THE ROLES AND MECHANISMS OF FOCAL ADHESION KINASE IN THE REGULATION OF ANGIOGENESIS

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THE ROLES AND MECHANISMS OF FOCAL ADHESION KINASE IN THE REGULATION OF ANGIOGENESIS

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Focal Adhesion Kinase (FAK) is a cytoplasmic tyrosine kinase that mediates signal transduction of integrins and other cell surface receptors in a variety of cells, including endothelial cells (ECs). Phosphorylation of FAK and its interactions with other signaling molecules have been shown to trigger several signaling pathways in the regulation of cellular functions, including cell migration, cell cycle progression and cell survival.

Consistent with its critical importance in the regulation of various cellular functions, deletion of the FAK gene in mice leads to death at embryonic day 8.5 (E8.5) due to defects in the axial mesodermal tissues including the cardiovascular system with incomplete development of both the blood vessels and the heart. Using a conditional mouse knock out approach, we and others have recently shown a role of FAK in vascular angiogenesis. Further studies with primary FAK deficient ECs showed that the essential function of FAK in the regulation of EC activities, including EC migration, proliferation and survival may contribute to the regulation of angiogenesis *in vivo* (Chapter 2).

The availability of the floxed FAK mice and ECs isolated from these mice allowed us to further investigate the role of specific FAK downstream pathways in EC functions by rescuing the various phenotypes with FAK mutants lacking specific interactions with its target both *in vitro* and *in vivo*. In this dissertation, we revealed a novel function of FAK in the regulation of centrosomal functions in a Ser-732

phosphorylation-dependent manner in ECs during mitosis, which plays a role in the regulation of EC proliferation and tubulogenesis *in vitro* and tumor angiogenesis *in vivo* (Chapter 3).

BIOGRAPHICAL SKETCH

Ann Yong-jin Park was born in Lincoln, Nebraska in January 1979 when her father studied at University of Nebraska pursuing his Ph.D. degree. She grew up in Busan, South Korea except for the period of time in Boston, where her father did a postdoctoral training at Harvard University. She entered Pusan National University, South Korea to study chemical engineering, but she decided to study Molecular Biology to pursue her curiosity in Biology. She graduated from the Department of Molecular Biology at the top of the graduation class of spring 2001. After her graduation, she worked at Dr. Byoung-hak Jeon's laboratory in the College of Pharmacy, Pusan Naional University to work on cell biology. In August of 2002, she came back to USA and entered graduate school through the field of Pharmacology in the Department of Molecular Medicine at Cornell University where she joined the lab of Dr. Jun-Lin Guan. She passed the Admission to Ph.D. Candidacy examination in December of 2005. She moved to Ann Arbor, Michigan in December of 2006 to continue her research under the guidance of her advisor Dr. Jun-Lin Guan, who has moved from Cornell University to University of Michigan, Ann Arbor.

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CHAPTER 1:

INTRODUCTION

A. Focal Adhesion Kinase (FAK)

A1. Characterization of FAK

Focal adhesion kinase (FAK) is a 125 kDa cytoplasmic tyrosine kinase that localizes to focal adhesions where FAK and other molecules transmit mechanical force and regulatory signals from extracellular matrix (ECM) to the cell interior. FAK was identified as a substrate of v-Src and as a highly tyrosine-phosphorylated protein in response to cell adhesion to ECM mediated by integrins (Guan and Shalloway 1992). Based on its amino acid sequence and structure, FAK represents a distinctive family of protein-tyrosine kinases (PTKs). FAK contains a catalytic domain that shows 31-41% sequence identity to the catalytic domains of other PTKs. However, FAK has a unique structure, containing large N- and C-terminal domains flanking the catalytic domain, but not SH2 and SH3 domains (Schaller et al. 1992). The FAK subfamily of nonreceptor PTK includes only one other member, Pyk2 (Avraham et al. 1995; Sasaki et al. 1995). Pyk2 shares 40% amino acid sequence identity with N- and C-terminal domain of FAK and 60% conservation in kinase domain of FAK. FAK is expressed in most tissues and cell types in many species, including human, rodent, chicken, Xenopus, zebrafish and Drosphila (Schaller et al. 1992; Parsons 2003) whereas Pyk2 is expressed mainly in neuronal and hematopoietic cell types (Avraham et al. 1995; Lev et al. 1995; Sasaki et al. 1995). Several studies showed that the deletion of FAK in primary fibroblasts or endothelial cells increases Pyk2 expression, suggesting the compensatory function of Pyk2 in FAK deficient cells (Sieg et al. 1998).

FAK localization to focal adhesions requires its C-terminal focal adhesion targeting (FAT) domain. Integrin-associated proteins paxillin and talin, have been

proposed to mediate FAK localization to focal adhesions. The C-terminal domain of FAK also has two proline-rich motifs, which connect FAK to SH3 domain-containing proteins such as p130Cas, endophilin A2, Graf (GTPase activating protein for Rho associated with FAK) and ASAP1 (ADP ribosylation factor [ARF]- GTPase-activating protein [GAP] containing SH3, ANK repeats, and PH domain). The FAK C-terminal domain termed FRNK (FAK-Related-Non-Kinase) has been known to be expressed as a separate mRNA transcript in several cells and function as a competitive inhibitor of FAK targeting to integrins and focal adhesions (Parsons 2003; Schlaepfer and Mitra 2004). The N- terminal domain of FAK contains a FERM (erythrocyte band four.1-ezin-radixin-moesin) homology domain followed by a proline-rich motif that has been shown as SH3 domain binding site for Src-family PTKs (Parsons 2003).

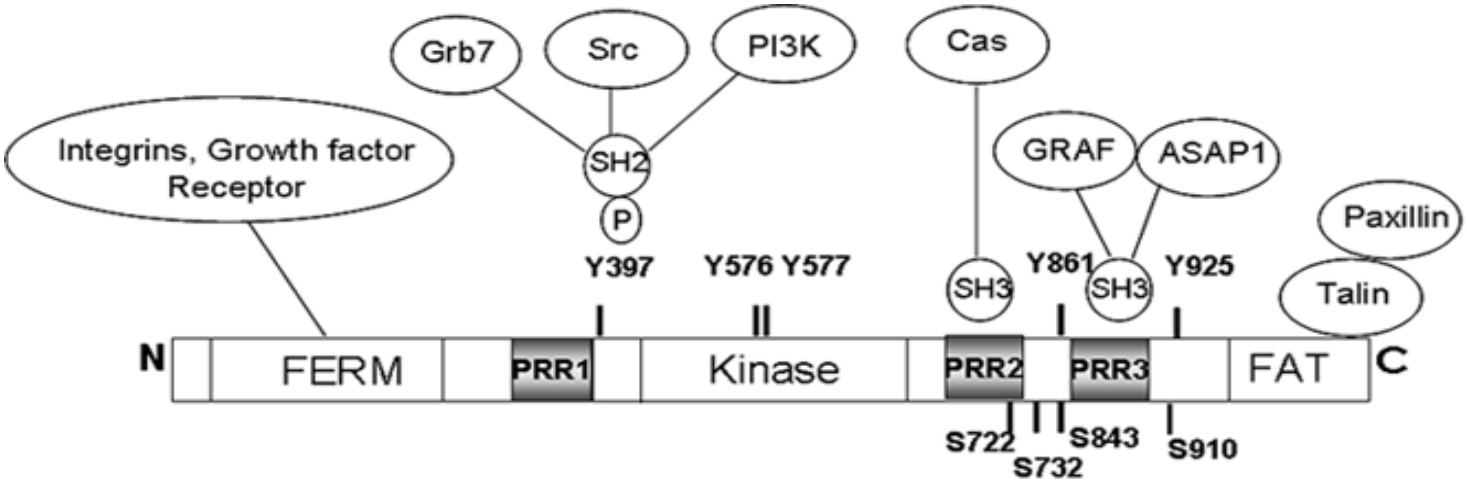
A2. Mechanisms of FAK activation

FAK is a major mediator of signal transduction by integrins and also participates in signaling by other cell surface receptors in a variety of cells (Schaller 2001; Parsons 2003; Schlaepfer and Mitra 2004). In most adherent cells, FAK is activated upon integrin-mediated cell adhesion to extracellular matrix (ECM) proteins through disruption of an intramolecular inhibitory interaction between its amino-terminal FERM domain and the kinase domain (Cooper et al. 2003; Lietha et al. 2007). Once it is activated, FAK undergoes autophosphorylation at Tyr397, which creates a binding site for several Src homology 2 domain–containing molecules including Src family kinases, p85 subunit of PI3K, phospholipase C-γ and Grb7 (Parsons 2003; Schlaepfer and Mitra 2004). The formation of FAK-Src complex has been demonstrated to activate tyrosine phoshorylation of additional sites on FAK.

Phsophorylation of FAK within the kinase domain activation loop at Tyr576 and Tyr577 leads to maximal FAK activation and tyrosine phosphorylation of FAK residues in its C-terminal domain at Tyr861 and Tyr925 creates the binding sites for SH2-domain containing proteins (Schlaepfer et al. 1994; Calalb et al. 1995; Owen et al. 1999). FAK also functions as a scaffold to mediate Src family kinase phosphorylation of several proteins, including paxillin (Burridge et al. 1992; Schaller and Parsons 1995), p130cas (Vuori et al. 1996; Ruest et al. 2001), and endophilin A2 (Wu et al. 2005), which bind to the carboxyl-terminal region of FAK. In addition, FAK has four known phosphorylatable serine residues in the C-terminal domain: Ser722, Ser732, Ser843, and Ser910. Several studies suggested that serine phosphorylation of FAK may play a role in modulating the interactions between downstream signaling proteins. However, the roles and specific mechanisms of serine phosphorylation of FAK in the regulation of cell functions are poorly characterized (Ma et al. 2001; Parsons 2003). This cascade of phosphorylation events and protein-protein interactions has been shown to trigger several signaling pathways in the regulation of a variety of cellular functions in different cells, which will be discussed in detail in Section A3.

Figure 1.1. Overview of FAK domain structure and its interacting proteins

The N-terminal FERM domain interacts with integrins, growth factor receptors and G protein-coupled receptor. Phosphoylation of FAK at Tyr397 creates SH2-domain binding sites for Src, PI3K and PLCγ. The central domain is the kinase domain and the phosphorylations at Tyr576 and Tyr577 promote maximal catalytic activity. The FAK C-terminal FAT domain binds to paxillin and talin and mediates FAK localization to focal adtheions. The C-terminal domain also contains two prolin-rich motifs that provide binding sites for SH3 domain-containing proeins including p130Cas, Graf and ASAP1. Additional sites of tyrosine and serine phosphorylation are indicated.


Grb7
Src
PI3K
Cas
Integrins, Growth factor Receptor
SH2
P
GRAF
ASAP1
Paxillin
Talin
Y397
Y576 Y577
SH3
Y861
SH3
Y925
N
FERM
PRR1
Kinase
PRR2
PRR3
FAT
C
S722
S732
S843
S910

A2.1 Integrin-mediated FAK activation

Integrins are the major transmembrane receptors that consist of two noncovalently bound glycoprotein subunits, α and β. They mediate interactions between the extracellular matrix (ECM) and the actin cytoskeleton and play important roles in regulating cell adhesion, spreading and migration, cell survival, and cell cycle progression. Since integrins lack intrinsic catalytic activity, signals from ECM are transmitted through the activation of integrin-associated proteins, including FAK.

Tyrosine phosphorylation and activation of FAK has been shown to be increased following cross-linking of integrins as well as plating of cells on ECM proteins including fibronectin, laminin, vitronectin, and typeIV collagen (Guan et al. 1991; Kornberg et al. 1992; Lipfert et al. 1992). Upon activation of integrins, FAK indirectly interacts with integrins through its C-terminal domain by binding to focal adhesion proteins, including paxillin and talin at the sites of integrin clustering, called focal adhesions (Parsons 2003). In addition, a direct interaction between the N-terminal domain of FAK and β1-integrin was reported in vitro (Schaller and Parsons 1995). Although specific mechanisms of FAK activation by integrins are unclear, several studies suggest that the cytoplasmic domains of β-integrins (β1, β3 and β5) are required for FAK activation (Schlaepfer and Mitra 2004).

A2.2 FAK activation by cell surface receptors

In addition to integrins, many growth factor receptors also mediate FAK signaling pathways in several different cell types. For example, HGF (Hepatocyte Growth Factor) activates FAK and promotes integrin-mediated cell migration (Lai et al. 2000). Also, FAK associates with the activated PDGFR (Platelet- Derived Growth Factor Receptor) and EGFR (Epidermal Growth Factor Receptor) through its N-

terminal FERM domain and promotes cell migration (Sieg et al. 1998). Interestingly, the kinase activity of FAK is not required to stimulate either PDGF- or EGF-mediated cell migration in $FAK^{-/-}$cells, although the same cells require the kinase activity of FAK to promote fibronectin-stimulated cell migration (Sieg et al. 1998). These results suggest that the mechanisms of FAK-mediated migration are different in integrin and growth-factor receptor mediated activation. VEGF (Vascular Endothelial Growth Factor) also has been shown to induce phosphorylation FAK at Tyr-397 and Tyr-861, and to promote the formation of a FAK-integrin αvβ5 complex, thereby induce angiogenesis (Eliceiri et al. 2002).

In addition, G protein- coupled receptor (GPCR) signal transduction increases tyrosine phosphorylation of FAK (Rozengurt 2007) and Tumor necrosis factor-α (TNF α) also activates FAK and mediates ERK2/mitogen-activated protein kinase (MAPK) activation and lipopolysaccharide-induced interleukin-6 (IL-6) production (Schlaepfer et al. 2007) .

A2.3 FERM domain regulation of FAK activation

Several studies have demonstrated that the N-terminal domain of FAK may play a role in the regulation of FAK phosphorylation and activity. Truncations of the N-terminal domain of FAK caused increased tyrosine phosphorylation and FAK activity, suggesting an inhibitory role for the N-terminal domain in FAK activation (Toutant et al. 2002; Cooper et al. 2003). Since it was reported that the N-terminal domain of FAK can bind to the kinase domain of FAK and inhibit FAK activity in trans (Cooper et al. 2003), FAK activation may be regulated by an autoinhibitory mechanism. Indeed, the crystal structure of the FAK fragment, which contains the N-termianl and kinase domain (31-686 residues) showed that the auto-inhibited state is

stabilized by the interaction between the N-termianl domain and the kinase C-lobe (Lietha et al. 2007). The N-terminal domain of FAK contains a region sharing some sequence identity (27%) with band 4.1 and the ezin/radixin/moesin (FERM) domain (Girault et al. 1998; Diakowski et al. 2006). Three subdomains (F1, F2, and F3) form a tertiary fold within the region, similar to those of known FERM structures. In the auto-inhibited state, the F2 subdomain binds to the C-lobe of the kinase domain and the F1 subdomain interacts with the linker segment containing the Tyr397 autophosphorylation site. Therefore, the release of N-terminal domain from the kinase domain will allow autophosphorylation of FAK at Tyr397 and FAK activation. However, the exact mechanisms that activate FAK by disrupting these interactions are still unclear.

A3. Control of cellular functions by FAK

A3.1 Cell migration

Cell migration needs the coordinated regulation of the dynamics of actin filaments and focal adhesions, which generate membrane protrusions and contraction force. Cell migration involves several cellular processes, including plasma membrane protrusions at the cell front, adhesions of cell front protrusions by integrins, the forward movement of the cell body, and release of adhesions at the cell rear (Lauffenburger 1996). FAK has been implicated in playing an important role in the regulation of cell migration. An early observation came from the detection of increased FAK expression in migrating keratinocytes of repairing epidermal wounds (Gates et al. 1994). Deletion of FAK in mice caused early embryonic lethality, showing mesodermal defects (Ilic et al. 1995) and FAK deficient cells from these

mutant embryos showed reduced cell migration in vitro. In addition, the inhibition of FAK activity by overexpressing FRNK reduced cell migration (Gilmore and Romer 1996) and overexpression of FAK in Chinese hamster ovary (Sharp et al.) cells increased cell migration(Cary et al. 1996).

A3.1.1 Signaling from FAK phosphotyrosine 397 in migration

Several studies suggested that Tyr-397 phosphorylation of FAK is essential for the regulation of migration. Re-expression of wild-type FAK, but not the Y397F FAK mutant increased cell migration in FAK-deficient cells. In addition, re-expression of the P712/715A FAK mutant which cannot bind to p130Cas failed to rescue cell migration as wild-type FAK did, suggesting that activation of p130Cas through FAK-Src complex is required for cell migration (Cary et al. 1996; Sieg et al. 1998). SH3 domain-mediated binding of p130Cas to FAK increases tyrosine phosphorylation of p130Cas, which can increase Crk adaptor protein binding to p130Cas. The interaction between p130Cas and Crk has been shown to promote cell migration by Rac activation mediated through the Rac GTPase exchange factor DOCK180 (Kiyokawa et al. 1998). Activated Rac plays a role in migration through enhanced lamellopodium extension (protrusion) at the leading edge (Ridley 2001). Paxillin is also phosphorylated by FAK-Src complex as mentioned in section A2 (page 4), and the activated paxillin plays a similar role as p130Cas in cell migration since it can also bind to Crk (Petit et al. 2000). Alternatively, the phosphorylated paxillin can interact with SH2 domains of p120RasGAP and release p190PhoGAP from p120RasGAP resulting in suppression of Rho activity thereby contributing to cell migration (Tsubouchi et al. 2002).

The p85 regulatory subunit of PI3K binds to FAK at phosphorylated Tyr-397 (See Section A2, page 4) and this interaction is also known to regulate cell migration. Inhibition of PI3K reduced FAK-promoted migration and the overexpression of the D395A FAK mutant that can bind to Src but not PI3K failed to promote cell migration (Reiske et al. 2000). PI3K signaling may regulate cell migration through the stimulation of protrusions at the leading edge, since PI3K has been shown to activate Rac (Reif et al. 1996).

Another downstream molecule of FAK that binds to phosphorylated Tyr-397 is Grb7. Phosphorylation of Grb7 by FAK has been shown to play a role in cell migration (Han et al. 2000). It was reported that the phosphorylation of Grb7 by FAK is dependent on PI3K activity, suggesting the cooperative regulation of Grb7 and PI3K in FAK-mediated cell migration (Shen et al. 2002).

Therefore, these studies suggest that FAK regulates cell migration through the activation of multiple downstream pathways that cause Rac activation and Rho suppression, resulting in the stimulation of lamellipodium extension. FAK-mediated Rac activation and Rho suppression have also been implicated in playing an important role in cell migration through their contribution to the focal adhesion disassembly, which will be discussed in more detail in the next section.

A3.1.2 Focal adhesion turnover

Focal adhesions are sites where integrins link the ECM to cytoplasmic actin cytoskeleton. The formation and turnover of focal adhesions is a dynamic process, whose coordinated regulation is essential for cell migration in response to a stimulus, such as ECM proteins, growth factors or cytokines. Focal adhesion dynamics in the migrating cell involve continuous assembly and disassembly of adhesion in a process

termed focal adhesion turnover at the cell front, center and rear. FAK has been demonstrated as a key trigger for focal adhesion turnover. FAK - deficient fibroblasts showed larger and more focal adhesions (Ilic et al. 1995) and the local increase of calcium concentration induces focal adhesion disassembly through increasing FAK recruitment at these sites (Giannone et al. 2004).

FAK and its downstream pathways mediated by Src have been shown to stimulate adhesion disassembly. One pathway is by increasing actomyosin contractility through the activation of ERK (extracellular signal regulated kinase) and MLCK (myosin light chain kinase). Src-mediated phosphorylation of FAK at Tyr 925 provides a SH2 domain binding site for Grb2 (growth factor receptor bound protein2) leading to ERK activation. The activation of ERK stimulates the phosphorylation of MLCK and thereby increases contractility, which causes adhesion disassembly (Webb et al. 2004). Another mechanism is through Rac activation or Rho inhibition by pathways mentioned in the previous section A3.1.1. Focal adhesion turnover is also regulated by the calcium - dependent protease, calpain (Schlaepfer and Mitra 2004). Calpain has been shown to localize to focal adhesions and cleave several proteins in focal adhesions, such as talin, paxillin, FAK, Src, α-actinin, and tensin. FAK has been demonstrated to stimulate caplain activity by recruiting both calpain and ERK to focal adhesion sites (Carragher et al. 2003).

A3.1.3 Serine phosphorylation of FAK in migration

In neuronal cells, cyclin-dependent kinase 5 (Cdk5), which plays an important role in neuronal migration during corticogenesis, directly phosphorylates FAK at Ser732 (Xie et al. 2003). Ser732-phosphorylated FAK was found predominantly in the distinct centrosome–associated microtubule structure that abuts the nucleus in

cultured neurons. Using the Ser732 to Ala mutant that can not be phosphorylated by FAK, it was shown that Ser732 phosphorylation is important to regulate the organization of a microtubule structure connecting the nucleus and the centrosome and, thereby, promote nuclear translocation during neuronal migration. Although specific mechanisms that link Ser732 phosphorylation of FAK and its regulation of microtubule structure are unknown, this study suggests that serine phosphorylation of FAK plays a role in neuronal migration.

A3.2 Cell proliferation and cell survival

A3.2.1 Function of FAK in G1 to S phase transition during cell cycle progression

It has been known that cell adhesions to the ECM are required for cell growth (Yancopoulos et al. 1998). Several studies suggest a role of FAK in cell cycle progression. Inhibition of FAK by overexpression of the C-terminal domain of FAK decreases cellular entry into S phase (Gilmore and Romer 1996). In addition, overexpression of the dominant-negative FAK mutant ΔC14, which can not localize to focal adhesions, blocks cell cycle progression at G1 phase whereas ovrexpression of wild-type FAK accelerates G1 to S phase transition (Zhao et al. 2003). Expression of Y397F FAK mutant, which cannot interact with Src, inhibits G1 to S phase transition and ERK activation. This result suggests the important roles of Y397 phosphorylation of FAK and ERK activation in cell cycle progression (Zhao et al. 2003). Consistent with this, fusion of the FAT sequence of FAK to Grb2 allows focal adhesion targeting of Grb2 and stimulates cell cycle progression as well as ERK activation (Shen and Guan 2001). Cell cycle progression from G1 to S phase is

regulated by distinct cyclin-dependent kinases that are regulated by various cyclins. The Ras-ERK signalling pathway has been demonstrated to activate cyclin D1 gene transcription. FAK was reported to regulate cyclin D1 expression through the EtsB binding site in the cyclin D1 promoter and ERK plays an important role in mediation of cyclin D1 expression (Zhao et al. 2003). These studies suggest that FAK regulates cell cycle progression through ERK-dependent stimulation of cyclin D1 gene transcription through its EtsB binding site.

A3.2.2 Serine phosphorylation of FAK during mitosis

It has been shown that phosphorylation of FAK at serine residues is increased during mitosis and this increase correlates with a decrease in FAK tyrosine phosphorylation and activation (Yamakita et al. 1999; Ma et al. 2001). During mitosis, focal adhesions are disassembled and integrin-mediated signalings are inactivated. After cytokinesis, cells start to reattach and integrin-mediated signaling is reactivated. This cycle of attachment and detachment during the cell cycle has been known to be critical for cell proliferation. It was reported that mitotic FAK shows reduced interaction with the integrin β1 subunit and FAK/Src/p130Cas signaling complex is dissociated during mitosis. Increased serine phosphorylaion of FAK during mitosis contributes to this dissociation between FAK and p130Cas, whereas tyrosine dephosphorylation of FAK disrupts FAK and Src interaction (Yamakita et al. 1999). Therefore, mitosis-specific serine phosphorylation of FAK contributes to the regulation of cell proliferation through the inactivation of integrin-mediated signaling during mitosis, showing the role of FAK in G2-M phase of cell cycle. However, specific functions and mechanisms of serine phosphorylation of FAK during mitosis are still undefined.

A3.2.3 Cell survival

It has been shown that the interaction between integins and ECM inhibits the detachment-induced apoptosis, anoikis. Both Tyr-397 phosphorylation and kinase activity of FAK have been demonstrated to play a role in the inhibition of anoikis, showing FAK is critical to this process (Frisch et al. 1996). One mechanism that contributes to cell survival is a FAK/p130Cas complex-mediated pathway, which activates c-Jun NH2-terminal kinase (JNK) through a Ras/Rac1/Pak1/MAPK kinase 4 (MKK4) pathway (Almeida et al. 2000). FAK- mediated activation of PI3K can also stimulate cell survival by Akt activation or by activation of NF-κB with the induction of inhibitor-of-apoptosis proteins (IAPs) thereby inhibiting apoptosis through blocking caspase-3 cascade (Sonoda et al. 2000; Bellas et al. 2002). In addition, the inactivation of FAK increases apoptosis through p53-dependent pathway, suggesting FAK-mediated signaling pathways may be involved in the inhibition of p53 activity (Sieg et al. 1998).

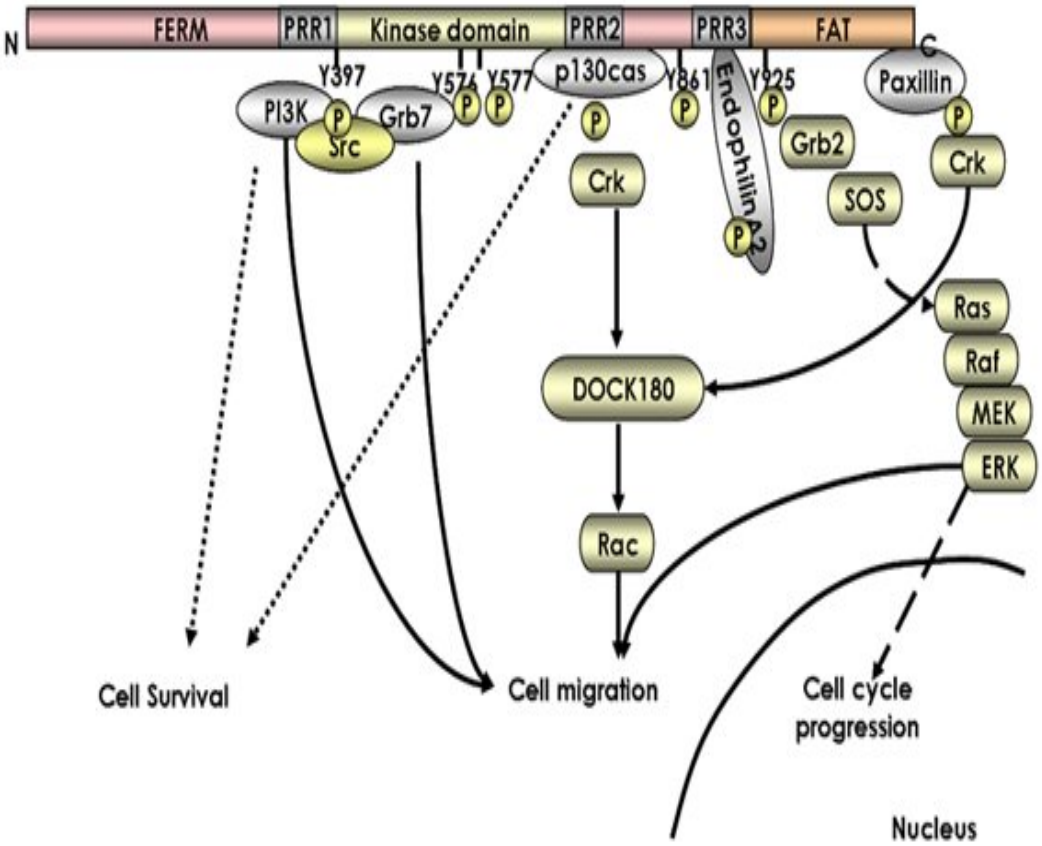
B. FAK and angiogenesis

B1. Angiogenesis

Blood vessel formation is a fundamental event in embryonic development and organogenesis, as well as in the pathogenesis of many diseases including coronary heart disease, retinopathies, and cancer (Dvorak 2003). Blood vessels are formed by two processes : vasculogenesis and angiogenesis (Risau 1991). Vasculogenesis is the *de novo* formation of new blood vessels from primitive cells (angioblasts) to form a primitive capillary network that occurs in the early embryogenesis, whereas angiogenesis, the sprouting of capillaries from preexisting blood vessels, is involved

Figure 1.2. FAK signals that regulate cell functions

Once FAK is activated, it is autophosphorylated at Tyr-397, which creates a binding site for several Src homology 2 domain–containing molecules including Src, p85 subunit of PI3K and Grb7. The formation of FAK-Src complex has been demonstrated to activate tyrosine phoshorylation of additional sites on FAK as well as other substrates, such as paxillin, p130cas and endophilin A2. FAK-induced activation of these signaling molecules has been shown to trigger several downstream signaling pathways that regulate several cellular functions. FAK - mediated activation of PI3K and p130Cas stimulates cell survival. Phosphorylation of FAK at Tyr 925 provides a binding site for Grb2 leading to ERK activation, which stimulates cell cycle progression from G1 to S phase through cyclin D1 gene transcription. FAK also has been shown to regulate cell migration through the activation of PI3K, Grb7, p130Cas and paxillin.


N
FERM
PRR1
Kinase domain
PRR2
PRR3
FAT
C
Y397
Y574
Y577
Y861
Y925
PI3K
Src
Grb7
p130cas
Endophilin A2
Paxillin
Grb2
Crk
SOS
Ras
Raf
MEK
ERK
DOCK180
Rac
Cell Survival
Cell migration
Cell cycle progression
Nucleus

in the late stage of embryogenesis and in the adult (Yancopoulos et al. 1998). Currently, the term angiogenesis has been used to indicate the growth, expansion and remodeling of primitive network into the mature vascular network, including the process of sprouting, bridging and intussusception (Carmeliet 2000). Angiogenesis is essential for physiological and pathological conditions including wound healing, chronic inflammation and carcinogenesis as well as embryonic development (Carmeliet et al. 1996; Yancopoulos et al. 1998). Complex and diverse cellular actions are implicated in angiogenesis, such as degradation and remodeling of the ECM, proliferation and migration of endothelial cells (ECs), and formation of a lumen completing vascular network (Section B1.1~1.3 and figure 1. 3)(Bussolino et al. 1997).

ECs line the inner surface of blood vessels, lymphatics, and the chamber of the heart and play an essential role in both vasculogenesis and angiogenesis. Their functions are tightly regulated by proangiogenic growth factors, ECM components and integrins (Yancopoulos et al. 1998). However, it is still unclear how these proangiogenic factors and integrin receptor-mediated events regulate angiogenesis and vasculogenesis in ECs. In addition to ECs, periendothelial cells (pericytes for small vessels and smooth muscle cells for large vessels) are required for vascular maturation during angiogenesis. They play a role in hemostatic control and protection of new endothelium lined vessels from regression (Benjamin et al. 1998).

B1.1 Regulation of vascular permeability and ECM degradation

The primary step in the early angiogenic process is the production and secretion of EC-specific VEGF into the extracellular environment. Several angiogenic stimuli have been shown to induce VEGF expression, including several growth

factors, cytokines, hormones, nitric oxide (NO), and hypoxia (Kimura et al. 2000). The secreted VEGF increases vascular permeability through its effects on the redistribution of intercellular adhesion molecules, such as platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)–cadherin, and induction of Src kinases (Eliceiri et al. 1999; Conway et al. 2001). Vascular permeability is tightly regulated by an anti-permeability factor, angiopoietin-1 (Ang1), which is a ligand for the endothelial receptor Tie2 (Gale and Yancopoulos 1999).

ECs exist in a quiescent state when they are bound to ECM, including basement membrane, which consist of molecules such as type IV, XV and XVII collagen, laminin, heparin-sulphate proteoglycans, and perlecans (Kalluri 2003). In response to angiogenic stimuli, including VEGF, bFGF, PDGF and chemokines, ECM has been shown to be degraded by several matrix-degrading enzymes, such as matrix metalloproteinases (MMPs), plaminogen activator, chymase and heparanase families. This ECM degradation leads ECs to proliferate and migrate into the adjacent tissues. In addition, it releases growth factors, such as VEGF, bFGF and Insulin-like growth factor-1 (IGF-1), which were sequestered within the matrix (Conway et al. 2001). Several studies have shown that MMP9 and MMP2 are required for the VEGF release from the matrix (Kalluri 2003). This MMP-mediated degradation of ECM produces cryptic domains of partially degraded collagen, which provide proangiogenic cues. It also produces fragments that have anti-angiogenic activity, such as endostatin, arrestin, canstatin and tumstatin (Kalluri 2003). Therefore, MMP-mediated ECM degradation acts as both an activator and an inhibitor during angiogenesis. In addition, angiopoietin-2 (Ang2), an antagonist of Ang1 plays a role in smooth muscle cell detachment and matrix loosening (Gale and Yancopoulos 1999).

B1.2 EC proliferation and migration

It has been shown that several factors, including VEGFs, angiopoietins, and FGFs play a role in the regulation of EC migration and proliferation during angiogenesis.

There are five VEGF homologues, VEGF-A ~D and placental growth factor (P1GF) in mammals and they interact with three receptor tyrosine kinases, VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1, and VEGFR-3/Flt-1 (Gale and Yancopoulos 1999). It was reported that VEGF-A stimulates EC proliferation, migration and sprouting in vitro. In vivo studies also showed that VEGF-A is critical for sprouting as well as lumen formation, vessel survival, and initial states of vasculogenesis. These effects of VEGF-A on angiogenesis are known to be mediated by the VEGFR-2 receptor (Carmeliet et al. 1996). The deletion of VEGFR-1 in mice leads to vascular disorganization in mutant embryos with increased number of ECs in the lumens of the abnormal vessels, suggesting a role of VEGFR-1 in the downregulation of VEGF activity to control numbers of ECs (Fong et al. 1995). VEGF-B knockout mice showed a reduced size of their hearts and vascular dysfunction after coronary occlusion, suggesting that it may play a role in the development or function of coronary vasculature (Bellomo et al. 2000). In addition, VEGF-C was reported to stimulate angiogenesis in adult mice and its receptor, VEGFR-3 is required for vascular remodelling and angiogenesis (Conway et al. 2001).

Several studies suggest that roles of angiopoietins in angiogenesis are different from VEGF, but it has been shown that angiopoietins work in coordination with VEGF to regulate angiogenesis. In vitro studies reveal that Ang1 stimulates EC sprouting, but it could not directly promote the EC growth and tube formation as VEGF did (Davis et al. 1996; Koblizek et al. 1998). The deletion of either Ang1 or its

receptor, Tie2, in mice caused severely impaired vessels, although the early stage of VEGF-dependent vasculogenesis is still normal. These defects were caused by disrupting the role of Ang1 in mediating interactions between the ECs and surrounding matrix and support cells (Suri et al. 1996). Consistent with the notion that Ang2 is an antagonist of Ang1, Ang2 transgenic mice showed similar disruption of blood vessel formation in the embryo to that caused by either Ang1 or Tie2 deletion (Maisonpierre et al. 1997). However, the effect of Ang2 on angiogenesis depends on the presence of VEGF. Ang2 has been shown to lead vessel regression in the absence of VEGF, whereas it stimulates angiogenesis in the presence of VEGF (Gale and Yancopoulos 1999).

Several other factors have been demonstrated to play a role in angiogenesis, including FGFs, PDGF, endothelial nitric oxide synthase (eNOS), and Eph/ephrins (See table 1.1 for additional factors).

B1.3 Vessel maturation

When ECs migrate into ECM, they form continuous connections between existing vessels and assemble into capillary tubes, and subsequently acquire a lumen. Lumen diameter has been demonstrated to be tightly regulated by several factors, including VEGF, Ang1, and integrins (Conway et al. 2001).

Stabilization of newly formed vessels is an important step in angiogenesis and this is accomplished by recruiting mural cells (pericytes and smooth muscle cells) and by generating ECM. Several factors have been shown to regulate this process, including PDGF-B, PDGF receptor (PDGFR)-β, Ang1/Tie2 and transforming growth factor (TGF)-β. PDGF-B promotes mural cell proliferation, migration, and incorporation into the vessel wall (Armulik et al. 2005). The deletion of PDGF-B in

mice also showed that its expression in ECs is important for the recruitment of pericytes and normal vessel wall formation (Enge et al. 2002). Also the Ang/Tie-signaling system plays an important role in vessel stabilization as mentioned in B1.2 section. In addition, TGF-β1 plays a role in vessel maturation through stimulating ECM production and inducing differentiation of mesenchymal cells to mural cells (Jain 2003). In vivo studies revealed a role of TGF-β1 and its receptors (TGF- β Rs I, II and endoglin) in mice embryonic vascular assembly and vessel maturation (Pepper 1997).

Vessel maturation involves the final patterning of vascular network for an organ by branching, remodelling and pruning. For this process, several ECM components and factors, which play a role in nervous system branching, such as ephrins and neuropilins, are involved in regulating EC and mural cell proliferation, survival, migration and differentiation (Jain 2003).

B2. Role of FAK in angiogenesis

Several studies suggest the importance of both integrins and growth factor receptors in the regulation of key aspects of angiogenesis as mentioned in section B1 (Eliceiri and Cheresh 2001). Since FAK mediates signaling from integrins as well as from growth factor receptors, these studies strongly suggest a potential role of FAK in angiogenesis and vasculogenesis. A potential role of FAK in angiogenesis and vasculogenesis has been suggested by several other studies. The pattern of FAK expression was examined during mouse development and it was found to be particularly abundant in the blood vessels (Polte et al. 1994). Also, increased EC migration into a wounded monolayer was correlated with the increased FAK activity (Romer et al. 1994). Moreover, the disruption of FAK in mice has demonstrated the

importance of FAK in vascular development. The deletion of the FAK gene in the mouse leads to death at embryonic day 8.5 (E8.5) due to defects in the axial mesodermal tissues including the cardiovascular system with incomplete development of both the blood vessels and the heart (Ilic et al. 1995). Several studies in vitro and in vivo (Section B2.1 and B2.2) have shown the important roles of FAK in angiogenesis, although how FAK signaling pathways are linked to the regulation of angiogenesis still remained unclear.

B2.1 FAK signaling in angiogenesis

FAK has been demonstrated to be involved in various angiogenic signaling pathways. Ang-1, which is known to stimulate EC sprouting, induces FAK phosphorylation in endothelial cells and the activated FAK plays a role in Ang-1 induced EC migration through PI3-Kinase activation (Kim et al. 2000). VEGF-A/VEGFR-2 signaling also activates FAK and the following PI 3-kinase activation by FAK is required for VEGF-A-stimulated migration of porcine aortic endothelial cells expressing VEGFR-2 (Qi and Claesson-Welsh 2001). In response to VEGF, Src-dependent phosphorylation of FAK at Tyr-861 has been shown to be significantly increased in HUVECs and this phosphorylation promotes the formation of a signaling complex containing FAK and integrin $\alpha v\beta 5$, which is essential for VEGF-stimulated angiogenesis (Eliceiri et al. 1999; Abu-Ghazaleh et al. 2001; Eliceiri et al. 2002). However, Src - dependent phosphorylation of FAK at Y861 is not involved in FAK activated PI3-kinase pathway, suggesting VEGF independently activates Src – dependent and – independent mechanisms to regulate angiogenesis.

Table 1.1. Angiogenesis activators and inhibitors

Activators	Function	Inhibitors	Function
Hypoxia	Stimulates activation of hypoxia-inducible factor, which activates angiogenesis-related genes, including VEGF and VEGFR	VEGFR-1, soluble VEGFR-1 and neuropilin-1 (NP-1)	Sink for VEGF, VEGF-B, PlGF (VEGFR-1)and for VEGF165 (NP-1)
VEGF, VEGF-C, PlGF and homologues	Stimulate angiogenesis, permeability; VEGF-C: stimulates lymphangiogenesis; PlGF: role in pathologic angiogenesis	Angiopoietin-2	Antagonist of Ang1: induces vessel regression in the absence of angiogenic signals
VEGF receptors (VEGFR)	VEGFR-2: angiogenic signaling receptor; VEGFR-3: (lymph) angiogenic signaling receptor; neuropilin-1 (NP-1): binds specifically VEGF165; coreceptor of VEGFR-2	Thrombospondin-1 (TSP-1)	Extracellular matrix protein; Type I repeats inhibit endothelial migration, growth, adhesion,survival; related TSP-2 also inhibits angiogenesis
Angiopoietin-1 (Ang1) and Tie2-receptor	Ang1: stabilizes vessels by tightening endothelial- smooth muscle interaction; inhibits permeability; Ang2: destabilizes vessels before sprouting	Meth-1, Meth-2	Inhibitors containing metalloprotease,thrombospondin and disintegrin domains

Table 1.1 (Continued).

PDGF-BB and receptors	Recruit smooth muscle cells	Angiostatin and related plasminogen kringles	Proteolytic fragments of plasminogen; inhibit endothelial migration and survival
TGF-β1, endoglin, TGF-β receptors	Stabilize vessels by stimulating extracellular matrix production	Endostatin	Fragment of type XVIII collagen; inhibits endothelial survival and migration
FGF, HGF, MCP-1	Stimulate angiogenesis (FGF, HGF) and arteriogenesis (FGF, MCP-1)	Vasostatin, calreticulin	Calreticulin and N-terminal fragment (vasostatin) inhibit endothelial growth
Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$	Receptors for matrix macromolecules and proteinases (MMP2)	Platelet factor-4	Heparin-binding CXC chemokine inhibits binding of bFGF and VEGF
VE-cadherin, PECAM (CD31)	Endothelial junctional molecules; essential for endothelial survival effect; antibodies block tumor angiogenesis	Tissue-inhibitors of MMP (TIMPs), MMP-inhibitors, PEX	Suppress pathologic angiogenesis; PEX: proteolytic fragment of MMP2, blocks binding of MMP2 to αvβ3
Ephrins	Regulate arterial/venous specification	Tissue-inhibitors of MMP (TIMPs), MMP-inhibitors, PEX	Suppress pathological angiogenesis

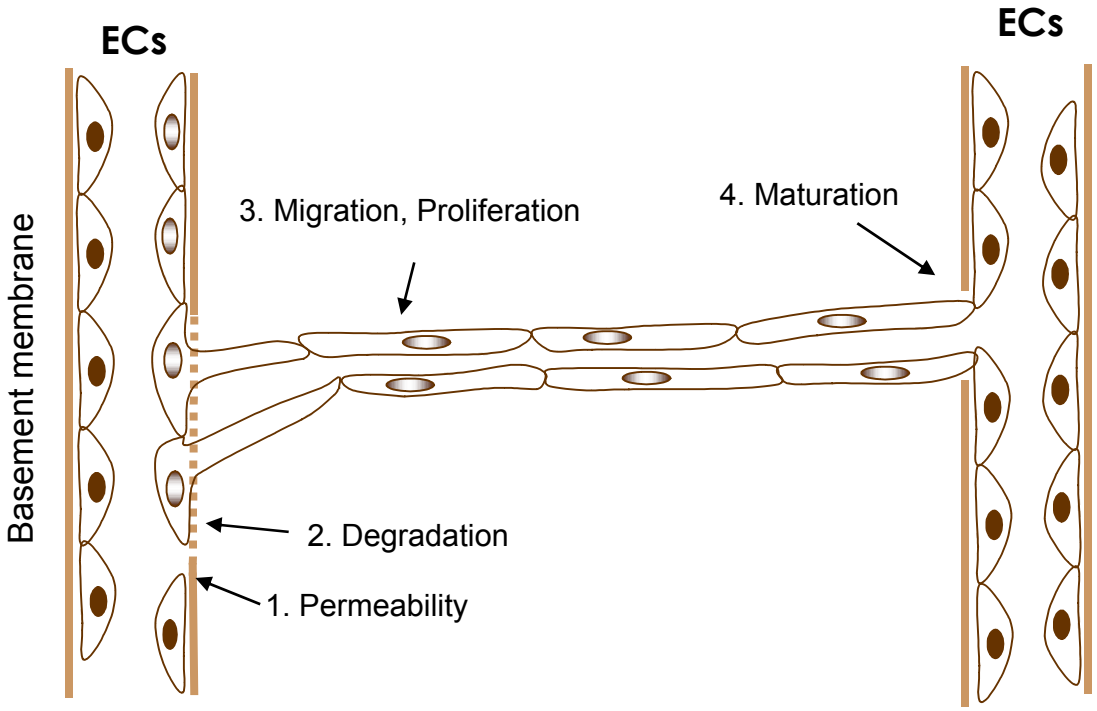
Table 1.1 (Continued).

Plasminogen activators, matrix metalloproteinases	Proteinases involved in cellular migration and matrix remodeling; liberate bFGF and VEGF from the matrix; activate TGF-β1; generate angiostatin	Interferon (IFN) α, β, γ ; IP-10, IL-4, IL-12, IL-18	Cytokines and chemokines, inhibiting endothelial migration; IFN downregulates bFGF
Nitric oxide synthase, cyclooxygenase-2	Nitric oxide and prostaglandins stimulate angiogenesis and vasodilation; Cox2 inhibitors suppress tumor angiogenesis	Prothrombin kringle-2, anti-thrombin III fragment	Fragments of the hemostatic factors suppress endothelial growth
Other activators	AC133 (orphan receptor involved in angioblast differentiation); chemokines c (pleiotropic role in angiogenesis); inhibitors of differentiation (Id1/Id3;helix-loop-helix transcriptional repressors)	Other inhibitors	16 kDa-prolactin (inhibits bFGF/VEGF); canstatin (fragment of the a2-chain of collagen IV); maspin (serpin); troponin-I (inhibits actomyosin ATPase); VEGI (member of TNF family); restin (NC10 domain of collagen XV);fragment of SPARC (inhibits endothelial binding and activity of VEGF); osteopontin fragment (contains RGD sequence)

Modified from *Conway et al. 2001*.

Figure 1.3. The processes of angiogenesis

Angioenesis is initiated by the activation of ECs in response to angiogenesis stimuli. Permeability across the EC layer increases (1) and the degradation of ECM (2) enable EC to proliferate and migrate (3) into the interstitial space. Finally, stabilization of new vessels is accomplished by recruiting mural cells and generating ECM (4).


ECs
ECs
Basement membrane
3. Migration, Proliferation
4. Maturation
2. Degradation
1. Permeability

B2.2 Analysis of FAK function for angiogenesis in mice

Consistent with its role in vitro, several studies in mice have demonstrated the importance of FAK in the regulation of angiogenesis. Transgenic mice, which overexpressed FAK in ECs under the control of the Tie-2 promoter and enhancer, show increased angiogenesis in both the wound-induced angiogenesis model and the ischemia skeleton muscle model (Peng et al. 2004). Moreover, recent studies of EC-specific FAK conditional knockout (CFKO) mice strongly suggest a role of FAK in angiogenesis during embryonic development (Shen et al. 2005; Braren et al. 2006). Analysis of EC-specific FAK conditional knockout (CFKO) embryos showed that FAK is required for the vascular development in late embryogenesis. Although the CFKO embryos developed normally in the formation of the vascular structures in early embryogenesis, FAK deletion in ECs led to multiple defects in late embryogenesis including reduced blood vessel arborization in the embryos, yolk sac and placenta, EC death and associated hemorrhage, edema, developmental delay in the embryos, and late embryonic lethality (Shen et al. 2005).

B2.3 The role of FAK in tumor angiogenesis

Angiogenesis plays a critical role in cancer progression as well as in physiological neovascularization. Tumor angiogenesis is necessary for tumor growth and metastasis and it is thought to depend on the balance between proangiogenic factors, generally VEGF and bFGF, and angiogenesis inhibitors (Hanahan et al. 1996). Tumor angiogenesis involves increased EC proliferation, migration, and tube formation into the tumor mass and these processes of angiogenesis require changes in cell adhesion, which are mediated by integrins, whose expression is increased during tumor angiogenesis (Silva et al. 2008). Several studies have demonstrated an

important role of integrins in tumor angiogeneis (Brooks 1996). The inhibition of αvβ3 or αvβ5 integrin effectively suppressed both tumor angiogenesis and tumor growth (Eliceiri et al. 1999), although genetic ablations of the genes encoding these integrins resulted in increased angiogenesis (Reynolds et al. 2002). In addition, the α6β4 integrin promotes tumor angiogenesis by promoting nuclear translocation of P-ERK and NF-κB (Nikolopoulos et al. 2004).

Several reports have shown increased levels of FAK expression in a wide range of human epithelial cancers. Levels of FAK expression correlate with the malignancy of tumors. In addition, FAK expression increased in the microvascular endothelial cells in astrocytic tumor and oral squamous cell carcinoma, implicating a role of FAK in tumor angiogenesis (Kornberg 1998; Haskell et al. 2003). However, the specific mechanisms of FAK in the regulation of tumor angiogenesis are still undefined. A recent study in 4T1 breast carcinoma cells showed a novel role for FAK in tumor angiogensis. Src phosphorylation of FAK at Tyr-925 followed by Grb2 adaptor protein binding to FAK and signaling to mitogen-activated protein kinase (MAPK) can enhance VEGF expression thereby promote tumor angiogenesis (Schlaepfer and Mitra 2004).

C. Centrosome

The centrosome is the microtubule organizing center (MTOC) that regulates microtubule-related functions, including cell migration, polarity, adhesion, maintenance of cell shape, intracellular transport and positioning of organelles as well as cell division (Doxsey 2001; Meraldi and Nigg 2002). It has been shown that the structure and number of centrosomes are tightly regulated throughout the cell cycle (Hinchcliffe et al. 1999; Doxsey 2001; Meraldi and Nigg 2002) and the centrosome is

increasingly being recognized for its significant contribution to cell cycle regulation (Hinchcliffe et al. 1999; Lange 2002). The centrosome regulates mitosis entry, anaphase onset, cytokinesis and G1/S transition, and monitors DNA damage (Schatten 2008).

A single centrosome consists of two centrioles that are surrounded by pericentriolar material (PCM). Each centriole has a barrel-shaped structure, which contains nine sets of triplet microtubules (Doxsey 2001). Within the centrosome, two non-identical centrioles are arranged perpendicular to one another and called the mother and daughter centrioles. Cenrioles play a role in the assembly of PCM and in the anchoring of the microtubule. The PCM is a lattice-like structure and consists of many coiled-coil scaffold proteins that serve as docking sites for other PCM components. The PCM is the major site for nucleation of microtubules. It contains γ-tubulin ring complexes (γTuRCs) that act as a template for microtubule nucleation (Meraldi and Nigg 2002).

C1. Centrosome cycle

The centrosome needs to be precisely duplicated in concert with the chromosomes in every cell cycle (Hinchcliffe et al. 1999). This centrosome duplication, or reproduction, can be divided into several steps integrated into cell cycle progression (fig.1.4). During mitosis, each centrosome contains a pair of centrioles and these centrioles are disassociated by the process of centriole disorientation, or centriole splitting, at the end of mitosis. Centriole duplication then starts during G1 and S phases through the nucleation of short daughter centrioles, or procentrioles, at the proximal wall of each parental centriole (Blagden and Glover 2003). These procentrioles then elongate during S and G2 phases until they reach the

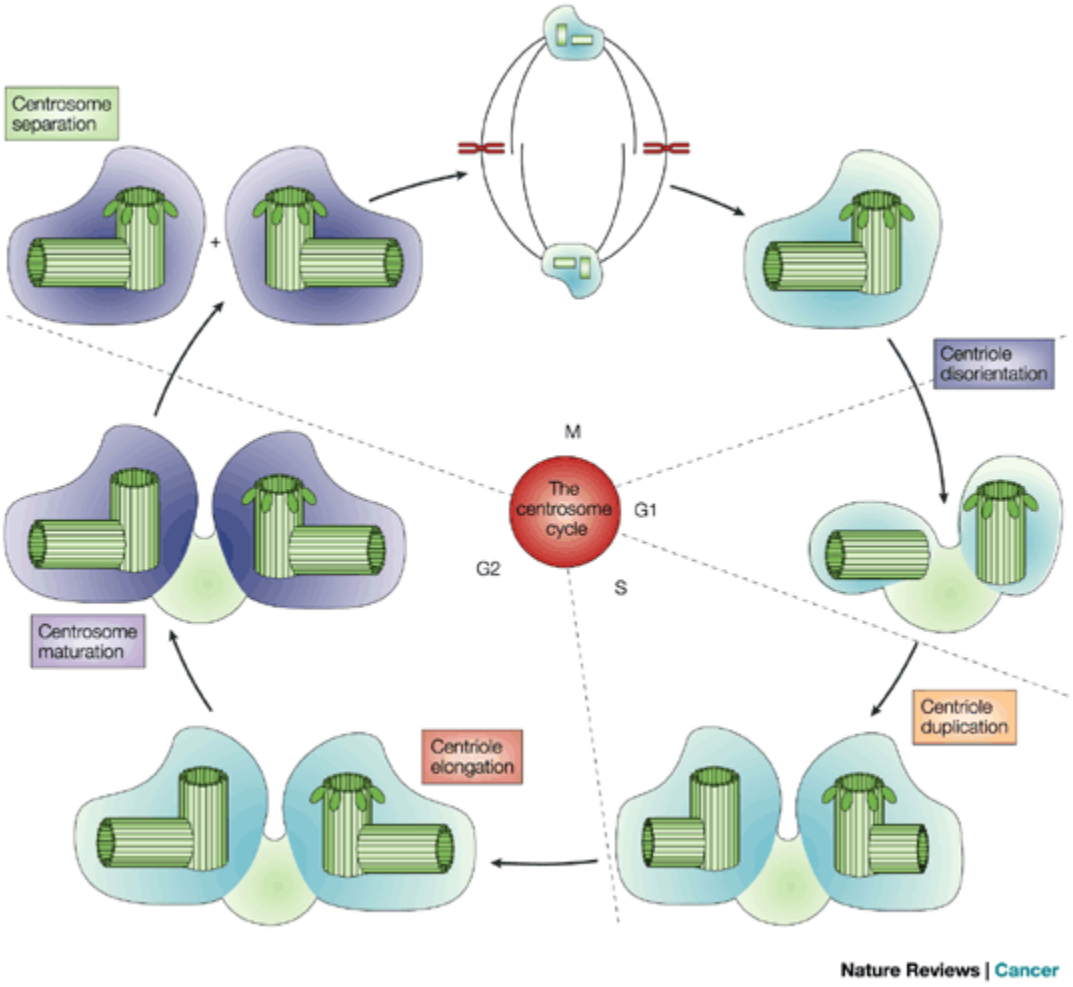
mature length. Elongation is complete when they obtain markers such as ninein and Cenexin/Odf2 (Lange and Gull 1995; Nakagawa et al. 2001). During G2 and M phases, centrosome maturation occurs. In order to nucleate a sufficient number of mitotic microtubules, centrosome maturation involves the expanding of PCM components and the recruitment of additional γTuRCs (Meraldi and Nigg 2002). At prophase, the centrosome undergoes disjunction, which leads to the loss of cohesion between two parental centrioles that were provided by the interconnecting bridge of the centriole-associated protein, C-Nap1 (Blagden and Glover 2003). Through the activation of microtubule-dependent motor proteins including dynein and kinesin, centrosomes then separate from each other and migrate around the nucleus to develop two centrosomes at the opposite sides (Sharp et al. 2000).

C2. Centrosome functions in cell division

The centrosome has been shown to play essential roles in the regulation of spindle bipolarity, spindle positioning and cytokinesis through its microtubule organizing capabilities during mitosis (Meraldi and Nigg 2002). Abnormality in centrosome numbers can disrupt bipolar spindle formation and chromosome segregation, suggesting the coordinated regulation of cell, centrosome and nuclear cycles. The presence of many regulatory molecules including cell cycle regulators on the centrosomes has demonstrated that the centrosome plays a role not only in the regulation of microtubule nucleation, but also in the coordination of centrosome duplication with cell cycle progression (Lange 2002). Some centrosome proteins are permanently associated with the centrosome core structure, including γ-tubulin, γTuRCs, and centrin whereas several centrosome proteins are temporarily associated with the centrosome core structure and may use centrosomes as a docking station to

Figure 1.4. The centrosome cycle

In early G1, the centrosome contains mother and daughter centrioles. These contrioles move apart and lose their orthogonal orientation during late G1 or early S phase (Centriole disorientation/splitting). Each centriole (parent centriole) then nucleates the new centiole (procentriole) during S phase (Centriole duplication). By mitosis, procentioles elongate until they reach their maximal length and centrosome maturation occurs. The two mature centrosomes are then separated through the action of microtubule-based motor proteins. Finally, each daughter cell acquires one centrosome after mitosis (Adapted from *Nature Reviews Cancer, vol. 2 (11), pp.818*) (Meraldi and Nigg 2002).


Centrosome separation
Centriole disorientation
M
The centrosome cycle
G1
G2
S
Centrosome maturation
Centriole duplication
Centriole elongation
Nature Reviews | Cancer

coordinate cell cycle specific functions such as NuMA (Nuclear Mitotic Apparatus) (Schatten 2008). However, much remains unclear about the coordinated regulation of the cell, centrosome and nuclear cycles and the functions of centrosomal proteins.

C2.1 Microtubule nucleation and anchoring

Microtubule organization is regulated by the microtubule nucleation and microtubule anchoring activities at the centrosome. Microtubules are nucleated by ring-shaped multiprotein complexes containing γ–tubulins, γTuRCs (as mentioned in previous section C.) and the pericentrin has been demonstrated to recruit γTuRCs at centrosomes (Doxsey 2001). Following microtubule nucleation, microtubules are either released to the cytoplasm or recaptured and anchored to the centrosomes. The sub-distal appendages of the mother centrioles are known as a major site for microtubule anchoring (Azimzadeh and Bornens 2007). It was reported that ninein and centriolin are associated with the sub-distal appendages of the mother centrioles and are also play a role in anchoring (Mogensen et al. 2000; Doxsey 2001). In addition, the dynactin, dynein activator complex is implicated in playing a role in anchoring microtubules at the centrosome through the interaction with microtubule associated protein EB1 (Askham et al. 2002).

The levels of γTuRCs at centrosomes are increased significantly before mitosis and at the same time many centrosomal proteins that are required for mitotic spindle formation, cell cycle progression, and centrosome duplication are recruited to centrosome. Centrosomal proteins, including pericentrin, PCM1 (pericentriolar satellites) and NuMA are known to be assembled to centrosomes from cytoplasmic pools for the centrosome function. Cytoplasmic dynein has been known to recruit

these proteins to centrosome through its interactions with many centrosomal proteins and microtubules (Zimmerman and Doxsey 2000; Doxsey 2001).

C2.2 Regulation of centrosome functions and its coordination with cell cycle

It has been shown that centrosome structure and function are regulated by several kinases during centrosome duplication in coordinated with chromosome cell cycle (Sankaran and Parvin 2006). Several important kinases that regulate centrosome functions will be discussed (Also see table 1-2 for additional kinases).

Cyclin-dependent kinase (cdk)

Cyclin and cdk have been demonstrated to play an important role in cell cycle regulation. Several studies revealed that cdk2 binding to cyclin E is required for initiating centrosome duplication as well as DNA replication (Sankaran and Parvin 2006). In some cell types, centrosome duplication has been shown to be regulated by cdk2-cyclin A (Meraldi and Nigg 2002). The specific mechanisms that initiate the centrosome duplication through cdk2 and cyclinE/A are still undefined. However, it was reported that nucleophosmin (NPM/B23), a phosphoprotein of nucleolus, can associate with unduplicated centrosomes and serve as a substrate of cdk2-cyclin E in centrosome duplication (Tokuyama et al. 2001). NPM/B23 dissociates from centrosomes through its phosphorylation mediated by cdk2-cyclinE, which is required for centrosomes to initiate duplication. In addition, recent studies suggest a role of centrosome in the G2 to M transition, showing cdk1- cyclinB as a key regulator in initiating mitosis (Schatten 2008). Although cyclinB is present throughout the cytoplasm, active cdk1-cyclinB is localized to centrosome during prophase, suggesting that centrosomes might serve as sites for integration of cell cycle regulators (Doxsey et al. 2005).

Aurora Kinases

Aurora kinases are a family of serine/threonine kinases that consists of three members, Aurora A, B, and C. Aurora A and B are essential for mitosis whereas Aurora C plays a role in the regulation of cilia and flagella (Ducat and Zheng 2004). Aurora A starts to localize at centrosomes in S phase and then, it is degraded in early G1. Aurora A has shown that it contributes to centrosome maturation, separation and bipolar spindle assembly. In contrast, Aurora B localized to chromosomes, centromeres and central spindles, playing a role in chromosomes segregation and cytokinesis (Dutertre et al. 2002).

Aurora A regulates several centrosome proteins for centrosome maturation during mitosis. Aurora A is known to directly bind and phosphorylate TACC (transforming acidic-coiled-coil-containing) protein, which recruits ch-TOG/XMAP215, a protein required for microtubule nucleation and stabilization, thereby contributing to centrosome maturation (Ducat and Zheng 2004). Several studies also showed that Aurora A regulates γTuRCs recruitment by another centrosomal protein, centrosomin (CNN). The interaction of Aurora A and CNN is required for the localization of both proteins to the centrosome and it enhances the microtubule nucleation through increasing its recruitment of γTuRCs at centrosome (Ducat and Zheng 2004). In addition, a serine-threonine kinase, LATS2 and NDEL are known to be phosphorylated by Aurora A and affect centrosome maturation (Barr and Gergely 2007).

Aurora A also plays a role in the regulation of centrosome separation and bipolar spindle assembly. Both centrosome separation and the assembly of mitotic spindle requires a coordinated regulation of microtubule based motor proteins, including the minus-end directed motor dynein and the plus-end directed motor

kinesin Eg5. Although specific molecular mechanisms are unclear, Aurora A has been implicated in the regulation of separation and bipolar spindle assembly through the coordination of these motor proteins (Barr and Gergely 2007). Aurora A phosphorylates the kinesin-related protein Eg5, which is required for both centrosome separation and spindle assembly and stability. Recent studies also showed that Aurora A is activated by RanGTP, which plays a role in spindle assembly. Since it was reported that RanGTP regulates spindle assembly in part through the activation of Eg5 indirectly, Aurora A might mediate RanGTPase signaling by directly phosphorylating Eg5 (Ducat and Zheng 2004).

In addition, Aurora A regulates cell cycle progression, entry into mitosis. During centrosome maturation, Aurora A contributes to centrosomal targeting of cdk1- cyclinB and it phosphorylates CDC25B, which in turn activates cdk1- cyclinB at the centrosome, thereby promoting entry into mitosis (Barr and Gergely 2007).

Polo-like kinase

Polo-like kinases (Plk 1-4) are a family of serine/threonine kinases, which have been recognized as key regulators of mitosis and cytokinesis. Plks are involved in the regulation of centrosome functions although its molecular mechanisms remain unclear. In mammalian cells, Plk1 is the best investigated Plk member so far, showing its roles in the centrosome maturation and separation, entry into mitosis, bipolar spindle formation and cytokinesis (Barr et al. 2004). Plk1 is known to be involved in the recruitment of γTuRCs during centrosome maturation. It was reported that Plk1 phosphorylates Nlp (ninein-like protein), which interacts with γTuRCs during interphase, and contributes to the dissociation of Nlp from the centrosome at the onset of mitosis. This dissociation may allow the increased recruitment of scaffold proteins that are required for centrosome maturation (Barr et al. 2004). Plk1 also plays a role

in the regulation of mitotic entry, through its ability to activate cdk1-cyclin B, which functions as a key regulator of mitotic entry (Barr et al. 2004). In addition, it may contribute to centrosome separation and bipolar spindle formation presumably through the cdk1 mediated activation of Eg5 (Wang et al. 2008).

NIMA-related kinase (Nek2)

Nek2 is a serine/threonine kinase that localized to centrosomes. Its activity is cell cycle regulated with peak levels in S/G2 phase. Nek2 has been demonstrated to phosphorylate C-Nap1 and displace C-Nap1 from the centrioles, thereby contributing to the splitting of centrolies at the onset of mitosis. Nek2 also phosphorylates protein phosphatase 1 (PP1), which in turn de-phosphorylates both C-Nap1 and Nek2 (Mayor et al. 2002). Therefore, these studies suggest that Nek2, C-Nap1 and PP1 may exist in cells as a ternary complex with Nek2 and PP1 antagonistically regulate the phosphorylations of both Nek2 and C-Nap1 (Fry 2002) .

C3. The potential function of FAK in centrosome functions

As previously described in section A3.2, FAK has been implicated in playing a role in cell cycle progression by the regulation of G1 to S transition. Furthermore, the levels of phosphoylations of FAK were reported to be changed during mitosis, suggesting a potential role for FAK in mitosis. A recent study showed that inhibition of integrin function disrupted centrosome functions, spindle assembly, and cytokinesis in mitotic cells (Reverte et al. 2006). Together with the report that described in section A3.1.3, showing a role of FAK Ser732 phosphorylation in the regulation of microtubule structure in neuronal cells (Xie et al. 2003), FAK may also possibly play a role in centrosome function regulating the microtubule structure, the

Table 1.2. Functions of mitotic kinase throughout the cell cycle

Kinase	Known substrate(s)	Role(s)
Cdk1	Cdc25 family, CAK, cyclin B1, Myt1, Wee1	Mitotic entrance, chromosome condensation, bipolar spindle assembly, nuclear envelope breakdown, APC/C regulation
Chk1/Chk2	ATM, ATR, Cdc25 family, Wee1, Plk3, p53, BRCA1	DNA damage checkpoint, mitotic entrance
Plk1	Cdc25 family, Cdk1, cyclin B1, p53, ATM/ATR, BRCA1, Chk1, Emi1	Mitotic entrance, centrosome maturation, bipolar spindle formation, APC/C regulation
Plk2	p53	Centriole duplication, spindle damage checkpoint
Plk3	ATM, Cdc25 family, Chk2, p53	DNA damage checkpoint, mitotic entrance
Plk4	Not known	Centriole duplication APC/C regulation
Aurora A	TPX2, p53	Spindle formation, centrosome separation
Aurora B	INCENP, survivin, borealin	Spindle assembly checkpoint, cytokinesis
Bub Family	Mps1, Mad1, Mad2, CENPE, Cdc20	Spindle assembly checkpoint, APC/C regulation
NIMA Family	C-Nap1	Centrosome assembly, maturation and separation, mitotic entrance

Adapted from *Schmit and Ahmad 2007*.

mitotic spindle, which plays an essential role during mitosis in non-neuronal cells. In this regard, it is

interesting to note that several other focal adhesion proteins, including HEF1 (Pugacheva and Golemis 2005), paxillin (Herreros et al. 2000), zyxin (Hirota et al. 2000), and ILK (Fielding et al. 2008) have been shown to localize and function in centrosomes. Therefore, these studies suggest that FAK may contribute to centrosome function as integrin and other focal adhesion proteins did, providing a mechanistic link for the control of the cell attachment and cell division.

D. Project overview

FAK is a cytoplasmic tyrosine kinase that plays an important role in signal transduction by integrins and other cell surface receptors in a variety of cells including endothelial cells (ECs). Phosphorylation of FAK on Tyr 397 and its binding to Src has been shown as a critical step to activate FAK itself and its downstream signaling molecules that mediate cellular functions of FAK, including cell migration, cell cycle progression and cell survival (Section A). Several studies suggest the importance of both integrins and growth factor receptors in the regulation of key aspects of angiogenesis (Section B1). Since FAK mediates signaling from integrins and growth factor receptors, these studies strongly suggest a potential role of FAK in angiogenesis. Moreover, targeted disruption of FAK in mice has demonstrated the importance of FAK in vascular development. FAK gene knockout in mice leads to death at embryonic day 8.5 (E8.5) due to defects in the axial mesodermal tissues including cardiovascular system. Recent studies of EC-specific FAK conditional knockout mice revealed that FAK is indeed required for the vascular development during embryogenesis (Section B2).

To investigate the underlying mechanims of FAK in the regulation of angiogenesis and vasculogenesis in mutant embryos, primary ECs will be isolated from FAK floxed mice and infected by recombinant adenoviruse encoding Cre recombinase (Ad-Cre) to delete endogenous FAK. Using these FAK-deficient ECs, the role of FAK in the regulation of EC function will be determined (Chapter 2). In order to study the specific mechanisms of FAK in angiogenesis, recombinant adenovirues encoding FAK mutants that are incapable of interacting with various targets of FAK were genereated (Appendix). For the *in vitro* study, FAK-deficient ECs will be reinfected with Ad-FAK and/or Ad-FAK mutants to examine their ability to rescue the various phenotypes upon the deletion of endogeneous FAK. In this dissertation, the differential requirement of FAK kinase acivity in FN- and VEGF-stimulated EC migration (Chapter 2) and the role of Serine 732 phosphorylation of FAK in EC proliferation and angiogenesis (Chapter 3) will be discussed. In addition, the role of FAK and FAK signaling pathways in vivo as a regulator of angiogenesis will be determined by infecting floxed FAK adult mice with recombinant adenoviruses using Marigel plug assay (Chapter 3).

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CHAPTER 2:

ESSENTIAL FUNCTION OF FAK IN THE REGULATION OF VARIOUS ENDOTHELIAL CELL FUNCTIONS*

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A. Abstract

Focal adhesion kinase (FAK) is a critical mediator of signal transduction by integrins and growth factor receptors in a variety of cells including endothelial cells (ECs). The analysis of EC-specific FAK knockout mice showed that FAK plays a role in vasculogenesis and angiogenesis (Shen et al. 2005; Braren et al. 2006). The inactivation of FAK in ECs caused reduced blood vessel network in the embryos, yolk sac, and placenta, EC death and associated hemorrhage, edema, and developmental delay, and embryonic lethality in late embryogenesis (Shen et al. 2005). To further investigate the mechanisms of the endothelial defects in mutant embryos, here we isolate primary ECs from homozygous floxed FAK mice and inactivate FAK by infecting recombinant adenoviruses encoding Cre recombinase. Consistent with the phenotypes *in vivo*, deletion of FAK in ECs showed reduced tubulogenesis, proliferation, and migration *in vitro*. Together, these results suggest that FAK is required for angiogenesis and vasculogenesis due to its essential function in the regulation of multiple EC activities.

B. Introduction

Blood vessel formation is fundamental to embryonic development and organogenesis, as well as to the pathogenesis of many diseases including coronary heart disease, diabetes, retinopathies, and cancer (Dvorak 2003). Blood vessels are formed by two processes: vasculogenesis, whereby a primitive vascular network is established during embryogenesis from the pluripotent mesenchymal progenitors, and angiogenesis, in which the growth of new capillaries occurs from the preexisting vessels (Yancopoulos et al. 1998). Endothelial cells (ECs) play a pivotal role in both vasculogenesis and angiogenesis, and function as both transducers and effectors of

local environmental signals for vessel formation. Multiple cell surface receptors and their ligands have been shown to play important roles in the regulation of blood vessel formation. These include proangiogenic growth factors and their receptors on EC, multiple integrins, and their ECM ligands (Yancopoulos et al. 1998; Hynes 2002). However, much less is known about the roles and mechanisms of the intracellular signaling pathways triggered by these receptors in the regulation of angiogenesis and vasculogenesis.

FAK is a cytoplasmic tyrosine kinase that plays a key role in integrin-mediated signal transduction pathways (Parsons 2003; Schlaepfer and Mitra 2004). Integrin-mediated cell adhesion leads to FAK activation and autophosphorylation in a variety of cell types. Activated FAK associates with a number of Src homology 2 domain–containing signaling molecules including Src family kinases, p85 subunit of PI3K, phospholipase C-γ, and Grb7 (Parsons 2003). FAK binding to Src family kinases contributes to the activation of both kinases, which leads to phosphorylation of several other sites on FAK and a number of other substrates including paxillin (Burridge et al. 1992; Schaller and Parsons 1995), p130cas (Vuori et al. 1996; Ruest et al. 2001), and Shc (Schlaepfer et al. 1998). FAK and its interactions with these signaling molecules have been shown to trigger several downstream signaling pathways that regulate cell spreading and migration, cell survival, and cell cycle progression (Parsons 2003; Schlaepfer and Mitra 2004).

Consistent with its critical roles in vitro, FAK gene knockout in mice resulted in an embryonic lethal phenotype due to defects in the axial mesodermal tissues and cardiovascular system (Ilic et al. 1995). Both vasculogenesis and angiogenesis of the vasculature were impaired and neither a normal heart nor fully developed blood vessels were present in the FAK-null embryos. These results suggested a crucial role

of FAK in the development of the vasculature. However, the relatively early (E8.5) embryonic lethality prevented analysis of the role of FAK in the late stage of embryonic development including angiogenesis in vivo.

A potential role of FAK in angiogenesis has also been suggested by a number of other studies. During the mouse embryo development, FAK expression became increasingly restricted to the blood vessels (Polte et al. 1994). Increased EC migration into a wounded monolayer was correlated with increased tyrosine phosphorylation and kinase activity of FAK (Romer et al. 1994). In addition, activation of VEGF receptor-2 by VEGF-A induced association of FAK with PI3-kinase, which is required for the stimulation of migration of porcine aortic ECs (Qi and Claesson-Welsh 2001). Angiopoietin-1, another angiogenesis stimulator, also increased FAK phosphorylation during angiogenesis in vitro (Kim et al. 2000). Lastly, several members of the integrin family play important roles in the regulation of angiogenesis (Eliceiri and Cheresh 2001). A recent report also showed that formation of a signaling complex containing FAK and integrin $\alpha v\beta 5$ in an Src-dependent manner is essential for VEGF-stimulated angiogenesis (Eliceiri et al. 2002). Given FAK's role in mediating signaling by integrins and growth factor receptors, these results also strongly suggest a potential role for FAK in vasculogenesis and angiogenesis.

To investigate the physiological role of FAK in vascular development and angiogenesis in vivo, we previously generated a strain of mice with FAK gene flanked by two loxP sites (floxed FAK) (Shen et al. 2005). To specifically inactivate the *FAK* gene in ECs, the floxed FAK mice were intercrossed with transgenic mice expressing Cre recombinase under the control of the Tie2 endothelial-specific promoter. In contrast to the total FAK knockout, deletion of FAK in ECs did not affect early embryonic development. The majority of conditional FAK knockout

(CFKO) embryos until E12.5 were seen normally in the overall gross appearance and development. However, a decreased number of CFKO embryos than the expected 25% Mendelian ratio was found at E13.5 and thereafter, suggesting that EC-specific deletion of FAK leads to a late embryonic lethality. Analysis of the CFKO embryos showed multiple defects in late embryogenesis including reduced blood vessel network in the superficial vasculature, hemorrhage, edema, developmental delay in the embryos, and abnormalities of blood vessels in both yolk sac and placental labyrinth. Histologically, ECs and blood vessels in the mutant embryos present a disorganized, detached, and apoptotic appearance. Therefore, these results strongly suggest a role of FAK in angiogenesis and vascular development in vivo. In the present study, we identify the critical cellular functions of FAK in the regulation of ECs.

C. Materials and methods

1. Culture of ECs and adenovirus infection

ECs with homozygous FAK floxed alleles were isolated from E12.5 embryos using the magnetic bead (Dyanbead M-450; Dynal Corp.) purification with rat anti–mouse PECAM-1 (BD Biosciences), as described previously (Cattelino, 2003; Peng, 2004). The endothelial nature of the cells was confirmed by FACS and immunofluorescence microscopy with antibodies to endothelial markers, PECAM-1 (1:100) and VE-Cadherin (1:50). Approximately 90% purity of ECs was routinely obtained in the preparations. Cells were cultured in high glucose DME with 20% FCS (Hyclone), EC growth supplement (5 μg/ml; Worthington), and heparin (100 μg/ml; Sigma-Aldrich) maintenance medium (Peng et al. 2004) on gelatin-coated tissue culture plates. MEFs with floxed FAK alleles were isolated from E12.5 *FAK*$^{flox/flox}$

embryos as described previously (Sage et al. 2000). The recombinant adenoviruses encoding Cre recombinase or lacZ control were purchased from Gene Transfer Vector Core (University of Iowa, Iowa City, IA). For most studies, 10^8 plaque-forming units were used for 10-cm dish. To increase efficiency, a second infection was performed after 9–12 h. The recombinant adenoviruses encoding FAK (Ad-FAK), its kinase-defective mutant (Ad-KD), or GPF control (Ad-GFP) were generated using the Adeasy-1 system (Stratagene) according to manufacturer's instruction. For the rescue experiments, cells infected with Ad-Cre were reinfected with Ad-FAK, Ad-KD, or Ad-GFP control at 10^8 plaque-forming units 2 d after infection of Ad-Cre to delete endogenous FAK. No detectable cell toxicity was observed.

2. Western blotting

Antibodies used are anti-FAK (C20; Santa Cruz Biotechnology, Inc.), anti-vinculin (Sigma-Aldrich), anti-Pyk2 (Zheng et al. 1998), anti-phospho-tyrosine397-FAK and anti-phospho-tyrosine118-paxillin (Upstate Biotechnology), anti-phospho-JNK (Cell Signaling Technology), or anti-phospho-Erk1/2 (New England Biolabs, Inc.).

3. Tube formation assay

ECs infected with Ad-LacZ or Ad-Cre were plated on a thin layer of Matrigel (BD Biosciences) at 10^4 cells/well of a 96-well plate in 10% FBS DME and allowed to form a tubular structure for 8 h to overnight. Cells were assessed on their ability to form simple tube structures and their morphology. The samples were examined on a microscope (model IX70; Olympus) with UplanF1 x10/0.3 objective lens and photographed with a progressive 3CCD camera (Sony) and Image-Pro Plus ver.

3.0.00.00 at RT. The length and branch points were determined as described previously (Haskell et al. 2003).

4. TUNEL assay

ECs infected with Ad-LacZ or Ad-Cre were assessed for apoptosis by TUNEL assay using the In Situ Cell Death Detection Kit (Roche), according to the manufacturer's recommendations.

5. BrdU incorporation assay

2 d after infection, ECs were serum starved for 18 h to arrest the cells in G0. BrdU incorporation assay was performed as described previously (Zhao et al. 2003) with the following modifications. In brief, cells were released from G0 by replating the cells in 10% FBS and 150 μM BrdU. After 48 h of growth, cells were fixed, treated with DNase I, and processed for double immunofluorescent staining with anti-BrdU and anti-PECAM-1, as described below. The percentage of BrdU(+)/ECs (PECAM-1) was determined for ~100 cells in multiple fields in each independent experiment.

6. Boyden chamber cell migration assay

Cell migration assays were performed using a Neuro Probe (Cabin John) 48-well chemotaxis Boyden chamber as described previously (Cary et al. 1996) with the following modifications. 7.5 x 10^3 cells were added in each upper well, and the bottom wells contained either 10 ng/ml VEGF or 10 μg/ml fibronectin as chemoattractant, or DME alone as a control. They were then incubated for 4 h in a

37°C humidified CO_2 incubator. At the end of the experiment, cells were fixed with methanol for 8 min and stained with modified Giemsa stain (Sigma-Aldrich).

7. Wound closure cell migration assay

Wound closure assays were performed essentially as described previously (Liang et al. 2007). Infected ECs or MEFs were plated (10^6 cells) on gelatin (for VEGF-stimulated cell migration) or FN-coated dishes (60 mm), allowed to adhere and spread for 4 h, and then used for assays.

8. Immunofluorescence staining

ECs infected with Ad-LacZ or Ad-Cre were processed for immunofluorescence staining as described previously (Cary et al. 1996). The primary antibodies used were anti-phosphotyrosine (PY20; 1:100), anti-vinculin (1:50), anti-BrdU (1:50), and anti-PECAM-1 (1:100). FITC-conjugated goat anti–rabbit IgG (1:150) and FITC-conjugated goat anti–mouse IgG (1:150) were used as the secondary antibodies. They were then mounted on Slowfade (Molecular Probes, Inc.) and examined under a microscope (model BX41; Olympus) with UplanF1 x20/0.5 objective lens at RT. The images were captured using a camera (model DP70; Olympus) with DP Controller ver. 1.2.1.108.

D. Results

1. Deletion of FAK in isolated primary ECs results in reduced capillary formation and multiple cellular deficiencies in vitro

To further understand the mechanisms of the endothelial defects in CFKO embryos (Shen et al. 2005), we isolated primary ECs from homozygous floxed FAK mice. The isolated floxed FAK ECs were infected by recombinant adenoviruses encoding Cre recombinase (Ad-Cre). As shown in Fig. 2.1A, Ad-Cre infection of the floxed FAK ECs led to a dose-dependent decrease in the expression of FAK protein concomitant with excision of exon 3 of FAK gene. As expected, infection of the cells with a control recombinant adenovirus encoding lacZ (Ad-lacZ) did not affect FAK protein expression or the flox allele of the FAK gene. These ECs are designated as CFKO and control ECs, respectively.

The effect of FAK inactivation in the isolated ECs was assessed by examining their ability to change morphology and form capillaries when cultured on Matrigel, which is a process mimicking sprouting and tube formation during angiogenesis in vivo. Fig. 2.1B shows the significantly reduced formation of tubules of CFKO ECs compared with the control ECs. Quantitation of multiple experiments indicated that both the length of the tubules and the number of branch points were reduced in the CFKO ECs (Fig. 2.1C and D). Interestingly, consistent with EC necrosis and apoptosis observed in the CFKO embryos (Shen et al. 2005), we noted that some of CFKO ECs, but few control ECs, appeared to be apoptotic under this condition. Indeed, the CFKO ECs showed reduced survival in serum-free condition when compared with the control ECs (unpublished data). TUNEL assays were then performed to test a possible role of FAK on EC apoptosis directly. Fig. 2.2A shows that inactivation of FAK resulted in increased apoptosis of the primary ECs. These

results suggested that increased apoptosis and decreased survival of ECs upon FAK KO may be responsible for the defective vascular development and associated hemorrhage and edema, and possibly also reduced angiogenesis in the CFKO embryos.

We also investigated the effects of FAK inactivation on the proliferation and migration of the primary ECs to determine the contribution of their possible changes to the in vivo vascular defects of CFKO embryos. Fig. 2.2B shows a decreased cell cycle progression of CFKO ECs upon serum stimulation in comparison to control ECs, as measured by the BrdU incorporation as described in Materials and methods. Analysis of cell migration using the Boyden chamber assays showed reduced migration of CFKO ECs in response to VEGF stimulation compared with control ECs. Surprisingly, however, little difference was observed between the CFKO and control ECs in their migration in response to FN (Fig. 2.2C). The important role of FAK in FN-stimulated migration is well described for many cell types, including ECs in previous studies (Parsons 2003; Schlaepfer and Mitra 2004). Therefore, we further investigated migration of CFKO ECs using the potentially more physiologically relevant wound closure assays. Fig. 2.2D shows that deletion of FAK reduced EC migration in response to both FN and VEGF. As both EC migration and proliferation are critical for angiogenesis, these data suggested that reduced proliferation and migration of ECs upon FAK deletion could both contribute to the defective angiogenesis in the CFKO embryos in vivo.

Figure 2.1. Defective tubulogenesis of the isolated FAK$^{-/-}$ primary ECs

(A) ECs isolated from homozygous floxed FAK mice (flox/flox) were infected with increasing amount of recombinant adenoviruses encoding Cre (Ad-Cre) or a control insert (Ad-lacZ), as indicated. Cell lysates were analyzed by Western blotting with anti-FAK or anti-vinculin (top two panels). Genomic DNA was analyzed by PCR (bottom two panels). (B–D) Primary ECs from floxed FAK mice and infected with Ad-Cre or Ad-lacZ were cultured on Matrigel as described in Materials and methods. Images of representative fields are shown in B. The length of the tubules (C) and branch points (D) were quantified from three independent experiments and shown as the relative ratio of the value ± standard error. *, P = 0.023 and **, P = 0.017 in comparison to value from Ad-lacZ–induced cells.

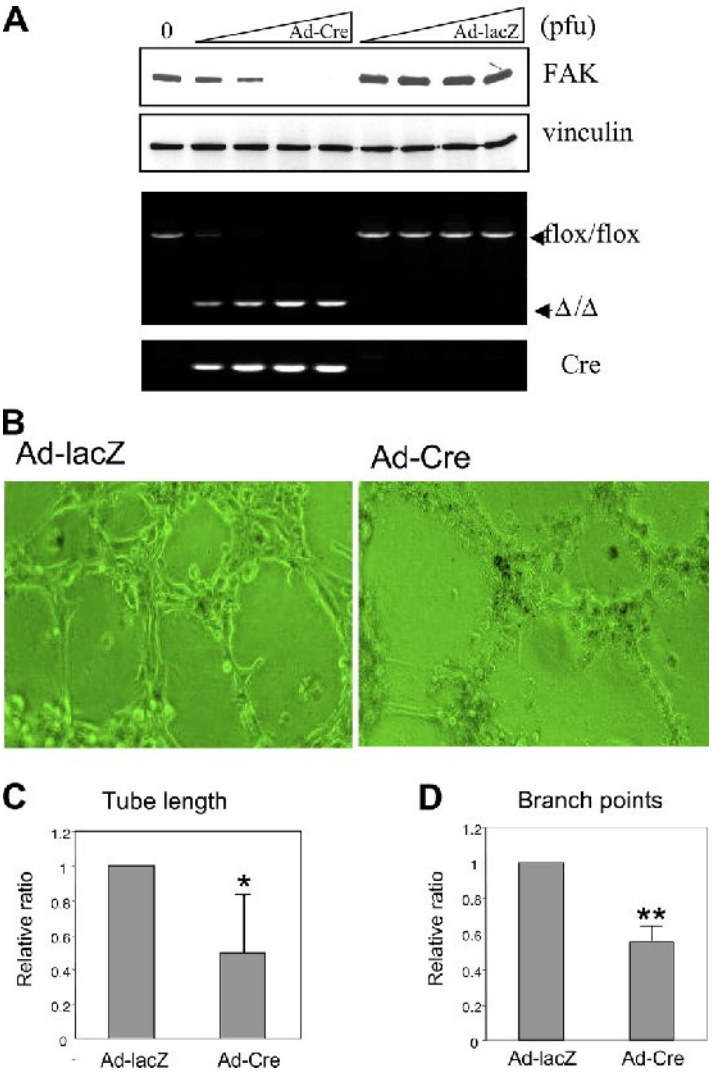
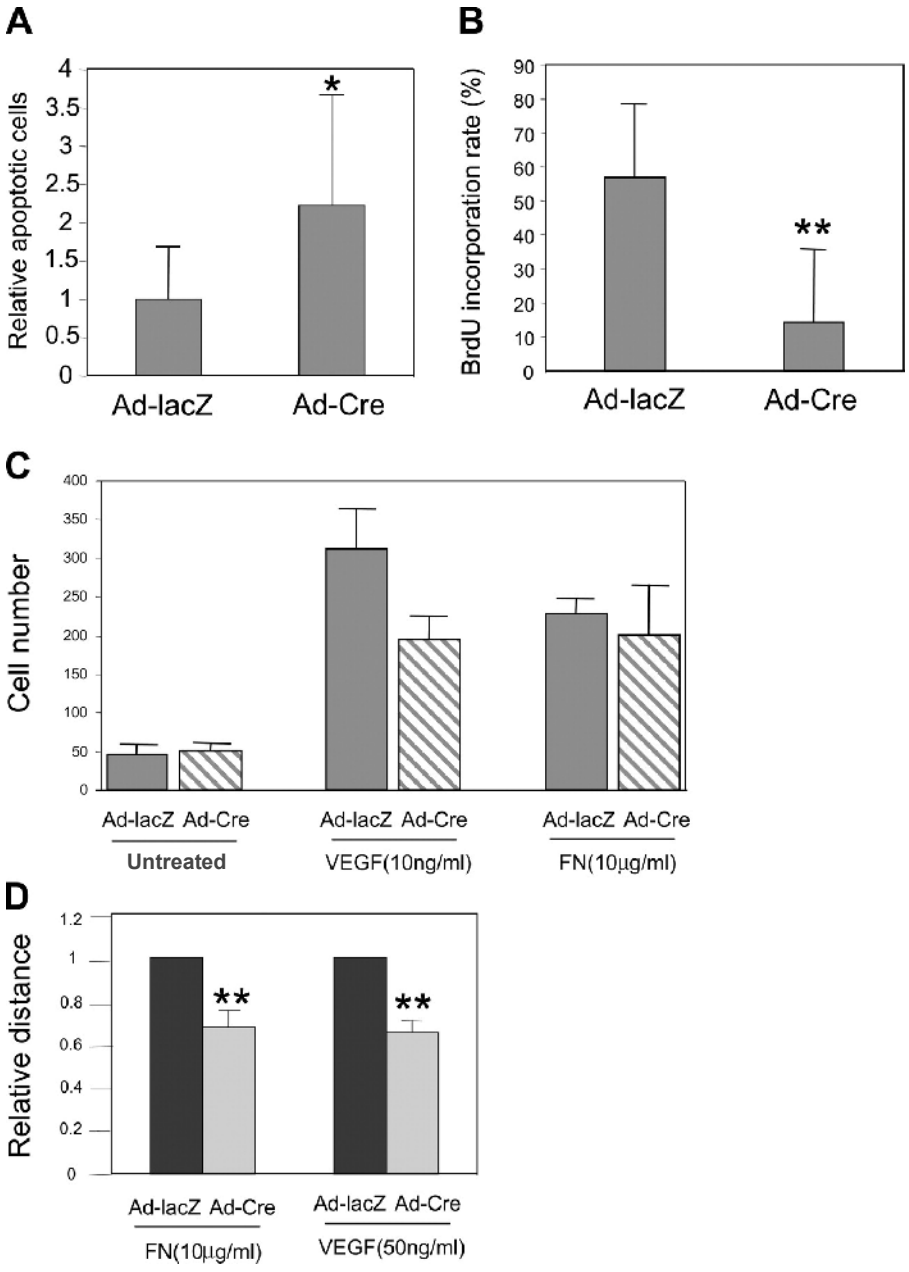

A
0
Ad-Cre
Ad-lacZ
(pfu)
FAK
vinculin
flox/flox
Δ/Δ
Cre
B
Ad-lacZ
Ad-Cre
C
Tube length
Relative ratio
1.2
1
0.8
0.6
0.4
0.2
0
*
Ad-lacZ
Ad-Cre
D
Branch points
Relative ratio
1.2
1
0.8
0.6
0.4
0.2
0
**
Ad-lacZ
Ad-Cre

Figure 2.2. Increased apoptosis, and reduced proliferation and migration of the isolated FAK$^{-/-}$ primary ECs

Primary ECs from floxed FAK mice and infected with Ad-lacZ or Ad-Cre were measured for apoptosis using TUNEL assay (A), proliferation by BrdU incorporation assay (B), and migration in response to VEGF or FN by Boyden chamber assay (C) and wound closure assay (D), as described in Materials and methods. The mean ± standard error from at least three experiments is shown. *, $P = 0.014$ and **, $P < 0.001$ in comparison to value from Ad-lacZ–infected cells.

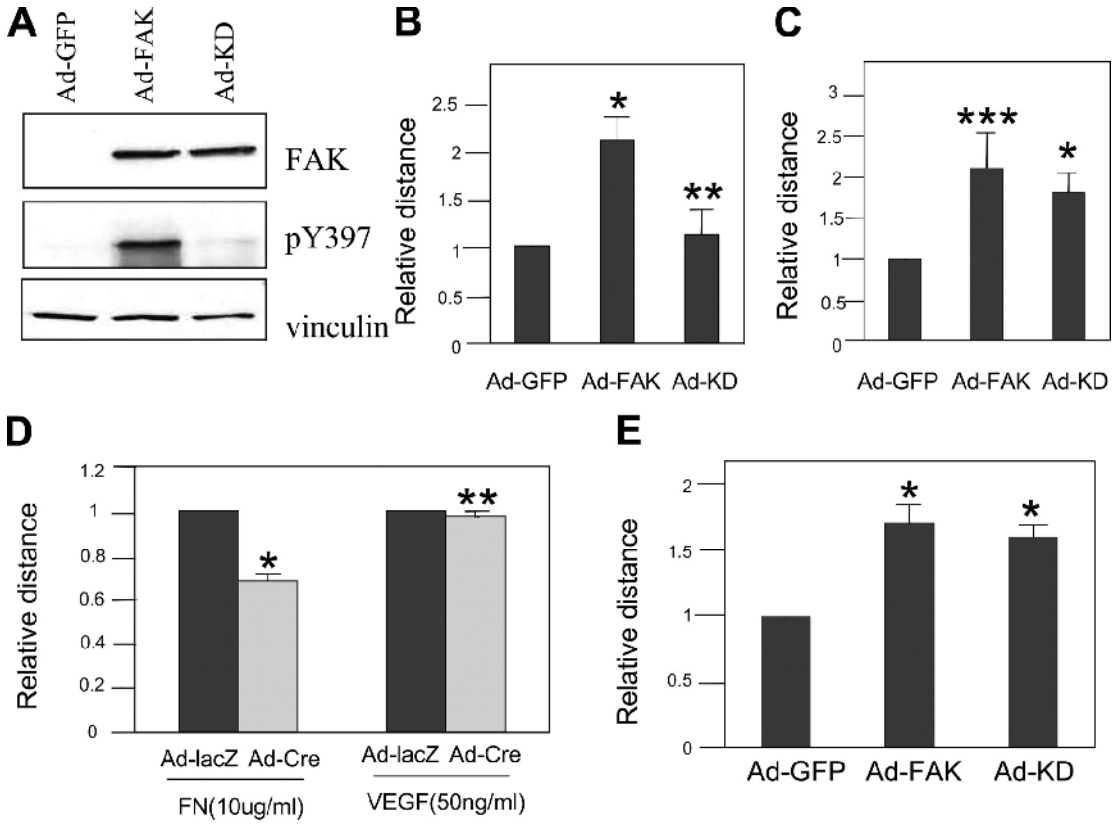

A
Relative apoptotic cells
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Ad-lacZ
Ad-Cre
B
BrdU incorporation rate (%)
90
80
70
60
50
40
30
20
10
0
**
Ad-lacZ
Ad-Cre
C
Cell number
400
350
300
250
200
150
100
50
0
Ad-lacZ Ad-Cre
Untreated
Ad-lacZ Ad-Cre
VEGF(10ng/ml)
Ad-lacZ Ad-Cre
FN(10μg/ml)
D
Relative distance
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Ad-lacZ Ad-Cre
FN(10μg/ml)
Ad-lacZ Ad-Cre
VEGF(50ng/ml)

2. Differential requirement of FAK kinase activity in VEGF-stimulated EC migration

To gain more insights into the role of FAK in EC migration in response to VEGF and FN as well as its potential differential function in various cell types, we prepared recombinant adenoviruses encoding FAK (Ad-FAK) and its kinase-defective mutant (Ad-KD) and examined their ability to rescue cell migration deficiency upon deletion of endogenous FAK. The isolated floxed FAK ECs were infected sequentially by Ad-Cre and Ad-FAK, Ad-KD, or a control recombinant adenovirus Ad-GFP, as described in Materials and methods. As shown in Fig. 2.3A, infection with Ad-FAK or Ad-KD, but not Ad-GFP, led to expression of the exogenous FAK in the CFKO ECs. Analysis of the exogenous FAK with anti-PY397 antibody (specific for the major FAK autophosphorylation site Y397) showed that FAK is phosphorylated at this site, whereas the KD mutant is not. As expected, reexpression of FAK rescued their deficiency in VEGF- and FN-stimulated migration (Fig. 2.3B and C). Interestingly, however, reexpression of FAK KD mutant rescued CFKO EC migration in response to FN (Fig. 2.3C), but not VEGF (Fig. 2.3B). We also isolated mouse embryonic fibroblasts (MEFs) from the floxed FAK mice and examined their migration upon deletion of FAK via Ad-Cre infection (CFKO MEF). In contrast to results from ECs, deletion of FAK only affected MEF migration in response to FN, but not to VEGF (Fig. 2.3D). As in the case of EC migration on FN, both wild-type and KD mutant FAK rescued CFKO MEF migration on FN (Fig. 2.3E). Together, these results suggest that FAK may play differential roles in migration of ECs and MEFs and that FAK activity is required for VEGF-stimulated EC migration, whereas it is dispensable for FN-stimulated migration of either ECs or MEFs.

Figure 2.3. Differential requirement of FAK kinase activity for VEGF-stimulated EC migration

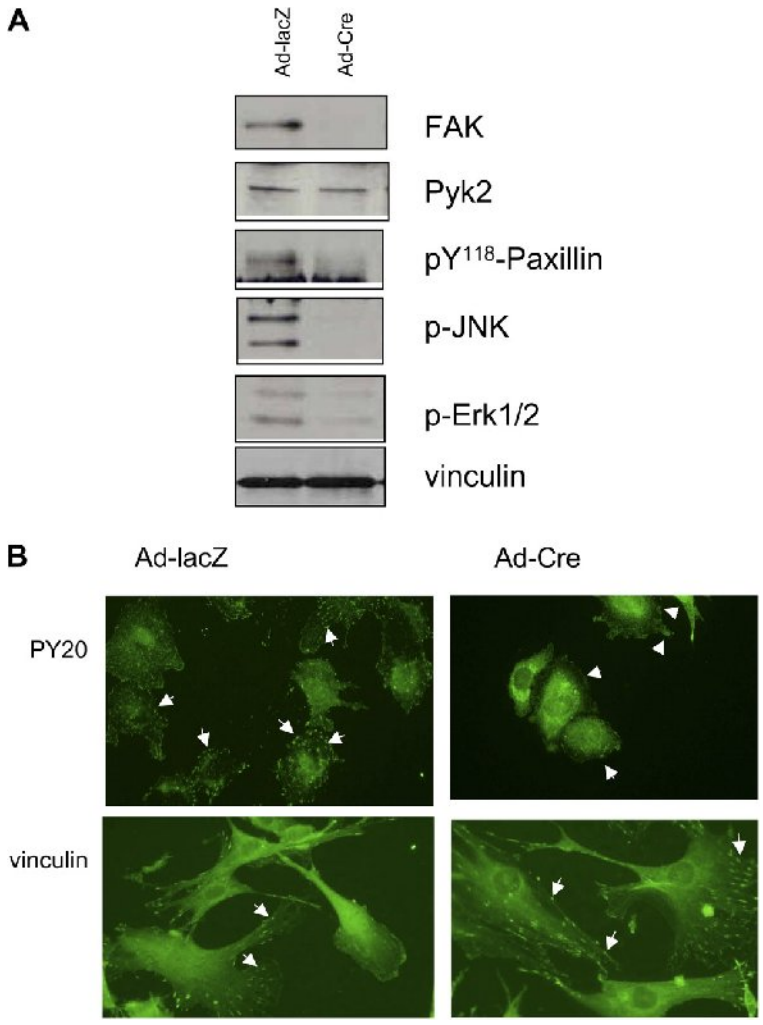
(A–C) Primary ECs from floxed FAK mice were infected with Ad-Cre to delete endogenous FAK followed by infection of Ad-FAK, Ad-KD, or the control Ad-GFP as indicated. Aliquots of lysates were analyzed by Western blotting using anti-FAK, anti-pY397, or anti-vinculin, as indicated (A). The infected cells were subjected to wound closure assay in response to VEGF (B) or FN (C), as described in Materials and methods. The mean ± standard error from at least three experiments is shown. *, $P < 0.005$; **, $P = 0.448$; and ***, $P = 0.012$ in comparison to value from Ad-GFP–infected cells. (D and E) MEFs were isolated from floxed FAK mice, and then infected with Ad-lacZ or Ad-Cre (D) or Ad-Cre followed by Ad-FAK, Ad-KD, or the control Ad-GFP (E), as indicated. The infected cells were then subjected to wound closure assays in response to VEGF (D as indicated) or FN (D as indicated and E). The mean ± standard error from at least three experiments is shown. *, $P < 0.01$ and **, $P = 0.17$ in comparison to value from (D) Ad-lacZ– or (E) Ad-GFP–infected cells.


A
Ad-GFP
Ad-FAK
Ad-KD
FAK
pY397
vinculin
B
Relative distance
2.5
2
1.5
1
0.5
0
*
**
Ad-GFP Ad-FAK Ad-KD
C
Relative distance
3
2.5
2
1.5
1
0.5
0
***
*
Ad-GFP Ad-FAK Ad-KD
D
Relative distance
1.2
1
0.8
0.6
0.4
0.2
0
*
**
Ad-lacZ Ad-Cre
Ad-lacZ Ad-Cre
FN(10ug/ml)
VEGF(50ng/ml)
E
Relative distance
2
1.5
1
0.5
0
*
*
Ad-GFP Ad-FAK Ad-KD

3. Reduced paxillin phosphorylation and decreased MAP kinase signaling in FAK-deficient ECs

Multiple targets and signaling pathways have been implicated in mediating regulation of cellular functions by FAK (Parsons 2003; Schlaepfer and Mitra 2004). The activation states of several FAK targets were examined. As shown in Fig. 2.4A, FAK expression is abolished in the CFKO ECs compared with the control ECs. In contrast to FAK-null cells from the total FAK KO embryos (Sieg et al. 1998), the FAK-related tyrosine kinase Pyk2 was not up-regulated in the absence of FAK in ECs. Furthermore, the major FAK target paxillin showed a significant decrease in phosphorylation at Tyr 118 in CFKO ECs that is critical for paxillin regulation of cell spreading and migration (Petit et al. 2000). Phosphorylations of JNK (C-Jun NH_2-terminal kinase) and Erk1/2 were also decreased in the CFKO ECs. Consistent with the absence of FAK and reduced paxillin phosphorylation, we found a marked reduction of tyrosine phosphorylation staining in focal contacts with a phosphotyrosine-specific antibody (PY20) (Fig. 2.4B). Staining of focal contacts with anti-vinculin showed an increased number of focal contacts in CFKO ECs than control ECs, as observed previously in the fibroblasts from total KO embryos (Ilic et al. 1995), suggesting that reduced phosphotyrosine staining is not due to a reduction in focal contacts in the CFKO ECs, per se. Together, these data suggest that deficiencies in paxillin phosphorylation and JNK and Erk signaling may be responsible for the cellular defects in the isolated primary CFKO ECs in vitro and defective angiogenesis and vascular development of CFKO embryos in vivo.

Figure 2.4. Effects of FAK deletion on downstream targets in the isolated primary ECs (A) Cell lysates of primary ECs from floxed FAK mice and infected with Ad-lacZ or Ad-Cre were analyzed by Western blotting with various antibodies as indicated. (B) Immunofluorescent staining of the above ECs (see A) with anti-phosphotyrosine antibody PY20 (top panels) or anti-vinculin (bottom panels). Focal adhesions are marked by arrows.


A
Ad-lacZ
Ad-Cre
FAK
Pyk2
pY118-Paxillin
p-JNK
p-Erk1/2
vinculin
B
Ad-lacZ
Ad-Cre
PY20
vinculin

4. EC-specific FAK transgene expression cannot rescue total FAK KO embryonic lethality

Together with the previous studies showing early embryonic lethality of FAK total KO embryos (Ilic et al. 1995), our results from the analysis of EC-specific FAK KO embryos suggested that FAK is required for both early and late embryogenesis perhaps in different cells/tissues (Shen et al. 2005). To further test this possibility, we examined whether restoration of FAK expression in ECs would rescue the early embryonic lethal phenotype of the total KO embryos. We have previously generated FAK transgenic mice with EC-specific expression of the FAK transgene under the control of Tie2 promoter/enhancer (Peng et al. 2004). These mice were crossed with $FAK^{\Delta/+}$ heterozygous mice to introduce Tie2-FAK transgene into the FAK-null background, which were then intercrossed for the generation of possibly rescued progeny with $FAK^{\Delta/\Delta}$; Tie2-FAK genotype. Of the 84 pups analyzed, we did not obtain any offspring with the rescued genotype, although the Mendelian ratio of this genotype for the crossing is 12.5%. These results suggest that introduction of EC-specific FAK gene could not rescue the early embryonic lethal phenotype of the total FAK KO embryos. They provide further support for the role of FAK in both early embryogenesis (perhaps for mesoderm cells) and vascular development in late embryogenesis.

E. Discussion

As a critical mediator of signaling by integrins and growth factor receptors, FAK has been implicated in playing an important role in the regulation and function of ECs by a number of studies in vitro (Romer et al. 1994; Kim et al. 2000; Qi and Claesson-Welsh 2001). Furthermore, vascular defects were observed in FAK total KO embryos and ECs from these embryos or cultured embryoid bodies (Ilic et al. 1995; Ilic et al. 2003). However, the early embryonic lethality of the total KO mice precluded in vivo analysis of potential roles of FAK in angiogenesis, which is an integral part of vascular development in late embryogenesis and adult organisms. Using a conditional KO approach that specifically inactivates FAK gene in ECs, we found that FAK expression is required for the vascular development in late embryogenesis (Shen et al. 2005). Although they developed normally, including formation of the vascular structures in early embryogenesis, the CFKO embryos showed multiple defects in late embryogenesis including defective angiogenesis in the embryos, yolk sac, and placenta, impaired vasculature and associated hemorrhage, edema, and developmental delay, and late embryonic lethal phenotype. Furthermore, here we showed that EC-specific expression of a FAK transgene could not rescue the early embryonic lethality of the total KO embryos. These studies demonstrate that FAK is required for angiogenesis and vascular development and integrity in late embryogenesis, and together with the previous total KO data (Ilic et al. 1995), they suggest that FAK plays a role in at least two different stages of embryonic development in multiple cell/tissue types.

A role for FAK expression in ECs for angiogenesis and vascular development and integrity in late embryogenesis is suggested by the observation of multiple vascular defects in the CFKO embryos (Shen et al. 2005). Consistent with other

studies of FAK using HUVEC cells (Romer et al. 1994; Gilmore and Romer 1996; Kim et al. 2000; Qi and Claesson-Welsh 2001; Ilic et al. 2003), we found that deletion of FAK in primary ECs led to increased apoptosis, reduced proliferation and migration, and reduced ability to form capillaries on Matrigel. As embryonic angiogenesis involves both EC proliferation and migration, the above cellular defects could contribute to the reduced angiogenesis in CFKO embryos in vivo. Interestingly, consistent with inactivation of FAK, tyrosine phosphorylation of paxillin at Tyr118 is significantly reduced in the CFKO ECs. Paxillin is a focal adhesion protein and major substrate for the FAK/Src complex, and has been shown to play important roles in the regulation of cell adhesion and migration (Turner 1998; Petit et al. 2000; Schaller 2004). We also observed decreases of JNK and Erk1/2 activities in CFKO ECs, which are consistent with previous studies showing regulation of cell cycle progression and migration by FAK via both of these pathways (Parsons 2003; Schlaepfer and Mitra 2004). Thus, reduced paxillin phosphorylation, JNK and/or Erk signaling could contribute to the reduced cell migration and proliferation in the primary CFKO ECs and defective angiogenesis in the CFKO embryos.

Our analysis of FAK and its kinase-defective mutant in their ability to rescue migration deficiency of primary ECs and MEFs suggested a potential kinase-independent function for FAK. We found that although FAK kinase activity is required for VEGF-stimulated EC migration, it is dispensable for FN-stimulated migration of either ECs or MEFs. The ability of KD mutant to rescue migration of EC and MEF on FN is consistent with our previous observation that it promoted migration of CHO cells as effectively as the wild-type FAK (Cary et al. 1996). This activity was attributed to trans-phosphorylation of the KD mutant by endogenous FAK in CHO cells, allowing it to function in a similar manner as wild-type and

phosphorylated FAK. In the case of ECs and MEFs here, however, endogenous FAK was deleted from these cells and the KD mutant in ECs was not phosphorylated on Y397. Therefore, these results suggest that promotion of migration of both EC and MEF on FN by FAK is independent of its kinase activity. They reveal potentially differential roles of FAK in mediating cell migration on ECM such as FN and growth factors like VEGF. Future studies will be needed to understand the potential kinase-independent function of FAK as well as the mechanisms underlying a differential role of FAK in EC migration on FN and VEGF.

In conclusion, these in vitro studies together with the analysis of EC-specific FAK KO mice demonstrate that FAK is required for angiogenesis and vascular development and integrity in late embryogenesis due to its important role in the regulation of EC functions.

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CHAPTER 3:

ROLE OF FOCAL ADHESION KINASE S732 PHOSPHORYLATION IN CENTROSOME FUNCTION DURING MITOSIS*

*Ann Y.J. Park, Tang-Long Shen, Shu Chien and Jun-Lin Guan. Role of Focal Adhesion Kinase Ser-732 Phosphorylation in Centrosome Function during Mitosis. Reproduced and Modified from Journal of Biological Chemistry, 2009, vol. 284(14), pp.9418-25

A. Abstract

Focal adhesion kinase (FAK) is the major cytoplasmic tyrosine kinase in focal adhesions and a critical mediator of integrin signaling in a variety of cells, including endothelial cells (ECs). Here we describe a new function for FAK in the regulation of centrosome functions in a S732 phosphorylation-dependent manner during mitosis. Deletion of FAK in primary ECs causes increases in centrosome numbers, multipolar and disorganized spindles, and unaligned chromosomes during mitosis. Re-expression of wild-type FAK, but not the S732A mutant, rescued these mitotic defects, suggesting a role for S732 phosphorylation in the regulation of centrosomal functions. Consistent with this possibility, S732-phosphorylated FAK was found to co-localize in centrosomes in mitotic cells. FAK also associated with cytoplasmic dynein in a S732-phosphorylation dependent manner. Further analysis in FAK-null primary ECs showed that S732A mutant could rescue EC migration, but not proliferation or tubulogenesis in vitro. Lastly, we showed that deletion of FAK in ECs reduced tumor angiogenesis in vivo, which could be restored by re-expression of wild-type FAK, but not S732A mutant. Together, these studies demonstrated a novel role for S732 phosphorylation of FAK in the regulation of centrosome function during mitosis, which may contribute to EC proliferation and angiogenesis.

B. Introduction

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that is a major mediator of signal transduction by integrins and also participates in signaling by other cell surface receptors in a variety of cells, including endothelial cells (ECs) (Schaller 2001; Parsons 2003; Schlaepfer and Mitra 2004; Siesser and Hanks 2006). In most adherent cells, FAK is activated upon integrin-mediated cell adhesion to extracellular

matrix proteins through disruption of an intramolecular inhibitory interaction between its amino-terminal FERM domain and the kinase domain (Cooper et al. 2003; Lietha et al. 2007). Once it is activated, FAK undergoes autophosphorylation at Y397, which creates a binding site for several SH2 domain-containing proteins including Src family kinases. The cascade of phosphorylation events and protein-protein interactions which are mediated by the FAK-Src complex has been shown to trigger several signaling pathways in the regulation of a variety of cellular functions in different cells (Parsons 2003; Schlaepfer and Mitra 2004).

Besides these well-characterized tyrosine phosphorylations, recent studies have identified FAK phosphorylation on several serine residues (Ma et al. 2001; Grigera et al. 2005). In the post-mitotic neurons, S732 has been shown to be phosphorylated by Cdk5, which plays an important role in microtubule organization and proper nuclear movement during neuronal migration (Xie et al. 2003; Xie and Tsai 2004). Indeed, S732-phosphorylated FAK is enriched in centrosome-associated microtubule fork that abuts the nucleus and a perinuclear region around the centrosome, consistent with its regulation of these functions in neurons. S732 of FAK has also been shown to be phosphorylated by Rho-dependent Kinase (ROCK) in ECs, which has been suggested to play a role in VEGF-stimulated EC migration (Le Boeuf et al. 2006). In addition to S732, the S722, S843 and S910 in the carboxy-terminal domain of FAK have also been found to be phosphorylated and regulate cell spreading and migration in recent studies (Hunger-Glaser et al. 2004; Bianchi et al. 2005; Grigera et al. 2005; Jacamo et al. 2007; Jiang et al. 2007; Villa-Moruzzi 2007). Despite these findings, our understanding of serine phosphorylation of FAK is very limited in contrast to the wealth of information on the regulation and function of tyrosine phosphorylation of FAK. In particular, it is not clear whether and how serine

phosphorylation is involved in the regulation of cell cycle progression and proliferation by FAK.

Focal adhesion localization of FAK in adherent cells is essential for its functions in the regulation of cell migration as well as proliferation (Parsons 2003; Schlaepfer and Mitra 2004). During mitosis, however, focal adhesion complexes dissociate as cells round up and detach from ECM. Interestingly, serine phosphorylation of FAK is increased during mitosis and this has been suggested to cause FAK dissociation from p130Cas and Src to inactivate signaling at focal adhesions (Yamakita et al. 1999), although the relevant sites of phosphorylation were not mapped in this study. It is not known whether FAK is localized to any specific sub-cellular structures and/or plays a role in mitosis and whether these are regulated by serine phosphorylation of FAK in mitotic cells.

Consistent with its critical importance in the regulation of various cellular functions, deletion of FAK gene leads to early embryonic lethality at embryonic day 8.5 (E8.5) due to defects in the axial mesodermal tissues including the cardiovascular system with incomplete development of both the blood vessels and the heart (Ilic et al. 1995). Using a conditional mouse KO approach, we and others have recently shown a role of FAK in vascular angiogenesis through its regulation of multiple functions of ECs including their survival, proliferation, migration, and tubulogenesis (Shen et al. 2005; Braren et al. 2006; Weis et al. 2008). The availability of the floxed FAK mice and ECs isolated from these mice also allowed us to investigate FAK signaling pathways involved in the regulation of EC functions and angiogenesis in vivo by a reconstitution strategy, where endogenous FAK is deleted via recombinant adenoviruses encoding Cre followed by re-expression of FAK or its various mutants in ECs both in vitro and in vivo. In this study, we present data showing a novel

function of FAK in the regulation of centrosomal functions in a S732 phosphorylation-dependent manner in ECs during mitosis, which plays a role in the regulation of EC proliferation and tubulogenesis in vitro and tumor angiogenesis in vivo.

C. Materials and methods

1. Recombinant adenoviruses

Recombinant adenoviruses encoding Cre recombinase or lacZ control were purchased from Gene Transfer Vector Core (University of Iowa, Iowa City, IA). The recombinant adenoviruses encoding FAK (Ad-FAK), its kinase-defective (Ad-KD), Y397F (Ad-Y397F), P712/715A (Ad-P712/715A) and S732A (Ad-S732A) mutants, or GPF control (Ad-GFP) were generated using the Adeasy-1 system (Stratagene) according to manufacturer's instruction.

2. Isolation and infection of ECs

ECs were isolated from 4- to 6-week-old homozygous FAK floxed mice using the magnetic bead (Dyanbead M-450; Dynal Corp.) purification protocol with rat anti-mouse PECAM-1 (BD Biosciences), as described previously (Cattelino et al. 2003; Peng et al. 2004; Shen et al. 2005). EC population was approximately 90% pure as determined by anti-PECAM-1 staining. Isolated ECs were infected at an m.o.i of 100 with Ad-lacZ or Ad-Cre. To increase efficiency, a second infection was performed after 9-12 h and incubated for 48 h. For the rescue experiments, cells infected with Ad-Cre were re-infected with recombinant adenoviruses encoding FAK, its mutants, or Ad-GFP 2d after infection of Ad-Cre at an m.o.i of 100. No detectable cell toxicity was observed.

3. Cell culture and transfections

Isolated ECs were cultured on a 0.1% gelatine (Sigma-Aldrich)- coated dish in high glucose DMEM supplemented with 20% FCS (Hyclone), Endothelial mitogen (Biomedical Technologies), and heparin (100 μg/ml; Sigma-Aldrich) (Peng et al. 2004; Shen et al. 2005). 293T, HeLa, MEF and Cos-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum. 293T cells were transfected with Cdk5, Rock1, or control shRNA (University of Michigan Comprehensive Cancer Center shRNA Core Facility) for 3 days by use of Lipofectamine following the manufacturer's instructions.

4. Flow cytometry Analysis

ECs were fixed with 70% ice cold ethanol at 4^{o}C for more than 2h. After fixation, cells were stained with 50 μg/ml propidium iodide (Sigma-Aldrich) with 100 μg/ml RNase A in PBS containing 0.1% Triton X-100. Flow cytometry analysis was performed by a Becton Dickinson BD-LSR II Flow Cytometer.

5. Immunofluorescence staining

Cells were processed for immunofluorescence staining as described previously (Cary, 1996). The primary antibodies used were anti-pS732 FAK (BioSource; 1:100), anti-α-tubulin (Zymed laboratories, Inc.; 1:50), anti-γ-tubulin (Sigma-Aldrich; 1:100), anti-dynein intermediate chain (Sigma-Aldrich; 1:100) and anti-BrdU (Sigma-Aldrich; 1:50). FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory; 1:200) and Texas red-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratory; 1:200) were used as the secondary antibodies. Cells were examined by a microscope (model BX41; Olympus) with UplanF1 x40/0.75

objective lens at RT. The images were captured using a camera (model DP70; Olympus) with DP Controller ver. 1.2.1.108**.**

6. Immunoprecipitation and Western blotting

Immunoprecipiataion and Western blotting analysis was performed as described previously (Cary, 1996). Antibodies used are anti-FAK (C20; Santa Cruz Biotechnology, Inc.), anti-vinculin (Sigma-Aldrich), anti-actin (Santa Cruz Biotechnology, Inc.), anti-myc (9E10; Santa Cruz Biotechnology, Inc.), anti-dynein intermediate chain (clone 70.1; Sigma-Aldrich), anti-cdk5 (C8; Santa Cruz Biotechnology, Inc.), and anti-Rock1 (H-85; Santa Cruz Biotechnology, Inc.).

7. BrdU incorporation assay

ECs were serum-starved for 18 h to arrest the cells in G0. BrdU incorporation assay was performed as described previously (Shen et al. 2005). Three independent experiments were performed and the percentage of cells positive for BrdU was quantified using a microscope (model BX41; Olympus) with UplanF1 x10/0.3 objective lens at RT. Approximately 100 cells were examined for each condition in each independent experiment.

8. Wound closure cell migration assay

ECs were plated on gelatin-coated dishes (60 mm) and stimulated with 50 ng/ml VEGF, and then subjected to assays. Wound closure assays were performed as described previously (Shen et al. 2005; Liang et al. 2007).

9. Tube formation assay

ECs were plated on a layer of Matrigel (Growh Factor Reduced, BD Biosciences) and allowed to form a tubular structure as described previously (Shen, 2005). Tubulogenesis in each condition was examined on a microscope (model IX70; Olympus) with UplanF1 x10/0.3 objective lens and photographed with a progressive 3CCD camera (Sony) at RT. The length and branch points were determined using Image-Pro Plus ver. 3.0.00.00 as described previously (Haskell et al. 2003).

10. Matrigel plug assays

Matrigel (Growth Factor Reduced, BD Biosciences) was supplemented with $5x10^8$ pfu/mL recombinant adenoviruses and $5x10^5$ B16F10 melanoma cells in a final volume of 0.5 mL. Matrigel mixture was then injected s.c. into the flank region of eight-week-old floxed FAK mice. Mice were sacrificed 10 d after injection and Matrigel weights were determined. Vascularization in Matrigel plugs was visualized by immunohistological examination using anti-PECAM-1 antibody (M-20, 1:200 dilution; Santa Cruz Biotechnology, Inc.) as described previously (Shen, 2005). They were then examined under a microscope (model BX41; Olympus) with UplanF1 x10/0.3 objective lens at RT, and the images were captured using a camera (model DP70; Olympus) with DP Controller ver. 1.2.1.108. Five representative images were obtained from each Matrigel plug, and vessel density was quantified using Image-Pro Plus ver. 3.0.00.00.

D. Results

1. Deletion of FAK causes spindle and centrosomal abnormalities in mitotic ECs

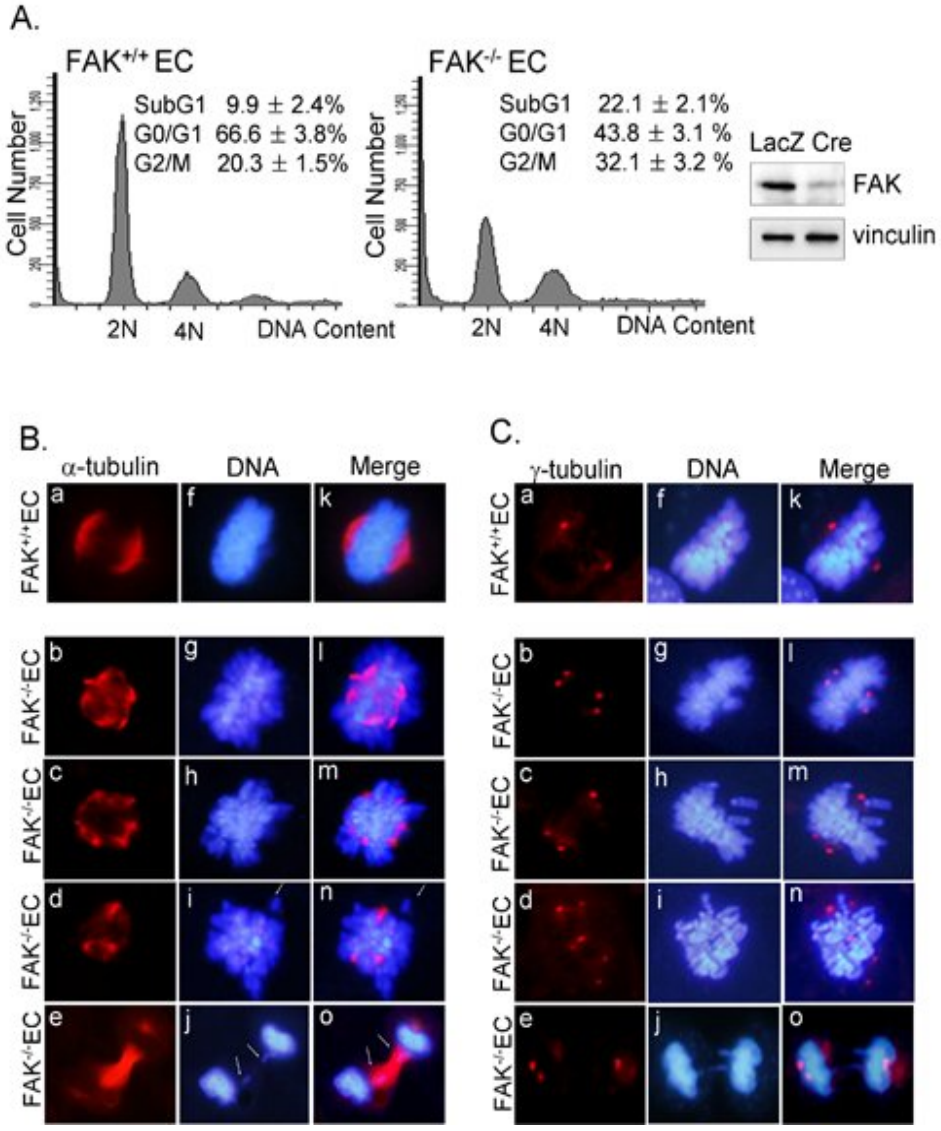
Previous studies of EC-specific FAK knockout mice and isolated ECs showed a role of FAK in angiogenesis and vascular development due to its essential role in the regulation of EC functions, including proliferation, migration, cell survival, and tubulogenesis (Shen et al. 2005). To further investigate mechanisms of FAK in the regulation of EC proliferation, we performed flow cytometric analyses in order to get more detailed information on the cell cycle profile in $FAK^{-/-}$ ECs. Primary ECs were isolated from floxed FAK mice and were infected by a recombinant adenovirus encoding Cre recombinase (Ad-Cre) to delete endogenous FAK or by a control recombinant adenovirus encoding lacZ to produce $FAK^{-/-}$ ECs or control $FAK^{+/+}$ ECs, respectively, as described previously (Shen et al. 2005). Analysis of these cells by flow cytometry showed an altered cell cycle profile for $FAK^{-/-}$ ECs compared to the control $FAK^{+/+}$ ECs (Fig. 3.1A). Consistent with our previous results showing increased apoptosis in $FAK^{-/-}$ ECs, a significant increase in SubG1 population was found for these cells compared to $FAK^{+/+}$ ECs (from about 10% to 22%). We also found a significantly increased G2/M population in $FAK^{-/-}$ ECs compared to $FAK^{+/+}$ ECs (from about 20% to 32%), suggesting a possibly increased mitotic arrest upon FAK deletion in ECs.

To investigate the mechanisms of mitotic abnormalities in $FAK^{-/-}$ ECs, we examined the effect of FAK deletion on mitotic spindle organization and chromosome alignment and segregation in these cells. As shown in Fig. 3.1B, normal mitotic spindles and chromosome alignment were detected in $FAK^{+/+}$ECs during mitosis by staining for α-tubulin and DNA, respectively (a, f, k). In contrast, the analysis of $FAK^{-/-}$ ECs revealed various defects including multiple and randomly

positioned spindles (b-d), loosely congregated chromosomes (g-i), and unaligned chromosomes (i, n, arrow heads). In some cells, anaphase proceeded with an unattached chromosome (j, o, arrows). Because centrosomes are the primary microtubule organization center and play an essential role in mitotic spindle organization and chromosome segregation, we next evaluated possible defects in centrosome organizations in $FAK^{-/-}$ ECs by immunostaining for γ-tubulin, a centrosome marker. Fig. 3.1C shows that whereas $FAK^{+/+}$ ECs have a typical staining of two centrosomes on the opposite sides of the condensed chromosomes during mitosis (a, f, k), deletion of FAK in ECs ($FAK^{-/-}$ ECs) caused various centrosomal defects in the number, size, and position of centrosomes (b-e), which are associated with abnormal chromosome condensation in metaphase (g-i) and segregation in anaphase (j). Quantitation of approximately 200 mitotic cells for each group showed centrosomal defects in about 60% of $FAK^{-/-}$ ECs compared to less than 10% of the control $FAK^{+/+}$ ECs (Fig. 3.2B). Together, these results suggest that FAK plays a role in the regulation of mitotic spindle assembly, chromosome alignment and centrosome integrity during mitosis and that the deregulation of these functions caused by the deletion of FAK may be responsible for mitotic arrest in $FAK^{-/-}$ ECs.

Figure 3.1. Mitotic defects of primary $FAK^{-/-}$ ECs

Isolated ECs from homozygous floxed FAK mice were infected with Ad-Cre ($FAK^{-/-}$ ECs) or the control Ad-lacZ ($FAK^{+/+}$ECs) and were analyzed after 3 days. (A) Cells were stained with propidium iodide for DNA content of cells by flow cytometry. Cell cycle profiles of $FAK^{+/+}$ECs and $FAK^{-/-}$ECs are shown. The experiments were performed three times in duplicate and the mean + standard error is shown as indicated (n=6 for each condition). Aliquots of lysates were analyzed by western blotting using anti-FAK and anti-vinculin (right panel). (B, C) $FAK^{+/+}$ECs (a, f, k) and $FAK^{-/-}$ECs (other panels) were immunostained with anti-α-tubulin antibody (B, a-e, red) for mitotic spindles, with anti-γ-tubulin antibody (C, a-e, red) for centrosomes, or stained with Hoechst (B and C, f-j, blue) for chromosomes, as indicated. Approximately 200 mitotic cells in three independent experiments were analyzed each and representative metaphase (a-d, f-i, k-n) and anaphase cells (e, j, o) are shown. Unaligned chromosomes in metaphase and anaphase are indicated by arrow heads and arrows, respectively (i, n, j, o).


A.
FAK+/+ EC
SubG1 9.9 ± 2.4%
G0/G1 66.6 ± 3.8%
G2/M 20.3 ± 1.5%
Cell Number
2N
4N
DNA Content
FAK-/- EC
SubG1 22.1 ± 2.1%
G0/G1 43.8 ± 3.1 %
G2/M 32.1 ± 3.2 %
Cell Number
2N
4N
DNA Content
LacZ Cre
FAK
vinculin
B.
α-tubulin
DNA
Merge
FAK+/+EC
FAK-/-EC
FAK-/-EC
FAK-/-EC
FAK-/-EC
C.
γ-tubulin
DNA
Merge
FAK+/+EC
FAK-/-EC
FAK-/-EC
FAK-/-EC
FAK-/-EC

2. Ser732 phosphorylation of FAK is required for its regulation of centrosome function during mitosis in primary ECs

To investigate the mechanisms of FAK regulation of centrosome function during mitosis, we generated recombinant adenoviruses encoding several FAK mutants and analyzed their ability to rescue the mitotic defects in ECs upon deletion of endogenous FAK. Primary ECs isolated from floxed FAK mice were infected by Ad-Cre to delete endogenous FAK followed by infection with recombinant adenoviruses encoding FAK (Ad-FAK), kinase-defective (Ad-KD), Y397 to F (Ad-Y397F), P712 and P715 to A (Ad-P712/715A) or S732 to A (Ad-S732A) mutant. As expected, infection of Ad-Cre, but not Ad-LacZ, resulted in the deletion of FAK (see Fig. 3.1A right panel). Re-infection of $FAK^{-/-}$ ECs with recombinant adenoviruses encoding FAK or its mutants led to expression of exogenous FAK and mutants to comparable levels in these cells (Fig. 3.2A). As expected, restoration of FAK expression in $FAK^{-/-}$ ECs significantly rescued the centrosomal abnormalities caused by deletion of endogenous FAK (Fig. 3.2B).

Consistent with previous studies on the critical roles of Y397 in FAK downstream signaling pathways initiated by autophosphorylation of this site, re-expression of either FAK Y397F or KD mutants did not rescue the centrosomal defects (Fig. 3.2B). In contrast to well characterized tyrosine phosphorylation of FAK, the role of serine phosphorylation of FAK is relatively less investigated although several serine residues of FAK have also been shown to be phosphorylated (Ma et al. 2001; Grigera et al. 2005). In particular, phosphorylation of S732 in FAK by Cdk5 has been shown to play a role in nuclear translocation during neuronal migration through regulation of microtubule networks (Xie et al. 2003). As centrosome-associated microtubule structure, mitotic spindle, plays a crucial role during mitosis

in non-neuronal cells, we examined the potential role of S732 phosphorylation in FAK regulation of centrosome function by analysis of FAK S732A mutant in the rescue experiments. We found that re-expression of S732A mutant did not rescue centrosomal abnormalities in $FAK^{-/-}$ ECs (Fig. 3.2B). Analysis of another FAK mutant, P712/715A which is deficient in binding to p130Cas, rescued centrosomal defects in $FAK^{-/-}$ ECs to a comparable level as the wild-type FAK (Fig. 3.2B), suggesting that FAK signaling through p130Cas is not involved in the regulation of centrosome function. Together, these mutational analyses suggest a novel role of S732 phosphorylation in mediating FAK regulation of centrosome functions during mitosis.

3. Localization of S732-phosphorylated FAK in centrosomes during mitosis

FAK is localized in focal adhesions in adherent cells, which are disassembled during mitosis. Previous studies have shown an increase of serine phosphorylation of FAK concomitant with cell rounding up and disassembly of focal adhesions in mitotic cells (Yamakita et al. 1999). It is not clear, however, whether serine phosphorylated FAK is evenly distributed in the cytoplasm or is localized to particular sub-cellular structures in mitotic cells. In light of the above observation suggesting a potential role of S732 phosphorylation of FAK in the integrity of centrosomes, we examined the possibility of a centrosomal localization of S732-phosphorylated FAK in mitotic ECs. Primary $FAK^{+/+}$ ECs at various phases of mitosis were subjected to double label immunofluorescent staining with antibodies against phospho-S732 of FAK (PS732) and the centrosomal marker γ-tubulin. As shown in Fig. 3.3A, S732-phosphorylated FAK was detected in the centrosomes throughout mitosis. The lack of staining in $FAK^{-/-}$ ECs by anti-PS732 confirmed the specificity of the antibody against FAK

Figure 3.2. Analysis of various FAK mutants in the regulation of centrosome function during mitosis

Primary ECs isolated from floxed FAK mice were infected with Ad-Cre to delete endogenous FAK followed by infection of Ad-FAK, Ad-Y397F, Ad-KD, Ad-P712/715A, Ad-S732A FAK, or the control Ad-GFP. (A) Lysates were analyzed directly by western blotting with anti-FAK, or anti-actin as indicated. (B) Infected cells were stained with Hoechst to reveal chromosomes and immunostained with anti-γ-tubulin antibody for centrosomes. A total of 200 mitotic cells were counted for each group in three independent experiments. The mean $\pm$ standard error is shown for mitotic cells with abnormal centrosomes in each group. *P <0.01, **P = 0.107, ***P = 0.074 in comparison to value from Ad-LacZ and Ad-GFP infected control cells.

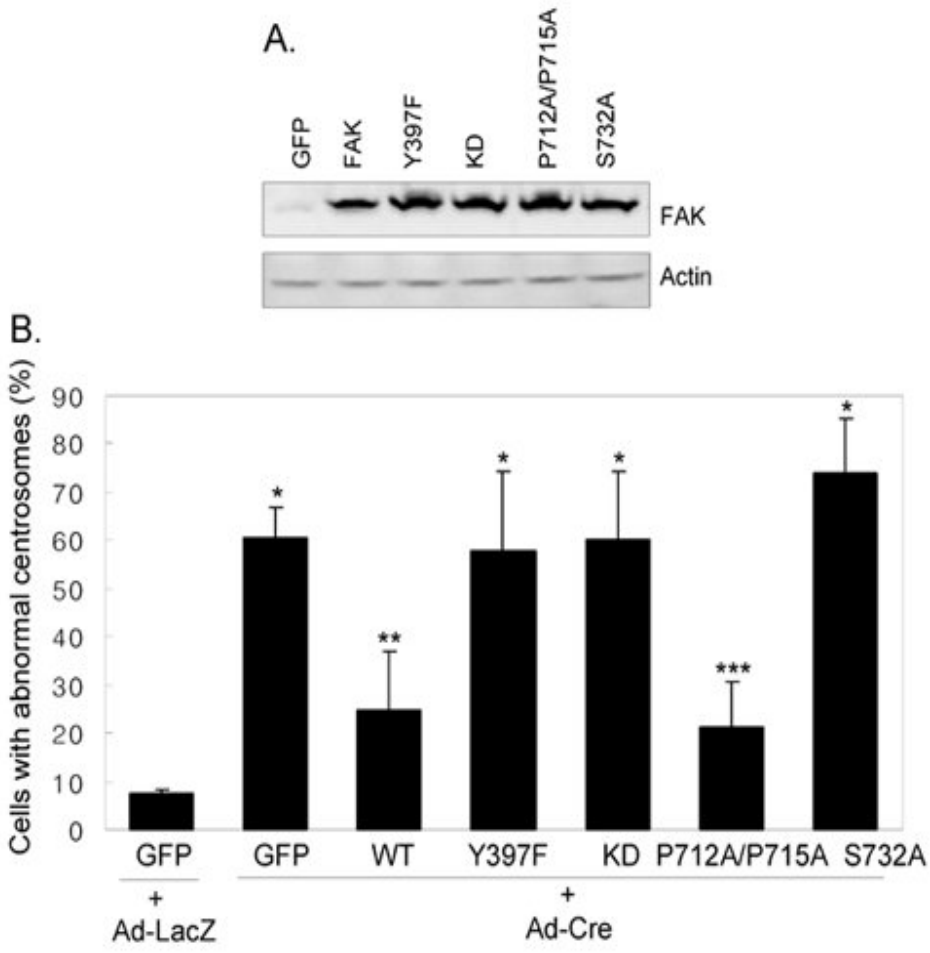
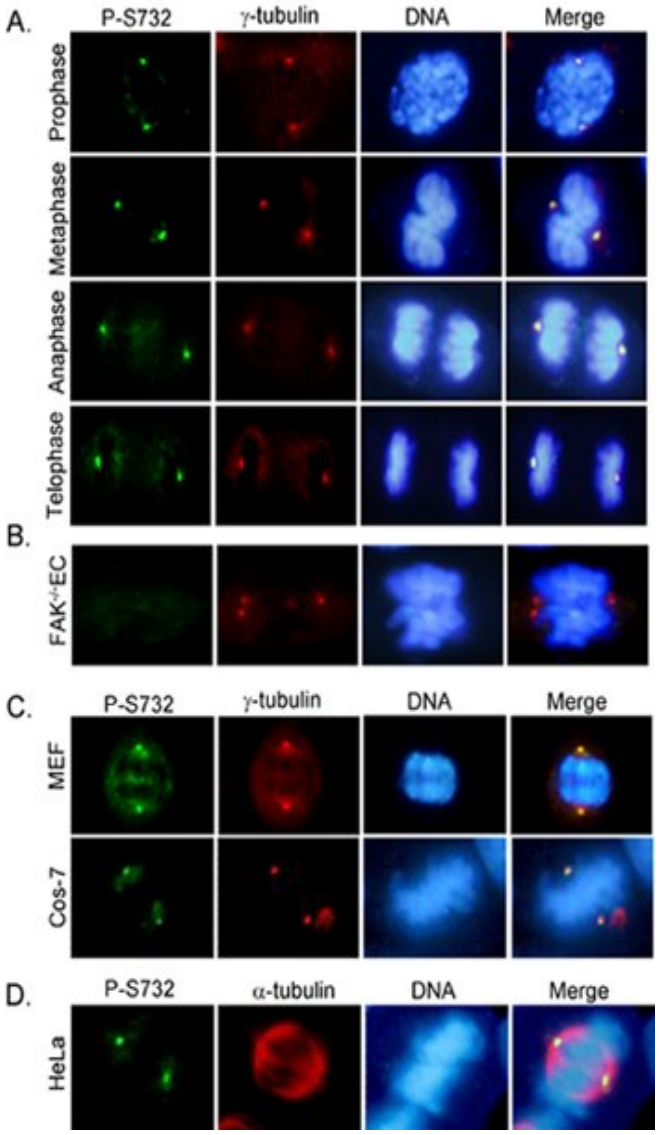

A.
GFP
FAK
Y397F
KD
P712A/P715A
S732A
FAK
Actin
B.
Cells with abnormal centrosomes (%)
90
80
70
60
50
40
30
20
10
0
*
*
**
*
*
***
*
GFP
GFP
WT
Y397F
KD
P712A/P715A
S732A
+
Ad-LacZ
+
Ad-Cre

Figure 3.3. S732-phosphorylated FAK is localized to the centrosomes

(A, B) Isolated ECs from floxed FAK mice were infected with Ad-lacZ ($FAK^{+/+}$ECs, panel A) or Ad-Cre ($FAK^{-/-}$ECs, panel B) and then co-stained with S732-phospho specific FAK antibody (green) and γ-tubulin antibody (red), as indicated. Representative images show the co-localization of S732-phosphorylated FAK with γ-tubulin at centrosome in the different phases during mitosis in $FAK^{+/+}$ECs (A), but not in $FAK^{-/-}$ECs (B and data not shown). Chromosomes were revealed by Hoechst staining (blue). (C) MEF (top panels) and Cos-7 (bottom panels) cells were processed for immunofluorescence staining with S732-phospho specific FAK antibody (green) and γ-tubulin antibody (red) as described in A. (D) HeLa cells were co-stained with S732-phospho specific FAK antibody (green) and α-tubulin antibody (red), as indicated. Representative metaphase cells were shown.


A.
P-S732
γ-tubulin
DNA
Merge
Prophase
Metaphase
Anaphase
Telophase
B.
FAK-/-EC
C.
P-S732
γ-tubulin
DNA
Merge
MEF
Cos-7
D.
P-S732
α-tubulin
DNA
Merge
HeLa

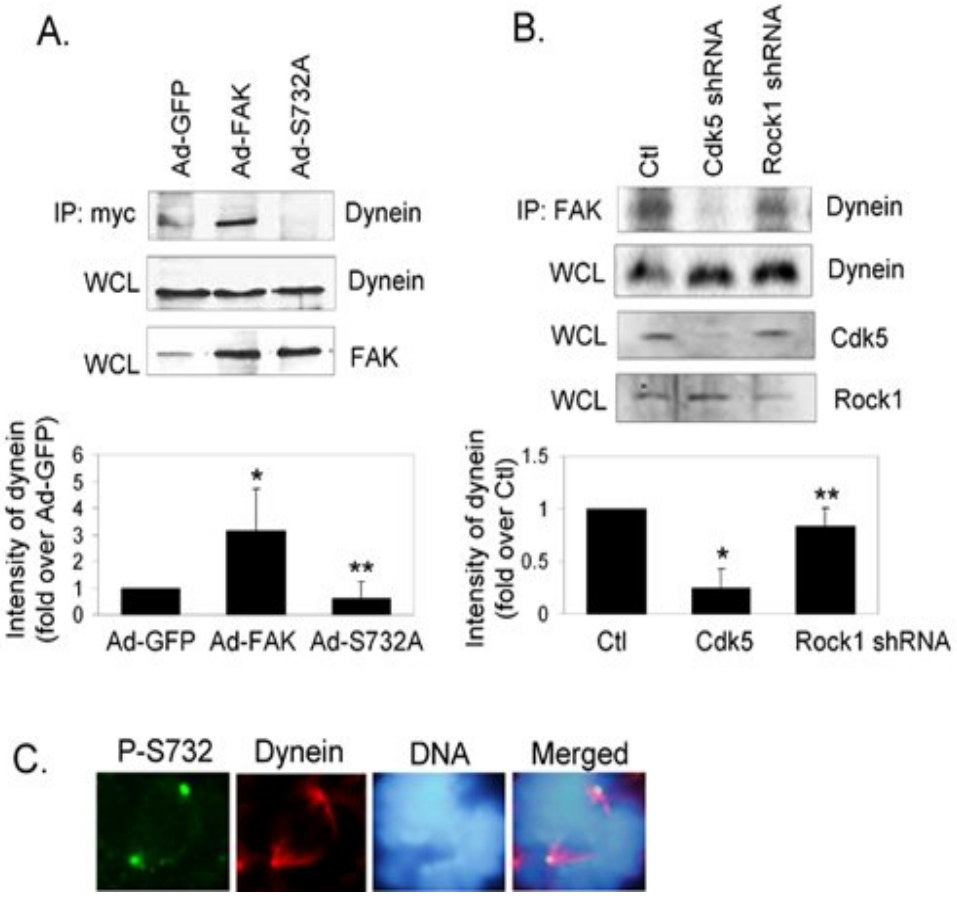
PS732 (Fig. 3.3B). Furthermore, localization of S732- phosphorylated FAK in the centrosomes was also detected in several other cell types, including murine embryonic fibroblasts, COS7 cells (Fig. 3.3C) and HeLa cells (Fig. 3.3D). These results suggest that FAK may regulate centrosome functions through acting on some components of centrosomes directly in a S732 phosphorylation-dependent manner during mitosis.

4. S732-phosphorylation dependent association of FAK with cytoplasmic dynein

To explore potential FAK targets, we examined various proteins localized in centrosomes for their potential association with FAK in a S732-phosphorylation dependent manner. $FAK^{-/-}$ ECs were infected with Ad-FAK, Ad-S732A or Ad-GFP as a control and lysates from these cells were immunoprecipitated by anti-Myc for the Myc-tagged FAK and S732A mutant and their associated proteins. Analysis of the immunoprecipitates by anti - dynein showed that it was associated with wild-type FAK, but not with the S732A mutant (Fig. 3.4A). Previous studies suggested that S732 of FAK can be phosphorylated by Cdk5 and ROCK1 in different cells (Xie et al. 2003; Le Boeuf et al. 2006). We therefore examined the effect of down-regulation of Cdk5 and ROCK1 on the association of FAK with cytoplasmic dynein. Lysates were prepared from 293T cells that had been transfected with vectors encoding Cdk5 shRNA, ROCK1 shRNA, or the vector alone control. Fig. 3.4B shows that interaction of FAK with cytoplasmic dynein was reduced by knockdown of expression of Cdk5, but not ROCK1, when compared with cells treated with control shRNA. Lastly, colocalization of S732 phosphorylated FAK with dynein at centrosomes was also confirmed by double-label immunofluorescent staining of mitotic cells (Fig. 3.4C). Together, these results suggest that Cdk5 - dependent S732 phosphorylation of FAK

Figure 3.4. FAK association with cytoplasmic dynein in a S732-phosphorylation dependent manner

(A) Floxed FAK ECs were infected with Ad-Cre to delete endogenous FAK followed by infection with Ad-FAK, Ad-S732A FAK, or a control Ad-GFP, as indicated. Cell lysates were immunoprecipitated with anti-myc. The precipitates or aliquots of cell lysates (WCL) were analyzed by Western blotting with anti-dynein and anti-FAK. The intensity of dynein bands in the precipitates were quantified by scanning densitometry and normalized to the intensity of the band in Ad-GFP infected precipitates (bottom panel). Three independent experiments were performed and the mean $\pm$ standard error is shown. *P <0.05, **P = 0.187 in comparison to value from Ad-GFP infected $FAK^{-/-}$ECs. (B) 293T cells were transfected with cdk5 shRNA, Rock1 shRNA, or control shRNA as indicated. Lysates were immunoprecipitated by anti-FAK followed by Western blotting with anti-dynein. WCL were also analyzed by Western blotting as indicated. The intensity of dynein bands in the precipitates were quantified and normalized to the intensity of the control band (bottom panel). Three independent experiments were performed and the mean $\pm$ standard error is shown as indicated. *P <0.05, **P = 0.205 in comparison to value from control shRNA transfected precipitates. (C) MEFs in metaphase were co-stained with S732-phospho specific FAK antibody (green) and α-dynein antibody (red), as indicated.


A.
Ad-GFP
Ad-FAK
Ad-S732A
IP: myc
Dynein
WCL
Dynein
WCL
FAK
Intensity of dynein (fold over Ad-GFP)
Ad-GFP
Ad-FAK
Ad-S732A
B.
Ctl
Cdk5 shRNA
Rock1 shRNA
IP: FAK
Dynein
WCL
Dynein
WCL
Cdk5
WCL
Rock1
Intensity of dynein (fold over Ctl)
Ctl
Cdk5
Rock1 shRNA

C.
P-S732
Dynein
DNA
Merged

and its binding to cytoplasmic dynein may play a role in the regulation of centrosome function during mitosis.

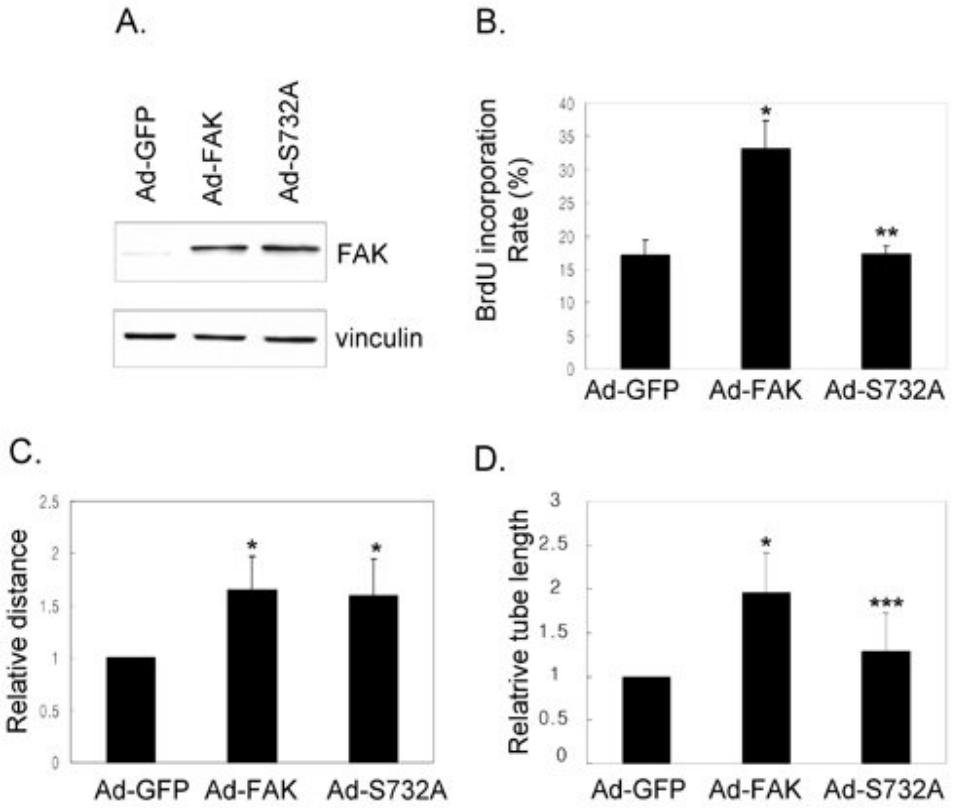
5. S732 phosphorylation of FAK is required for cell proliferation and tubulogenesis in primary ECs

Our previous studies showed that inactivation of FAK in primary ECs caused increased apoptosis, reduced proliferation and migration, and reduced capillary formation on Matrigel, suggesting an essential function of FAK in the regulation of multiple EC activities (Shen et al. 2005). The inability of S732A mutant to rescue the centrosomal defects in $FAK^{-/-}$ ECs raised the possibility that this mutant will not be able to rescue the deficiency in proliferation of these cells, consistent with a role for S732 phosphorylation of FAK in the regulation of EC proliferation. To test such a possibility, $FAK^{-/-}$ ECs were infected with Ad-FAK or Ad-S732A, and the re-expression of FAK and its mutant to comparable levels were verified in these cells (Fig. 3.5A). The cells were then subjected to analysis for proliferation by BrdU incorporation assays. As expected, re-expression of FAK in $FAK^{-/-}$ ECs rescued their deficiency in proliferation compared to those cells infected by Ad-GFP control virus. In contrast, however, re-expression of S732A mutant did not restore the reduced proliferation of $FAK^{-/-}$ ECs (Fig. 3.5B), suggesting a role of S732 phosphorylation for FAK regulation of cell cycle progression.

We next examined whether S732 phosphorylation is required for FAK stimulation of EC migration and/or tubulogenesis by analysis of S732A mutant in $FAK^{-/-}$ ECs. As shown in Fig. 3.5C, re-expression of S732A mutant in $FAK^{-/-}$ ECs restored migration of these cells to a comparable level as wild-type FAK, suggesting that Ser732 phosphorylation of FAK is not necessary for regulation of EC migration

Figure 3.5. Requirement of S732 phosphorylation of FAK in EC proliferation and tubulogenesis

Isolated ECs from floxed FAK mice were infected sequentially with Ad-Cre and Ad-FAK, Ad-S732A, or the control Ad-GFP. Cell lysates were analyzed by western blotting using anti-FAK, or anti-vinculin antibody (A). Proliferation rates of the infected ECs were determined by BrdU incorporation assay (B). Cell migration in response to VEGF was measured by wound closure assay (C). The infected cells were cultured on Matrigel and the lengths of the tubules were quantified (D). Each bar represents the mean ± standard error of at least three independent experiments in duplicate (n=6~8). *P <0.05, **P = 0.458, ***P = 0.332 in comparison to value from Ad-GFP infected cells.


A.
Ad-GFP
Ad-FAK
Ad-S732A
FAK
vinculin
B.
BrdU incorporation Rate (%)
40
35
30
25
20
15
10
5
0
*
**
Ad-GFP
Ad-FAK
Ad-S732A
C.
Relative distance
2.5
2
1.5
1
0.5
0
*
*
Ad-GFP
Ad-FAK
Ad-S732A
D.
Relatrive tube length
3
2.5
2
1.5
1
0.5
0
*
***
Ad-GFP
Ad-FAK
Ad-S732A

by FAK. In contrast, re-expression of S732A in FAK $^{-/-}$ ECs was not able to rescue tubulogenesis deficiency of FAK$^{-/-}$ ECs (Fig. 3.5D). Together, these results demonstrate that S732-phosphorylation dependent functions of centrosomal-localized FAK in mitotic cells is important for FAK regulation of proliferation and tubulogenesis of primary ECs.

6. Role of FAK S732 phosphorylation in tumor angiogenesis

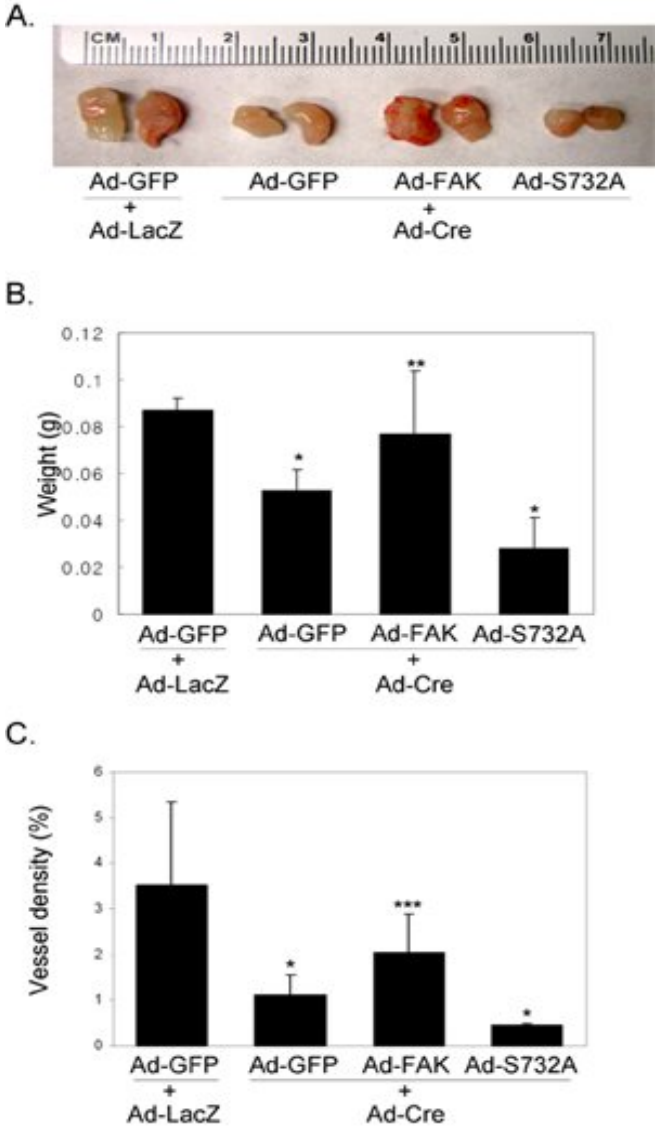
Previous studies using EC-specific FAK knockout mice indicated a role for FAK in embryonic angiogenesis *in vivo* (Shen et al. 2005; Braren et al. 2006). However, the embryonic lethality of these mice prevented their usage for analysis of a role for FAK in angiogenesis in adult mice. A mouse model with inducible EC-specific deletion of FAK in adult mice was generated very recently and used to demonstrate a role for FAK and its related kinase Pyk2 in angiogenesis using inhibitors for FAK and Pyk2 (Weis et al. 2008). Because of the compensatory up-regulation of Pyk2 in these mice, this inducible EC-specific FAK-knockout mouse model could not be used to assess the specific role of FAK and its mutants. Therefore, in order to examine a potential role of S732 phosphorylation of FAK in angiogenesis *in vivo*, we developed a tumor angiogenesis assay using floxed FAK mice, in which Ad-Cre is included in Matrigel to induce Cre-mediated deletion of endogenous FAK in ECs migrating into the Matrigel plugs in response to angiogenic stimulation of Matrigel containing tumor cells. Floxed FAK mice were subcutaneously injected with Matrigels containing Ad-Cre or Ad-lacZ as a control as well as B16F10 melanoma cells to induce angiogenesis, as described in the Materials and Methods. Ten days later, the Matrigel plugs containing tumors were dissected and analyzed. As shown in Fig. 3.6A, Matrigel plugs from mice with Ad-Cre infection were smaller and

appeared less red when compared to those from mice infected by the Ad-LacZ control. Quantification of multiple samples showed a significant reduction in weight (Fig. 3.6B) as well as size (data not shown) for the plugs containing Ad-Cre than those with Ad-LacZ control. The plugs were also sectioned and subjected to immunohistochemical analysis using anti-PECAM-1 antibody to detect blood vessels. Consistent with the reduced tumor growth, we found a significantly reduced density of blood vessels in the Matrigel plugs containing Ad-Cre than those with Ad-LacZ control (Fig. 3.6C). Together, these results suggest that Ad-Cre mediated deletion of the floxed FAK in ECs significantly reduced tumor angiogenesis and growth *in vivo*.

We then assessed the role of S732 phosphorylation of FAK in tumor angiogenesis by re-expression of FAK or S732A mutant in $FAK^{-/-}$ ECs using this floxed FAK mouse model. Ad-FAK or Ad-S732A was included in Matrigel containing Ad-Cre as well as B16F10 melanoma cells injected into floxed FAK mice. As shown in Fig. 3.6, re-expression of wild-type FAK restored tumor growth as well as angiogenesis in Matrigel, as expected. In contrast, re-expression of S732A mutant did not rescue the decreased tumor growth or angiogenesis caused by deletion of endogenous FAK in ECs. Therefore, consistent with results from *in vitro* analysis, S732 phosphorylation of FAK is required for angiogenesis *in vivo* due to its role in the regulation of centrosome functions and proliferation in ECs.

Figure 3.6. Role of FAK S732 phosphorylation in tumor angiogenesis

Ad-lacZ and Ad-Cre were added to Matrigel with B16F10 melanoma cells and injected s.c. into floxed FAK mice. For rescue experiments, Ad-FAK, S732A FAK, or GFP was added to Ad-Cre containing Matrigel plug. After 10 days, Matrigel plugs were removed and evaluated by gross examination (A). Matrigel plug weights were determined (B) and sections were prepared for histochemical analysis. Quantitation of the vascularization in the Matrigel plug was performed by immunohistological examination using anti-PECAM-1 antibody (C). PECAM-1-positive vessels were evaluated in five different 10x fields in each Matrigel plug. The mean $\pm$ standard error from three independent experiments is shown (n=8 in each group). *P <0.05, **P = 0.466, ***P =0.239 in comparison to value from Ad-lacZ infected cells.


A.
CM
1
2
3
4
5
6
7
Ad-GFP
+
Ad-LacZ
Ad-GFP
Ad-FAK
+
Ad-Cre
Ad-S732A
B.
Weight (g)
0.12
0.1
0.08
0.06
0.04
0.02
0
**
*
*
Ad-GFP
+
Ad-LacZ
Ad-GFP
Ad-FAK
+
Ad-Cre
Ad-S732A
C.
Vessel density (%)
6
5
4
3
2
1
0
***
*
*
Ad-GFP
+
Ad-LacZ
Ad-GFP
Ad-FAK
+
Ad-Cre
Ad-S732A

E. Discussion

As the principal cytoplasmic tyrosine kinase located in focal adhesions, FAK is well established as a major mediator of signaling cascades triggered by clustering of integrins in these sites in the regulation of various cellular functions, including G1-S transition in cell cycle (Parsons 2003; Schlaepfer and Mitra 2004). In this report, we present data suggesting a novel function for FAK in the regulation of centrosome integrity, spindle pole formation, and chromosome segregation during mitosis in primary ECs. Besides being required for G1-S transition, cell adhesion to ECM was known to control other phases of cell cycle such as cytokinesis (Orly and Sato 1979; Ben-Ze'ev and Raz 1981; Winklbauer 1986). Indeed, a recent study showed that inhibition of integrin function disrupted centrosome functions, spindle assembly, and cytokinesis in mitotic cells (Reverte et al. 2006). Thus, FAK may play a role in both focal adhesions and centrosomes during different phases of cell cycle progression. In this regard, it is interesting to note that several other focal adhesion proteins, including HEF1 (Pugacheva and Golemis 2005), paxillin (Herreros et al. 2000), zyxin (Hirota et al. 2000), and Integrin-linked kinase (Fielding et al. 2008) have been shown to localize and function in centrosomes. Given the known connections with FAK for at least some of these molecules (Schaller 2001; Parsons 2003; Schlaepfer and Mitra 2004; Siesser and Hanks 2006), FAK may work together with these other focal adhesion proteins to provide a mechanistic link for the control of mitotic events in the nucleus by integrins localized on the plasma membrane.

Centrosomes are composed of two paired centrioles surrounded by pericentriolar material (PCM), which comprised hundreds of structural and signaling proteins. They undergo structural modifications during cell cycle, including duplication, maturation, and separation, which are tightly coordinated with the

chromosome duplication and segregation (Meraldi and Nigg 2002). The abnormal centrosomal phenotype in $FAK^{-/-}$ ECs could result from deregulation of centrosomal duplication, incomplete centrosome separation, or loss of cohesion in mitotic centrosomes, resulting in premature splitting of mother and daughter centrioles. Centrosome number and splitting are regulated by protein kinases, including Cdk2/cyclinE, the Polo-like kinases, Aurora-A, and Nek2 (Hinchcliffe et al. 1999; Nigg 2001; Faragher and Fry 2003; Marumoto et al. 2005). Two focal adhesion-associated proteins, HEF1 and ILK, may regulate centrosomal functions through Aurora-A, as previous studies showed that HEF1 associates with and activates Aurora-A and that ILK regulates spindle organization by modulating Aurora A/TACC3/ch-TOG interaction (Pugacheva and Golemis 2005; Fielding et al. 2008). However, we found that deletion of FAK in primary ECs did not affect Aurora-A activation (data not shown), suggesting that FAK may use a different mechanism from HEF1 and/or ILK in its regulation of centrosome functions.

Our results showed that centrosomal-localized FAK is phosphorylated on S732 and that S732 phosphorylation is required for FAK to rescue the centrosomal defects in FAK-/- ECs, suggesting that FAK phosphorylation at S732 is crucial for its distinct functions in G2/M phase of mitosis. Interestingly, S732 of FAK was identified as a physiological substrate for Cdk5, and its phosphorylation was shown to promote organization of microtubule structures in the post-mitotic neurons (Xie et al. 2003; Xie and Tsai 2004). Centrosomes are the major microtubule-organizing center in mammalian cells, which regulate spindle bipolarity, spindle positioning, and cytokinesis (Meraldi and Nigg 2002). Therefore, S732-phosphorylated FAK may regulate these mitotic events through its functions in the microtubule organization in the proliferating primary ECs in a similar manner as its regulation of nuclear

translocation in the post-mitotic neurons. Indeed, our preliminary studies showed that treatment of ECs with roscovitine, a specific Cdk5 inhibitor, abolished S732 phosphorylation of FAK and induced similar centrosomal abnormality as that in FAK-/- ECs (data not shown).Although the potential target proteins localized in centrosomes that mediate FAK regulation of centrosomal functions are not clarified, we have found an interaction of FAK with cytoplasmic dynein in a S732-phosphorylation dependent manner. Interestingly, Nudel, a substrate for Cdk5, and its binding partner Lis1, form a complex with cytoplasmic dynein localized around the centrosomes and in the growth cones of neuronal cells (Niethammer et al. 2000). Perturbation of cytoplasmic dynein or Lis1 has been shown to cause various defects in chromosome alignment, spindle organization and centrosome separation (Busson et al. 1998; Faulkner et al. 2000; Yang et al. 2007). In addition, Nudel participates with Lis1 in the regulation of cytoplasmic dynein function via Cdk5 phosphorylation (Niethammer et al. 2000). Together with our findings of disruption of FAK interaction with cytoplasmic dynein upon down-regulation of Cdk5, these results raised the possibility that Cdk5-induced FAK phosphorylation at S732 could stimulate activation of cytoplasmic dynein and participate in cytoplasmic dynein function. Future studies will be necessary to determine how interaction of FAK with cytoplasmic dynein regulates cytoplasmic dynein function in mitosis and also to identify potentially other target proteins of FAK in centrosomes that mediate centrosome functions of FAK during mitosis.

Consistent with a role of S732-phosphorylated FAK in centrosome functions, S732 phosphorylation of FAK was found to be required for its promotion of cell cycle progression and tubulogenesis of primary EC in vitro, as well as tumor angiogenesis in vivo. However, we found that S732 phosphorylation is dispensable for FAK

stimulation of migration in the primary ECs, although Le Boeuf et al. reported recently that S732 can be phosphorylated by ROCK and that S732A mutant was unable to stimulate migration of HUVEC and MEF as wild-type FAK (Le Boeuf et al. 2006). It is not clear whether the use of different cells is responsible for the discrepancies. Nevertheless, our data suggest that a defective cell cycle progression, caused by deregulation of centrosome functions, rather than migration, contributes to the inability of S732A mutant to promote EC tubulogenesis in vitro and tumor angiogenesis in vivo.

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CHAPTER 4:

CONCLUTIONS AND PROSPECTS

Conclusion and Prospects

FAK has been demonstrated as a major mediator of signal transduction by integrins and other cell surface receptors in a variety of cells. Phosphorylation of FAK and its interactions with other signaling molecules has been shown to trigger several signaling pathways that regulate cellular functions, including cell migration, cell cycle progression and cell survival (Parsons 2003; Schlaepfer and Mitra 2004). Phosphorylation of FAK on Tyr 397 and its binding to Src has been shown as a critical step to activate FAK itself and its downstream signaling molecules. Despite the well characterized tyrosine phosphorylations of FAK in the regulation of its cellular functions, the function and the specific mechanism of FAK mediated by serine phosphorylations is relatively undefined.

Several studies suggest the importance of both integrins and growth factor receptors in the regulation of angiogenesis and several cellular functions, including cell migration, proliferation and survival, are involved in the process of angiogenesis (Chapter1 in section B). Since FAK mediates signaling from integrins and growth factors and regulates those cellular functions, FAK has been implicated to play a role in angiogenesis. Indeed, several studies in vitro and in vivo have shown the important role of FAK in angiogenesis, in addition to the early observation in FAK knockout mice, which suggested a potential role of FAK in angiogenesis during embryogenesis (Ilic et al. 1995). Recent studies of EC-specific FAK conditional knockout mice revealed that FAK is required for the vascular development in angiogenesis (Shen et al. 2005; Braren et al. 2006). Further studies with FAK deficient ECs showed essential function of FAK in the regulation of EC activities, including EC migration, proliferation and survival may contribute to the regulation of angiogenesis (Chapter 2). However, the specific mechanisms that link FAK to the regulation of angiogenesis

are still unclear. Given the multiple targets of FAK for different cellular functions, it will be necessary to further clarify the roles of various FAK downstream signaling pathways in the regulation of various EC functions in vitro and angiogenesis and vascular development in vivo. A potentially powerful approach is to use various FAK mutants defective in interaction with specific targets to rescue the EC and embryonic phenotypes of CFKO.

The embryonic lethality of EC-specific FAK knockout mice prevented the analysis of a role for FAK in angiogenesis in adult mice (Shen et al. 2005; Braren et al. 2006). In addition, a recent study with inducible EC-specific FAK knockout mice showed that such an approach could not be used to address the role of specific FAK downstream pathways in angiogenesis because of the compensatory upregulation of Pyk2 in these mice (Weis et al. 2008). Therefore, we developed a tumor angiogenesis assay using floxed FAK adult mice in which Ad-Cre is included in Matrigel to induce Cre-mediated deletion of FAK in ECs migrating into the Matrigel plugs in response to angiogenic stimulation of Matrigel-containing tumor cells (Chapter 3). We found that Ad-Cre mediated deletion of the floxed FAK in ECs significantly reduced tumor angiogenesis in vivo. To further investigate the mechanisms of FAK in the regulation of EC functions, we performed rescue experiments with several FAK mutants in FAK deficient ECs. In this dissertation, we revealed the role of Ser732 phosphorylation of FAK in the regulation of centrosome function during mitosis, which may contribute to EC proliferation and angiogenesis in vivo (Chapter 3). Future studies will be necessary to test more FAK mutants that are incapable of interacting with various targets of FAK, which contribute to cell proliferation, migration and survival. In addition, a recent study from our lab showed that FAK may enhance the ability of cancer cell to degrade ECM through its interaction and phosphorylation of endophilin

A2 leading to MT1-MMP accumulation on the cell surface (Wu et al. 2005). Since the ECM degradation plays an essential role in the process of angiogenesis as described in Chapter 1 (Section B1.1), it will be interesting to determine the role of FAK signaling through endophilin A2 in angiogenesis.

FAK is well established as a key mediator of signaling pathways triggered by integrins in focal adhesions in the regulation of several cellular functions, including G1 to S transition during cell cycle. In this dissertation, we reveal a novel localization and function of FAK in centrosomes during mitosis (Chapter 3). Consistent with our results, a recent study showed that the inhibition of integrin disrupted centrosome functions, spindle assembly and cytokinesis in mitotic cells (Reverte et al. 2006). In addition, several other focal adhesion proteins, including HEF1 (Pugacheva and Golemis 2005), paxillin (Herreros et al. 2000), zyxin (Hirota et al. 2000), and ILK (Fielding et al. 2008) have been shown to function in centrosomes. Therefore, these studies suggest that focal adhesion proteins, including FAK may provide a mechanistic link for the control of events in the nucleus by integrins localized on the plasma membrane.

Centrosome has been shown to play an essential role in the regulation of bipolar spindle formation through its microtubule organizing capability during mitosis and the structural modifications of centrosomes during centrosome cycle are tightly coordinated with the chromosome and cell cycle (Chapter1 in section C2). Our results in Chapter 3 showed that FAK plays a role in cell cycle progression through the regulation of centrosome integrity and functions, including bipolar spindle formation and chromosome segregation during mitosis, besides its known role in G1 to S phases transition during cell cycle. The abnormal centrosomal phenotype in $FAK^{-/-}$ ECs could result from the disruption of centrosome function during

centrosome cycle, including deregulation of centrosomal duplication, incomplete centrosome separation, or loss of cohesion in mitotic centrosomes, resulting in premature splitting of mother and daughter centrioles. Although we found the interaction of FAK with cytoplasmic dynein, which has been shown to regulate centrosome separation as described in Chapter1 (Section C1), it will be necessary to study how interaction of FAK with cytoplasmic dynein regulates the cytoplasmic dynein function in centrosome during mitosis. In addition, further study should be carried out to identify other potential target proteins localized in centrosomes that mediate FAK regulation of centosome functions. Although much remains unclear about the functions of centrosomal proteins, several important kinases are well established as key regulators for centrosome functions, including cdk2-cyclinE/A, cdk1-cyclinB, Aurora-A, Polo-like kinase and Nek2 (Chapter 1 in section C2.2). Therefore, it will be interesting to examine whether FAK mediate centrosome functions through these kinases during mitosis.

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APPENDIX:

GENERATION OF RECOMBINANT ADENOVIRUSES ENCODING FAK AND FAK MUTANTS USING THE ADEASY SYSTEM

The inactivation of FAK in ECs using a conditional KO approach showed that FAK expression is required for the vascular development in embryogenesis (Shen et al. 2005; Braren et al. 2006). Consistent with the phenotypes *in vivo*, deletion of FAK in ECs showed reduced tubulogenesis, proliferation, and migration *in vitro* (Chapter 2). Given the multiple targets of FAK in the regulation of different cellular functions, it will be necessary to further investigate the roles of FAK downstream signaling pathways in the regulation of EC functions. A powerful approach will be to use various FAK mutants defective in interaction with specific FAK targets to rescue the EC and embryonic phenotypes of CFKO.

Recombinant adenoviruses are replication-defective adenoviral vectors that have proven useful for gene transfer. Since high-titer preparations of adenoviruses can be prepared and used to get a high level of transgene expression in a broad spectrum of host cells and tissues, including non-dividing cells, recombinant adenoviruses are appealing vectors for gene transfer both *in vitro* and *in vivo* (Luo et al. 2007). The most commonly used adenoviral vectors are derived from human adenovirus serotypes 2 and 5. The viral genome consists of 36-kb double-stranded linear DNA and the DNA length greater than 38 kb cannot be efficiently packaged into competent viral particles. Viral transcription units are conventionally referred to as early (E1, E2, E3, and E4), delayed early (proteins IX and Iva2) or late genes (L1-L5). The early gene products are involved in viral gene transcription, DNA replication, host immune suppression and inhibition of host cell apoptosis, whereas the late gene products are required for viron assembly (Luo et al. 2007). The complexity of the viral transcription units causes problems for recombinant manipulation, which therefore is usually limited to specific regions not essential for viral production, including E1, E2A, E3 and E4. First-generation adenoviral vectors

replaced E1 genes with the desired transgene and these vectors could be propagated in cell lines that express E1 gene products, such as HEK-293 cells (Graham et al. 1977). However, these vectors had relatively limited packaging capacity (Bett et al. 1993). The second-generation adenoviral vectors accommodated larger transgenes, reduced the cytotoxic effects in host cells and reduced the ability to elicit host immune response. In the extreme case, the whole adenoviral genome (except inverted terminal repeat sequences and the packaging signal sequences) was replaced by exogenous sequences and the gene products required for viral replication and packaging were provided *in trans*. These adenoviral vectors accommodated up to 35kb foreign DNA, showing significantly reduced host immune responses (Luo et al. 2007). The most widely used method to generate recombinant adenoviruses involves homologous recombination in mammalian cells or in microorganisms. This method needs a two-vector system, 'shuttle' and 'backbone' plasmids (Graham and Prevec 1995). The shuttle vector usually contains the adenoviral genome, in which E1 and other non-essential genes are replaced with a transgene. This shuttle vector is subsequently recombined into the 'backbone' vector which provides adenovirus genome except genes essential for virus propagation in naturally occurring cells. Through recombination, a single DNA encoding all genes required for the virus production is produced in packaging cells. Since homologous recombination in mammalian cells showed low efficiency, yeast and bacterial systems have been explored for generating adenoviral vectors (Ketner et al. 1994; Chartier et al. 1996; Luo et al. 2007). In this dissertation, the AdEasy system, which exploits the high efficiency of homologous recombination in a specific bacterial strain, is used for generating recombinant adenoviruses encoding FAK and FAK mutants to investigate

the specific mechanisms of FAK in the regulation of angiogenesis both in primary ECs and *in vivo* model.

The AdEasy system can be used with any of four shuttle vectors (Fig.A.1A). pAdTrack and pAdTrack-CMV allow convenient tracing of all step in viral production through an incorporated GFP reporter. The pShuttle is the basic vector with the greatest capacity for accommodating foreign genes that are used when particularly large transgenes must be expressed. Two adenoviral backbone vectors can be used for adenovirus production. The commonly used pAdEasy-1 (Fig. A.1B) is an E1 and E3 double-deletion vector. pAdEasy-1-derived recombinant adenoviruses can be propagated in E1-expressing packaging cells, such as HEK-293 cells (E3 is not necessary for viral production).

The overall strategy for the generation of recombinant adenoviruses is diagrammed in Fig.A.2 and it involves three steps. First, the gene of interest is cloned into a shuttle vector. In order to generate recombinant adenoviruses encoding FAK and FAK mutants, EcoRV-KpnI fragments from pBS (pBluescript)-FAK and pBS-Y397F FAK, pBS-KD FAK, pBS-D395A FAK, pBS-P712/715A FAK, pBS-FRNK and pBS-S732A FAK are cloned into the pAdTrack-CMV through EcoRV and KpnI sites. Second, the resultant constructs are cleaved with a restriction endonuclease (PmeI) to linearize them and transformed into *E.coli* strain BJ5183, which contains the adenoviral backbone plasmid pAdEasy-1. Recombinants are selected with kanamycin and screened by restriction endonuclease digestion. Third, the recombinant adenoviral construct is cleaved with PacI to expose its inverted terminal repeats (FigA.3A) and transfected into a packaging cell line (HEK-293 cells). Transfections and viral productions can be monitored by GFP expression and comet-like adenovirus-producing foci became apparent at 6-8 days (Fig.A.3.B). Presence of

the recombinant adenoviruses was confirmed by western blotting as shown in Fig.A.3C and Fig. 3.2A.

Figure A.1. Vectors used in the AdEasy system

Shuttle vectors (A) and adenoviral backbone vector pAd-Easy-1 (B). Approximate locations of ΔE1 and/or ΔE3 are shown. Adapted from *Luo et al. 2007*.

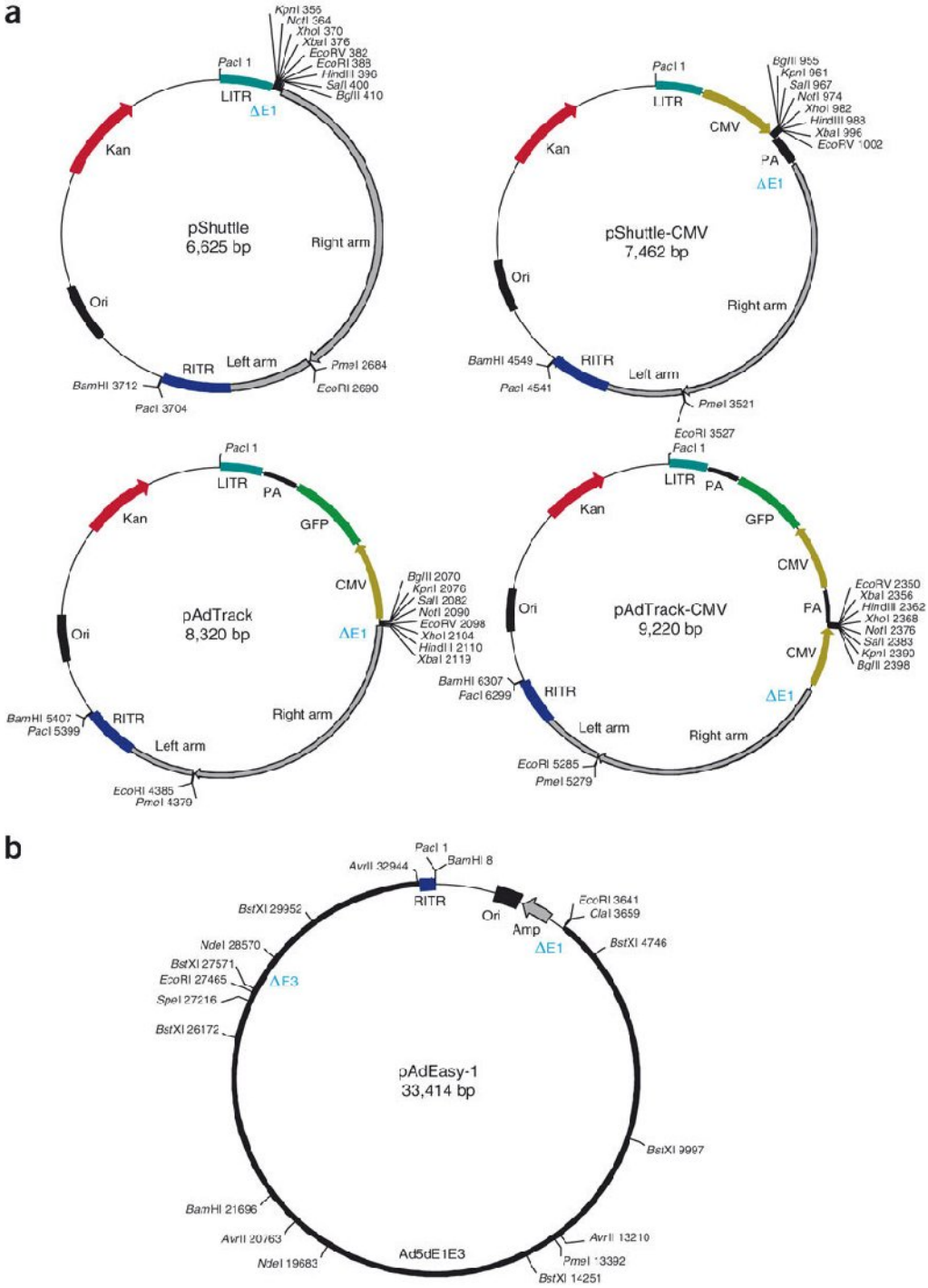

a
pShuttle
6,625 bp
Kan
Ori
LITR
ΔE1
Right arm
Left arm
RITR
PacI 1
KpnI 355
NotI 364
XhoI 370
XbaI 376
EcoRV 382
EcoRI 388
HindIII 396
SalI 400
BglII 410
PmeI 2684
EcoRI 2690
BamHI 3712
PacI 3704
pShuttle-CMV
7,462 bp
CMV
PA
BglII 955
KpnI 961
SalI 967
NotI 974
XhoI 982
HindIII 988
XbaI 996
EcoRV 1002
BamHI 4549
PacI 4541
PmeI 3521
EcoRI 3527
pAdTrack
8,320 bp
GFP
BglII 2070
KpnI 2076
SalI 2082
NotI 2090
EcoRV 2098
XhoI 2104
HindIII 2110
XbaI 2119
BamHI 5407
PacI 5399
EcoRI 4385
PmeI 4379
pAdTrack-CMV
9,220 bp
EcoRV 2350
XbaI 2356
HindIII 2362
XhoI 2368
NotI 2376
SalI 2383
KpnI 2390
BglII 2398
BamHI 6307
PacI 6299
EcoRI 5285
PmeI 5279
b
pAdEasy-1
33,414 bp
Ad5dE1E3
PacI 1
BamHI 8
AvrII 32944
RITR
Ori
Amp
ΔE1
ΔE3
EcoRI 3641
ClaI 3659
BstXI 4746
BstXI 29952
NdeI 28570
BstXI 27571
EcoRI 27465
SpeI 27216
BstXI 26172
BstXI 9997
BamHI 21696
AvrII 20763
NdeI 19683
AvrII 13210
PmeI 13392
BstXI 14251

Figure A.2. Schematic outline of the AdEasy system

The gene of interest is cloned into the shuttle vector, pAdTrack-CMV. The resultant plasmid is cleaved with a restriction endonuclease (PmeI) to linearize it and transformed into *E.coli* strain BJ5183, which contains the adenoviral backbone plasmid pAdEasy-1. Recombinants are selected with kanamycin and screened by restriction endonuclease digestion. Finally, the recombinant adenoviral construct is cleaved with PacI to expose its inverted terminal repeats and transfected into a packaging cell line (HEK-293 cells). Recombinant adenoviruses are typically generated within 7-10 days. The 'left arm' and 'right arm' represent the regions mediating homologous recombination between the shuttle vector and the adenoviral backbone vector. Alternative homologous recombination between two *Ori* sites is shown with dotted lines. PA: polyadenylation site; LITR: left-hand ITR and packaging signal; RITR: right-hand ITR. Adapted from *Luo et al. 2007*.

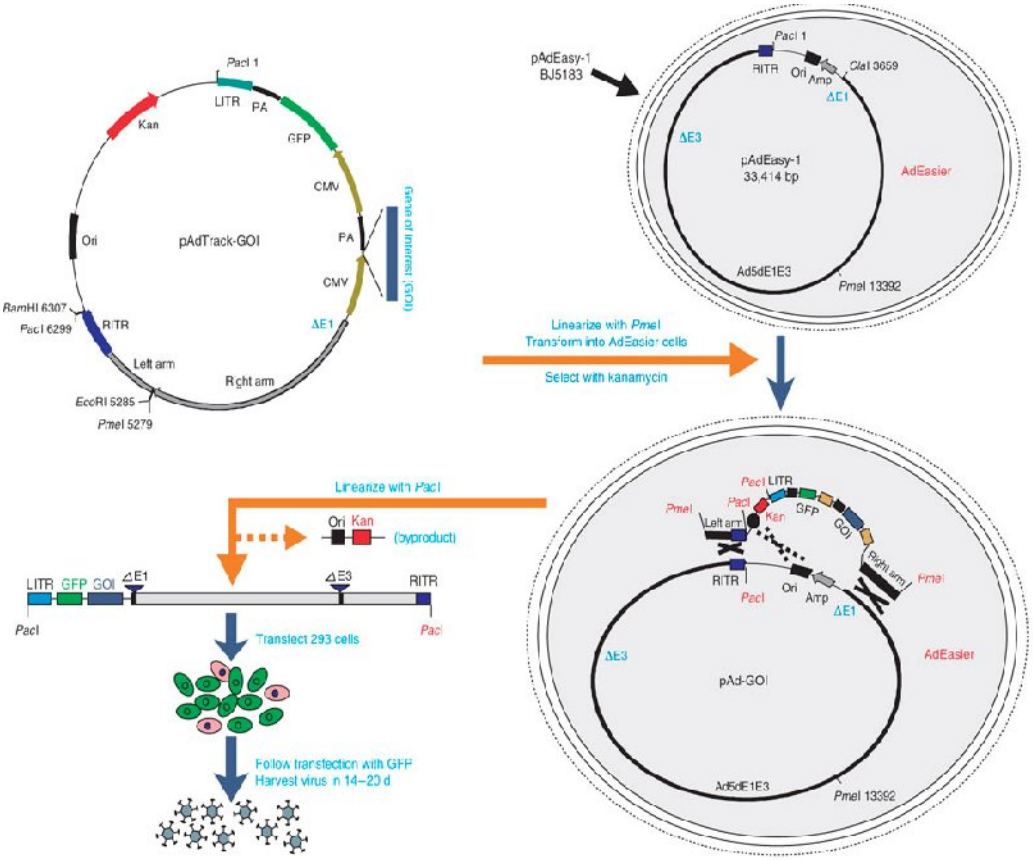
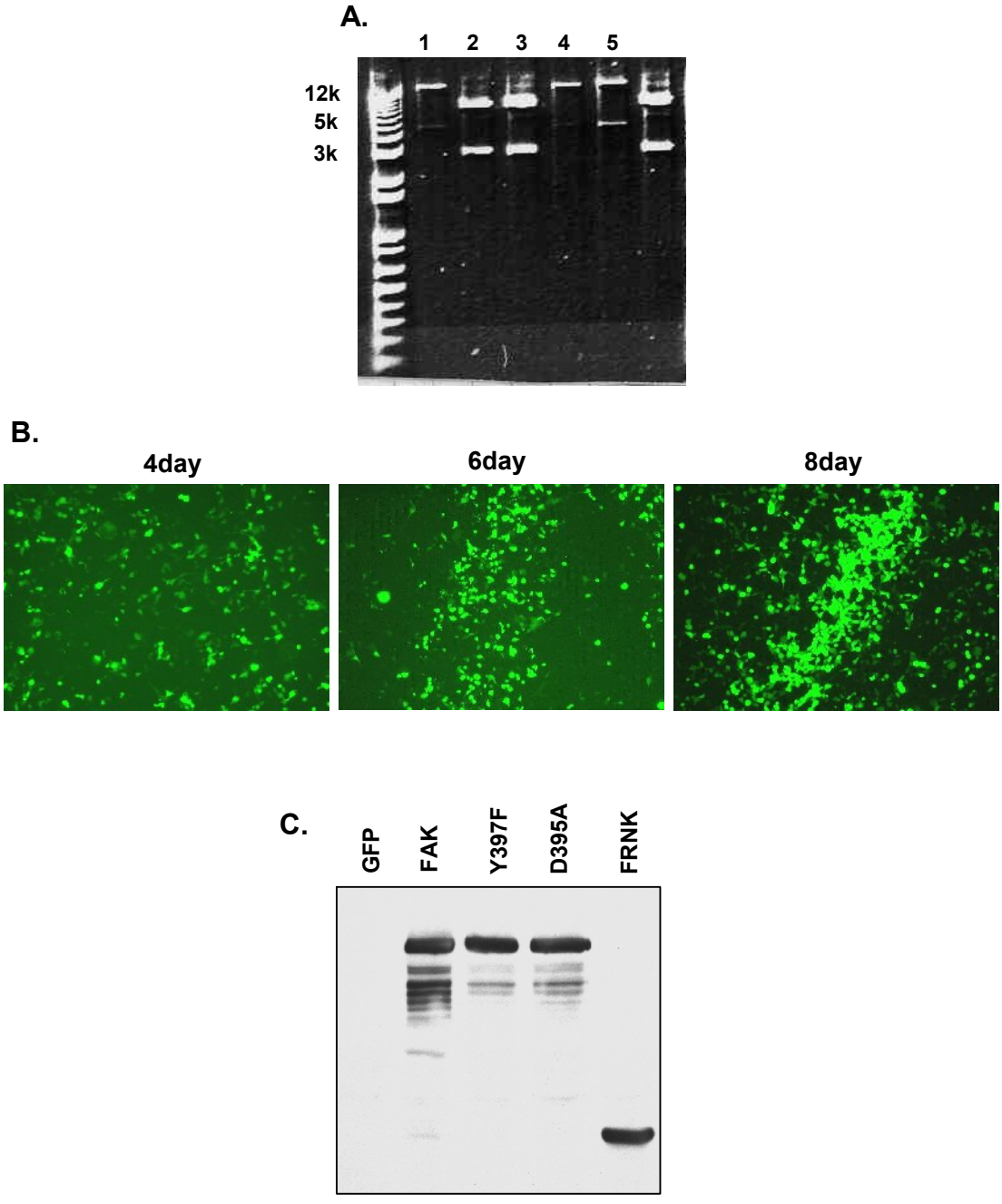

PacI 1
LITR
PA
GFP
CMV
PA
CMV
ΔE1
RITR
Left arm
Right arm
EcoRI 5285
PmeI 5279
RamHI 6307
PacI 6299
Ori
Kan
pAdTrack-GOI
Gene of interest (GOI)
pAdEasy-1
BJ5183
PacI 1
RITR
Ori
Amp
ClaI 3659
ΔE1
ΔE3
pAdEasy-1
33,414 bp
AdEasier
Ad5dE1E3
PmeI 13392
Linearize with PmeI
Transform into AdEasier cells
Select with kanamycin
Linearize with PacI
Ori
Kan
(byproduct)
LITR
GFP
GOI
ΔE1
ΔE3
RITR
PacI
PacI
Transfect 293 cells
Follow transfection with GFP
Harvest virus in 14–20 d
PacI LITR
PmeI
PacI
Left arm
Kan
GFP
GOI
Right arm
PmeI
RITR
PacI
Ori
Amp
ΔE1
ΔE3
AdEasier
pAd-GOI
Ad5dE1E3
PmeI 13392

Figure A.3. Selection and characterization of recombinant adenovirus plasmids

(A) PacI restriction endoculease digestion of candidate recombinants encoding FAK. All of the six candidate clones were validated. Three of the six clones (#1,4 and 5) released a 4.5-kb fragment after PacI digestion, and the other three (#2,3 and 6) released a 3-kb fragment. (B) Adenovirus-producing foci after transfection of 293 cells. Comet-like adenovirus-producing foci became apparent at 6-8 days. (C) Presence of the recombinant adenoviruses was confirmed by western blotting.


A.
1
2
3
4
5
12k
5k
3k
B.
4day
6day
8day
C.
GFP
FAK
Y397F
D395A
FRNK

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