VEGF</td>
<td>Oh et al., 1999, Holash et al., 1999</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Chord formation in vitro, Proliferation of SMCs and PCs, vessel stabilization</td>
<td>Heldin et al., 1999</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tube formation in vitro (low doses), vessel stabilization, angiogenesis in vivo in inflammatory environment</td>
<td>Pepper, 1997</td>
</tr>
<tr>
<td>TNF-α</td>
<td>EC migration, tube formation in vitro, angiogenesis in vivo</td>
<td>Jackson et al., 1997</td>
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<tr>
<td>EGF</td>
<td>EC proliferation, angiogenesis in vivo</td>
<td>Sato et al., 1993 ; Bussolino et al., 1996</td>
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<td>CSFs</td>
<td>EC proliferation, migration</td>
<td>Bussolino et al., 1993</td>
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<td>Angiogenin</td>
<td>EC proliferation</td>
<td>Badet, 1999</td>
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<tr>
<td>Angiotropin</td>
<td>EC migration, tube formation, angiogenesis in vivo</td>
<td>Hockel et al., 1987; 1988</td>
</tr>
<tr>
<td>IGF-1</td>
<td>↑ Plasminogen activators, ↓EC apoptosis EC proliferation, induction of VEGF</td>
<td>Sato et al., 1993; Bar et al., 1988</td>
</tr>
<tr>
<td>HGF</td>
<td>Migration and proliferation of EC and SMC, angiogenesis in vivo</td>
<td>Rosen et al., 1997; Bussolino et al., 1996</td>
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<tr>
<td>PECAM-1</td>
<td>EC aggregation, tube formation, EC migration, vessel stabilization, essential for FGF induced angiogenesis</td>
<td>Yang et al., 1999</td>
</tr>
<tr>
<td>Integrins</td>
<td>EC attachment, EC migration, essential for FGF induced angiogenesis, ↓EC apoptosis</td>
<td>Kim et al., 2000; Eliceiri and Cheresh, 1999</td>
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<tr>
<td>VE-Cadherin</td>
<td>Vessel stabilization, angiogenesis in vivo, ↓EC apoptosis</td>
<td>Carmeliet et al., 1999</td>
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<tr>
<td>MMPs</td>
<td>ECM degradation</td>
<td>Rundhaug, 2005</td>
</tr>
<tr>
<td>NO</td>
<td>↑ Permeability, EC proliferation, FGF release</td>
<td>Jackson et al., 1997</td>
</tr>
<tr>
<td>Prostaglandin E₁, E₂</td>
<td>EC differentiation</td>
<td>Gullino, 1995; Chiarugi et al., 1998</td>
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Table Contd.
### ANGIOSTATIC FACTORS

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<thead>
<tr>
<th>Factor</th>
<th>Biological Functions</th>
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<tr>
<td>Angiostatin</td>
<td>( \downarrow ) EC proliferation, migration and tube formation, ( \uparrow ) EC apoptosis, angiostatic ( \textit{in vivo} )</td>
<td>O’Reilly et al., 1994; Cao, 1999</td>
</tr>
<tr>
<td>Endostatin</td>
<td>( \downarrow ) EC proliferation, migration (?) ( \uparrow ) EC apoptosis (?) ( \textit{in vivo} ) Inhibition of MMPs, angiostatic ( \textit{in vivo} )</td>
<td>O'Reilly et al., 1997</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>Vessel destabilization by antagonizing angiopoietin-1 signaling, ( \uparrow ) EC apoptosis</td>
<td>Maisonpierre et al., 1997</td>
</tr>
<tr>
<td>TGF-( \beta )</td>
<td>( \uparrow ) EC apoptosis, TIMPs ( \downarrow ) EC proliferation, migration and plasminogen activators</td>
<td>Pepper et al., 1991</td>
</tr>
<tr>
<td>TNF-( \alpha )</td>
<td>Inhibition of FGF induced EC proliferation ( \textit{in vitro} ), ( \uparrow ) EC apoptosis</td>
<td>Frater-Schroder et al., 1987</td>
</tr>
<tr>
<td>CXC chemokines</td>
<td>Inhibition of FGF and VEGF(_{165}) binding to their receptors, ( \textit{angiostatic in vivo} )</td>
<td>Distler et al., 2003</td>
</tr>
<tr>
<td>TSP-1, TSP-2</td>
<td>( \downarrow ) EC migration, ( \uparrow ) EC apoptosis, ( \textit{angiostatic in vivo} )</td>
<td>Iruela-Arispe and Dvorak, 1997</td>
</tr>
<tr>
<td>MMPs and TIMPs</td>
<td>Generate angiotatin ( \textit{angiostatic in vivo} )</td>
<td>Gomez et al., 1997</td>
</tr>
<tr>
<td>PEDF</td>
<td>( \downarrow ) EC proliferation, migration ( \textit{angiostatic in vivo} )</td>
<td>Dawson et al., 1999</td>
</tr>
</tbody>
</table>

**EC-Endothelial cell, SMC-smooth muscle cell, \( \text{PC-pericytes, ECM-} \) extracellular matrix, NO-nitric oxide, PEDF-pigment epithelium derived factor, MMPs-matrix metalloproteinases, TIMPs-tissue inhibitor of MMPs, TSP-thrombospondin, EGF-epidermal growth factor, PDGF-platelet derived growth factor, CSFs-colony stimulating factors, TGF-\( \beta \)-transforming growth factor-\( \beta \), TNF-\( \alpha \)-tumor necrosis factor-\( \alpha \), VEGF-vascular endothelial growth factor, FGF-fibroblast growth factor, PECAM-1-platelet endothelial cell adhesion molecule-1, HGF-hepatocyte growth factor, IGF-1- Insulin-like growth factor-1.**

Excessive angiogenesis occurs in conditions such as hemangiomas (proliferation of capillary endothelium with accumulation of mast cells, fibroblasts and macrophages), psoriasis, Kaposi’s sarcoma, ocular neovascularisation associated with diabetic retinopathy, rheumatoid arthritis, endometriosis, atherosclerosis, tumor growth and metastasis (Liekens et al., 2001). Insufficient angiogenesis occurs in situations such as myocardial ischemia, peripheral ischemia, cerebral ischemia, ulcer and reconstructive surgery (Liekens et al., 2001).
1.2. Molecular Events and Components Involved in Angiogenesis

Blood vessels consist of endothelial cells that are in direct contact with the blood and sub-endothelially located pericytes, smooth muscle cells, fibroblasts, BM, and extracellular matrix (ECM) (Figure: 1.1.). Depending on the location in the body, the organ microenvironment, the cellular constituents, BM and ECM of the vasculature differ in phenotype, composition and function (Rajotte et al., 1998). In general, the process of angiogenesis is regulated by a number of components that include the cells, the soluble factors, membrane bound factors, mechanical forces, the components of the extracellular matrix (ECM) and the matrix degrading proteolytic enzymes. Hypoxia is an important environmental factor that leads to neovascularization.

![Cross-section of a blood vessel](image)

**Figure: 1.1. Cross-section of a blood vessel:** Blood Vessels consist of three layers; intima (ECs and the underlying BM), media (smooth muscle cells) and adventitial layer (fibroblasts and ECM).

### 1.2.1. Cells

ECs line the entire inner surface of the blood vessels in an adult human and are actively involved in several regulatory processes in the body. Depending on the vessel type and vascular bed, activated ECs that are migrating and proliferating, forming anastomotic connections with each other, become variably surrounded by layers of peri-endothelial cells, pericytes for small vessels and smooth muscle cells for large vessels.
Although ECs are the key players of the angiogenic process, they should interact with a variety of peri-endothelial cells such as the pericytes and smooth muscle cells during angiogenesis and remodeling. Activated pericytes appear to take the lead in establishing not only pathways of invasion but also the formation of actual tubes suggesting that pericytes have an early role in angiogenesis (Ozerdem and Stallcup, 2003; Nehls et al., 1992; McDonald and Choyke, 2003; Gerhardt and Betsholtz, 2003) in addition to their later roles in the process (Hirschi et al., 1999; Beck and D'Amore, 1997; Sato and Rifkin, 1989).

One of the first events in angiogenesis is the weakening of stable cell-cell contacts between ECs in the parent vessels and the transition of quiescent ECs to dynamic migratory state (Ausprunk and Folkman, 1997). Following proteolytic degradation of the ECM, "leader" ECs start to migrate through the degraded matrix (Figure: 1.2.). They are followed by the proliferating ECs, which are stimulated by a variety of growth factors, some of which are released from the degraded ECM (Lamalice et al., 2007). The proliferating ECs migrate along a gradient of chemotactic agents, generated by a local collapse of the ECM, through the disintegrated BM into the remodeled and softened perivascular space (Distler et al., 2003). When the ECs reach the area with reduced vessel density, the ECs arrange in a monolayer, synthesize a new BM and form tube-like structures. In order to form a lumen, the polarity of the ECs, luminal versus abluminal, has to be established by the cell adhesion molecules. To stabilize the vascular tube, mesenchymal cells in the surrounding tissue proliferate and migrate to the abluminal surface of the premature vessels followed by their differentiation into pericytes, which are located within the BM or into vascular smooth muscle cells, which are found abluminal of the BM (Kurz, 2000). Finally, when sufficient neovascularisation has occurred, angiogenic factors are downregulated or the local concentration of inhibitors increases. As a result, the ECs become quiescent and the vessels remain or regress if no longer needed. During invasion and migration, ECs interact with the ECM and the final phases of the angiogenic process additionally involve cell-cell interactions.

1.2.2. Soluble Factors

The soluble factors regulating the process may be either angiogenic or angiostatic. The angiogenic mediators include growth factors (VEGF, FGF, PDGF, transforming growth factor-β and angiopoietins), cytokines (TNF-α, IL-1, IL-8) and
Figure: 1.2. Molecular events in angiogenesis:- Angiogenesis depends on the coordination of several independent processes. Removal of pericytes from the endothelium and destabilization of the vessel by angiopoietin-2 (ang-2) shift ECs from a stable, growth-arrested state to a plastic, proliferative phenotype. Vascular endothelial growth factor (VEGF)-induced hyperpermeability allows for local extravasation of proteases and matrix components from the bloodstream. ECs proliferate and migrate through the remodeled matrix in the presence of VEGF and fibroblast growth factor (FGF), and then they form tubes through which blood can flow. Mesenchymal cells proliferate and migrate along the new vessel and differentiate into mature pericytes. Establishment of EC quiescence, strengthening of cell-cell contacts and elaboration of new matrix stabilize the new vessel. (Ng and D'Amore, 2001)
VEGF\textsubscript{121} and VEGF\textsubscript{165} are secreted into extracellular environment whereas VEGF\textsubscript{189} and VEGF\textsubscript{206} and to some extent VEGF\textsubscript{165}, remain cell- or matrix-associated via their affinity for heparin sulfates (Houck et al., 1992). The biological activity of VEGF is also reported to be modulated at the transcriptional, post-transcriptional and post-translational levels (Neufeld et al., 1999; Semenza, 2001; Xiong et al., 1998), the most important post-translational modification that influences its angiogenic potency being poly ADPribozylation (PAR) (Xiong et al., 1998).

VEGF binds to at least three known tyrosine kinase receptors: Flt-1 (VEGFR-1) (de Vries et al., 1992), KDR/Flk-1 (VEGFR-2) (Terman et al., 1992) and Flt-4 (VEGFR-3) (Pajusola et al., 1992). VEGF increases EC permeability by enhancing the activity of vesicular-vacuolar organelles (Kohn et al., 1992) and by loosening adherens junctions between ECs in a monolayer via rearrangement of cadherins/catenin complexes (Esser et al., 1998; Kevil et al., 1998). VEGF induces a balanced system of proteolysis that can remodel ECM components necessary for angiogenesis (Pepper, 1997; Unemori et al., 1992). VEGF also enhances EC migration, inhibits EC apoptosis and thus acts as a survival factor (Gerber et al., 1998; Dimmeler et al., 2000).

The initial phases of angiogenesis seem to be dependent on VEGF signaling. Angiogenesis is rapidly initiated in response to hypoxic or ischemic conditions and VEGF production is found to be under control of hypoxia inducible factor (HIF), which together suggests an early involvement of VEGF in the angiogenic response (Kimura et al., 2001). VEGF binding induces homodimerization of the VEGFR-1 and VEGFR-2 receptors, followed by its autophosphorylation and activation of the downstream signaling cascade (Rossant and Howard, 2002). VEGFR-1 undergoes little detectable phosphorylation when bound to VEGF, whereas VEGF binding to VEGFR-2 results in its autophosphorylation at four major sites, followed by the activation of the Ras/MAP kinase pathway (Matsumoto and Claesson-Welsh, 2001). Neuropilin can bind specific splicing isoforms of VEGF and such binding enhances the VEGF-VEGFR-2 dependent migration of ECs (Soker et al., 1998) thus contributing to angiogenesis possibly by the formation of a complex between neuropilin and VEGFR-2 (Whitaker et al., 2001). VEGFR-1 or Flt-1 is thought to be a negative regulator of VEGF signaling in the early embryo acting to modulate VEGF levels by sequestering ligand (Hiratsuka et al., 1998; Fong et al., 1999).

VEGF stimulates DNA synthesis and proliferation via VEGFR-2 and Ras-Raf-
Chapter 1  
Angiogenesis: Role of FN- A Review

MEK-ERK pathway (Parenti et al., 1998; Pedram et al., 1998). VEGF binding to VEGFR-2 activates PI3-K/Akt leading to cell survival (Gerber et al., 1998). VEGF/VEGFR-2 also induces cell migration by activating FAK/PI3-K/Akt (Qi and Claesson-Welsh, 2001) and p38 MAPK pathway (Rousseau et al., 1997). Being an essential signaling pathway in angiogenesis, VEGF pathway is a key target for clinical intervention in situations such as preventing tumor angiogenesis and promoting revascularization after tissue ischemia (Ferrara and Alitalo, 1999). The transcriptional activation factors downstream of the VEGF signal transduction pathways shown to be functional in endothelial cells are AP-1, nuclear factor of activated T cells (NFAT) and nuclear transcription factor ETS (Chen et al., 1997). Whereas AP-1 and NFAT switch on genes regulating tissue factor expression upon VEGF activation, ETS regulates the expression of u-PA, MMPs, and integrin β3 (Iwasaka et al., 1996; Oda et al., 1999).

1.2.2.2. Fibroblast Growth Factor (FGF): - Members of FGF family are directly-acting pro-angiogenic molecules. Basic and acidic FGFs are ubiquitously expressed 18 to 25 kDa polypeptides that play important roles in normal angiogenesis (Thomas, 1987). FGF-2 or basic FGF consists of an 18- kDa low-molecular weight form and a 22- to 24-kDa high-molecular weight form (Givol and Yayon, 1992; Fantl et al., 1993). During angiogenesis, low molecular weight FGF-2 binding to endothelium induces FGF receptor (FGF-R) down-regulation, increased motility, proliferation and proteinase activity, and modulates integrin levels. High-molecular weight FGF-2 may cause endothelial cell proliferation after nuclear translocation in the endothelial cells (Gleizes et al., 1995; Klein et al., 1997). A secreted FGF-2-binding protein can bind FGF-2 that is normally inactive due to strong adherence to heparan sulfate proteoglycans in the ECM. The displaced FGF-2 molecules are thus released to mediate biological function (Griffioen and Molema, 2000).

FGFs stimulate proliferation (Gospodarowicz et al., 1989) and migration of ECs (Terranova et al., 1985) as well as the production of plasminogen activator and collagenase by ECs (Presta et al., 1986). Thus FGFs appear to induce processes involved in angiogenesis. Nevertheless, FGF is not specific for ECs but it stimulates proliferation of most, if not all, cells derived from embryonic mesoderm and neuroectoderm including pericytes, fibroblasts, myoblasts, chondrocytes, and osteoblasts (Thomas, 1987). Most FGF remains cytoplasmic or is bound to the ECM (Healy and Herman, 1992; Folkman et al., 1988) because of an intrinsic affinity for
heparin, but FGF may be released upon cell disruption by an injury and might have a role in local reparative angiogenesis following tissue injury where it is deposited in the ECM. Two types of receptors have been identified for FGF: high affinity tyrosine-kinase FGF receptors (Johnson and Williams, 1993; Rusnati and Presta, 1996) and low affinity heparan sulfate proteoglycans.

1.2.2.3. Angiopoietins: - The angiopoietins are ~70 kDa secreted ligands for Tie-2 receptors that are crucial for sprouting and branching of vessels characteristic of angiogenesis. The vessel branching and remodeling stimulated by Ang-1 signaling appears to be mechanistically related to its ability to increase the girth and stability of endothelium in newly formed angiogenic sprouts (Papetti and Herman, 2002). Ang-2, a structural homolog of Ang-1 that bound Tie-2, was first characterized as an antagonist of Ang-1 (Maisonpierre et al., 1997). Ang-2 antagonizes Ang-1 in the vasculature and may act as a check on Ang-1/Tie-2-mediated angiogenesis to prevent excessive branching and sprouting of blood vessels by promoting destabilization of blood vessels (Maisonpierre et al., 1997). In addition, vessel destabilization induced by Ang-2 may allow angiogenic sprouts to be plastic and sensitive to remodeling factors.

The Tie/Ang pathway mediates EC chemotaxis, cell survival/apoptosis protection, EC-ECM interactions and stimulation of vessel organization and sprouting, which makes it a key player in the complex process of vascular remodeling (Loughna and Sato, 2001). The Tie receptor forms homodimers on binding ligand, becomes autophosphorylated and interacts with a docking protein (Dok-R) (Jones et al., 1999b) and several other proteins including Grb7, Grb14, p85, Grb2 and Shp2 (Jones and Dumont, 2000). Activated Tie-2 might activate Akt through PI3-kinase (Papapetropoulos et al., 2000). Angiopoietin's effect on vessel maturation may be partly brought about by signaling the EC to secrete PDGF-B (Hellstrom et al., 1999).

1.2.2.4. Other Growth Factors: - Platelet-derived growth factor (PDGF), a 45 kDa dimeric protein, is suggested to play a role in recruitment of pericytes to preformed capillaries or in inducing the proliferation of pericytes previously recruited by a PDGF-independent mechanism and thus it helps maintain capillary wall stability (Lindahl et al., 1997; Hellstrom et al., 1999; Papetti and Herman, 2002). Transforming growth factor-β (TGF- β), a 25 kDa dimeric cytokine (Massague, 1990), is produced by both ECs and pericytes (Antonelli-Orlidge et al., 1989; Sato et al., 1995) and they possess TGF- β receptors. TGF- β regulates EC survival, migration, proliferation and
formation of tube-like structures in a dose-dependent manner (Pepper et al., 1991; Myoken et al., 1990).

1.2.2.5. Nitric Oxide: - NO synthesized by endothelial nitric oxide synthase has been implicated as a crucial signaling molecule and regulator in angiogenesis. NO donors promote EC proliferation and migration which are important steps in angiogenesis (Ziche et al., 1994). Morbidelli et al. (Morbidelli et al., 1996) and Ziche et al. (Ziche et al., 1997) have established that NO lies downstream from and mediates the effects of vascular endothelial growth factor (VEGF). VEGF has also been shown to upregulate NO production and eNOS protein (Hood et al., 1998; Papapetropoulos et al., 1997). Murohara and co-workers (Murohara et al., 1998) have suggested that eNOS modulates angiogenesis in response to tissue ischemia and have demonstrated impaired angiogenesis in the ischemic hindlimb of mice lacking the eNOS gene. Noiri et al. (Noiri et al., 1998) has suggested that in the endothelial cells NO induces a switch from a stationary to a locomotive phenotype, resulting in directional migration.

1.2.2.6. Prostaglandins: - Prostaglandins (PGs) are synthesized from arachidonic acid (AA) by the action of cyclooxygenases (COX), which are the products of two distinct genes, referred to as COX-1 and COX-2. PGs participate in angiogenesis by several mechanisms, such as promoting endothelial cell tubular formation (Ziche et al., 1982). PG E1, PG E2 and TX A2 are reported to promote angiogenesis (Ziche et al., 1982; Form and Auerbach, 1983; Daniel et al., 1999). In contrast, 15-deoxy-D12,14-PGJ2, a dehydration product from PG D2, induces endothelial cell apoptosis by activation of PPAR γ (Bishop-Bailey and Hla, 1999) and inhibits angiogenesis (Xin et al., 1999). PGs have been known to modulate EC proliferation, migration and capillary formation associated with angiogenesis (Graeber et al., 1990). PG E2 by itself has been shown to influence angiogenesis in vivo and induces the expression of VEGF (Ben-Av et al., 1995; Seno et al., 2002). PG E2 may also contribute to angiogenesis by activating the NO/cGMP signaling pathway through PKA/PI3-K/Akt-dependent increase in eNOS activity in HUVECs (Namkoong et al., 2005).

1.2.2.7. Other Soluble Factors: - Many other soluble factors have been proposed to function in angiogenesis including TNF-α, EGF, TGF-α, CSFs, angiotropin, angiogenin etc. Still other natural factors have been demonstrated to inhibit angiogenesis such as IFNs, cortisone, protamine, angiostatin, platelet factor-IV, thrombospondin etc. TNF-α shares its angiogenic properties with TGF-β (Frater-
Schroder et al., 1987). EGF and TGF-α are mitogenic for ECs and induce angiogenesis (Schreiber et al., 1986). G-CSF and GM-CSF induce migration and proliferation of ECs to a limited extent (Bussolino et al., 1989). Angiotropin stimulates angiogenesis (Hockel et al., 1987; 1988) whereas the role of angiogenin is rather controversial (Soncin, 1992; Fett et al., 1985). IFN-α was the first endogenous angiogenesis inhibitor showed to inhibit EC chemotaxis (Brouty-Boyé and Zetter, 1980). Interferons generally have been shown to inhibit angiogenesis in vivo. A subset of interleukins (lymphokines) has been found to affect blood vessel growth. ILs having a Glu-Leu-Arg (ELR) motif at the NH₂ terminus, such as IL-8, enhance angiogenesis, and those that lack this sequence, such as IL-4, inhibit it (Strieter et al., 1995). Angiostatin, 38 kDa internal fragment of plasminogen, that is a potent inhibitor of capillary EC growth in vitro (Cao et al., 1996; O'Reilly et al., 1994). Endostatin is a 20 kDa fragment of type XVIII collagen that has been identified as a factor produced by hemangioendothelioma cells that specifically inhibit EC proliferation (O'Reilly et al., 1997), angiogenesis in vivo and irreversibly halt tumor progression (Boehm et al., 1997).

1.2.3. Membrane-Bound Factors

In addition to factors that are secreted from cells and act at a distance from their sites of synthesis, several membrane-bound proteins play prominent roles in angiogenesis. These molecules require close cell-cell or cell-matrix contact for their effects to be felt. Members of all the four families of cell adhesion molecules viz., the selectins, the immunoglobulin supergene family, the cadherins and the integrins, are implicated in neovascularisation. In addition, ephrins and hedgehogs and notch are membrane bound factors involved in angiogenesis.

P-selectin and E-selectin promote adhesion of leukocytes to cytokine-activated vascular endothelium and have been shown to play a role in angiogenesis (Koch et al., 1995). E-selectin induces EC migration and tube formation (Nguyen et al., 1993). VCAM-1 and ICAM-1, members of the immunoglobulin supergene family, are expressed on quiescent endothelium but are upregulated after TNF-α, IL-1 or IFN-γ stimulation (Brooks, 1996). VCAM-1 can induce chemotaxis and angiogenesis (Koch et al., 1995).

ECs possess two cadherins: VE-cadherin, which is localized to adherens junctions exclusively on ECs (Lampugnani et al., 1992) and N-cadherin, which is not found at cell-cell contacts (Salomon et al., 1992). The intracellular domain of cadherins
mediates a linkage to the cytoskeleton by binding to β-catenin and plakoglobin, which when released from cadherins, can translocate to the nucleus and regulate gene transcription (Behrens et al., 1996; Gumbiner, 1996). VEGF-mediated enhancement of endothelial permeability is accompanied by tyrosine phosphorylation and dissociation of VE-cadherin (Esser et al., 1998; Kevil et al., 1998). VE-cadherin also mediates contact inhibition of EC growth (Caveda et al., 1996) thus ensures that ECs maintain a patent, stable monolayer in the vessel wall. VE-cadherin inhibits EC apoptosis and transmits EC survival signal by VEGF.

Integrins are heterodimeric transmembrane receptors involved in mediating cell-matrix and cell-cell interactions (discussed in detail in a subsequent section). αVβ3 integrin was found to be particularly important during angiogenesis as it acts as a receptor for von Willebrand factor, vitronectin, fibronectin, osteopontin and fibrin (Cheresh, 1987). This receptor is undetectable on quiescent endothelium but is highly upregulated during cytokines (TNF-α, FGF-2, VEGF) or tumor induced angiogenesis. It binds MMP-2 thereby localizing MMP-2 mediated matrix degradation to the EC surface. αVβ3 integrin also mediates in vitro EC attachment, spreading and migration (Leavesley et al., 1993). αVβ3 ligation also induces mitogen-activated protein kinase activation (Eliceiri and Cheresh, 1999) and suppresses apoptosis (Stromblad et al., 1996) in ECs. Another receptor, α5β1 integrin has been implicated in VEGF induced angiogenesis (Friedlander et al., 1995). In addition, the collagen receptors, α1β1 and α2β1 integrins are induced by VEGF (Senger et al., 1997). α5β1 integrin has also been implicated in angiogenesis (Kim et al., 2000). Thus specific cytokines stimulate angiogenesis by distinct signaling pathways that may be mediated by specific integrins. Given the diverse functions of integrins in angiogenesis, including adhesion to ECM, localization of proteases to capillary sprouts, and enhancement of EC survival, EC expression of a variety of integrins may stimulate distinct intracellular pathways that contribute to the progression of angiogenesis.

A unique class of receptor/ligand pair, eph receptors and ephrin ligands play a prominent role in blood vessel development. Eph receptors belong to the largest known family of receptor tyrosine kinases (Yancopoulos et al., 2000). An ephrin expressed on the surface of one cell binds and activates its cognate eph receptor on another cell, but through a reciprocal signaling mechanism the ephrin is also activated upon receptor engagement (Holland et al., 1996). One member of the ephrin family, ephrin-B2, is
expressed on arterial ECs of the developing embryo, and its receptor, eph-B4, is exclusively localized to venous ECs; ephrin-B2 co-localizes with eph-B4 at arterial/venous interfaces, after vasculogenesis has established the primary capillary plexus but before angiogenesis has remodeled it (Wang et al., 1998). Establishment of contact and signaling between arterial and venous compartments mediated by ephrin-B2 and eph-B4 is necessary for remodeling of the established primary capillary plexus during angiogenesis (Wang et al., 1998). Other members such as ephrin-A1 and ephrin-2A/eph-2A also are important for EC events involved in angiogenesis (Pandey et al., 1995; Ogawa et al., 2000).

Notch signaling is a highly conserved pathway that is important in vasculogenesis. There are four Notch receptors and five ligands (Jagged-1 and -2, Delta-1, -3, -4) (Iso et al., 2003). The activation of Notch results in cleavage at an intermembrane site that releases the intracellular domain, which translocates to the nucleus to activate transcription factors (Bicknell and Harris, 2004). This pathway is involved in the remodeling of the primary capillary plexus, vascular maintenance and determines artery versus venous fates. In vitro studies of EC function have shown an important negative role of Notch signaling in angiogenesis.

Hedgehogs are a class of 19 kDa proteins that interact with heparan sulphate on the cell surface through an N-terminal basic domain and are tethered to the surface through cholesterol and fatty acyl modification. The three human homologues, sonic hedgehog (Shh), desert hedgehog (Dhh) and Indian hedgehog (Ihh) and their signaling through Patched 1 receptor have been reported to be involved in angiogenesis. It seems that they may have a role in the spatial-temporal production of angiogenic growth factors during embryonic and post-natal angiogenesis, working upstream of VEGF, which in turn operates upstream of Notch (Bicknell and Harris, 2004).

Sprouty (Spry) is an intracellular protein localized to the inner leaflet of the plasma membrane by a cysteine-rich domain. There are four known isoforms of Spry in mammals. Spry has been shown to function negatively in angiogenesis (Bicknell and Harris, 2004).

1.2.4. Biomechanical Forces

In addition to the soluble and membrane-bound molecules, mechanical forces acting on vascular endothelium also contribute to the pruning and remodeling
processes characteristic of normal angiogenesis. The mechanical forces mediated by
blood flow have profound effects on vessel growth. Vessels that are not perfused with
blood eventually regress (Risau, 1997). Fluid shear stress induces a dramatic increase
in EC stress fiber expression (if flow is laminar) (Franke et al., 1984), promotes ECs to
divide (if flow is turbulent) (Davies et al., 1986), and stimulates the transcription of
genes for PDGF and TGF-β (Resnick and Gimbrone, 1995), which promote
angiogenesis.

1.3 Extracellular matrix

Extracellular matrix (ECM) is a complex network of macromolecules that
underlies all epithelia and endothelia and surrounds connective tissue cells. ECM has
an instructive role in directing the embryonic development and morphogenesis and it
influences many cellular processes in addition to its crucial role in maintaining the
structural integrity. ECM influences the behavior and pattern of gene expression of the
cells, which are in contact with it. The composition of the ECM interacting with the cell
surface has profound influence on the structure and function of the cells as it provides
signals which affect the morphology, motility, gene expression and survival of
adherent cells (Lukashov and Werb, 1998; Boudreau and Bissell, 1998; Ruoslahti,
1999). The diverse biological roles of ECM were explored by in vitro studies using the
isolated pure individual components of ECM, genetic studies using knockouts or
spliced segments of ECM proteins or using specific antibodies against the protein
components.

1.3.1 Composition of ECM

ECM comprises of both interstitial stroma and the basement membrane or basal
lamina. BM is composed of fibrils arranged in a meshwork and a granular matrix. It is
situated at the base of the epithelia that lines the circulative, digestive, reproductive,
respiratory and urinary tracts, where it separates the epithelia from the connective
tissue (Hay, 1991). ECM is composed of fibrous proteins (like collagens and elastins),
proteoglycans, glycoproteins (like fibronectin, laminin, entactin, nidogen, tenascin
etc) and proteolytic enzymes involved in degradation and remodeling of the ECM. The
macromolecules of the ECM are secreted locally by all cells and the exact composition
of the matrix depends on the cell type, its state of differentiation and its metabolic
status.
Collagens, a highly specialized family of glycoproteins, play structural roles as well as numerous developmental and physiological functions as antigens, chemotactic agents and also defective components in numerous pathological conditions. Elastin, a major protein of the elastic fiber, provides elasticity and resilience to tissues, promotes cell adhesion and is chemotactic (Mecham and Heuser, 1991; Fransblau and Faris, 1981). Fibronectin (FN), a prominent adhesive glycoprotein, is found in most ECMs, plasma and in other body fluids. It mediates various aspects of cellular interactions with ECM and is primarily involved in cellular migration during development and in wound healing (Mosher, 1989; Hynes, 1990). Laminins (LN) provide a link between cells and structural components and play key roles in development, differentiation and migration (Thyboll et al., 2002; Dziadek and Timpl, 1985).

Vitronectin, a cell adhesion and spreading factor found in plasma and ECM, participates in homeostasis, phagocytosis, tissue repair and immune function (Preissner, 1991). Tenascin is involved in cell matrix adhesion, cell migration and in modulation of growth and differentiation during morphogenesis (Erickson and Bourdon, 1989). Entactin plays a crucial role in linking collagen IV and LN together in the BM (Mann et al., 1989). Thrombospondin (TSP), which is secreted continuously by many cell types, regulates cell adhesion, cell migration, proliferation and growth (Lawler, 1986; Frazier, 1987). Proteoglycans, another major component of the ECM, exhibit both structural and functional diversity. They act as tissue organizers, influence cell growth and maturation of specialized tissues and affect tumor growth and invasion.

1.3.2. Matrix Receptors

The adhesion of cells to their surrounding ECM has vital roles in embryonic development, inflammatory responses, wound healing and adult tissue homeostasis. Cells attach to ECM by specific cell surface receptors which help mediate the diverse biological effects of ECM. Although some cell surface receptors bind cells directly to collagen, proteoglycans or hyaluronic acid, anchorage of cells to the ECM depends primarily on a group of plasma membrane receptors that are specialized to recognize and bind linkers such as FN and LN. The primary class of these receptors is transmembrane proteins of a receptor family called integrins. There are also non-integrin receptors.

1.3.2.1. Integrins:- Integrins are heterodimeric glycoprotein complexes composed of α and β subunits that are receptors for ECM proteins and membrane-
bound polypeptides on other cells (Figure: 1.3.). Integrins can exist in both active and inactive forms. Acquisition of active site requires divalent cations (Hynes, 1992), which can influence ligand binding characteristics (Pytela et al., 1987; Kirchhofer et al., 1991; Grzesiak et al., 1992). Binding specificity and activity of integrins are also found to be controlled by RNA splicing (Bray et al., 1990), post-translational modifications (Cierniewski et al., 1989), phosphorylation (Shaw et al., 1990), the membrane lipid environment (Conforti et al., 1990), growth factors (Heino et al., 1989) and agonists such as ADP, thrombin and phorbol ester (Philips et al., 1988).

There are 18 different α and 8 different β subunits which assemble to form 24 different heterodimers. A single α subunit of integrin can bind more than one β subunit and vice versa producing the diversity of integrins. Several different integrins can bind to the same ECM protein and a single integrin may be recognized by more than one ECM protein. The tripeptide sequence, Arg-Gly-Asp (RGD) has been identified as an essential sequence recognized by the integrins on most of the ligands including LN, FN, vitronectin, fibrinogen, von Willebrand factor and osteopontin (Ruoslahti and Pierschbacher, 1987; Oldberg et al., 1986). Many “counter-receptors” are ligands of integrins, reflecting their role in mediating cell-cell interactions.

Integrin engagement of an ECM ligand typically activates a signaling pathway(s) (Giancotti and Ruoslahti, 1999; Hecker and Gladson, 2003; Hynes, 1992) that initiate cellular activities such as division, secretion and gene expression. Integrin mediated signaling includes Ca\(^{2+}\) influx, H\(^+\) exchange, protein tyrosine and non-tyrosine phosphorylation, alterations in phosphoinositide metabolism, activation of mitogen activated protein kinases (MAPKs), changes in gene expression and growth stimulation (Yamada and Miyamoto, 1995). Integrins can function in additive or synergistic fashion with many growth factors (McNamee et al., 1993). Cytosplasmic tails of integrins interact with cytoskeletal elements through adapter proteins leading to the “outside-in” signaling (Otey et al., 1990; Ingber, 1991). The cytosolic effector binding propagates the conformational changes from the cytoplasmic domains of integrins to the extracellular binding site in response to intracellular signaling events leading to “inside-out” signaling (Dedhar and Hannigan, 1996; Williams et al., 1994).

1.3.2.2. Non-Integrin Receptors: - There are a number of non-integrin receptors involved in cell-matrix interactions. Many of them are hydrophobic integral membrane proteins that can interact with cytoskeleton. A hydrophobic integral
membrane protein of MW 68-70 kDa binds to LN and also interacts with cytoskeleton (Clement et al., 1990). A cell surface receptor complex which binds to elastin, β-galactosides, LN and actin is also discovered (Smith and Wood, 1992). Anchorin is a collagen receptor and is an integral membrane protein (Mollenhauer et al., 1984). AGp 110 is a non-integrin membrane glycoprotein that interacts with FN in an RGD independent manner (Stamatoglou et al., 1990). Cell surface proteoglycans can also serve as receptors for matrix proteins like FN (Hook et al., 1984).

Figure: 1.3. Schematic structure of an integrin: Integrins are heterodimeric transmembrane proteins consisting of an α and a β subunit. Internal disulfide bonds within the subunits are shown. Dark blue regions in the head segment of the α subunit represent homologous repeats and red circles represent EF-hand consensus sequences that bind divalent metal ion. Arrow indicates the region where post-translational modifications take place.
1.3.3. Matrix Remodeling

Matrix metalloproteinases are neutral, cation dependent endopeptidases directing the matrix proteolysis and activating other pro MMPs. ECM is extensively modified and remodeled by proteases. As a result of the activity of these proteases, important changes in cell-cell and cell-ECM interactions occur, and new signals are generated from the cell surface. These signals affect gene expression and ultimately influence critical cell behavior such as proliferation, survival, differentiation and motility (Mott and Werb, 2004). Proteases are expressed as inactive proforms that become activated through a variety of mechanisms that often involves a close collaboration among several families of proteases (Murphy et al., 1999; Nagase, 1997). MMPs are secreted together with their inhibitors, ensuring a stringent control of local proteolytic activity, in order to preserve normal tissue structure (Liekens et al., 2001; Blavier et al., 1999).

1.3.4. Role of ECM in Angiogenesis

Migration of ECs and development of new capillary vessels are dependent on not only the cells and cytokines present but also the production and organization of ECM components. In the capillaries, ECs are attached to the basal lamina, which contains various ECM components synthesized by the endothelium. These macromolecules include mainly type IV collagen and laminin, but also fibronectin, proteoglycans (PGs), entactin and thrombospondin-1 (Colville-Nash and Scott, 1992; Diaz-Flores et al., 1994; Montesano et al., 1992). But the ECM surrounding the newly formed capillaries contains mainly type I collagen as well as other ECM components including fibronectin, fibrinogen, vitronectin, tenascin and thrombospondin-1 (Diaz-Flores et al., 1994; Montesano et al., 1992; Canfield and Schor, 1995). During angiogenesis, ECs must adhere to the ECM to proliferate, migrate, establish polarity, form tubes and maintain an appropriate cell shape. EC survival depends upon cell–ECM interactions. The migratory response of cells to ECM is biphasic. High or low concentrations of matrix proteins are suboptimal for cell migration, while intermediate concentrations promote optimal migration (Hocking and Chang, 2003; Palecek et al., 1997; DiMilla et al., 1993). In addition, cell-ECM interactions are required for the mitogenic response of cells to soluble growth factors (Guadagno et al., 1993; Assoian and Zhu, 1997; Hansen et al., 1994; Fang et al., 1996). The ECM regulates angiogenesis by providing scaffold support and signaling roles. They also
Angiogenesis: Role of FN- A Review

serve as a reservoir and modulator for growth factors by sequestering angiogenic factors, such as FGF 2 and heparin-binding forms of VEGF. ECM components play a role in the direction of ECs during capillary formation. ECM degradation by MMPs or plasmin can promote angiogenesis by stimulating EC migration (Carmeliet and Collen, 2000; Hangai et al., 2002; Pepper, 2001). This stimulatory effect on migration may be due to decreasing the density of ECM proteins and/or by exposing cryptic binding sites within matrix molecules that promote migration (Hangai et al., 2002; Xu et al., 2001). ECM molecules also play an important role in stabilizing blood vessels.

Role of ECM in EC function has been studied by using individual components of ECM and by molecular dissection of each component. Individual components of ECM have been shown to promote EC adhesion, growth and migration. LN promotes EC adhesion, growth, migration, proliferation and multicellular organization during angiogenesis (Aumailley and Smyth, 1998; Gonzalez et al., 2002). Fibronectin is chemotactic for ECs and promotes the elongation of micro vessels in explant cultures in vitro (Colville-Nash and Scott, 1992; Nicosia et al., 1993). Collagen I stimulates EC migration. Collagen IV has also been shown to regulate angiogenesis. Cleavage of collagen IV by MMP exposes a cryptic epitope within collagen IV, whose presence is required for angiogenesis and tumor growth in vivo (Hangai et al., 2002; Xu et al., 2001). Heparan sulphate proteoglycans are necessary for stable binding of the pro-angiogenic growth factor bFGF to its receptor and is involved in signaling (Ornitz et al., 1992; Rapraeger et al., 1991; Steinfeld et al., 1996). Intact perlecan is pro-angiogenic whereas the carboxyl-terminal fragment has been shown to have anti-angiogenic properties (Ioizzo, 2005; Mongiat et al., 2003). A transmembrane chondroitin sulfate proteoglycan known as NG2 has been shown to promote EC spreading and migration, as well as the proliferation of microvascular ECs in response to PDGF stimulation (Ozerdem and Stallcup, 2003). Additional pro-angiogenic actions of NG2 include its ability to bind angiostatin (kringles 1-4 of plasminogen), which apparently leads to the sequestration and inhibition of this anti-angiogenic molecule (Chekenya et al., 2002).

Intact TSP-1 and TSP-2 are both potent inhibitors of angiogenesis (Armstrong and Bornstein, 2003; Adams and Lawler, 2004). Increased expression of pigment epithelial-derived factor (PEDF) has been linked to decreased microvessel density and suppression of tumor growth (Abe et al., 2004; Hosomichi et al., 2005). Tenascin-C can promote de-adhesion (Murphy-Ullrich, 2001) and may also function in a pro-
Angiogenesis: Role of FN-A Review

angiogenic manner. Osteopontin over-expression has been shown to promote angiogenesis and tumor growth (Hirama et al., 2003). Over-expression of SPARC (secreted protein acidic and rich in cysteine) also known as BM40 or osteonectin has been observed in tumors such as human oesophageal carcinoma and cutaneous malignant melanoma (Porte et al., 1998; Massi et al., 1999). Furthermore, transient expression of SPARC during EC injury and cellular activation indicated a role in tissue repair, remodeling and angiogenesis (Jendraschak and Sage, 1996).

As indicated before, cells interact with the different components of ECM through integral cell surface integrin receptors. Of the sixteen integrins reported to be involved in vascular biology (Drake and Little, 1998), seven are known to be expressed in ECs (α1β1, α2β1, α3β1, α5β1, α6β1, αvβ3, αvβ5) at varying times. Because angiogenesis involves invasion of the ECM and migration of ECs through it, the cell-matrix interactions mediated by integrins seem likely to play important roles in vascular remodeling. Two non-receptor cytoplasmic tyrosine kinases, cellular Src and focal adhesion kinase (FAK) are activated in a relatively rapid manner upon integrin clustering in the cell membrane during EC attachment (Giancotti and Ruoslahti, 1999; Kornberg et al., 1992). This activation of FAK and cellular Src facilitates the interaction of FAK with cellular Src, as well as the activation of phosphatidylinositol-3-kinase (PI3-K) and the phosphorylation of downstream signaling molecules, such as CAS family members and ERK, leading to EC migration and proliferation (Giancotti and Ruoslahti, 1999; Brakebusch et al., 2002; Chakravarti et al., 2004; Ding et al., 2005; Wang et al., 2000). Integrin ligation in ECs has been implicated in cell survival and regulation of gene transcription. Moreover, integrin receptors act in conjunction with growth factor receptors such as VEGF, PDGF etc (Mahabeleshwar et al., 2007).

It has been generally assumed that proteases are necessary to degrade the ECM during the process of angiogenesis. Cell invasion requires matrix-degrading proteolytic enzymes, such as MMPs and PA. The release of these enzymes by EC promotes the degradation of BM during sprout formation (Rundhaug, 2005). The interaction of urokinase-type plasminogen activators (uPA) with its receptor concentrates the enzyme activity to the so-called focal attachment sites on the cell surface and stimulates signal transduction through the uPAR, leading to induction of cell migration and invasion (Blasi, 1997). Matrix metalloproteases (MMPs) are crucial for angiogenesis, being necessary to degrade the ECM to make room for migrating ECs. MMPs also contribute to angiogenesis by detaching pericytes from vessels.
undergoing angiogenesis, by releasing ECM-bound angiogenic growth factors, by localizing on the cell surface of the invading tips of migrating ECs, by exposing cryptic, pro-angiogenic integrin binding sites in the ECM, by generating pro-migratory ECM fragments and by cleaving VE-cadherin to break endothelial cell-cell adhesions (Rundhaug, 2005). MMPs can also contribute negatively to angiogenesis through the generation of endogenous angiogenesis inhibitors by proteolytic cleavage of certain collagen chains and plasminogen and by modulating cell receptor signaling by cleaving off their ligand-binding domains (Rundhaug, 2005). These extracellular endopeptidases are secreted as zymogens that become activated in the extracellular compartment and subsequently selectively degrade components of the ECM (Stetler-Stevenson, 1999). MMP activity and, hence, angiogenesis is counteracted by the family of tissue inhibitors of metalloproteinase (TIMPs) (Gomez et al., 1997; Valente et al., 1998). Tissue inhibitor of metalloproteinases (TIMP-1), an angiostatic compound exhibits its inhibitory activity by blocking the action of MMP in this process. Thus a balance between cell surface-associated proteases and their natural inhibitors is important during angiogenesis.

1.4. Fibronectin

Fibronectin (FN) is a large multi-domain glycoprotein found in connective tissues, on cell surface and in plasma and other body fluids. It interacts with a variety of macromolecules including components of the cytoskeleton and the ECM, circulating components of the blood coagulation, acute phase and complement systems, and with cell-surface receptors on a variety of cells including fibroblasts, neurons, phagocytes and bacteria. FN also interacts with itself, forming fibrillar entities. In addition, it binds several small molecules such as gangliosides, sugars and Ca^{2+} ions. These diverse recognition functions are located on distinct domains, many of which have been expressed in recombinant form or isolated from proteolytic digests with retention of specific binding properties. FN is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting and cell migration/adhesion. It exists in two main forms: as an insoluble polymeric fibrillar glycoprotein network that serves as a linker in the ECM and as a soluble disulphide linked dimer found in the plasma (plasma FN).

FN sometimes serves as a general cell adhesion molecule by anchoring cells to collagen or proteoglycan substrates. It also can serve to organize cellular interaction
with the ECM by binding to different components of the ECM and to membrane-bound FN receptors on cell surfaces. The importance of FN in cell migration events during embryogenesis has been documented in several contexts (Mosher, 1989; Hynes, 1990; Johansson et al., 1997; Campbell, 2003; Wierzbicka-Patynowski and Schwarzbauer, 2003; Wagner and Hynes, 1979; Mosher and Furcht, 1981; Kornblith et al., 1996; Davis and Senger, 2005).

1.4.1. Structure of Fibronectin: Functional Domains

Fibronectin is a 550 kDa multifunctional adhesive glycoprotein. It is a dimer composed of two identical high molecular weight subunits or closely related polypeptides joined by a pair of disulfide bonds near their carboxyl termini. FN is a rod-like molecule, composed of three different types of internally homologous, repeating modules, Types I, II and III (Figure: 1.4.). These modules, though present in the same amino acid chain, can be envisioned as "beads on a string," each one joined to its neighbours by short linkers. Each module constitutes an independently folded unit existing in isolation from their neighbours, often referred to as a domain. The stability of individual modules is often affected by interactions with neighbouring modules and such interactions have important implications for the overall shape of the protein. The native soluble protomer is believed to have a globular configuration (Erickson and Carrell, 1983; Rocco et al., 1987), while an elongated form would be adopted during polymerization to fibrils (Williams et al., 1982; Tooney et al., 1983).

Twelve type I modules, each made up of ~45 amino acids, constitute the amino-terminal and carboxy-terminal region of the molecule and are involved mainly in fibrin and collagen binding (Potts and Campbell, 1996). Two type II modules, ~60 amino acids in length, are also found in FN. They are instrumental in binding collagen. The most abundant module in FN is Type III (Bork and Doolittle, 1992), each module being ~ 90 amino acids in length. Two regions in F3 domain possess cell binding activity: III10 and III14-V. The 10F3 module contains the Arg-Gly-Asp (RGD), the FN receptor (integrin) binding motif (Pierschbacher and Ruoslahti, 1984) along with binding sites for other integrins and heparin. Depending on the tissue type and/or cellular conditions, the FN molecule is made up of 15-17 type III modules. In addition, there is a module that does not fall into any of these categories, called IIICS. This module, along with EDB and EDA (both type III modules), is regulated through alternative splicing of FN pre-mRNA. FN molecules can form two disulphide bridges.
at their carboxy-termini, producing a covalently-linked dimer.

Digestion of FN with a variety of proteases generates a collection of fragments that are referred to as functional domains because they retain the ability to interact with other macromolecules (Hynes and Yamada, 1982). When FN is digested with thermolysin, an N-terminal 29 kDa Fib-1/Hep-1 fragment (I₁,₃) is obtained that binds to fibrin, heparin and some bacteria and it is important for fibronectin matrix assembly. This is followed by a 42 kDa gelatin binding fragment (GBF, I₆₂I₇₂I₈₂) that binds to denatured collagen (gelatin). Then comes a small ∼9 kDa fragment (III-1) that binds weakly to heparin and is thought to be important for self-association and fibril formation. The large 110 kDa central cell-binding fragment (III₂-₁₀) contains the tripeptide RGD. This is followed by either a 30 or 40 kDa Hep-2 fragment (III₁₂₁₄ or III₁₂₁₅) which contains the high-affinity heparin binding site. From the C-terminus, a

![Figure: 1.4. Domain structure of FN: Repeated arrangement of the three module types, as well as key binding sites.](image-url)
second fibrin binding fragment, 19 kDa Fib-2 (I_{10-12}) is also obtained. The variably spliced V region, also referred to as the type III connecting strand (CS) contains two sites for recognition by αβ₃ integrin; however, it is not recovered intact from proteolytic digests. Its presence in only one chain of plasma FN and its vulnerability to proteolysis account for the occurrence of 30 and 40 kDa Hep-2 fragments as well as a small 8 kDa fragment consisting of module III-15 whose function is unknown.

1.4.2. Binding Characteristics

Most cell types in the body synthesize and secrete FN into the extracellular space where it interacts with itself and other macromolecules such as collagens, proteoglycans, tenasin, fibulin and thrombospondin. The electrostatic long range interactions between regions of opposite net charge within or between FN chains hold the molecule in a relatively compact conformation. Gelatin, denatured form of collagen, interacts well with FN (Engvall and Ruoslahti, 1977) and this interaction serves as the basis of the most common method of purification of the whole protein as well as collagen binding fragments following protease digestion (Engvall and Ruoslahti;1977, Miekka et al., 1982). FN tends to co-distribute with collagen in the ECM and polymerization of collagen into fibrils is diminished in the absence of FN (Velling et al., 2002). However, the extent to which FN binds to native triple helical collagen molecules (or their fibrils) is unclear. Heparin interacts with FN through the Hep-2 domains located in the C-terminal region of each polypeptide chain. The N-terminal region of FN, Fib-1/hep-I fragment that contains the first five type I modules also interacts with heparin. It seems that both sites could contribute to interaction with heparin-like GAGs present in the ECM. The same may be true for other much weaker heparin-binding sites located in module III-1 (Litvinovich et al., 1992) and in the N-terminal half of 110 kDa CBF (Gold et al., 1983), which may add to the overall strength of interaction of FN with an ECM that is rich in HSPGs. However, the interaction of FN with heparin in vivo is less likely except in the blood of patients on heparin therapy. Heparan sulfate (HS) is the more relevant GAG as far as FN is concerned since it is the one covalently attached to proteoglycans that co-distribute with FN in the ECM. As HS is not as highly sulfated as heparin, its affinity for FN and other heparin binding proteins is lower than that of heparin. Other GAGs that have been reported to bind to FN under various conditions include dermatan sulfate, chondroitin sulfate and hyaluronic acid. In most cases their affinities tend to be qualitatively lower than for
heparin and little quantitative information is available. Binding of FN to immobilized chondroitin sulfate (at low ionic strength) appears to be mediated by the same Hep-2 determinants that mediate binding to heparin (Barkalow and Schwarzbauer, 1994). Fibrillar FN, the form prevalent in the ECM, may present an array of positively charged domains to the polyanionic GAGs leading to a cooperative multipoint attachment.

The shorter forms of Tenascin-C (Tn) bind much more effectively to FN and preferentially co-distribute with FN fibrils in the ECM suggesting that the FN binding site is masked in the longer forms (Chiquet-Ehrismann et al., 1991). The binding site for FN was reported to be in the 3rd FN III module of Tn. The location of the Tn binding site on FN is controversial. Tn-C was reported to bind to module III-13 in the Hep-2 region of FN, thereby competing with syndecan-4 and blocking signals for cell adhesion (Huang et al., 2001). This is in conflict with a more recent study that failed to detect an interaction with the Hep-2 fragment but instead produced evidence for a cryptic binding site in the N-terminal fib-1/hep-1 domain with a Kd ~ 1 mM (Ingham et al., 2004). Perlecan, a large HSPG, binds via its core protein to FN (Hopf et al., 1999), co-localizes with FN fibrils in the matrix and seems to enhance the binding of Tn to those fibrils, presumably via its GAG moiety serving as an additional bridge between FN and Tn (Chung and Erickson, 1997). Fibulin-1 interacts with FN and seems to suppress FN-mediated adhesion and motility (Twal et al., 2001). Fibulin-2 also binds FN along with several other matrix proteins (Sasaki et al., 1995). At least two regions which may be cryptic within TSP appear to be able to bind FN (Dardik and Lahav, 1989).

FN interacts with several other molecules. Some of these are of questionable physiological significance, little more than in vitro curiosities. FN is reported to bind actin (Keski-Oja et al., 1980). The non-covalent interaction of transglutaminase to the gelatin-binding region of FN may play a role in disposing of transglutaminase (TGase) that is released into the blood by lysed red cells and it also helps in matrix assembly. Dipeptidylpeptidase IV (DPP-IV or CD26) mediates binding of FN on hepatocyte surfaces and may facilitate an interaction of these cells with the ECM. DPP-IV on ECs serves as an adhesion receptor for FN multimers on the surface of metastatic breast cancer cells (e.g. MTF7 cells) (Cheng et al., 1998; 2003). Low density lipoprotein receptor related protein (LRP) binds FN, but the physiological significance of this interaction is not clear but LRP could play a role in the removal of
excess FN during tissue remodeling and embryogenesis. CD9 associates with integrins and affects cell behavior on FN surfaces (Cook et al., 1999).

1.4.3. Synthesis, Fibril Formation and Matrix Assembly

The plasma form is synthesized by hepatocytes and the ECM form is made by various cell types including fibroblasts, chondrocytes, endothelial cells, macrophages, platelets as well as certain epithelial cells. The FN gene is about 75 kb long, contains around 50 exons and is transcribed from a single promoter into a single primary transcript. Albeit the product of a single gene FN exists in a number of variant forms. The diversity observed among FN is due to both alternative splicing and post-translational modifications.

Different FN polypeptide variants arise mainly through a complex pattern of alternative mRNA splicing of certain type III exons. Whereas F1 and F2 modules are encoded by one exon each, two exons encode most F3 modules. There are five sites of alternate splicing of FN mRNA. The first two splicing through exon skipping results in the insertion of extra type III domains, EDA (also called EIIIA or EDI) and EDB (also called EIIIB or EDII) after modules III-11 and III-7 respectively. These modules are virtually absent from adult tissue but are differentially expressed during embryonic development and again in malignant or injured tissue and during angiogenesis (George et al., 2000; Peters et al., 2003) and has been used as a marker for angiogenesis and for certain types of cancer (Santimaria et al., 2003; Manabe et al., 1997). The third site of splicing is in the variable (V or IIICS) region. In plasma FN, the V region is fully incorporated into one chain but entirely absent from the other. A fourth site of splicing occurs primarily in cartilage where the dominant form of FN lacks not only the entire V region but modules III-15 and I-10 as well (MacLeod et al., 1996). A truncated single chain form of FN (Zhao et al., 2001), has been reported in humans (Liu et al., 2003).

The post-translational modifications of FN take place in the Golgi apparatus (Hynes and Yamada, 1982; Paul and Hynes, 1984). FN undergoes glycosylation that appears to stabilize FN molecules against proteolysis and influence the interaction with other molecules and cells. FN is glycosylated at about 4-6 sites within the protein via arginine (N-linked), which accounts for 4-5% of the molecular weight of the molecule. Sites of N-linked glycosylation include the 2<sup>nd</sup> type II module, the 8<sup>th</sup> type 1 module, the 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> type III modules and the V region, which also contains O-linked carbohydrate. Major sugar residues in glycosylation units are mannose,
galactose, glucosamine and sialic acid. The extent and type of glycosylation varies depending on the tissue source. Amniotic fluid FN contains about twice as much carbohydrate as the plasma form and this includes the presence of polylactosamine in the gelatin-binding domain (Zhu and Laine, 1985). FN isolated from malignant human tissues, fetal tissues or placenta also contains a unique "oncofetal" epitope, involving N-acetylgalactosaminylation of a threonine in the V region (Matsuura et al., 1989). The actual function of the various carbohydrate groups on FN, beyond protecting certain domains from proteolysis (Bernard et al., 1982) or stabilizing them toward heat (Ingham et al., 1995), remains unclear. Other post-translational modifications of FN include phosphorylation and sulfation. FN is phosphorylated on a serine residue near the carboxy terminus. Certain FN variants are found to be tyrosine-linked sulfated (Paul and Hynes, 1984).

FN secreted into the extracellular space interacts with other matrix molecules and assemble to form a highly ordered structure. The formation of an insoluble fibrillar meshwork from a relatively compact soluble protomer is a highly regulated process occurring at the cell surface that utilizes integrins and possibly other cell-surface receptors (Mosher et al., 1992) including uPAR (urokinase-type plasminogen activator receptor) (Monaghan et al., 2004) and cell-surface proteoglycans (Woods and Couchman, 1994). Since major ECM components such as collagens, fibulins and proteoglycans (Hedman et al., 1984; Roman and McDonald, 1993) may be dependent on interactions with FN for their organized deposition into the matrix, the polymerization reaction of soluble FN protomers to insoluble networks is central for matrix formation. \( \alpha_5\beta_1 \) integrin was the first integrin identified to be involved in FN network formation. It localizes to the sites where FN fibrils are in contact with the cell (Roman et al., 1989). \( \alpha_4\beta_1 \) integrin (Wennerberg et al., 1996) has also been shown to have this potential, although \( \alpha_4\beta_1 \) integrin was markedly less efficient than \( \alpha_5\beta_1 \) integrin.

The polymerization process can be separated into two phases, initiation (nucleation) and extension. The fibrils grow only at one end, indicating that they are polarized (Winklbauer and Stoltz, 1995). Alternatively, the direction of fibril growth may reflect the migration of the cell. The fibrils often appear to be anchored at both ends to cell surfaces, either on one cell or on two different cells. The monomeric FN chains are unable to become incorporated into the fibrils (Schwarzbauer, 1991). Several regions in FN are involved in one or the other phases of polymerization,
through binding to cell surface components or to a neighboring FN molecule. Binding of the RGD region to integrins may be the initial event which triggers the subsequent reactions (Fogerty et al., 1990), possibly by induction of an altered FN conformation. The FN fibril formation in the ECM involves an intermolecular strand swapping between FN domains that are partially unfolded in response to mechanical stress (Schlunegger et al., 1997). Such a mechanism could reconcile the failure to identify self-association sites on native proteolytic fragments and would be consistent with the emerging evidence for interactions involving cryptic sites in type III domains.

1.4.4. Biological Functions of Fibronectin

Since expression of FN is widespread in embryos and in adult tissues and is altered in physiological and pathological processes including cancer, hemostasis and thrombosis, fibrosis and wound healing, it is widely believed that FN plays important roles in development and in adult physiology. FN plays a vital role in cellular adhesion and migration, oncogenic transformation, wound healing and hemostasis. Synthesis of FN is stimulated during wound healing, which typically involves migration on a FN-fibrin matrix (Clark, 1988; Ffrench-Constant et al., 1989; Hynes, 1990). During embryogenesis, FN appears before or at the onset of gastrulation in all vertebrates examined, and it is abundant at times and sites of cell migration during gastrulation, neural crest cell migration and the migration of primordial germ cells (Adams and Watt, 1993). The expression of integrins has also been demonstrated to be developmentally regulated, where some of the FN receptor subunits are continuously expressed, while others are not (Sutherland et al., 1993). Injection of antibodies to FN or RGD containing peptides inhibits gastrulation of several species, indicating that the interaction with FN is important during that particular stage of development (Boucaut et al., 1984a; 1984b; Darribere et al., 1988; Bronner-Fraser et al., 1991).

FN-null mouse embryos die at embryonic day 8.5 and they have defects in the development of mesoderm and mesoderm-derived structures such as neural tube, notochord, somites, heart and blood vessels (George et al., 1993; Georges-Labouesse et al., 1996). A number of studies have shown that the multimeric ECM form of FN has properties distinct from soluble, protomeric FN (Mercurius and Morla, 1998; Sottile et al., 1998; Morla et al., 1994; Pasqualini et al., 1996; Hocking et al., 2000). Further, FN matrix polymerization is important for regulating cell proliferation, cell migration and ECM remodeling (Bourdoulous et al., 1998; Clark et al., 1997; Wu et al., 1998;
Hocking et al., 2000; Hocking and Chang, 2003; Hocking and Kowalski, 2002; Sechler and Schwarzbauer, 1998). Agents that inhibit FN matrix deposition also block the deposition and retention of other proteins in the ECM, including TSP-1 (Sottile and Hocking, 2002).

Since there are several receptors for FN, the total effect of the FN-null mutation is likely to be made up of separate effects due to lack of binding between FN and its individual receptors. The inner cell mass requires $\beta_1$ integrins for survival (Johansson et al., 1997). The knockout of the $\alpha_5$ subunit resulted in nearly the same defects as the FN-null mutation did (Yang et al., 1993), suggesting that the majority of the defects seen in the fibronectin-null embryos probably are due to the lack of $\alpha_5\beta_1$ integrin/FN binding. It seems that $\alpha_5\beta_1$ integrin is required for proper formation and maintenance of blood vessels, while other FN receptors are involved in initial steps of vasculogenesis (Yang et al., 1993). Lack of $\alpha_4$ results in defective development of the kidney, indicating a role for $\alpha_4\beta_1$ integrin in kidney morphogenesis (Fässler et al., 1996; Hynes, 1996). The $\alpha_4$-null mice die on the first day after birth, probably as a cause of vascular haemorrhage (Fässler et al., 1996; Hynes, 1996). The $\alpha_4$ containing integrins have a wide range of ligands in addition to FN and this complicates the interpretation of the results with regard to FN and its functions.

Taken together, these knock-out experiments of both FN and its integrin receptors clearly demonstrate that these proteins are of vital importance for the organism. They also show that the different integrins have distinct functions and distribution, and that they in most cases do not compensate for each other. Nevertheless, $\alpha_5$ integrins have been found to be able to compensate for $\alpha_5\beta_1$ integrin function with regard to adhesion to FN and FN matrix assembly in vitro (Wennerberg et al., 1996; Yang and Hynes, 1996).

1.4.5. Molecular Mechanism of FN Action

FN can bind to 10 different integrin receptors (Table: 1.2.) and different intracellular signals are generated by the protein depending on which integrin it interacts with. FN binding integrins contain ion coordination sites; different $\alpha$ subunits contain three or four homologous sequences (DXDXDGXXD) which are similar to the $\text{Ca}^{2+}$-binding EF-hand motif (Gulino et al., 1992) and a second type of direct cation binding motif, DXSXS, is located in the $\beta$ subunits (Lee et al., 1995; Tozer et al., 1996).
\( \alpha_\beta \) integrin was the first to be identified as a prototype FN receptor (Pytela et al., 1985) and in contrast to most other FN binding integrins, it is specialized for binding this ligand. It is expressed by many cell types and is probably the major FN receptor in several of these. The RGD loop in III\(_{10} \) is the critical recognition site for \( \alpha_\beta \) integrin (Pierschbacher and Ruoslahti, 1984), but the synergy site PHSRN in III\(_{9} \) is required for high affinity binding (Aota et al., 1994). Similarly, both the RGD site and the synergy site contribute to the binding of FN to \( \alpha_\beta \) integrin (Bowditch et al., 1994; Mohri et al., 1995), through two separate binding sites. There is also evidence that this integrin recognizes a site in the hep-2 region of FN (Mohri et al., 1996).

The \( \alpha_\beta \) integrin is expressed in white blood cells and in several types of adherent cells (Albelda et al., 1990; Rosen et al., 1992; Stepp et al., 1994). It mediates cell-matrix contacts through FN (Humphries et al., 1995). Activating signals are required to induce strong binding of \( \alpha_\beta \) to FN (Alon et al., 1995; Berlin et al., 1995). \( \alpha_\beta \) integrin interacts primarily with the III\(_{14}-V \) region in FN. \( \alpha_\beta \) integrin can be induced to recognize the RGD region of FN also (Sanchez-Aparicio et al., 1994). The interaction is detectable only in the presence of integrin activating antibodies, hence its physiological relevance remains to be determined. \( \alpha_\beta \) integrin, expressed mainly in epithelial cells of skin, ECs of the digestive tract and kidney mesangial cells, shows a weak binding to FN (Wayner et al., 1988; Wayner and Carter, 1987; Elices et al., 1991; Plantefaber and Hynes, 1989). It does not function as a FN receptor in intact cells (Weitzman et al., 1993; Delwel et al., 1994). \( \alpha_\beta \) integrin has been suggested to serve as a secondary receptor with post adhesion functions (DiPersio et al., 1995). \( \alpha_\beta \) integrin, expressed mainly in epithelial cells, smooth muscle cells, myofibroblasts, and embryonic neural cells (Bossy et al., 1991; Schnapp et al., 1995a), has been shown to bind to RGD sites in FN (Schnapp et al., 1995b) and mediate cell adhesion and neurite outgrowth (Muller et al., 1995; Varnum-Finney et al., 1995). \( \alpha_\beta \) integrin is present on the surface of some cell lines and appears to recognize FN as its major ligand (Vogel et al., 1990; Zhang et al., 1993). \( \alpha_\beta \) integrin is abundantly expressed by many cultured cells, but its distribution in vivo is restricted mainly to activated ECs, osteoclasts and tumor cells (Varner et al., 1995). It has been demonstrated to bind FN and is important for angiogenesis and is therefore a potential target for inhibition of tumor growth (Friedlander et al., 1995; Brooks et al., 1996; Varner and Cheresh, 1996). FN appears to be the main ligand for \( \alpha_\beta \) (Busk et al., 1992), which is expressed by epithelial cells.
Chapter 1  

Angiogenesis: Role of FN- A Review

during development and wound healing and in many epithelial tumors (Breuss et al., 1995). Both \( \alpha_5\beta_1 \) and \( \alpha_5\beta_4 \) integrins bind FN via the RGD site and do not require the synergy site in FN (Bowditch et al., 1994; Akiyama et al., 1995; Chen et al., 1996).

Integrin binding to FN causes focal adhesion complex formation that links to actin cytoskeleton through structural components such as talin, \( \alpha \)-actinin, vinculin, paxilin and filamin (Figure: 1.5.). Integrin activation and clustering also induces an intracellular signaling cascade involving a variety of kinases and adapter molecules linking integrins to other kinases, members of the GTPase families, lipid kinases and phospholipases and ion channels (Ivaska and Heino, 2000; Parsons et al., 2000). Focal adhesion kinase (FAK) plays a central role in assembling complexes of signaling proteins at the cell surface.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Main binding site in FN</th>
<th>Additional binding sites in FN</th>
<th>Other protein ligands</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_5\beta_1 )</td>
<td>RGD</td>
<td></td>
<td>LN5</td>
<td></td>
</tr>
<tr>
<td>( \alpha_4\beta_1 )</td>
<td>LDV in CS1</td>
<td>H1, CS5, (RGD)</td>
<td>VCAM-1</td>
<td></td>
</tr>
<tr>
<td>( \alpha_5\beta_1 )</td>
<td>RGD</td>
<td>PHSRN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha_4\beta_1 )</td>
<td>RGD</td>
<td></td>
<td>VN, Tn</td>
<td></td>
</tr>
<tr>
<td>( \alpha_6 )</td>
<td>RGD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha_5\beta_1 )</td>
<td>RGD</td>
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</tr>
<tr>
<td>( \alpha_4\beta_1 )</td>
<td>LDV in CS1</td>
<td></td>
<td>MAdCAM-1, VCAM-1</td>
<td>Johansson et al., 1997</td>
</tr>
<tr>
<td>( \alpha_8 )</td>
<td>CS1</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 1.2.- Fibronectin-binding integrins-FN-fibronectin; FG-fibrinogen; LN-laminin; MAdCAM-mucosal addressin cell adhesion molecule; OP-osteopontin; Tn-tenascin; TSP-thrombospondin; VCAM-vascular cell adhesion molecule; VN-vitronectin; vWF-von Willebrand factor.

Recently, changes in plasma membrane cholesterol levels in cells have been shown to modify the \( \alpha_5\beta_1 \) integrin and FN-mediated signaling pathways that regulate the cell shape, adhesion and motility in L27 cells by changes in the organization of actin cytoskeleton involving FAK, paxilin and ERK 1/2 MAPK (Ramprasad et al., 2007b). Further, the potential interaction of phosphatidyl choline molecules in cell membrane with FN in the ECM is aided by cholesterol and \( \alpha_5\beta_1 \) integrin (Ramprasad et al., 2007a).
Chapter 1  

Angiogenesis: Role of FN - A Review

Certain heparin-binding sequences in FN seem to cooperate with its integrin $\alpha_\beta_1$-binding domain (modules III$_{8,10}$) to enhance the spreading of cells (Woods et al., 1986; Moyano et al., 2003). The Hep-2 domain exerts its effects by binding to one or more cell-surface heparan sulfate proteoglycans (HSPGs) to induce additional signals through activation of PKC (Couchman and Woods, 1999) (Figure 1.5.). The cell surface HSPG, syndecan-4 interacts with FN via its heparan sulfate (HS) chains, and contributes additional signals through the ability of its unique cytoplasmic domain to bind and activate protein kinase C-$\alpha$ (Woods et al., 2000; 1986; Saoncella et al., 1999; Jeong et al., 2001). A direct interaction between syndecan-4 and FN contributes to cell spreading and survival. A fully developed adhesive response to FN thus requires the coordinated extracellular interactions of integrin and HS with the "cell-binding" and "heparin-binding" domains of FN, respectively (Woods et al., 2000; 1986; Saoncella et al., 1999; Jeong et al., 2001).

Figure: 1.5. Molecular mechanism of FN action:-- FN through its cell binding domain interacts with the integrin receptors and mediates an intracellular signaling cascade involving FAK-Src leading to biological effects. In addition to this, heparin binding domain of FN interacts with cell surface HSPGs like syndecans and may mediate a PKC dependent intracellular pathway. The cooperative effect of these two interactions results in cell spreading and migration. Adapted from http://www.rbej.com/content/2/1/3
1.4.6. Fibronectin in Angiogenesis

In the vasculature, FN has been localized to the ECM underlying ECs and is also found in the medial and adventitial layers of the vessel wall (Hynes, 1990; Jensen et al., 1983; Takasaki et al., 1991; Labat-Robert et al., 1985). FN is produced locally by ECs and vascular smooth muscle cells (Hynes, 1990). The main integrin receptors of FN expressed in ECs include $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$. FN has been suggested to promote vasculogenesis (Risau and Lemmon, 1988; George et al., 1993). FN affects EC adhesion, growth, migration and survival, and is also important for in vivo angiogenesis (Bourdoulous et al., 1998; George et al., 1993; 1997; Wijelath et al., 2002; 2006; Re et al., 1994; Ilic et al., 1998; Hynes, 1990). Inhibiting FN matrix deposition and/or disrupting a preexisting FN matrix inhibit the growth of ECs (Bourdoulous et al., 1998; Clark et al., 1997; Mercurius and Morla, 1998; Wu et al., 1998). FN promotes EC survival (Re et al., 1994; Ilic et al., 1998; Kim et al., 2002) and migration (Kim et al., 2000). In addition, FN binds to VEGF and enhances VEGF-induced endothelial cell migration and MAP kinase activation (Wijelath et al., 2002). Further, both FN and the $\alpha_5\beta_1$ integrin receptor are up-regulated in the vascular ECM following treatment with angiogenic factors in vivo (Kim et al., 2000). Interaction of central cell binding domain of FN and $\alpha_5\beta_1$ integrin is central to the contribution of these two molecules in angiogenesis. Moreover, $\alpha_5\beta_1$ integrin-mediated NF-κB signaling has been shown to be important in angiogenesis by controlling the EC gene expression during the process (Klein et al., 2002).

In addition to directly regulating EC function, FN may also regulate angiogenesis by controlling ECM remodeling events. Agents that regulate the rate and extent of FN matrix polymerization (Zhang et al., 1997; Wu et al., 1998; Allen-Hoffmann et al., 1988; Ignotz and Massague, 1986; Sommers and Mosher, 1993; Burrige et al., 1997; Zhong et al., 1998) may play an important role in regulating the composition and stability of the ECM and in regulating EC functions that are critical for angiogenesis. Multimeric FN produced in vitro has been shown to inhibit cell migration, enhance cell adhesion, inhibit tumor growth and inhibit tumor associated angiogenesis (Morla et al., 1994; Yi and Ruoslahti, 2001). Hence, the effects of FN on EC function are likely to depend on the local concentration of soluble FN as well as the density of the FN matrix. The importance of FN in the vasculature is underscored by the ability of FN to regulate in vivo angiogenesis in an animal model (Kim et al., 2000), and
by the phenotype of mice lacking FN (George et al., 1993; 1997; Francis et al., 2002). FN- null mice die during embryogenesis with defects in blood vessel development and/or maintenance (George et al., 1993; 1997).

An alternatively spliced form of FN has been identified that contains an extra-domain B (EDB), which is a complete 91-amino acid type-III repeat (Carnemolla et al., 1989). This alternatively spliced FN is not expressed in normal adult tissues, but is found in fetal and neoplastic tissues (Carnemolla et al., 1989; Castellani et al., 2002). In situ hybridization studies have demonstrated that this alternatively-spliced FN is synthesized by the vascular cells in tumors (Castellani et al., 2002). The expression of this alternatively spliced FN protein in the ECM of vessels appears to be a precise diagnostic marker of tumors (Carnemolla et al., 1989; Castellani et al., 2002; Santimaria et al., 2003; Tarli et al., 1999). Exposure of cells to EDB peptide increased VEGF expression, endothelial proliferation and tube formation. EDB is involved in EC proliferation and vascular morphogenesis, findings which may provide novel avenues for the development of anti-angiogenic therapies. As α₅β₁ integrin is required for tumor angiogenesis, the interaction of α₅β₁ integrin with this alternatively-spliced FN is likely to play an important role in this process.

FN is susceptible to proteolysis during the angiogenic process and the FN fragments have activities not found in the intact molecule. FN fragments have been identified at sites of inflammation, injury and metastasis where they affect proliferation, adhesion, spreading and migration of vascular ECs (Grant et al., 1998; Huebsch et al., 1995). Recently, FN and its heparin binding domains have been shown to bind VEGF and thus can profoundly influence the biological activity of VEGF (Wijelath et al., 2006). These VEGF binding domains of FN are required for the specific association of the FN receptor α₅β₁ integrin with the VEGF receptor, Flk-1. Consequently this can affect the behavior of ECs through their coordinated effects on Flk-1 and α₅β₁ integrin.
1.5. Objective of the Present Study

It is evident from the review of literature discussed above that FN is a multifunctional ECM protein, influencing cell adhesion, proliferation, migration, differentiation and cell growth by interacting with specific cell surface receptors. These events are important in the process of angiogenesis in which the quiescent ECs in the capillary endothelium get activated, detach from the underlying BM, migrate, proliferate, differentiate, adhere to newly synthesized ECM and form capillary-like tubes. ECs encounter a FN rich provisional matrix as they migrate and invade into the surrounding ECM and undergo transition to angiogenic phenotype. ECs interact with FN through specific cell surface integrin receptors. Although FN is known to be pro-angiogenic in nature, the molecular mechanisms involved in modulating the transition of ECs to angiogenic phenotype are not clear. The role of FN in influencing EC functions relevant to angiogenesis is also not clear. Therefore, the broad objective of the study was to understand the molecular mechanisms involved in the angiogenic effect of FN.

The present study was therefore aimed at understanding the role of FN in modulating certain EC functions that are relevant to its angiogenic behavior using an in vitro model system of human umbilical vein endothelial cells as indicated below.

i) Since ECs interact with FN during migratory phases of angiogenesis, the contribution of FN to survival of ECs and the molecular mechanisms thereof were examined.

ii) As ECs elaborate their migratory response, they require a steady supply of growth factors and so the influence of FN on the production and biological potency of angiogenic factors particularly VEGF was studied in vitro.

iii) In addition to growth factors, several soluble factors such as prostaglandins and nitric oxide play important roles in the transition of ECs to angiogenic phenotype. It was therefore proposed to study the influence of FN matrix on the expression and activity of cyclooxygenases and nitric oxide synthase in ECs, the enzymes involved in the synthesis of these angiogenic modulators.

iv) FN, being a multi-domain protein, can interact with cells through its heparin binding domain and the cell surface HSPGs, apart from its
cell binding domain-integrin interactions. FN is reported to undergo proteolytic fragmentation in angiogenesis related to several pathological conditions. These fragments have activities distinct from the parent protein. So, the role of integrin-independent interaction of cells with FN through its heparin binding domain in regulating EC functions relevant to angiogenesis was also studied.

The results of these investigations have been described in the following chapters.