STUDY OF FOCAL ADHESION KINASE (FAK) FUNCTIONS IN DEVELOPMENTAL ANGIOGENESIS AND MAMMARY TUMORS UTILIZING KNOCKIN TECHNOLOGY

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STUDY OF FAK FUNCTIONS IN DEVELOPMENTAL ANGIOGENESIS AND MAMMARY TUMORS UTILIZING KNOCKIN TECHNOLOGY

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Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase involved in integrin-mediated signal transductions. In adherent cells, FAK co-localizes with integrins in focal contacts, and integrin binding to their extracellular ligands leads to FAK activation and autophosphorylation in a variety of cell types. FAK phosphorylation and its interactions with other molecules trigger several downstream signaling pathways which can regulate various cell functions, including cell migration, proliferation, cell survival and adhesions. Although many in vitro studies utilizing overexpression of FAK mutants have been done to illustrate the functions of the kinase activity or the second C-terminal proline-rich motif of FAK in different cellular functions, very little is known about their roles in vivo either during development or under pathological conditions, like cancer. This thesis utilized genetic knockin technology and for the first time illustrated both kinase-independent and –dependent roles of FAK during developmental angiogenesis and the functions of the second C-terminal proline-rich motif of FAK in mammary tumor growth in vivo.

FAK plays an essential role in vascular development as endothelial cells (ECs)-specific conditional knockout of FAK (i.e. CFKO mice) leads to embryonic lethality caused by increased apoptosis and other defects in ECs. In order to investigate the mechanisms by which FAK regulates vascular development and angiogenesis in vivo, we created and analyzed an EC-specific conditional FAK kinase-defective mutant

knockin (CFKI) mouse model (Chapter 2). Our data suggested that kinase-independent functions of FAK can support EC survival in vascular developmental through E13.5, but is insufficient for maintaining EC functions to allow for completion of embryogenesis.

FAK is also important for mammary tumorigenesis and metastasis. Specific deletion of FAK in mammary epithelial cells suppressed mammary tumor formation, growth and metastasis. Here we studied the specific roles of the second C-terminal proline-rich motif of FAK in vivo with knockin mouse models. Our studies revealed that PA mutations of this motif in mammary epithelial tumor cells led to increased cell contact inhibition; whereas in endothelial cells, the specific knockin of this motif resulted in formation of mal-functioning blood vessels (Chapter 3). In summary, by generating various conditional knockin mice models, we identified that FAK could promote mammary tumor growth through distinct mechanisms in different systems.

BIOGRAPHICAL SKETCH

Xiaofeng Zhao was born and raised in ChengDe, a small yet beautiful city northwest to Beijing, China. She was enrolled in a 7-year program of clinical medicine and graduated from the medical school of Nankai University in Tianjin, China in 2004. After graduation, the author was recruited to the field of "Biochemistry, Molecular and Cell Biology" in the department of Molecular Biology and Genetics to pursue her graduate study in Cornell University in Ithaca, NY. In 2005, she joined the laboratory of Dr. Jun-Lin Guan in the department of Molecular Medicine, where she started studies on angiogenesis and oncology. Her projects mainly focus on the roles of FAK in developmental/tumor angiogenesis and mammary tumor progression/metastasis using in vivo genetic approaches, combined with in vitro analyses.

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CHAPTER 1

INTRODUCTION

1.1 Focal adhesion Kinase (FAK) and its activation

1.1.1 Characterization of FAK

Focal adhesion kinase (FAK) [1, 2] belongs to the subfamily of non-receptor protein tyrosine kinases (PTKs) that also includes PYK2 [3, 4]. Parsons and colleagues identified a protein with a molecular weight of around 125kD, whose phosphorylation content is elevated in v-Src-transformed chick embryo cells [5]. Meanwhile, two other studies showed that integrin/ligand interactions can trigger tyrosine phosphorylation of a protein of Mr 120,000 [6, 7], which is identical to the phosphorylation substrate of Src characterized by Parsons et al., and found to be focal adhesion-associated. This protein was hereafter named as Focal Adhesion Kinase, or FAK, which was identified to play important roles in integrin and other molecules-mediated signaling transductions [5, 8].

FAK was mapped to the mouse chromosome 15 and human chromosome 8 and has a wide and conserved expression pattern in a variety of species and tissues [9]. The deduced amino acid sequences from different species, including chicken, mouse, human and Xenopus, showed an identity of over 90% [8, 10-12]. The cDNA of FAK encodes a protein with a predicted molecular weight of 119-121kDa. One feature of FAK is its subcellular localization to specialized submembranous structures called focal adhesions (FAs) which occur where actin filaments terminate and cells attach to the extracellular matrix upon intergrin engagement and aggregation. Therefore, FAs can mediate cell adhesion and migration on the cellular substrate [1, 2, 12]. As mentioned above, in addition to integrin-mediated cell adhesion, FAK tyrosine

phosphorylation is also induced by v-Src transformation as well as a variety of other stimuli, such as ligands for receptor tyrosine kinases and for seven transmembrane domain G-protein-coupled receptors. Therefore, FAK is considered as one of the important mediators to transduce biochemical signals from the extracellular matrix to the cell interior for modulation of various cellular functions [6, 13].

FAK contains three major domains (Figure 1.1) [6, 14, 15]. The N-terminal domain of FAK exhibits homology with FERM (Band 4.1, ezrin-radixin-moesin) domains, which are present in structural proteins such as talin and the ezrin-radixin-moesin (ERM) family of proteins, as well as in signaling proteins such as the JAK family of tyrosine kinases and several tyrosine phosphotases [14, 16]. FERM domain mediates protein-protein interactions in two different paradigms of interaction. One is intermolecular interaction to dock with the cytoplasmic tails of transmemebrane proteins, the other is intramolecular or homophilic intermolecular interactions [16]. Similar to that of previously identified PTKs, FAK has a kinase domain with catalytic activity in the middle. The C-terminal domain of FAK contains a focal adhesion targeting sequence (FAT) comprising the C-terminal 140 amino acids of the protein and the region between the kinase domain and the FAT sequence. An additional FAK-related cDNA, referred to as FRNK (FAK-related nonkinase) exists naturally in many cell types [17]. The FRNK-encoded protein, $p41^{FRNK}$, derives from mRNAs that encode only the C-terminal domain of $pp125^{FAK}$. $p41^{FRNK}$ localizes to focal adhesions, but lacks the kinase activity. Thus FRNK could act as a dominant negative mutant to inhibit FAK signaling [17]. Unlike other non-receptor tyrosine kinase proteins, FAK does not contain Src homology 2 or 3 (SH2 or SH3) domains that mediate specific interactions

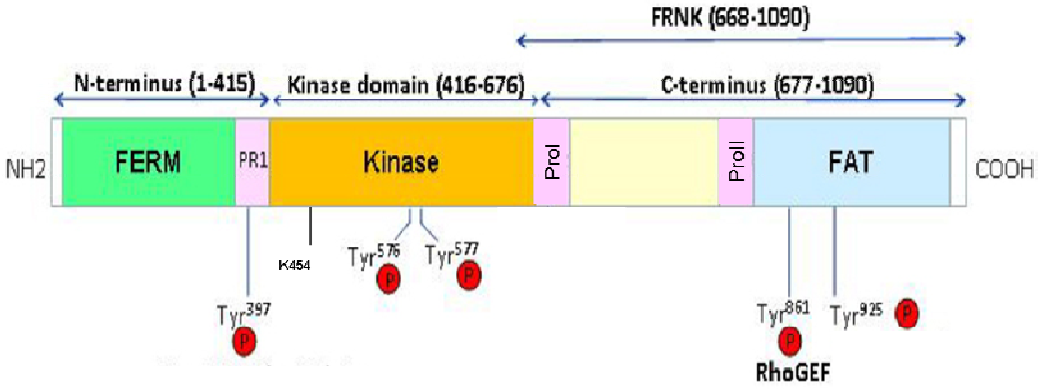

Figure 1.1 | Focal adhesion kinase (FAK) Structural Features. FAK has three major domains: the N-terminal domain, the central kinase domain and the C-terminal domain. Important tyrosine phosphorylation sites of FAK are shown. The N-terminal domain contains the Tyr397 autophosphorylation site. The kinase domain harbors the Tyr576/577, crucial for FAK kinase activity. The C-terminal domain has Tyr861 and Tyr925 phosphorylation sites and FAT region. FAK contains three proline-rich regions (PR1 and ProI, ProII), with PR1 in N-terminal and ProI-II in the C-terminal domain. FAT: focal adhesion targeting; FERM: protein 4.1, ezrin, radixin and moesin homology; FRNK: FAK related non-kinase. (†From [15], courtesy of the author)

with phosphotyrosine containing sequences or proline-rich sequences respectively [18]. However, the sequence between the kinase domain and the FAT sequence contains docking sites for SH3 domain-containing proteins, which serve as a scaffold for the recruitment of signaling proteins. FAK also has several sites of tyrosine phosphorylation, which could bind with SH2 domain-containing proteins so as to play important regulatory roles in FAK. Within the catalytic kinase domain there are two tyrosine residues, tyrosine 576 and 577, which regulate catalytic activity in the activation loop. Tyrosine 397, which lies just N terminal to the kinase domain, is the major site of FAK autophosphorylation. Phospho-Y397 also serves as a binding site for Src family kinases. The binding of FAK to Src will further phosphorylate FAK on several other tyrosine residues, including tyrosine 576,577,861 and 925, resulting in full activation of FAK. Therefore the autophosphorylation of FAK by its kinase domain or the tyrosine phosphorylation of tyrosine 397, is considered to be the most important for FAK functions. Other than Y397, FAK has other substrates including Grb7 and N-wasp [19, 20]. So the kinase activity of FAK could mediate more cellular functions than that mediated by autophosphorylation of Y397.

1.1.2 FAK activation

1.1.2.1 Integrin-mediated cell adhesion to ECM

Proper cellular function regulation, like proliferation and differentiation, requires specific external signals. These can be presented to target cells in the form of soluble peptide hormones, proteins retained on the surface of adjacent cells, or as part of the extracellular matrix (ECM) [21]. There are various ECM proteins, such as fibronectin, vitronectin, laminin and collagens. All of them bind to a family of heterodimeric transmembrane proteins called integrins. Integrins convey anchorage-dependent signals regulating cell proliferation and survival [22]. Integrins exist in α and β-

subunit heterodimers at the cell surface with short cytoplamic domains that functionally link change in ECM composition to alterations in the intracellular actin cytoskeleton network [23-25]. About 16α and 8β subunits can heterodimerize to form more than 20 different integrin receptors. These receptors cluster at focal adhesions, where cells make contact with the ECM. One of their roles is to link the interior actin cytoskeleton of cells with ECM proteins that comprise a complex multi-protein scaffolding and signaling unit that mediates cell adhesion and tensile forces important for cell motility. This structural linkage between the actin cytoskeleton and integrins is believed to be mediated by cytoskeleton-associated proteins such as talin and alpha-actinin which can bind to integrin cytoplasmic domains [26]. In addition to a structural role, integrin binding to ECM results in the activation of a number of signal transduction pathways similar to those stimulated by growth factors and cytokines. As integrin cytoplasmic domains do not possess intrinsic catalytic activity, signaling events must be mediated by associated proteins. Protein-tyrosine kinases (PTKs) play important roles in mediating signals generated by integrin clustering. The cytoplasmic PTK FAK associates with integrins at FAs and is activated by integrin clustering. Thus, FAK is regarded as one of the key components downstream of integrin signal transduction.

In fibroblasts, tyrosine phosphorylation of FAK requires that cells are adherent to the ECM and it is reduced in suspended cells [27]. FAK activation and phosphorylation apparently depend on clustering of integrins, and can be seen when suspended cells are incubated with anti-integrin antibodies or replated on ECM substrates. Enhanced phosphorylation and activation do not occur when cells are plated on poly-L-lysine, indicating that specific signals from ECM, transduced by integrins, can trigger FAK phosphorylation and activation. The presence of an ECM-

integrin-FAK cascade has been further supported by the evidence that FAK and paxillin can bind directly to peptides mimicking β integrin cytoplasmic domains [28].

The co-localization of FAK with integrins in FAs, which is mediated through FAT domain binding to proteins such as paxillin, is required for cell adhesion-dependent activation of FAK. FAK containing mutations in the FAT sequence fail to become tyrosine phosphorylated and activated in response to cell adhesion to fibronectin [29, 30]. On the other hand, Pyk2 which shares similar structural and sequence homology with FAK, shows a diffuse subcellular localization in the cytoplasm, and the tyrosine phosphorylation of Pyk2 is only weakly stimulated upon cell adhesion [31-33].

Upon the engagement of integrins to the ECM, enhanced protein-tyrosine phosphorylation [34-36], including that of FAK, occurs. This can lead to the activation of mitogen-activated proteins (MAP)-ERK [37-40] and c-jun N-terminal (JNK) MAP kinase cascades [41]. The actin cytoskeleton is also essential for tyrosine phosphorylation of FAK induced by integrin-dependent cell adhesion [42, 43]. As key regulators of the actin cytoskeleton, the Rho family of GTP-binding proteins and Rho itself promote contractility and the formation of stress fibers and focal adhesions [44]. The activation of Rho could induce tyrosine phosphorylation of FAK and inhibition of Rho blocks the tyrosine phosphorylation of FAK in response to integrin.

1.1.2.2 Other components that activate FAK

Src family kinases are another group of key components of FAK activation. In addition to the phosphorylation of Y397, an important event in the enzymatic activation of FAK is the phosphorylation of the tyrosine residues in the activation loop: Y576 and Y577. These residues, unlike Y397, are not substrates of

autophosphorylation of FAK, but are phosphorylated by Src family kinases [45]. Inhibition of Src signaling results in a reduction of the FAK phosphotyrosine content and FAK activation.

Overall, cell adhesion-dependent tyrosine phosphorylation of FAK occurs in two phases. The first, autophosphorylation phase, occurs in the absence of Src and results in the tyrosine phosphorylation of FAK residue 397. The second phase occurs following the recruitment of Src into forming a complex with FAK and results in the phosphorylation of FAK at other tyrosine residues leading to the full activation of FAK and transmission of downstream signals.

Along with integrin ligands and oncogenic forms of Src, several other agents also stimulate increases in the phosphotyrosine content of FAK, including mitogenic neuropeptides [46-48], lysophosphatitic acid [49-52], plateletderived growth factor [52], activated Rho [53], and v-Crk [5]. Thus, FAK is a point of convergence in the actions of a variety of factors known to influence cell morphology, locomotion, growth, and differentiation.

1.1.3 Binding partners of FAK and regulation of FAK activity

The N-terminal FERM domain of FAK shares a low level of sequence conservation with other FERM domain-containing proteins [14, 54], but the FAK FERM crystal structure revealed that it forms a predicted 3-lobed (F1-F3) structure [55]. The FAK FERM domain has been suggested to contain the putative integrin binding site. The truncations of the FERM-domain of FAK yield proteins with increased tyrosine phosphorylation and associated activities [56, 57], which suggested a negative regulatory role of FERM. A direct auto-inhibitory mechanism for FAK regulation was proposed by our lab. According to this model, the FAK FERM domain could bind the

FAK kinase domain and inhibit FAK activity in a trans manner [58].This model was further supported by the crystal structure of FAK residues 31-686 encompassing the FERM and kinase domain [59]. There are two major points of regulatory contact: the F1 FERM lobe binding to a linker segment containing Y397 and a hydrophobic pocket within the F2 FERM lobe to F596 within the FAK kinase domain. FAK FERM-mediated inhibition of FAK kinase activity therefore results from steric inhibition of target protein access to the catalytic cleft and to the Y397 autophosphorylation site. The speculation is that the release of FAK FERM binding to the catalytic domain, most likely by binding to proteins through the FERM domain and changing the conformation, will allow for FAK autophosphorylation at Y397, recruitment of Src-family PTKs by SH2 binding to Y397 site [59] and full activation of FAK. The significance of FAK Tyr397 phosphorylation may be a means by which FAK can recruit Src-family tyrosine kinases to focal adhesion structures. The association between Src and FAK could be detected in vivo in both Src transformed cells [60], and in non-transformed fibroblasts upon integrin stimulation by fibronectin [13].

FAK has five tyrosine phosphorylation sites (Tyr397,407,576,577 and 925) [13, 45]. Tyr397 is an autophosphorylation site that generates a high-affinity binding site for the SH2 domain of Src family PTKs, or PI3K, Grb7, etc. The interaction of Src with the FAK autophosphorylation site then leads to phosphorylation of Tyr407, 576 and 577, which maximizes the kinase activity of FAK in vitro [45]. Interacting of FAK with Src also results in the phosphorylation of Tyr925 on FAK, which creates a Grb2-binding site and therefore links FAK to the Ras/mitogen-activated protein kinase (MAPK) pathway [13]. These data suggested that by being phosphorylated, FAK can bind and integrate multiple signaling pathways in response to signals from the environment.

In addition to the phosphorylated tyrosine residues, FAK also has other motifs that mediate the binding between FAK and cellular proteins. The FAK C-terminal domain contains conserved proline-rich motifs. It has been shown that the SH3 domain of $p130^{cas}$ binds to a proline-rich motif (residue 711-717) in FAK [61]. Tyrosine phosphorylation of p130cas seems to be a key step in integrin-activated signaling by serving primarily as a docking protein to recruit additional signaling proteins containing SH2 domains into the focal adhesion complex. One such protein is c-Crk. In turn, c-Crk could promote Ras activation through a SH3-mediated association with guanine nucleotide-exchange proteins C3G ad SOS [62-64]. This would represent an adhesion-induced route to Ras activation distinct from the proposed FAK-Grb2 pathway [13]. Another potential $p130^{cas}$ interacting SH2 protein is the actin-binding protein tensin. The recruitment of tensin, together with FAK, to sites of integrin clustering has been suggested as an early step in the cascade of events involved in integrin signaling.

The second Pro-rich motif of FAK is also the binding site for several cellular proteins, including Graf (GTPase regulator associated with FAK) [65], ASAP (ADP ribosylation factor-GTPase activating protein) [66], and amphiphysin [67]. By binding to these proteins, FAK regulates various cellular functions, especially cytoskeleton remodeling, as well as migration and proliferation. Our lab recently identified that endophilinA2, one of the endophilin family proteins, binds to the second Pro-rich motif of FAK. Endophilins are a family of evolutionarily conserved proteins that can bind to the cytoplasmic side of membranes [68, 69]. There are two subgroups in this family, endophilin A and endophilin B. The A subfamily consists of A1, A2 and A3 members, but only endophilin A2 is ubiquitously expressed [70]. The critical role of endophilins is best characterized with endophilin A1, which is to regulate early and

late stages of endocytosis [71-73]. Both the N-terminal BAR domain and C-terminal SH3 domain of endophilin A1 are critical for its function in endocytosis. BAR domain encodes lysophosphatidic acid acyl transferase (LPAAT) activity and the SH3 domain can associate with synaptojanin-1 and dynamin. These protein-protein interactions are required for endocytosis [68, 69]. However, the role of endophilin A2 and A3 in endocytosis is less well understood. A previous study from our laboratory showed that in v-Src transformed MEF cells, endophilin A2 can bind to the Pro-rich motif of FAK via its SH3 domain and subsequently becomes phosphorylated by Src at Y315. PhosphorylatedendophilinA2 will reduce the endocytosis of MT1-MMP of v-Src transformed fibroblasts, so as to promote cell invasion [70]. This study suggested that FAK could potentially regulate tumor growth and metastasis through its interaction with endophilin A2, affecting endocytosis of MT1-MMP and possibly other proteins.

Through protein-protein interaction between FAK and various molecules (Figure1.2) [74], FAK transduces signals from the outside to the inside of cells and regulates diverse cellular functions.

1.1.4 Cellular functions regulated by FAK

1.1.4.1 Focal adhesion turnover

FAK was originally thought to function in the focal adhesion assembly. However, the study using v-src transformed fibroblasts suggested that FAK might perform the opposite function, i.e., promote the disassembly of focal adhesions [75]. Larger focal adhesions and defective actinin disassembly found in FAK-/- fibroblasts also supported this theory [76, 77]. The activity of Rho is found to be different between wild type FAK and FAK-null fibroblasts. In addition, by inhibiting Rho activity, FAK -/- fibroblasts can resemble wild-type in both morphology and focal adhesion

assembly [77]. This suggests that FAK regulates focal adhesion turnover by modulating Rho activity. The mechanism for FAK inhibiting Rho signaling could involve the FAK-binding partner GRAF (GAP for Rho associated with focal adhesion kinase), a negative inhibitor of Rho (RhoGAP) [65]. The phosphorylation of Graf by MAP kinase or related kinases has been suggested to be a mechanism by which growth factor signaling modulates Rho-mediated cytoskeletal changes in cells [78].

1.1.4.2 FAK and cell motility

Cell migration is a dynamic process requiring the integration of multiple signaling pathways. Migration of cells on ECM proteins requires the reiterative process of lamellipodia extension, integrin mediated adhesion, focal complex formation and the transition of focal complexes to focal adhesions, and finally the release of adhesion complexes at the rear of the cell [79]. Numerous experiments have demonstrated that FAK plays a central role in the dynamic regulation of cell adhesion structures. FAK has been shown to regulate the motility of cells in response to ECM proteins, a process named hapotaxis [76, 80]. Integrin signaling through FAK has been suggested to be involved in cell migration. Increased FAK phosphorylation and activity have been observed in migrating endothelial cells, and inhibition of FAK activity by the tyrosine inhibitor tyrphostin blocks their migration into wounded monolayers of cells [81]. In the repair of burn wounds, FAK is localized and activated in rapidly migrating and proliferating keratinocytes [82]. Furthermore, increased levels of FAK expression have been correlated with the invasive and metastatic potential of human tumors [83, 84]. Lastly, embryonic cells from FAK-deficient mice exhibited a decreased migration in culture, which was suggested to be responsible for a defect in mesodermal migration resulting in an embryonic lethality of the FAK-deficient mice [76]. Taken

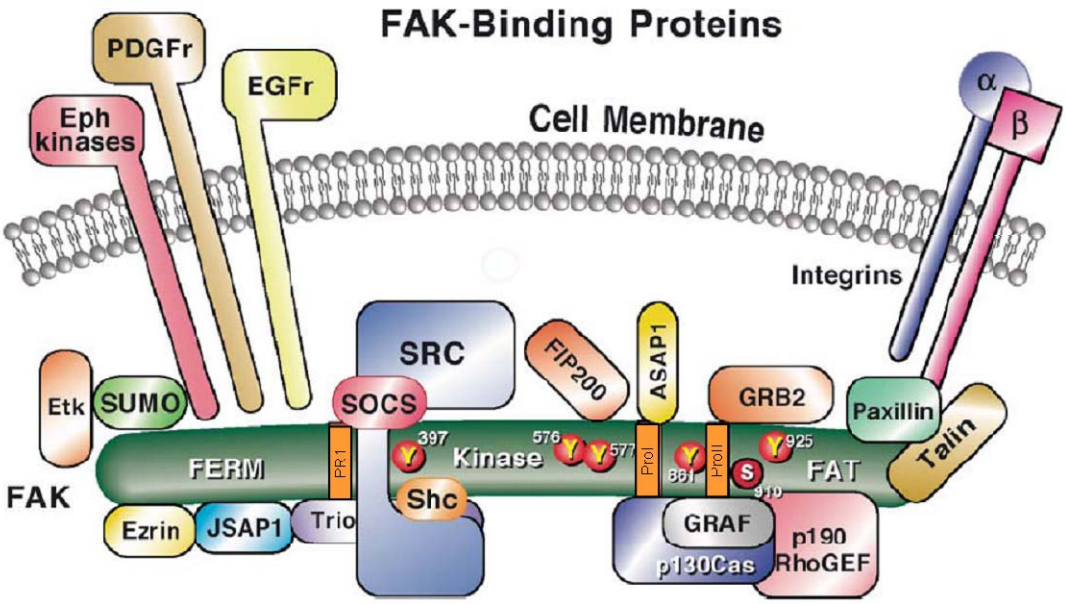

Figure 1.2 | Binding sites for FAK-associated proteins. The N-terminal FAK FERM domain is important for signal integration from growth factor receptor and integrins. The FAK C-terminal FAT domain binds cytoskeletal proteins paxillin and talin and mediates FAK localization to integrin-enriched focal adhesions. The FAT domain also makes direct contacts to p190RhoGEF. SH3 containing proteins such as p130cas, Graf, ASAP1 and endophilin A2 can bind to FAK C-terminal ProI and ProII motifs. Phosphorylation of FAK at Tyr397 creates high-affinity SH2 binding sites for Src-family PTKs and the Shc adaptor proteins, as well as FIP200, which inhibits FAK catalytic activity. Tyr-925 phosphorylation promotes the SH2 mediated binding of Grb2 adaptor protein. († From [74], courtesy of the author.)

together, all of these data strongly suggest that after stimulation with different growth factors or integrin engagement, increased expression and/or activation of FAK play a role in cell migration and invasiveness in biological processes such as embryonic development, wound healing and cancer [76, 81, 83, 84]. By expressing different FAK mutants in rescue experiments to dissect the signaling pathways involved, the autophosphorylation of FAK at Y397 and its subsequent association with Src were found to be crucial for FAK regulated migration [80, 85, 86]. Binding to growth factor receptors with N-terminus and targeting to focal adhesion by C-terminus are also indispensable [87]. The FAK binding partner Cas appears to function as an important 'molecular switch' for the induction of migration signals via its binding to the SH2/SH3-adapter protein Crk [35]. FAK is likely to play a direct role in regulating signals to the small GTPase Rac via interactions with Cas/Crk and paxillin/Crk and paxillin/PLK/PIX and to PI3K kinase by its direct binding to FAK. Both Rac and PI3K kinase are intimately involved in the regulation of cortical actin and lamellipodia and are essential for the process of cell migration [88]. However, the molecular mechanism of FAK-mediated motility differs depending upon the primary cell stimulus [89].

1.1.4.3 FAK and cell proliferation (or cell cycle progression) and apoptosis

The ECM transmits a cell survival signal in many cells. When cells are cultured in the absence of ECM, they undergo anoikis [90]. Inhibition of FAK, either by microinjection of the C-terminal fragment or by a monoclonal antibody, caused cell cycle arrest and apoptosis [91, 92]. However, overexpression of FAK can enhance DNA synthesis in fibroblasts [93, 94], and prevent anoikis and apoptosis induced in response to other stimuli [92, 95-101]. Integrin-mediated cell adhesion has been found

to regulate several cyclins and cyclin-dependent kinases (CDKs) [102-104]. These findings support the positive role of FAK in transmitting a cell survival signal. Several approaches have been taken to elucidate the mechanism of action of FAK in promoting cell survival. In MDCK cells, the association of FAK with PI3-kinase was specifically shown to be a prerequisite for the inhibition of apoptosis induced by ultraviolet light [98]. In HL60 cells, tyrosine 925, which binds Grb2 and links FAK to the Ras/MAPK pathway, was also required to inhibit apoptosis [101]. Further analysis using mutants of FAK and FRNK demonstrated that the p130cas binding site was required to prevent apoptosis of fibroblasts in the absence of serum [97]. Additional evidence also suggested that JNK was activated by FAK through recruiting and phosphorylating p130cas, which in turn signals to Rac, Pak1 and MKK4 [97]. In this system, expression of activated Akt alone failed to block apoptosis induced by overexpressing the FAT domain of FAK, suggesting that the PI3-kinase signaling alone was insufficient for blocking apoptosis [96, 97]. An alternative mechanism for the inhibition of apoptosis by FAK was demonstrated in HL60 cells. FAK overexpression appears to enhance NFκB activity and stimulate expression of cIAP-1, cIAP-2 and X-IAP, which are endogenous inhibitors of caspases and thus potent inhibitors of apoptosis [101, 105]. Recently, a study about the function of nuclear FAK revealed that FAK could regulate the degradation of p53 in a FERM domain dependent manner [106], and more detailed mechanisms on the function of FAK in regulating apoptosis would shed light on the design of strategies to inhibit FAK-promoted cell survival in experimental and pathological scenarios.

Compelling evidences from in vitro experiments have shown that FAK regulates numerous cellular functions (Figure 1.3). Recently, several mouse models modulating FAK expression or activity were created and FAK is found to be critical for processes

ranging from embryo development to cancer progression [86, 107-110]. More detailed information and mechanisms involved in FAK regulation of various biological processes will be achieved by the studies on these mouse models.

1.2 FAK in vascular development and blood vessel functions

1.2.1 Angiogenesis during development

During embryonic development, the first organ system that forms is the cardiovascular system [111], which involves two stages: vasculogenesis and angiogenesis [112, 113]. Vasculogenesis is the development of blood vessels from in situ differentiating endothelial cells, and it seems to be restricted to early developmental periods. Endothelial cells derive from the successive differentiation of mesodermal cells into hemangioblasts, which leads to the formation of the first vascular structures that are called primitive blood islands. The hemangioblasts from the center of the islands give rise to the hematopoietic stem cells, whereas the peripheral hemangioblasts differentiate into angioblasts, the precursors of mature endothelial cells. Under the influence of VEGF, the angioblasts and newly formed endothelial cells migrate on a matrix consisting mainly of collagen and hyaluronanm, allowing the fusion of the blood islands, their remodeling into tubular structures, and the formation of the first primitive vascular plexus. These tubules remodel through vasculogenesis into large vessels, leading to vascularization of the embryo [114]. Angiogenesis refers to the sprouting of capillaries from pre-existing vessels and in contrast to vasculogenesis, angiogenesis occurs not only in the early embryo but also during the entire lifespan, including during wound healing, tissue regeneration, and tumor growth [113].

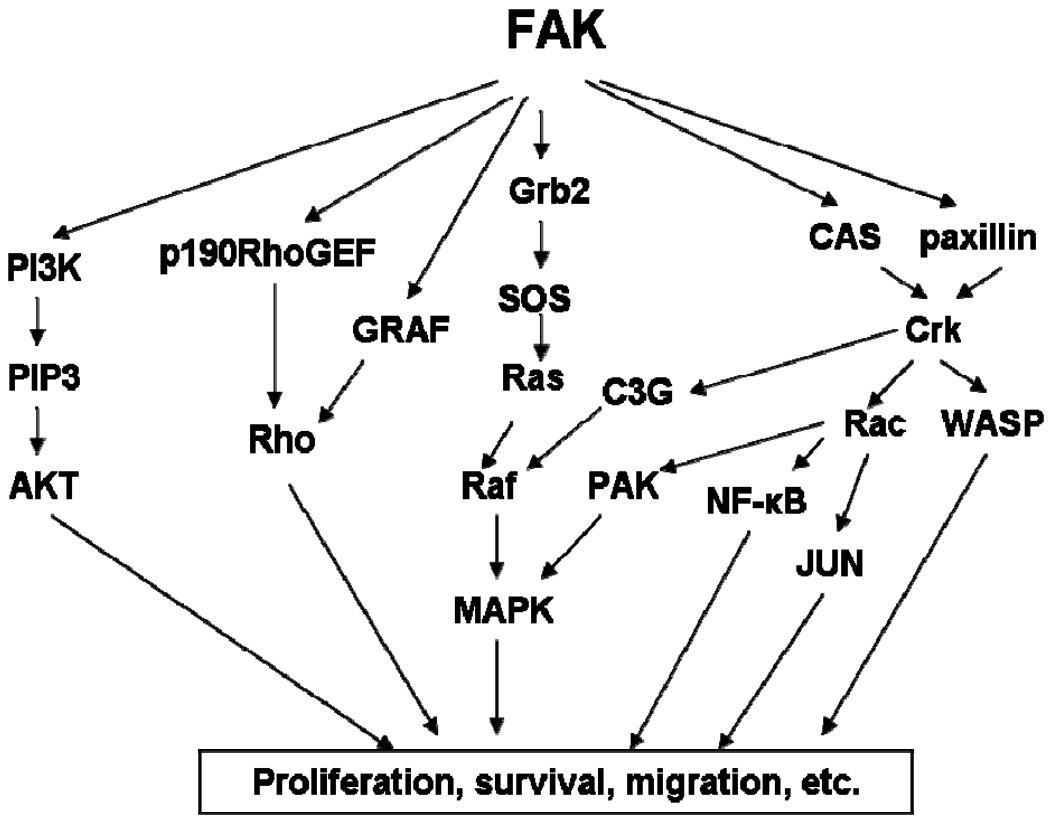

Figure 1.3 | Downstream signaling pathways and cellular functions regulated by FAK. Upon its activation and autophosphorylation in cell adhesion, FAK can lead to activation of several downstream signaling pathways. Cellectively, these downstream pathways regulate various cellular functions controlled by integrin-mediated cell adhesion or other stimulations.

The turnover of endothelial cells in the normal adult humans is very low, in the order of years [115]. And this process is tightly controlled when it happens, for example, during reproduction and wound healing. In contrast, persistent uncontrolled angiogenesis occurs during solid tumor growth, retinopathies and other pathological disorders that have been called angiogenic diseases [116]. The mechanism involved in the regulation of angiogenesis might be best studied during embryonic development because the vascular pattern is reproducible and forms in the absence of inflammatory processes. These studies could shed light on the mechanisms regulating angiogenesis under pathological conditions.

1.2.2 FAK and angiogenesis

A potential role of FAK in angiogenesis has been suggested by a number of studies. During the mouse embryo development, FAK expression becomes increasingly restricted to the blood vessels [47]. The first FAK total knockout mouse was created in 1995 [76]. The early embryonic lethality of these FAK-null mice confirmed the essential role of FAK in mouse development, for the mesenchymal cells and cardiovascular system in particular [76]. Studies in a number of other model systems also support the role of FAK as a key regulator in angiogenesis [86, 107, 117]. Inhibition of FAK signaling in endothelial cells in culture using dominant negative, anti-sense or knockout strategies impairs the ability of the cells to form tubules in matrigel [118]. Furthermore, a transgenic model with FAK overexpression in vascular endothelial cells was created and these mice exhibit increased angiogenesis in both hind limb ischemia and wound-induced angiogenesis models [117]. The compelling evidence supporting FAK's role in angiogenesis comes from the endothelial cell conditional knockout mice created in our lab as well as another lab [86, 107]. Our studies showed that the conditional knockout mice die around E13.5, in contrast with

the FAK null mice which die by E8.5 [76]. Despite the loss of FAK expression in the conditional knockout in endothelial cells by E8.5, because of the early expression of Tie2 Cre recombinase, the vasculature looks normal before E11.5, but exhibits severe phenotypes at a later stage of development (mid-late gestation). These include the reduced vasculature on the yolk sac and head region, various sizes of hemorrhages and systemic edema. This suggests that the establishment of the vasculature can occur relatively normally in the absence of FAK expression in the endothelium, but vascular remodeling and elaboration of the vascular system via angiogenesis fails to occur without FAK. Further, characterization of these animals and in vitro cellular function assays on FAK deficient endothelial cells revealed that increased apoptosis and defective motility may be responsible for these phenotypes. Using a different Tie2-Cre strain, Braren et al. reported that FAK in ECs is required for early embryogenesis [107]. Deteriorated vessels in the conditional knockout mouse and vessel regression in embryonic explants from mutants indicated that FAK provides survival and stabilizing signals to ECs, thereby sustaining vascular integrity [107]. Recently two different FAK mutant knockin mouse models were created to further explore the role of FAK in development. One involves a mutant FAK with exon 15 deleted, which encodes the autophosphorylation site, Tyr-397. This mutant FAK (FAKΔ) expresses at normal levels and acts as an active kinase. $FAK^{\Delta/\Delta}$ embryos displayed hemorrhages, edema, delayed artery formation, vascular remodeling defects, multiple organ abnormalities, and overall developmental retardation at E13.5–14.5, and died thereafter. This study demonstrates that FAK autophosphorylation is necessary for normal vascular development. The other mouse model has homozygous point-mutation within the catalytic domain (lysine454 to arginine, R454), which resulted in defects in blood vessel formation as determined by lack of yolk sac primary capillary plexus formation and disorganized endothelial cell patterning in embryos [119]. This indicates that FAK

kinase activity is important in the process of embryonic vascular network formation. However, neither of these studies further analyzed the role of FAK autophosphorylation and kinase activity in the endothelial cells. Therefore their roles in angiogenesis are still not clearly understood.

1.2.3 FAK regulates several EC functions

1.2.3.1 Survival

FAK has been shown to be a key molecule in transducing survival signal for endothelial cells from ECM. In vitro studies on the FAK null (by deleting FAK flox allele utilizing adenovirus-Cre infection) endothelial cells carried in our lab showed that FAK is essential for protecting endothelial cells from undergoing apoptosis [86]. However, the mechanism involved in the regulation of apoptosis has not been clearly revealed. A number of different mechanisms of FAK-dependent cell survival in several cell types have been proposed. One mechanism involves the FAK/p130cas complex, which signals through the Ras/Rac1/Pak1/MKK4 pathway to activate JNK to promote cell survival [97]. A second mechanism involves the activation of PI3 kinase which can promote survival by activating Akt or by increasing the expression of inhibitors of apoptosis (IAPs) in an NFκB-dependent manner [101, 120]. In endothelial cells, mechanisms that impact p53 are of particular interest, since serum starved endothelial cells undergo apoptosis in the absence of FAK via a p53-dependent pathway [96]. The N-terminal FERM domain FAK was also proposed to bind directly to the transactivation domain of p53 in vitro, though not in endothelial cells [121]. In fibroblasts, the FERM domain of FAK functions as a scaffold by binding both p53 and mdm2, promoting p53 ubiquitination and thus regulating the degradation of p53 [106]. This mechanism operates to promote cell survival in fibroblasts treated with cisplatin.

1.2.3.2 Proliferation

FAK promotes cell proliferation in cultured endothelial cells [91, 122]. Multiple mechanisms of regulation are proposed and the major pathways converge upon the cyclin dependent kinases and are active in late G1. In endothelial cells in culture, VEGF stimulation induces the degradation of the p21 and p27 cyclin dependent kinase inhibitors, which promotes progression through G1 into S phase. Inhibiting FAK blocks p21 and p27 degradation and the elevated levels of p21 and p27 impair VEGF induced cell proliferation [122]. In this scenario, FAK regulates p27 degradation through the F-box protein Skp2 and p21 degradation via an unknown Skp2-independent mechanism (which is proven to regulate the p21 and p27 by FAK in other cells). Note that under certain conditions FAK may act as an inhibitor of proliferation. Poorly adherent endothelial cells and fibroblasts fail to proliferate in the presence of serum. Inhibition of FAK by expression of a dominant negative mutant in endothelial cells promotes proliferation and FAK null fibroblasts also proliferate under these conditions [123].

1.2.3.3 Migration

Endothelial cell migration is an essential component of angiogenesis. This process requires a tight regulation of the contractility of the cells. Directed migration of EC is divided into six phases: (i) sensing of a chemoattractant, (ii) extension and protrusion at the leading edge, (iii) attachment by assembly of focal contacts, (iv) contraction of the cell body by formation of stress fibers, (v) release of the rear edge by disassembly by focal contacts, and (vi) recycling of adhesive and signaling components [124]. All six stages are highly regulated by a variety of signaling cascades. The vascular endothelium is supported by an ECM that is assembled by endothelial cells, pericytes, and supporting smooth muscle cells. Vascular endothelial cells should adhere to ECM

to migrate either dependently or independently of chemoattractants. This ECM is critical for endothelial cell migration. Endothelial cells are connected to ECM at focal adhesions. They are assembled following the recruitment of signaling molecules such as FAK and paxillin and of structural and membrane actin-anchoring proteins such as vinculin and α-actinin, which links the microfilament network to integrins at their site of clustering [24, 125]. In migrating endothelial cells, focal adhesions and actin stress fibers are aligned in the direction of migration, supporting their participation in the process of actin-based motility [81]. Moreover, in migrating endothelial cells, the forward movement is tightly associated with the rapid assembly/disassembly of the focal adhesions, which allows the adhesion/de-adhesion processes inherent to migration.

Endothelial cell migration is essential for blood vessel morphogenesis and angiogenesis and may be very sensitive to FAK levels. There were several studies suggesting a role for FAK in EC migration. Several inhibitors of angiogenesis have been reported to reduce tyrosine phosphorylation of FAK and inhibit cell migration [126]. Overexpression of dominant-negative FAK in ECs decreases VEGF induced PI3K activation and cell migration [118, 127]. Antisense inhibition of FAK mRNA severely reduces migration of HUVEC and endothelial EaHY 926 cells [118, 128]. Knocking-down of FAK by infection with adenovirus carrying siRNA in HUVECs resulted in reduced motility and wound healing. Furthermore, tube formation induced by type I collagen was also inhibited in these FAK-knockdown HUVECs [129].FAK was also shown to regulate the migration of human umbilical cord blood mesenchymal stem cell (hMSC) upon hypoxia [130]. Finally, deletion of FAK in ECs isolated from conditional FAK deficient mice leads to reduced migration [86].

Several mechanisms have been proposed for the role of FAK in regulating of cell migration. Major factors that promote formation of focal adhesion and assembly of actin stress fiber are small GTPases of the Rho family. FAK may suppress Rho activity to promote focal adhesion disassembly [77], as well as other Rho family proteins, like Rac. Rac is activated in a p130cas-dependent manner downstream of FAK and cdc42 may be regulated by FAK modulation of N-WASP [20]. FAK has also been shown to interact with the Arp2/3 complex [87].The mechanisms of modulating the actin cytoskeleton by FAK are likely to contribute to its control of cell migration.

A number of studies utilized other cell types than endothelial cells, thus it remains to be determined if all of these mechanisms for regulating cell survival, proliferation or migration, also operate in endothelial cells. As these mechanisms were observed in cells in culture, it is also necessary to establish the mechanisms operating in vivo under physiological and pathological conditions.

1.2.3.4 Endothelial permeability (blood vessel integrity)

The endothelium provides a barrier between the blood and surrounding tissues, and the permeability of this barrier changes in response to physiological and pathological stimuli. Defective permeability regulation would lead to various phenotypes, including hemorrhages and edema, and embryonic lethality of mice in severe conditions. Various soluble ligands and cell-cell interactions modulate permeability and FAK may play a role in regulating permeability in both scenarios, though it is still controversial if FAK plays either a positive or negative role. In some studies, FAK was shown to promote barrier function, since blocking FAK with a dominant negative mutant increases the permeability of endothelial cell monolayers under steady state conditions [131]. Inhibition of FAK activity also resulted in blocked recovery of junctional

opening initially induced by low doses of H_2O_2 in one study [132]. A second study reports the exact opposite, that inhibition of FAK reduces H_2O_2 induced monolayer permeability [133]. In isolated porcine coronary venules and monolayers of endothelial cells, VEGF induces an increase in permeability, which is attenuated by expression of a dominant negative mutant of FAK [134, 135], suggesting that FAK might promote permeability.

The regulation of endothelial permeability has important consequences. Vasogenic edema, which can occur in the heart following myocardial infarction or in the brain following stroke, is caused by increased endothelial permeability and may be induced by local production of VEGF in response to ischemia [136]. FAK was noted to be regulated by VEGF in a Src-dependent manner, and it is likely that FAK also plays a role in regulating vascular permeabilization following ischemia. Another important physiological process regulated by endothelial permeability is the transendothelial migration of leukocytes to inflammatory sites from the underlying tissue. When activated neutrophils attach to the endothelium of isolated vessels or to endothelial cell monolayers, permeability is increased. Expression of a FAK dominant negative mutant inhibits the increase in permeability induced by neutrophil attachment [137]. The explanation for the obvious opposite roles for FAK in regulating permeability is not clear, but it is possible that different cell types, or different conditions could lead to different experimental results. One of the possibilities is that, different stimuli might promote differential phosphorylation of FAK, i.e., phosphorylation on different tyrosine residues, and the assembly of different protein complexes, e.g., FAK/αvβ5. This could result in differential subcellular localization and/or direct FAK towards different substrates, which could lead to the transmission of different signals that either promotes barrier function or permeability. Another issue

is that, in some of the studies, a dominant negative strategy to inhibit FAK was used and the results could be confounded by off target effects, e.g., by inhibition of the FAK-related kinase Pyk2, which has been implicated in promoting cell permeability [138]. Inhibition of FAK by additional experimental approaches would provide important insights.

1.2.4 FAK and tumor angiogenesis

Solid tumor growth and survival requires a functioning vascular network, which enables rapid proliferation of tumor cells by the delivery of oxygen and other nutrients and facilitating the development of metastatic disease. More and more evidence suggested that tumor growth is dependent on angiogenesis. After the early phase of solid tumor growth, every increase in tumor cell population must be preceded by an increase in new capillaries converging on the tumor. The tumor vasculature is derived from sprouting of local blood vessels (angiogenesis) and circulating vasculogenic progenitor cells derived from the bone marrow (vasculogenesis). The new vessels are often irregular and leaky due to the loss of adherence between endothelial junctions, as well as lack of the pericyte cover, with the result that tumor cells can penetrate them more easily. As a result, the onset of angiogenesis also contributes to metastasis, though the capacity of tumor cells to induce angiogenesis does not always correlate with their degree of malignancy. Neovascularization permits the shedding of cells from the primary tumor [139], and decreased angiogenesis is associated with a decreased rate of metastasis.

Several sequential steps can be highlighted during tumor angiogenesis. In mature capillaries, the vessel wall is composed of an endothelial cell lining, a basement membrane and a layer of pericytes which partially surround the endothelium. The pericytes are contained in the same basement membrane as the endothelial cells and

occasionally make direct contact with them. Angiogenic factors produced by tumor cells bind to endothelial cell receptors and initiate the angiogenesis. Once the endothelial cells are stimulated to grow, they secrete protease, heparanase, and other digestive enzymes that can digest the basement membrane surrounding the vessel. Matrix metalloproteinases (MMPs), a family of metallo-endopeptidases secreted by tumor cells or surrounding cells, play important roles in degradation of basement membrane and ECM surrounding pre-existing capillaries. The dissolution of ECM also allows the release of proangiogenic factors bound to them [140]. The junctions between endothelial cells become altered, cell projections pass through the space created, and the newly formed sprout grows toward the source of the stimulus. Endothelial cells invade ECM and begin to migrate and proliferate into the tumor mass, where newly formed endothelial cells organize into hollow tubes (canalization) and create new basement membrane for vascular stability. The formation of the lumen during canalization is driven by important interactions between cell-associated surface proteins and the ECM. Some of the surface proteins identified in this interaction include galectin-3, PECAM-1, and VE-cadherin [141, 142]. However, the detailed mechanism controlling the balance between pro- and anti-angiogenesis is still poorly understood.

Knowledge of molecular mediators of angiogenesis is fundamental in understanding the mechanisms that control its pathways and may ultimately be useful in developing therapies for angiogenesis-related diseases, including cancer. Several proteins are known to activate endothelial cell growth and movement such as angiogenin, VEGF, FGF, estrogen, interleukin 8, TNF-α and prostaglandin E1 and E2. In addition, there are many other gene products, ranging from transcription factors to the Notch family members that are essential during new vessels formation. However,

the presence of angiogenic factors is not enough to initiate the new vasculature growth. The influence of proangiogenic factors is counterbalanced by a number of inhibitory agents, such as endostatin, angiostatin, thrombospondins and tissue inhibitors of metalloproteinases. The net result of these opposing factors on the vascular endothelial cell determines the outcome of angiogenesis homeostasis. In most tissues, the overall lack of angiogenesis in physiological situations results from the balance of a complex multifactorial system consisting of stimulators and inhibitors of angiogenesis. An imbalance in any one of these proteins may lead to a switch toward an angiogenetic phenotype. Owing to the multitude of angiogenetic signals triggered by tumor cells, it is unlikely that a straightforward inhibition of angiogenic stimuli will be an effective approach for cancer therapy. A better understanding of the mechanisms could possibly lead an effective treatment to suppress angiogenesis and therefore tumor growth.

The role of FAK in the tumor angiogenic switch has been proposed and studied because of the close correlation between elevated FAK expression and an aggressive tumor phenotype [143]. FAK was shown to be recruited and activated upon VEGF binding to VEGFR-2 [127]. Additional support for a role of FAK in tumor angiogenesis comes from studies of the involvement of integrins, the major upstream activators of FAK in the process. It has been shown that the blockade of integrins αvβ3 with monoclonal antibodies or small molecules inhibits tumor angiogenesis in a variety of animal models [144-146]. Moreover, Mitra et al. found that inhibiting FAK activity via stable FRNK expression in 4T1 breast carcinoma cells resulted in small avascular tumors in mice upon injection [147]. FRNK inhibited a FAK-Grb2-MAPK-signaling linkage regulating VEGF expression without interfering cell proliferation or anchorage-independent cell survival. Reconstitution experiments using Src-transformed FAK-null fibroblasts confirmed that FAK catalytic activity and Y925

phosphorylation are essential to promote MAPK- and VEGF-associated tumor growth. Inhibition of FAK expression in breast, prostate and neuroblastoma cells also resulted in reduced VEGF expression. This study reveals that FAK plays an important role in tumor angiogenesis and strongly supports the idea of developing inhibitors for FAK as antitumor agents. TAE226, an inhibitor of FAK was used in therapy experiments and FAK inhibition by TAE226 significantly reduced tumor burden and prolonged survival in tumor-bearing mice [148]. The therapeutic efficacy was related to the reduced pericyte coverage, induction of apoptosis of tumor-associated endothelial cells and reduced microvessel density. Nevertheless, a direct demonstration of FAK in tumor angiogenesis in mouse models is still lacking due to the embryonic lethality, albeit late, of the EC-conditional KO mice. A recent study established inducible inactivation of FAK in ECs, but it was found that the FAK related kinase Pyk2 was up-regulated and compensated for the loss of FAK in this mouse model [149], which kept the role of FAK in adult angiogenesis from being fully understood, and more work needs to be done for further exploration.

1.3 The role of FAK in mammary gland cancer

1.3.1 FAK and Mammary gland development

Mammary gland development is a dynamic process in which proliferation, apoptosis, differentiation, and migration are essential for the formation of highly organized branched ductal network of epithelial cells. Mouse breast tissue undergoes continuous changes throughout the lifespan of reproductively active females, mediated by interactions between the mammary epithelium and the surrounding mesenchyme. Although the mammary gland is already present in embryos, most of the branching morphogenesis that is required for the development of the ductal tree occurs postnatally, when terminal end buds appear, and the process of ductal elongation

commences [150]. At the onset of puberty, the mammary ducts begin to branch, and alveolar buds appear at the ends of these branches. These alveolar buds are the precursors of the secretory units called alveoli. During pregnancy and lactation, the mammary gland further differentiates into a fully branched ductal network that creates a system of ducts that collects milk produced by alveoli. After weaning, the mammary gland undergoes involution during which the mammary gland represses to an almost pre-pregnant state [151]. Structurally, the mature mammary gland is a ramified tubuloaveolar gland (parenchyma), embed within a heterogeneous connective tissue (mesenchyma or stroma). Mammary parenchyma is composed of two types of differentiated epithelial cells: secretory luminal epithelial and contractile myoepithelial cells. These two types of cells together form the functional units of the mammary gland, the acini. The luminal cell lineage can be further subdivided into ductal and alveolar luminal cells that line the ducts and constitute the alveolar units that arise during pregnancy, respectively. In contrast, the myoepithelial cells are specialized, contractile cells surrounding the luminal epithelial cells, and are bound through integrin receptors to the laminin-rich basement membrane [152]. The complex signaling by growth receptors, endocrine receptors, and cell adhesion receptors leads to the coordinated myoepithelial and luminal epithelial cell biological process, including cell contraction, proliferation, survival, differentiation resulting in the restructuring of acini, and the development of functional lactating alveoli [153, 154].

Like other epithelial and endothelial cells, mammary epithelial cells require adhesion to the extracellular matrix for their survival and proliferation. As the major cell surface receptors that mediate adhesion between cells and the exracellular matrix (ECM), Integrins have key roles in mammary gland development and the maintenance of homeostasis during adulthood. They are present in all cell types within the gland

and control many aspects of cell phenotype, including cell adhesion, spreading and migration, cellular differentiation, cell survival, and cell cycle progression [106, 155]. The mechanisms by which integrins regulate cellular responses involve both organization of the cytoskeleton and activation of kinase- and GTPase-mediated signaling pathways; in many instances they are essential for the optimal signaling of growth factor receptors [106, 156]. The cytoplasmic tails of most integrins are short and devoid of enzymatic features. Therefore, integrins rely on the association with adaptor proteins to link them to the actin cytoskeleton or an interaction with cytoplasmic enzymes for their signaling activity.

FAK is a critical component that integrates cell-ECM interactions due to its ability to bind many adaptor proteins and other signaling enzymes. specific deletion of FAK in mammary gland epithelium resulted in reduced branch points and mild retardation of mammary ductal elongation in virgin mice [109, 152]. During pregnancy and lactation, FAK deletion causes severe lobulo-alveolar hypoplasia. Proliferation of mammary epithelial cells stimulated by 17β-estradiol benzoate and progesterone in these conditional knockout mice is greatly reduced [109], though the receptors for both estrogen and progesterone are not affected.

1.3.2 The role of FAK in breast cancer

Breast cancer is the most commonly diagnosed cancer among women in the United States and worldwide, and is the second leading cause of cancer-related death in women [157]. Although there has been significant progress in the understanding of the molecular and cellular mechanisms of breast cancer over the past several decades, the mechanisms involved in tumor formation and metastasis are still not fully understood.

Among the different types of local cues for epithelial fate decisions, cell adhesion to ECM is of central importance. Using three-dimensional culture models of mammary ductal structure and tumorigenesis, it has been shown that integrins, which mediate cellular adhesions to ECM, play a substantial role in regulating the malignant phenotype and growth of epithelial cells [158, 159]. Moreover, it has been demonstrated that targeted deletion of β1-integrin in the mammary epithelium results in inhibition of mammary tumorigenesis, and the β1-integrin is required for continued tumor cell proliferation [160]. Focal adhesions (FAs) are sites of integrin-clustering and are composed of a large complement of scaffolding and signaling proteins that link the actin cytoskeleton to the ECM [161]. In addition to providing physical attachment to the ECM, these complexes also act as a signaling node from which multiple signaling cascades emanate to regulate cell proliferation, survival, and migration [88, 162-165]. Furthermore, different growth factors and their receptors are shown to play a role in carcinoma development and metastasis, including the epidermal growth factor (EGF) and insulin-like growth factor (IGF) family of receptors. These receptors also co-localize and participate in signaling cross talk with integrins at FAs [89, 166-169].

As a key mediator of signal transduction from ECM and a point of convergence between integrin and growth factor signaling, FAK has been suggested by a large body of evidence to play a role in breast cancer. The levels of FAK mRNA and protein are found to be significantly elevated in invasive and metastatic breast tumor specimens in comparison to paired normal tissues, suggesting a role of FAK in promoting breast cancer invasion and metastasis [84]. Subsequent analysis revealed that FAK overexpression is not restricted to the invasive phenotype, but rather appears to be an early event in breast tumorigenesis [170, 171]. The evolution of a normal,

finite-lifespan somatic epithelial cell into an immortalized metastatic cell requires deregulation of multiple cellular processes, including genome stability, proliferation, apoptosis, motility and angiogenesis [172], and FAK could regulating some or all of these processes.

Recent studies using both xenograft mouse models and conditional KO mice have provided more direct evidence for a causative role of FAK in cancer. Such in vivo data was initially obtained in a xenograft mouse model in which a constitutively active recombinant FAK (CD2-FAK) was found to transform MDCK cells and induce tumor formation in immunodeficient mice [95]. Several subsequent studies provided additional support for the requirement of FAK in both tumor formation and progression in xenograft mouse models of tumors [165, 168, 173, 174]. Overexpression of FAK in U-215MG human malignant astrocytoma cells leads to an increase in tumor volume and cell number which are attributed to increased tumor cell proliferation [175]. Correspondingly, epidermis-specific deletion of FAK reduces chemically-induced papilloma formation and blocks malignant conversion [176]. Consistent with these findings, expression of FAK inhibitory protein, FAK related nonkinase (FRNK) in rat mammary adenocarcinoma cells results in decreased tumor volume and significantly decreases the number of lung metastases in a rat xenograft model [177]. In recent years, mice with mammary epithelial-specific loss of FAK were generated, and the role of FAK in mammary tumor development and progression was further confirmed [110, 178-180]. Targeted disruption of FAK in mammary tumor cells can delay the tumor initiation and block the progression of hyperplasias to full carcinomas in PyVT mouse models. This block in tumor progression was further shown to be correlated with a dramatic reduction in the ability of the mammary tumor cells to proliferate [178]. The retardation of tumor initiation and suppression of tumor

growth were further confirmed by other investigations on PyVT mouse models with more efficient deletion of FAK in the mammary tumor cells [110, 179]. Furthermore, FAK deficiency of mammary tumor cells resulted in less efficient metastases of those cells to lungs. Disruption of FAK was proven to attenuate cell migration, in addition to proliferation and deregulate metastasis-associated protein activities, including Src and p130Cas [110, 180-182]. More detailed studies of the role of FAK in mammary epithelial cell transformation and tumor progression were carried out by transplantation assay [179], and the results suggested that FAK is required to maintain the oncogenic potential of PyMT-transformed cells. Further studies showed that FAK sustains the core functions that underlie tumorigenesis by enabling Src-mediated phosphorylation of p130Cas. Not limited to PyMT, the requirement of FAK during mammary tumorigenesis is confirmed in Neu and Ras transformed mammary tumor cells too, though to a lesser extent [179].

All of these observations provide compelling evidence that FAK is an integral and necessary component in the network of signaling interactions that initiate and support mammary tumorigenesis. However, the molecular and cellular mechanisms involved are not well understood.

1.3.3 FAK and mammary cancer stem cells

Stem cells have long been investigated for their central role in organ development, but recent studies have shown that cells with stem and/or progenitor characteristics play critical roles as well in tumor formation and progression [183]. Stem cells have been characterized as having low proliferation rates, existing as minority populations within tissues in defined niches, and having responses to extracellular stimuli that are distinct from those of the more differentiated cells within the organ [184]. Stem cells exhibit self renewal and can divide symmetrically, to produce additional stem cells, or

asymmetrically, to generate progenitor cells that can subsequently differentiate into the many different cell types within the organ. The breast tumors are comprised of phenotypically diverse populations of breast cancer cells. Functional assays show that only a specific subset of cells within a tumor is able to propagate tumor growth in immunodeficient mice whereas other cells are unable to propagate tumor growth. It appears that these cancer cells responsible for tumor propagation are able to self-renew as well as give rise to cells that can not propagate tumor growth. Thus, the "cancer stem cell theory" states that these tumor forming cells have two main properties, i.e., self-renewal and differentiation [185, 186]. For tumors containing a subpopulation of cancer stem cells, there are at least two ways that the cancer stem cells could have arisen. In the first, oncogenic mutations may inactivate the constraints on normal stem cell expansion, resulting in cancer stem cells that originate from normal stem cells. For example, mutations could affect pathways that keep stem cells dependent on the niche for self-renewal. Alternatively, cancer stem cells could produce factors that recruit niche-forming cells into the tumor, resulting in an effective expansion of the niche itself. In the second, oncogenic mutations may allow transit-amplifying cells to continue to proliferate without entering a postmitotic differentiated state. This could imbue transit-amplifying progenitor cells with stem-cell-like properties, therefore creating a pool of self-renewing cells in which further mutations can accumulate. This pool ultimately may give rise to cancer stem cells that originated from a more differentiated cell.

Recently, it was shown that a single breast stem cell could regenerate the entire breast tissue [187, 188]. The ability to prospectively identify tumorigenic cancer stem cells will facilitate the elucidation of pathways that regulate their growth and survival. Because these cells drive tumor development, strategies designed to target this

population may lead to more effective therapies. Markers that were useful for the isolation of normal stem cells were investigated for the study of breast cancer stem cells. Shackleton et al. identified a group of cells as $CD29^{hi}$ $CD24^{+}$ within the mouse breast tissue that contain cells capable of regenerating a mouse mammary gland called mammary stem cells (MaSCs). Stingl et al. described cells with similar mammary gland forming characteristics as mammary repopulating units (MRUs) in vivo, and identified as being CD24 $^{med}CD49f^{hi}$. Additional makers were identified, including $Thy1^{+}$ $CD24^{+}$ cells are found to be highly enriched for tumor forming cells and referred to as tumorigenic (TG) cells [189]. Other markers commonly used in characterizing mammary stem cells include smooth muscle antigen, common acute lymphoblastic leukemia antigen, p63, vimentin, CD44, keratin 14 (K14), K18, K19, CD133, MUC1, and ErbB2 [190].

Several lines of evidence suggest that FAK can play a role in breast cancer through regulating mammary stem cells (MaSCs) and mammary cancer stem cells (MaCSCs). Firstly, high levels of specific integrins expression in mouse MaSCs are acknowledged, and in addition, a high level of β1 integrin has also been shown as a marker of MaCSCs. A recent study has revealed that ablation of β1 integrin in the basal compartment of mammary epithelium impaired the regenerative potential of MaSCs [191]. As a major mediator of integrin signaling, FAK is more active in MaCSCs, suggesting a possibly essential role of FAK in MaSCs and MaCSCs. Secondly, MaSCs, progenitor cells and MaCSCs are able to evade anoikis and survive in an anchorage-independent manner, by the indication of forming mammospheres in serum-free suspension culture [192-194]. Since FAK has been shown to be important for the resistance to anoikis in several cell lines, the activity of FAK could be one of the regulators to control self-renewal/survival of MaSCs and MaCSCs under

anchorage-independent conditions in vitro and possibly in vivo. Lastly and more importantly, by using a conditional FAK knockout model that specifically ablates FAK in MaECs during early embryonic state, our lab has shown that FAK ablation in the PyMT breast cancer model has a severe impact on mammary cancer stem/progenitor cells. FAK deletion reduced the pool of cancer stem/progenitor cells in primary tumor development in FAK targeted mice, decreased their self-renewal and migration in vitro, and compromised their tumorigenicity and maintenance in vivo [180]. These studies raised the possibility that the defects in mammary cancer stem/progenitor cells induced by FAK deletion may contribute at least in part to the suppressed mammary tumorigenesis and progression in these FAK targeted mice. This suggests that FAK may serve as a potential target to eliminate breast cancer stem cells, leading to a cure of breast cancer.

1.4 Project overview

The roles of FAK have been well-characterized by numerous studies in the past two decades, and it has been proven to be critical for many physiological and pathological processes via both in vitro and in vivo methods. The functions of the kinase activity (K454) and the second C-terminal proline-rich motif of FAK have been explored too, by transient or stable expression of genetically modified FAK constructs in cells in vitro. Due to the possible inconsistency of results generated from in vitro experiments and that from in vivo, the endogenous roles played by this site/motif during development or under pathological conditions remain unclear. My project has focused on the analysis of FAK kinase activity and its second C-terminal proline-rich motif in developmental angiogenesis and mammary tumor progression, respectively.

To investigate the role of FAK kinase activity in developmental angiogenesis, I generated EC specific knockin mouse models expressing kinase-defective FAK

mutant, which did not affect FAK kinase activity in other systems. As detailed in Chapter 2, I found that in contrast to the embryo lethality at E13.5 caused by FAK deletion in ECs, embryos carrying kinase-defective FAK died about two days later, at E15.5. I further showed that expression of the kinase-defective FAK reversed the increased apoptosis in CFKO embryos and FAK-null primary ECs in vitro, but not vessel dilation and defective angiogenesis of CFKO embryos. Finally, in vitro studies suggested that the FAK could mediate resistance to apoptosis through down-regulating p21 in a kinase-independent manner; the phosphorylation of VE-cadherin, which was necessary for the maintenance of blood vessel integrity, required the kinase activity of FAK. Together, our study for the first time revealed the role of FAK kinase activity in blood vessel formation and functioning during development.

Although a large amount of researches have indicated a critical role of FAK in mammary tumorigenesis and metastasis, the role of its second C-terminal proline-rich motif in this process, as well as in development, was unknown. Thus I also analyzed the specific roles of this motif of FAK in mammary tumor formation and development by generating different knockin mouse models. As described in Chapter 3, I found that PA mutations in mammary epithelial cells and endothelial cells both resulted in reduced mammary tumor growth rate and metastasis. Further investigations indicated that the second C-terminal proline-rich motif of FAK could be important for the loss of contact inhibition of mammary tumor cells and changes in β-catenin expression and JNK phosphorylation could contribute to this process. Moreover, PA mutations in the ECs led to massive necrosis, even in areas rich of vascularization, which suggested the mal-functioning of those blood vessels. In summary, by generating various conditional knockin mice models, I identified the roles of the second C-terminal proline-rich motif of FAK in vivo and showed that this motif was indispensable for the role of FAK in

promoting mammary tumor growth and metastasis. This study also suggested that the second C-terminal proline-rich motif of FAK could regulate this process via distinct mechanisms in different cells/systems.

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[1]Chapter 2

ROLE OF KINASE-INDEPENDENT AND DEPENDENT FUNCTIONS OF FAK IN ENDOTHELIAL CELL SURVIVAL AND BARRIER FUNCTION DURING EMBRYONIC DEVELOPMENT

2.1 Abstract

Focal adhesion kinase (FAK) plays an essential role in vascular development as endothelial cells (ECs)-specific conditional knockout of FAK (i.e. CFKO mice) leads to embryonic lethality caused by increased apoptosis and other defects in ECs. Here, we report on the differential roles of kinase-independent and dependent functions of FAK in vascular development by creating and analyzing an EC-specific conditional FAK kinase-defective mutant knockin (CFKI) mouse model. CFKI embryos showed apparently normal development through E13.5 while the majority of CFKO embryos die of vascular defects at the same stage. Expression of the kinase-defective FAK reversed the increased apoptosis in CFKO embryos and FAK-null primary ECs *in vitro* through suppression of up-regulated cyclin-dependent kinase inhibitor p21. In contrast to EC survival, vessel dilation and defective angiogenesis of CFKO embryos were not rescued in CFKI embryos. Moreover, primary ECs with FAK knockout or only kinase-defective FAK both had increased permeability as well as abnormal distribution of VE-cadherin and reduced basal phosphorylation of its Y658 residue. Together, our data suggest that kinase-independent functions of FAK can support EC survival in vascular developmental through E13.5, but is insufficient for maintaining EC functions to allow for completion of embryogenesis.

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2.2 Introduction

Endothelial cells (ECs) play central roles in the development of vasculature essential for organogenesis and embryonic survival throughout different developmental stages [1, 2]. The survival and function of ECs in embryogenesis and adult organisms are regulated by complex interactions among growth factor receptors, integrin receptors, and their extracellular ligands, which can trigger multiple intracellular signaling pathways through cytoplasmic kinases, small GTPases and other adaptor molecules. Focal adhesion kinase (FAK) is a major mediator of signal transduction by integrins and also participates in signal transduction by growth factor receptors such as VEGF receptors in ECs [3-7]. An essential role of FAK in vascular development has been established by recent findings that EC-specific deletion of FAK results in embryonic lethality associated with multiple vascular defects due to decreased survival and other defects of ECs [8, 9]. A critical role of FAK in adult vasculature is also implicated by the functional compensation of the related Pyk2 kinase in mice with inducible deletion of FAK in ECs [10]. However, little is known about the mechanisms by which FAK exerts its regulatory functions through multiple target molecules and signaling pathways as characterized in studies *in vitro* for embryonic development *in vivo*.

Recent studies suggest that FAK functions not only as a kinase but also through its kinase-independent activities in different cellular processes [8, 11]. Nevertheless whether kinase activity of FAK is required for survival and/or function of ECs in vascular development and embryogenesis is not very clear yet. Here we address this issue directly by creating a FAK knockin mouse model with kinase-defective mutant allele in the endogenous FAK gene in ECs. Analysis of the EC-specific FAK mutant knockin embryos and isolated ECs revealed both kinase-dependent and kinase-independent functions of FAK in EC barrier function and their survival, respectively,

which are required for vascular development and embryogenesis at different stages of development.

2.3 Material and Methods

Construction of the targeting vector and generation of CFKI mice

Based on available mouse genome sequences in the Ensembl database, an isogenic 129SvJ mouse BAC genomic clone containing FAK exon 16 (where the key K454 residue is encoded) and flanking sequences were obtained from BACPAC Resources Center. The presence of exon 16 in the BAC clone was verified by PCR using two pairs of primers surrounding exon 16 as well as Southern blotting. A targeting vector containing a mutated exon 16 (K454 to R) and a neomycin cassette was then constructed for homologous recombination. Gene targeting in 129P2/OlaHsd-derived E14Tg2a mouse embryonic stem cells [12] was performed as described previously using ESGRO (Millipore) [13]. Chimeric mice were identified by coat color and then bred to C57BL/6J mice. Transmission of the germ line was identified by PCR (see next section) and confirmed by Southern blotting. Heterozygous targeted mice bearing the KD[neo] allele (FAK+/KD[neo] mice) were obtained and then crossed with EIIa-Cre mice (The Jackson Laboratory), which express Cre in the very early stage of embryogenesis [14], to delete the neomycin cassette to avoid its possible interference with FAK gene expression. The progenies with neomycin cassette removed (FAK+/KD;EIIa-Cre mice) were crossed with C57BL/6J mice to segregate the FAK KD allele from the heterozygote EIIa-Cre transgene. The resulting heterozygous FAK knockin mice (FAK+/KD mice) were identified by PCR analysis and confirmed by sequencing of tail DNA. Tie2-Cre transgenic mice and CFKO mice have been described previously [8, 15]. All mice used in this study were bred and maintained at

the University of Michigan under specific pathogen-free conditions in accordance with institutional guidelines.

Genotyping by PCR

Mice and embryos were genotyped by PCR analysis of genomic DNA. Isolation of genomic DNA was described previously [16].Primers used to genotype flox and Δ FAK alleles were 5'-GCTGATGTCCCAAGCTATTCC-3' and 5'-AGGGCTGGTCTGCGCTGACAGG-3', as described previously [8]. Primers used to genotype the FAK KD knockin allele were P1 5'-TCAACAGCATGTAACTTCCC-3' and P2 5'-GGCATTCCAGTGCAAAACAC-3'. PCR was performed for 30 cycles of 94°C for 3 min, 67°C (for flox allele) or 60°C (for KD allele) for 2 min, and 72°C for 4 min. CreF (5'-GCAGAACCTGAAGATGTTCGCGATTA-3') and CreR (5'-TCTCCCACCGTCAGTACGTGAGATATC-3') primers were used to detect the Cre transgene, which was performed for 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Morphological, histological, and immunohistochemical analysis

Timed matings were set up between male FAK+/KD;Tie2-Cre and female FAKflox/flox mice to generate CKFI and control mice or male FAK+/flox;Tie2-Cre and female FAKflox/flox mice to generate CFKO and control mice, respectively. Embryos, yolk sacs, and placenta were harvested between E12.5 and E15.5, fixed in 4% PFA in PBS at 4°C for 4–16 h, and transferred to 70% ethanol. They were examined for gross morphology and photographed on a dissecting microscope (model S6D; Leica) with a progressive 3CCD camera (Sony) and Image-Pro Plus version 3.0.00.00 (Media Cybernetics) at RT. The embryos and placenta were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin (H&E) or nuclear

dye Hoechst 33258 (Sigma-Aldrich) or used for immunohistochemistry. The slides were examined under a microscope (model BX41; Olympus) with UplanF1 10× NA 0.3 or UplanF1 20× NA 0.5 objective lenses at RT, and the images were captured using a camera (model DP70; Olympus) with DP Controller version 1.2.1.108 (Olympus). For immunohistochemical analysis, the slides were subjected to staining using primary antibodies against PECAM-1, VE-cadherin, β-catenin (all from Santa Cruz Biotechnology, Inc.) followed by biotinylated and peroxidase-conjugated secondary antibodies. They were then processed using the DAB immunostaining assay kit (Santa Cruz Biotechnology, Inc.) according to the instructions. The samples were usually counterstained with hematoxylin before mounting on coverslips.

For the whole-mount staining with anti–PECAM-1 antibody, embryos were fixed in 4% PFA/PBS. After dehydration by a series of methanol, they were treated with 1% H2O2 (diluted in MeOH and DMSO mixed 4:1) to quench endogenous peroxidases. Samples were rehydrated by methanol to PBS and blocked in 4% BSA with 0.1% Triton X-100 in PBS. They were then incubated with anti–PECAM-1 (rat monoclonal MEC13.3; 1:50 dilution; BD) diluted 1:10 in 4% BSA in PBS + 0.05% Tween at 4°C overnight followed by peroxidase-conjugated secondary antibodies. The embryos were developed in 0.25% DAB with H2O2 in PBS. They were examined and photographed on a dissecting microscope (model S6D; Leica) with a progressive 3CCD camera (Sony) and Image-Pro Plus version 3.0.00.00 at RT. Culture of ECs and adenovirus infection Primary ECs from hearts and lungs of FAKflox/flox, FAK+/flox, and FAKflox/KD mice were isolated using magnetic bead (Dynabead M-450; Invitrogen) purification with rat anti–mouse PECAM-1 (BD), as described previously [8, 17]. In brief, lungs and hearts were harvested, minced, and then digested with 2 mg/ml of type I collagenase (Worthington Biomedical) at 37°C for 45 min. The digested tissue was

mechanically dissociated using vigorous flushing through a metal cannula, passed through a 70-μm filter (BD), and then centrifuged at 400 g for 8 min at 4°C. The cells were resuspended in cold Dulbecco's PBS and then incubated with rat anti–murine CD31 (PECAM-1; clone MEC 13.3; BD)–coated magnetic beads (M-450; sheep anti–rat IgG Dynabeads; Invitrogen) at 15 μl/ml for 10 min at RT. The beads were washed several times in cold isolation medium (high glucose Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, penicillin, streptomycin [at standard concentrations], and 0.02 M Hepes), resuspended in growth medium (isolation medium supplemented with 100 μg/ml heparin, 100 μg/ml EC mitogen [Biomedical Technologies], l-glutamine, nonessential amino acids, and Na pyruvate at standard concentrations), and cultured in 0.1% gelatin (Sigma-Aldrich)-coated 100-mm tissue culture dishes at 37°C in 5% CO2. The recombinant adenoviruses encoding Cre recombinase or lacZ control were purchased from Gene Transfer Vector Core (University of Iowa, Iowa City, IA) and used to infect the primary ECs as described previously [8]. For most experiments, 108 plaque-forming units were used for 10-cm dishes. To increase efficiency, a second infection was performed after 9–12 h. The recombinant adenoviruses encoding FRNK (Ad-FRNK) or GPF control (Ad-GFP) were generated using the AdEasy-1 system (Agilent Technologies) according to manufacturer's instruction. No detectable cell toxicity was observed.

Western blotting

Lysates from primary ECs were prepared and analyzed by Western blotting analysis as described previously [8, 17]. In brief, ECs isolated from mice were washed three times with ice-cold PBS and then lysed with modified radioimmunoprecipitation assay buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% Na deoxycholate, 1 mM Na vanadate, 10 mM Na pyrophosphate, 10 mM NaF, 1% Triton X-100, 0.5%

SDS, 0.1% EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). Lysates were cleared by centrifugation, and total protein concentrations were determined using the Bio-Rad Laboratories protein assay. Antibodies used were anti-FAK, anti–phospho-tyrosine397–FAK, anti-Pyk2, anti-p21, anti-p53, anti-Myc, anti–VE-cadherin, and antiactin (all from Santa Cruz Biotechnology, Inc.); anti–phospho-tyrosine402–Pyk2, anti–phospho-serine473–Akt, and anti–phospho-tyrosine417–Src (all from Cell Signaling Technology); and anti–phospho-tyrosine658–VE-cadherin (Biosource International).

TUNEL assay

For detection of apoptotic cells in vivo, paraffin-embedded embryo sections (6 μm) were deparaffinized, incubated in methanol containing 0.3% H2O2 for 30 min, washed, and incubated with 20 μg/ml proteinase K in PBS for 15 min at RT. Apoptotic cells were detected as described in the ApopTag Peroxidase In Situ Apoptosis Detection kit (Millipore). In brief, equilibration buffer was applied on each slide for 20 s. After taping off excess liquid, all slides were incubated at 37°C with TdT enzyme for 1 h. The reaction was stopped by stop/wash buffer. The slides were then incubated with antidigoxigenin peroxidase conjugate at RT for 30 min. The staining was made by DAB for 5 min, and the counterstaining was made by methyl green. The slides were examined under a BX41 microscope with UplanF1 10× NA 0.3 objective lens at RT, and the images were captured using a DP70 camera with DP Controller version 1.2.1.108.

For detection of apoptosis of primary ECs infected with Ad-lacZ or Ad-Cre in vitro, the cells were fixed and analyzed using In situ Cell Death Detection kit, TMR red (Roche), according to the manufacturer's instructions. In brief, cells were fixed in 4% PFA in PBS, pH 7.4, for 1 h at RT, followed by incubation in 0.1% Triton X-100

in 0.1% Na citrate for 2 min on ice. Cells were rinsed with PBS, and an aliquot of TUNEL reaction mixture was placed, followed by incubation for 1 h at 37°C in humidified atmosphere in the dark. After washing with PBS, the nuclei were counterstained with Hoechst. Samples were embedded with antifade and subjected to observation under a BX41 microscope with a UplanF1 20× NA 0.5 objective lens at RT. The images were captured using a DP70 camera with DP Controller version 1.2.1.108.

Transwell permeability assay

Permeability across the EC monolayer was measured by using type I collagen–coated Transwell units (6.5-mm diameter and 3.0-μm pore size polycarbonate filter; Corning), essentially as described previously [18]. In brief, ECs infected with adenovirus were plated at 105 cells in each well and cultured for 2 d to get confluent before experiments. After cells were serum starved in medium 199 containing 1% BSA for 1 h, permeability was measured by adding 1 mg of FITClabeled dextran (molecular weight, 42,000)/ml to the upper chamber. After incubation for 30 min, 50 μl of sample from the lower compartment was diluted with 300 μl PBS and measured for fluorescence at 520 nm when excited at 492 nm with a spectrophotometer (Synergy HT; BioTek).

Immunofluorescence staining

ECs infected with Ad-lacZ or Ad-Cre were processed for immunofluorescence staining using anti–VE-cadherin (BD) or β-catenin or p120-catenin (both from Santa Cruz Biotechnology, Inc.), as described previously [8]. Texas red–conjugated mouse anti–rat IgG (1:200), FITC conjugated goat anti–rabbit IgG (1:200), and FITC-conjugated goat anti–mouse IgG (1:200) were used as the secondary antibodies. They

were then mounted on Slowfade (Invitrogen) and examined under a BX41 microscope with a UplanF1 20× NA 0.5 objective lens at RT. The images were captured using a DP70 camera with DP Controller version 1.2.1.108. For detection of EC apoptosis in vivo, brain sections were subjected to TUNEL assay as described above, followed by immunofluorescent staining using anti–PECAM-1 and Hoechst. Images of stained sections were captured using a laser-scanning confocal microscope (FluoView 500; Olympus) and a CCD camera at RT.

2.4 Results and Discussion

2.4.1 Generation of kinase-defective FAK mutant knockin mice

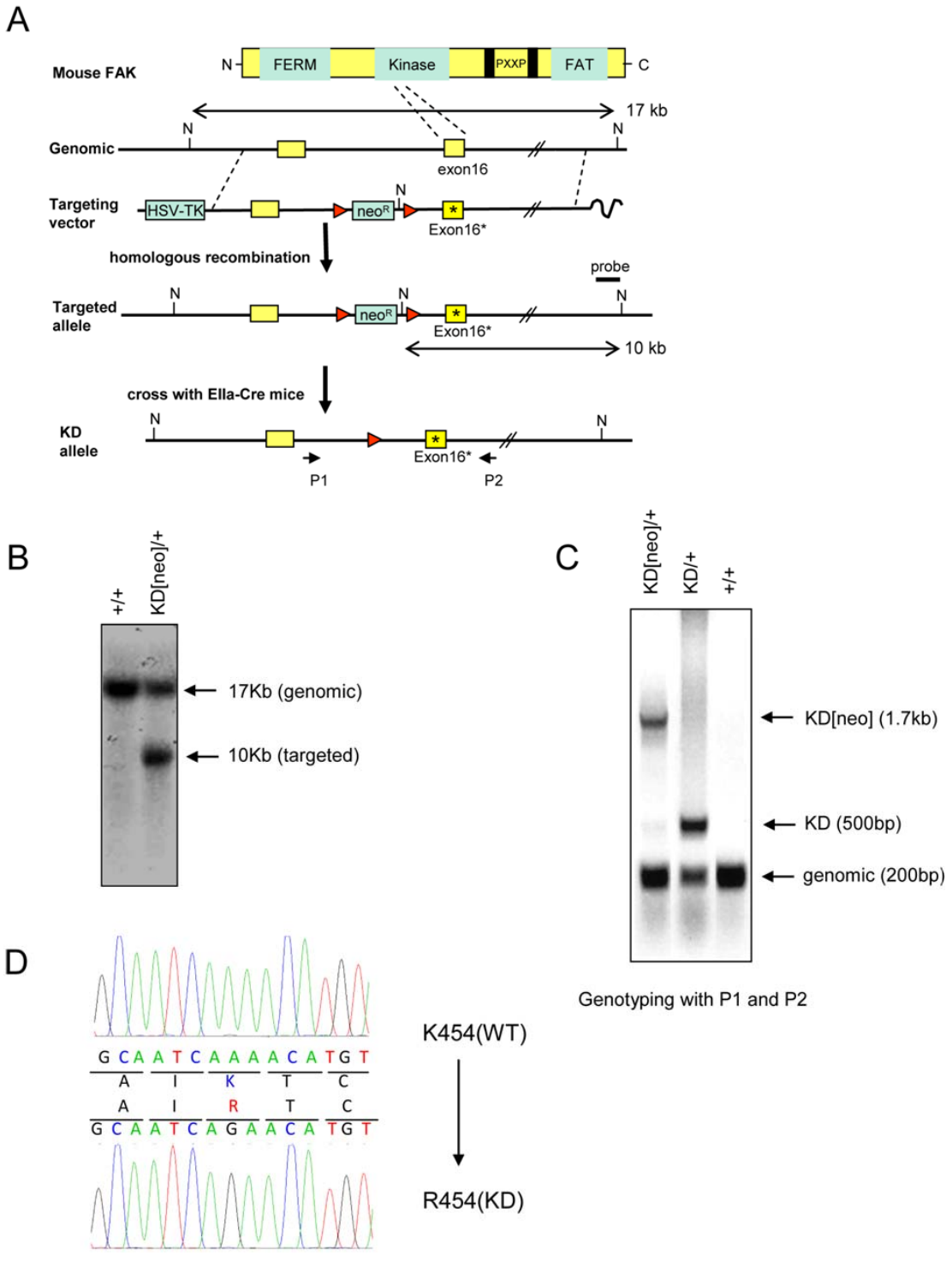
To study the potential role of kinase-independent functions of FAK *in vivo*, we generated a kinase-defective mutant FAK allele in the endogenous FAK gene utilizing a gene knockin approach via homologous recombination. The K454 to R mutation abolishing FAK kinase activity was created in exon 16 of FAK genomic DNA, as described in Materials and Methods. A targeting vector containing the mutated exon 16 (K454R) and a neo cassette with flanking LoxP sequences (designated as KD[neo] allele) was then constructed and used to generate mutant mice containing the knockin mutant allele employing standard homologous recombination methods (Figure 2.1A). Southern blotting analysis of DNA from targeted embryonic stem cells verified the presence of the KD[neo] allele in the cells (Figure 2.1B). Heterozygous targeted mice bearing KD[neo] allele ($FAK^{+/KD[neo]}$ mice) were obtained and then crossed with EIIa-Cre mice, which express Cre in the very early stage of embryogenesis [14], to delete the neomycin cassette in order to avoid its possible interference with FAK gene expression (see Figure 2.1A). The progenies with neomycin cassette removed ($FAK^{+/KD}$;EIIa-Cre mice) were crossed with C57BL/6J mice to segregate the FAK KD allele from the heterozygote EIIa-Cre transgene. The resulting heterozygous FAK

knockin mice ($FAK^{+/KD}$ mice) were identified by PCR analysis (Figure 2.1C) and confirmed by sequencing (Figure 2.1D) of tail DNA, as described in Materials and Methods. All $FAK^{+/KD}$ mice are viable, fertile and indistinguishable from wild type mice, confirming that one functional FAK allele is sufficient for normal mouse development and function and that the KD mutant allele (in the endogenous gene and not over-expressed) did not exhibit any dominant negative effect severe enough to result in embryonic lethality and/or sterility for the mice.

Matings between heterozygous $FAK^{+/KD}$ mice yielded wild type (i.e. $FAK^{+/+}$) and $FAK^{+/KD}$ mice at the expected 1:2 Mendelian ratio, but no homozygous FAK knockin (i.e. $FAK^{KD/KD}$) mice were detected after more than 400 pups were screened (data not shown).

Analysis of embryos from intercrosses of $FAK^{+/KD}$ mice did not detect any live $FAK^{KD/KD}$ embryos beyond E10.5 (data not shown). These results suggested that the kinase activity of FAK is required for embryogenesis and that the kinase-independent functions of FAK are not sufficient to rescue the early embryonic lethality of FAK KO mice.

Figure 2.1 | Generation of the KD FAK knockin allele. (A) Schematic of mouse FAK protein, targeting vector, and the genomic and targeted allele in FAK loci. Large closed triangles represent loxP sites. The K454 to R mutation (i.e., KD) in the knockin allele is in exon 16 (marked by asterisks). Horizontal lines with arrows on both sides indicate the expected sizes of DNA bands in Southern blotting analysis of the genomic or targeted alleles as detected by the probe (thick horizontal bar) just 3'end downstream from the targeting vector. Crosses of the mice with targeted allele to EIIa-Cre mice result in the deletion of the floxed neomycin cassette to create the FAK KD knockin allele. The relevant restriction sites (NheI [N]) and primers (P1 and P2) for PCR genotyping are indicated. (B) Southern blotting analysis of the tail DNA from representative mice after digestion with NheI. (C) PCR genotyping of the tail DNA from representative mice, using primers P1 and P2 as indicated in A. (D) The PCR fragments corresponding to the wild-type (WT) and KD FAK that were amplified in C were sequenced to confirm the mutation of the codon for K454 to R.


A
Mouse FAK
N
FERM
Kinase
PXXP
FAT
C
17 kb
Genomic
exon16
Targeting vector
HSV-TK
neoR
Exon16*
homologous recombination
probe
Targeted allele
10 kb
cross with Ella-Cre mice
KD allele
P1
P2
B
+/+
KD[neo]/+
17Kb (genomic)
10Kb (targeted)
C
KD[neo]/+
KD/+
+/+
KD[neo] (1.7kb)
KD (500bp)
genomic (200bp)
Genotyping with P1 and P2
D
G C A A T C A A A A C A T G T
A I K T C
A I R T C
G C A A T C A G A A C A T G T
K454(WT)
R454(KD)

2.4.2 Kinase-defective FAK is sufficient to rescue vascular developmental defects in CFKO mice through E13.5

Previous studies identified a critical role of FAK in the regulation of vascular development as EC-specific deletion of FAK led to embryonic lethality due to various defects in vascular development around E12.5 and E13.5 [8]. To investigate the potential role of kinase-independent functions of FAK in vascular development *in vivo*, we crossed $FAK^{+/KD}$ mice to Tie2-Cre mice expressing Cre recombinase in ECs [15] to generate $FAK^{+/KD}$;Tie2-Cre mice, which were then crossed with $FAK^{flox/flox}$ [8] mice to produce $FAK^{flox/KD}$;Tie2-Cre mice as well as control mice with three other genotypes. The $FAK^{flox/KD}$;Tie2-Cre mice express only kinase-defective FAK protein in ECs from the knockin KD mutant allele (flox allele is converted to deleted allele by Tie2-Cre), but contain functional FAK (i.e. the flox allele) in other cells, and therefore are designated as conditional FAK knockin (CFKI) mice. Parallel crosses between $FAK^{flox/+}$;Tie2-Cre and $FAK^{flox/flox}$ were also performed to generate $FAK^{flox/flox}$;Tie2-Cre mice (i.e. CFKO mice in which both FAK alleles are converted to deleted allele thus inactivating FAK completely in ECs) [8] for comparison with CFKI mice in order to determine the potential role of kinase-independent functions of FAK in vascular development. As shown in Table 1, both CFKI and CFKO embryos were recovered at near-Mendelian ratios of 1:1:1:1 at E12.5, which is consistent with previous observations that the majority of embryos with FAK inactivation in ECs develop normally till about E12.5 [8]. At E13.5, CFKI embryos were still found at the expected Mendelian ratio and the majority of the embryos observed appeared normal in their overall gross appearance and development (Figure 2.2A). In contrast, significantly fewer CFKO embryos than the expected Mendelian ratio were recovered and most of them showed various vascular phenotypes, including multifocal

superficial scattered hemorrhages and superficial edema, as described previously [8]. At E14.5 and E15.5, reduced CFKI embryos were observed and a significant fraction of embryos exhibited various vascular defects as well. However, no live CFKO embryos were found at these stages. Lastly, we did not detect any live pups for either CFKI or CFKO mice out of more than 200 newborn mice examined for each.

Table 1. Genotypes of progeny from crosses to generate CFKI and CFKO mice

Genotypes	E12.5	E13.5	E14.5	E15.5	E16.5	Born
Progeny from crosses between $FAK^{+/KD}$;Tie2-Cre and $FAK^{flox/flox}$ mice						
flox/KD	7	36	25	24	10	79
flox/KD;Tie2-Cre (CFKI)	9	40	9	4	1	0
flox/+	8	35	27	19	12	92
flox/+;Tie2-Cre	6	37	30	19	10	85
Progeny from crosses between $FAK^{+/flox}$;Tie2-Cre and $FAK^{flox/flox}$ mice						
flox/flox	12	33	13	ND	ND	72
flox/flox;Tie2-Cre (CFKO)	12	15	0	ND	ND	0
flox/+	12	37	14	ND	ND	71
flox/+;Tie2-Cre	16	40	16	ND	ND	68

Previous studies showed various vascular developmental defects including impaired angiogenesis and vascular stability, hemorrhage in the embryos, and decreased placenta vascularization in CFKO mice [8, 9]. Consistent with observation by gross morphology of the embryos, histological examination also showed rescue of various vascular defects in CFKI embryos. As shown in Figure 2.2B, hemorrhages of different sizes were found in the CFKO embryos (panel b), whereas all red blood cells were restricted inside the intact vessels in the control embryos (panel a), as shown previously [8]. Interestingly, this phenotype was not found in CFKI embryos (Figure 2.2, panel c), suggesting that these vascular defects were rescued by the kinase-independent functions of FAK. The fetal placental tissues increase noticeably with

significant vascularization of the vital organ in midgestation [19]. As observed previously [8], defective vascularization caused significant decrease in the thickness of the labyrinth layer of CFKO placenta compared with control placenta at E13.5 (Figure 2.2C). Analysis of CFKI placenta at the same developmental stage showed restored thickness of their labyrinth layer, suggesting that the kinase-independent functions of FAK were able to support placenta vascularization through E13.5. Consistent with the measurements of the labyrinth layer thickness, the lack of vessels containing nucleated red blood cells (i.e. fetal vessels; Figure 2.2B, d-f, marked by arrows) in CFKO placenta was also rescued in CFKI placenta.

Taken together, these results suggest that the kinase-independent functions of FAK are able to support vascular development through E13.5 by rescuing the deficiency caused by deletion of FAK in ECs. Nevertheless, they also indicate that the kinase activity of FAK is required for late stages of vascular development and functions to support the completion of embryogenesis.

Figure 2.2 | Kinase-independent functions of FAK are sufficient to support normal embryonic development through E13.5. (A) Gross examination of whole embryos at various stages with or without intact yolk sac. Note the significantly reduced vascular network in CFKO embryos at E13.5, which was restored in CFKI embryos (arrows). The edema (blue arrow) and hemorrhages (blue arrowhead) observed in CFKO at E13.5 are also absent in CFKI embryos. The CFKO embryos found at E14.5 and E15.5 were smaller and with marked abnormalities caused by embryonic death, whereas a significant fraction of CFKI embryos were alive with more limited defects. (B) H&E staining of skin and labyrinth layer of placenta sections of control, CFKO, and CFKI embryos at E13.5. In a–c, arrows mark red blood cells within vessels for control and CFKI embryos (a and c) and outside of vessels because of a hemorrhage in CFKO embryos (b). In d–f, note the reduced number of vessels that contain the nucleated red blood cells from fetal embryos (arrows) in CFKO mice (e) compared with control and CFKI mice (d and f). Arrowheads mark maternal vessels containing enucleated red blood cells that are present in all three samples. (C) Placenta sections from control, CFKO, and CFKI embryos at E13.5 were stained with H&E. Mean ± SEM of calculated thickness of labyrinth layer from three independent experiments in multiple fields is shown as a percentage of the value in control embryos.

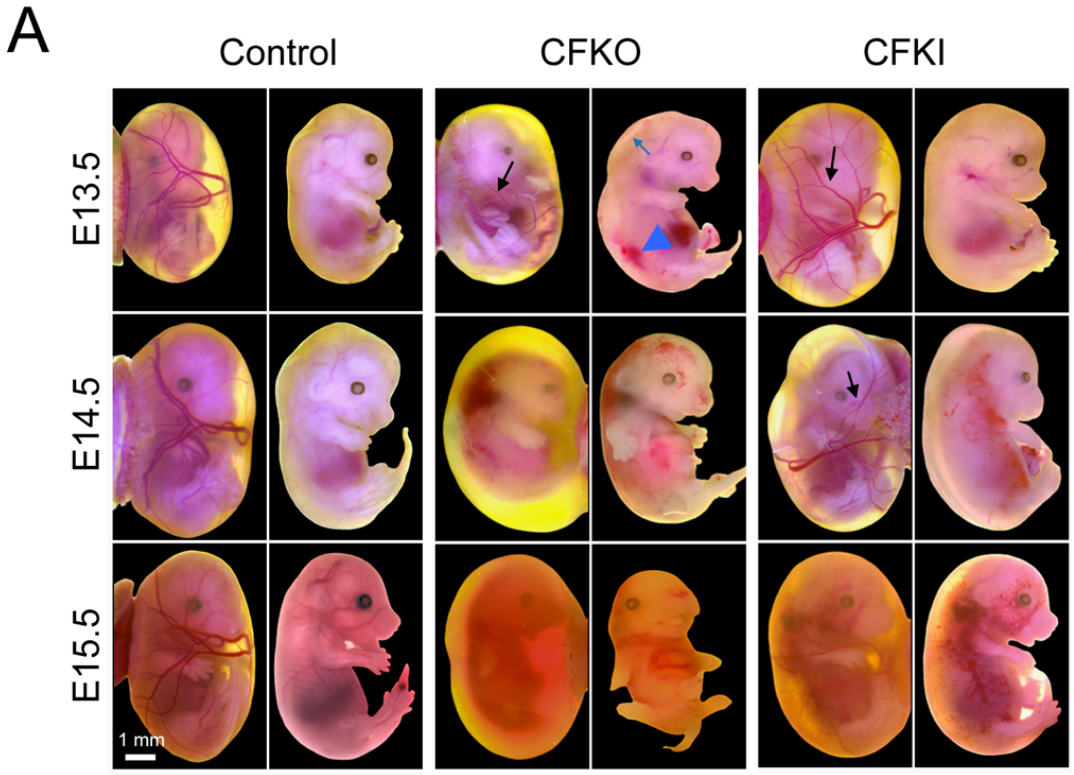
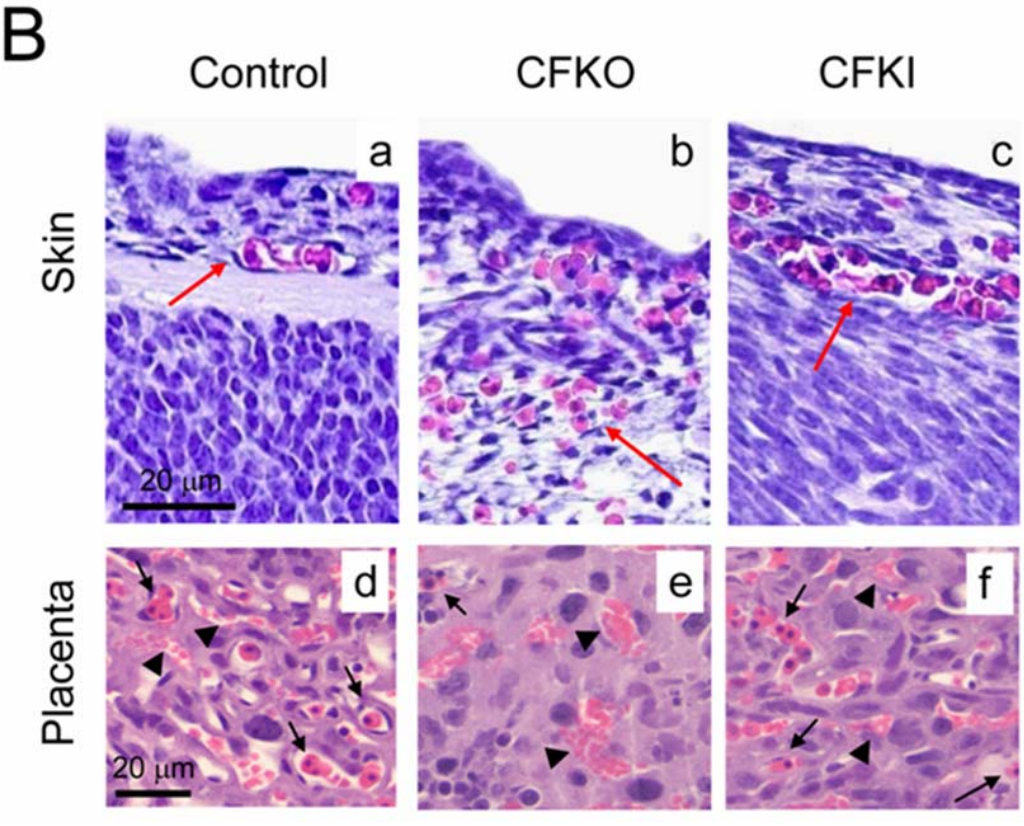
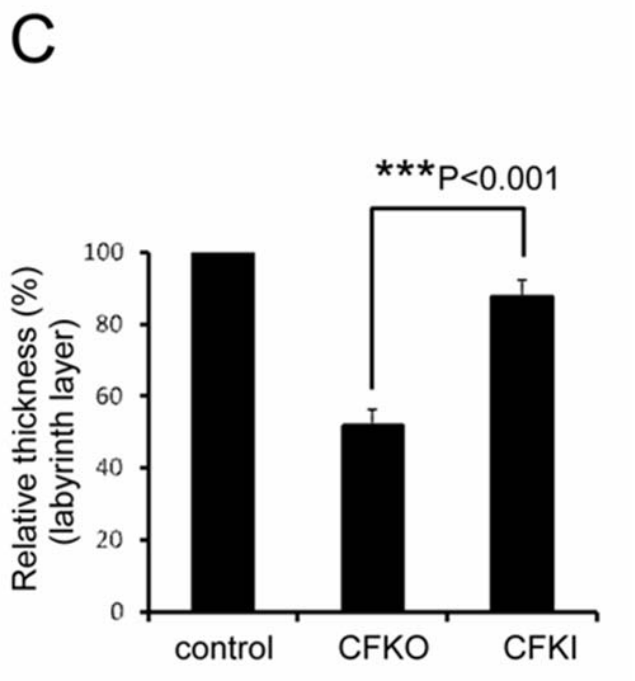

A
Control
CFKO
CFKI
E13.5
E14.5
E15.5
1 mm

Figure 2.2 (Continued)

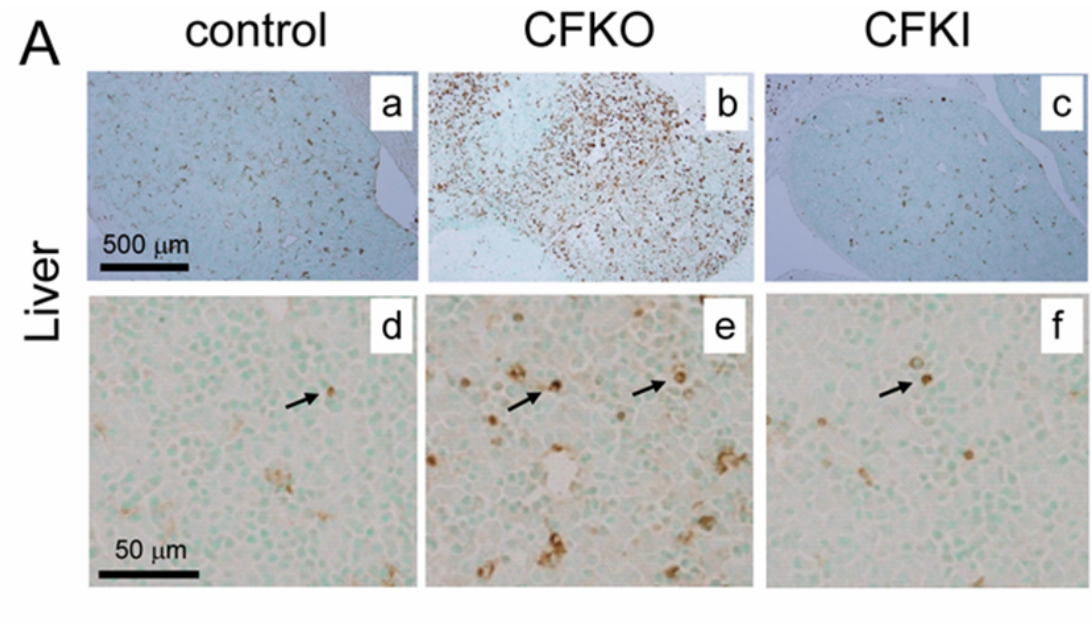




2.4.3 Kinase-defective FAK promotes EC survival through regulation of p21

Previous studies using primary ECs showed differential requirement of kinase activity of FAK for VEGF-stimulated cell migration, but not for cell migration on FN [8]. Although the kinase-independent function of FAK in EC migration on FN may contribute to the rescue of some aspects of vascular development in CFKI embryos, we also examined whether kinase-defective FAK can rescue the increased apoptosis upon deletion of FAK in ECs because the reduced cell survival have been suggested as a major cause of vascular defects in CFKO mice [8, 9]. First, TUNEL assays were used to evaluate apoptosis in the CFKO and CFKI embryos at E13.5. As shown in Figure 2.3A, significantly increased apoptosis was found in the liver sections in CFKO embryos (panels b and e) compared to that from the control mice (panels a and d). Interestingly, the liver sections of CFKI embryos showed only slightly elevated apoptosis (panels c and f), which is much lower than that in CFKO embryos. Qualification of data from multiple experiments indicated that the kinase-defective FAK expressed in CFKI embryos virtually abolished the increased apoptosis upon EC-specific FAK deletion (Figure 2.3B). The increased apoptosis in the placenta and skin of CFKO embryos was also rescued in CFKI embryos (Figure 2.3, C-E). Double staining of brain sections revealed the presence of apoptotic ECs in the blood vessel of CFKO embryos but not CFKI or control embryos (Figure 2.3F). Similar results were found in skin sections (Figure 2.4A and not depicted).These results suggest that kinase-defective FAK was able to rescue the defective survival of ECs caused by deletion of FAK in embryos.

Figure 2.3 | Kinase-independent functions of FAK rescue the increased apoptosis induced by FAK deletion. (A–E) Sections from livers, skins, or placenta of control, CFKO, or CFKI embryos at E13.5 were analyzed by TUNEL assays. (A and C) Representative fields with arrows marking apoptotic cells. (B, D, and E) Mean ± SEM of relative number of apoptotic cells per field from three experiments. (F) Brain sections from control, CFKO, or CFKI embryos at E13.5 were analyzed by TUNEL assays followed by staining using PECAM-1 and Hoechst. ECs and blood cells (both green) are marked by arrowheads and asterisks, respectively. Apoptotic cells are marked by arrows (red). Note the presence of apoptotic ECs in the vessel of CFKO embryos but not control or CFKI embryos.


A
control
CFKO
CFKI
Liver
a
b
c
d
e
f
500 μm
50 μm

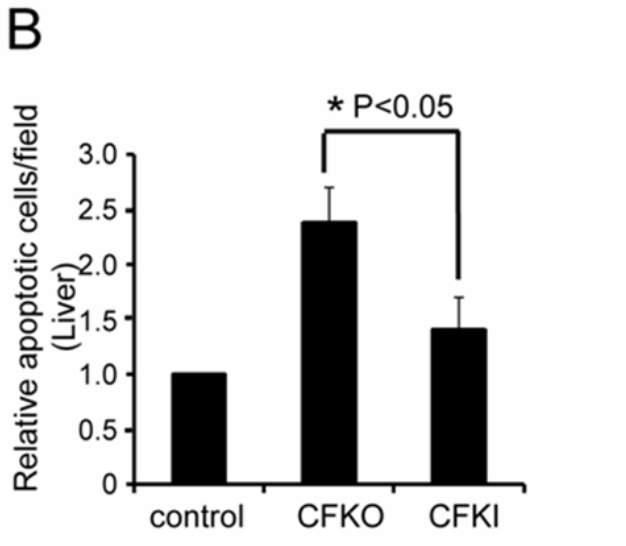
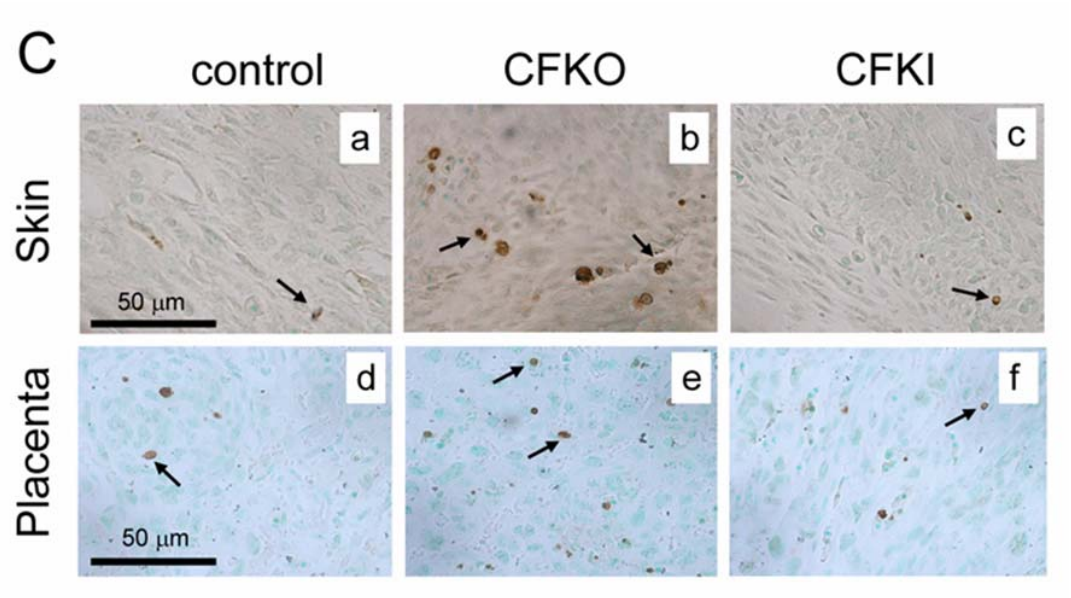

B
* P<0.05
Relative apoptotic cells/field (Liver)
3.0
2.5
2.0
1.5
1.0
0.5
0
control
CFKO
CFKI

Figure 2.3 (Continued)



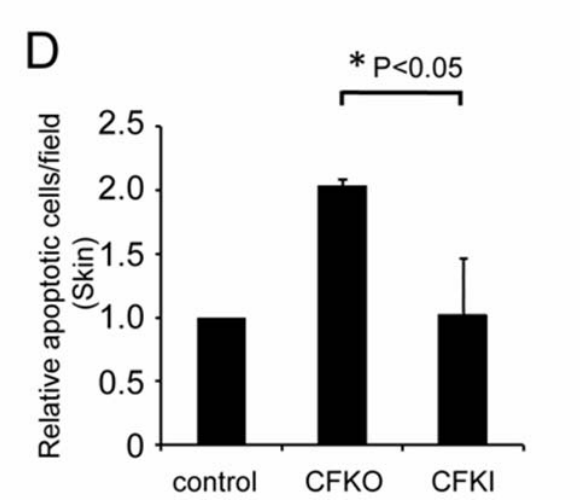
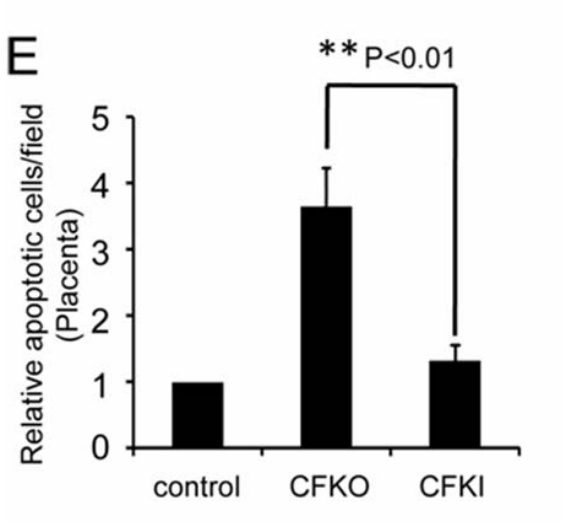
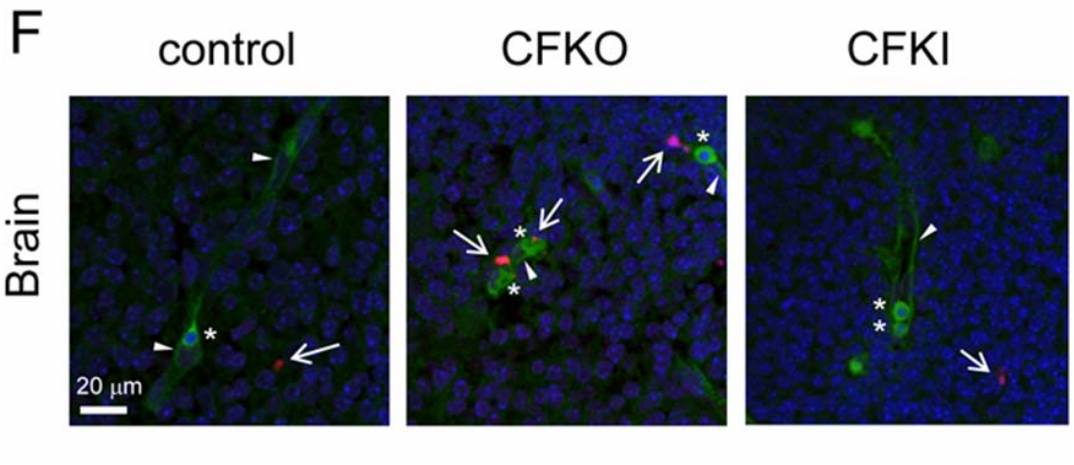


Figure 2.3 (Continued)





To further define the role of kinase-independent functions of FAK in EC-autonomous survival and apoptosis, primary ECs were isolated from $FAK^{flox/flox}$, $FAK^{flox/KD}$ and $FAK^{+/flox}$ mice (Figure 2.4, B and C) and infected by recombinant adenoviruses encoding Cre recombinase (Ad-Cre), as described previously [8]. As expected, Ad-Cre infection of the primary ECs led to conversion of the flox allele to deleted allele *in vitro* (Figure 2.4D). Analysis of the cells by TUNEL assay showed that Ad-Cre infection of $FAK^{flox/flox}$ ECs but not ECs from $FAK^{+/flox}$ mice led to their increased apoptosis (Figure 2.5A, panel a-f, and Figure 2.5B). Interestingly, the presence of kinase-defective FAK in the Ad-Cre-infected primary ECs from $FAK^{flox/KD}$ mice (with one allele deleted) reversed the increased apoptosis (Figure 2.5A, panels g-l; Figure 2.5B). These results suggested that kinase-independent functions of FAK are sufficient for EC survival, which may account for the rescued vascular development in CFKI embryos through E13.5.

To study possible mechanisms by which the kinase-defective FAK rescued the increase of EC apoptosis in FAK-null ECs, lysates were prepared from the primary ECs and analyzed for the activation status of FAK downstream targets. As shown in Figure 2.5C, deletion of the flox FAK allele by Ad-Cre infection eliminated expression of FAK in ECs from $FAK^{flox/flox}$ mice (lanes 1 and 2), but did not affect FAK expression in ECs from $FAK^{+/flox}$ and $FAK^{flox/KD}$ mice because of the remaining wild type or KD allele (lanes 3-6). Moreover, similar FAK autophosphorylation at Y397 was detected in ECs infected by the control Ad-lacZ and $FAK^{+/flox}$ ECs infected by Ad-Cre (Figure 2.5C, lanes 1, 3-5). As expected, kinase-defective FAK in $FAK^{flox/KD}$ ECs infected by Ad-Cre was not autophosphorylated at Y397 (lane 6). No significant difference in either the expression or activation of the FAK related kinase Pyk2 was observed in the primary ECs with FAK deletion or expression of the KD

Figure 2.4 | Analysis of embryonic liver sections in vivo and preparation and analysis of primary ECs in vitro. (A) Liver sections of CFKO embryos at E13.5 were subjected to TUNEL analysis (left) followed by immunofluorescent staining using anti–PECAM-1. Note the high nonspecific background signal in the liver. Arrows mark possible apoptotic EC in the skin area. (B and C) Primary ECs were isolated as described previously (Shen et al., 2005). They were analyzed by staining with VE-cadherin (green) and Hoechst (blue; B) or flow cytometry using PECAM-1–phycoerythrin (PE) and ICAM-2–FITC (C) to verify their EC natures. (D and E) Primary ECs from FAKflox/flox, FAK+/flox, and FAKflox/KD mice were infected with Ad-Cre to delete the floxed FAK allele or Ad-lacZ as a control, as indicated. Genomic DNA was then analyzed by PCR. The expected positions of flox, +, and deleted alleles are marked on the right (D). Total RNAs were extracted for analysis of p21 mRNA levels by quantitative RT-PCR. The mean ± SEM of relative values of three experiments (normalized to lacZinfected cells) is shown (E). (F) Primary ECs from control mice were infected with Ad-lacZ or Ad-FRNK, as indicated. Lysates from the infected cells were analyzed by Western blotting using antibodies against p21 (top), FAK (detecting both FAK and the Myc-tagged FRNK as marked by arrows on the right), Myc (for Myc-FRNK), or actin, as indicated.

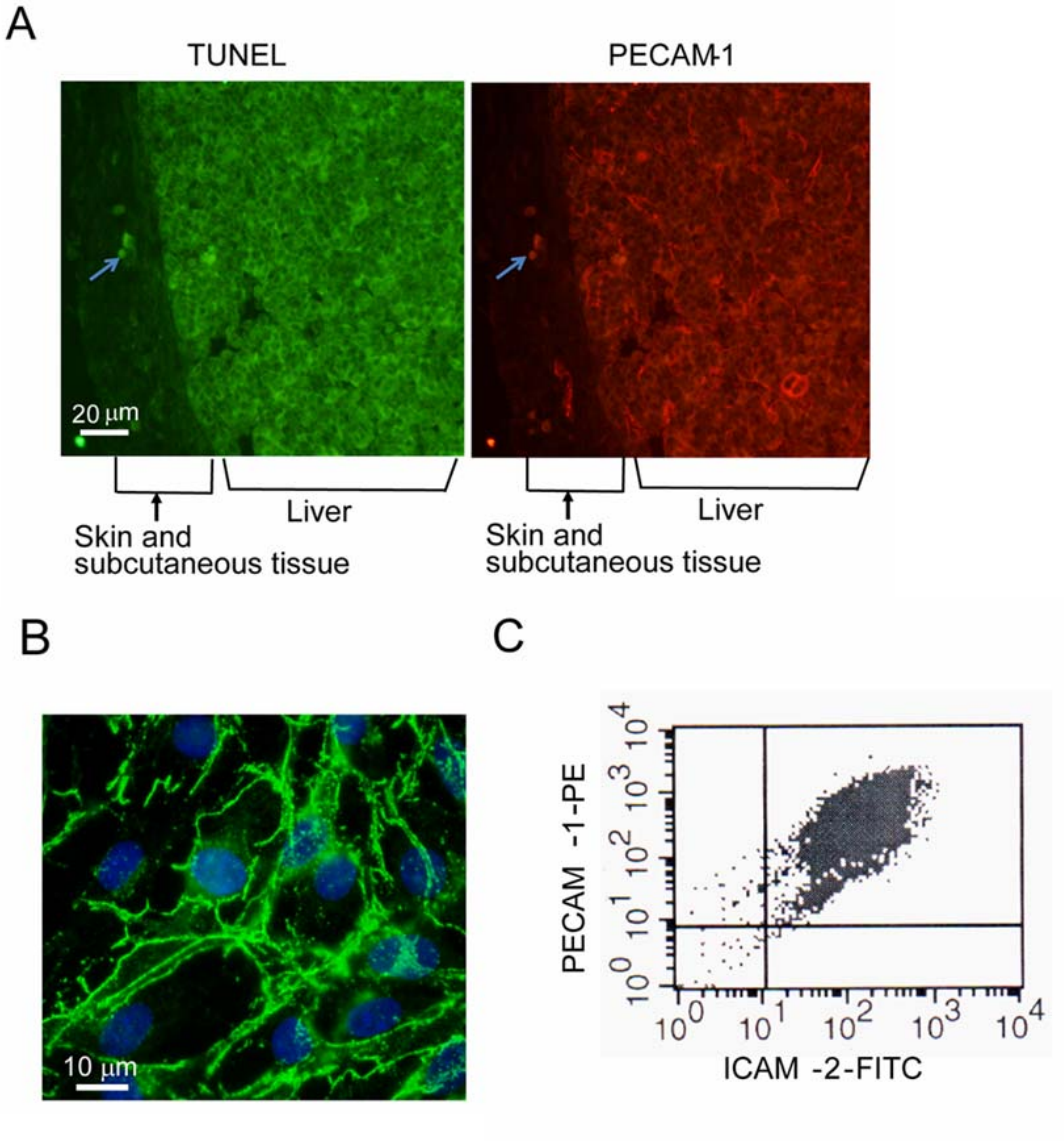

A
TUNEL
PECAM-1
20 μm
Liver
Skin and
subcutaneous tissue
Liver
Skin and
subcutaneous tissue
B
10 μm
C
PECAM -1-PE
ICAM -2-FITC

Figure 2.4 (Continued)

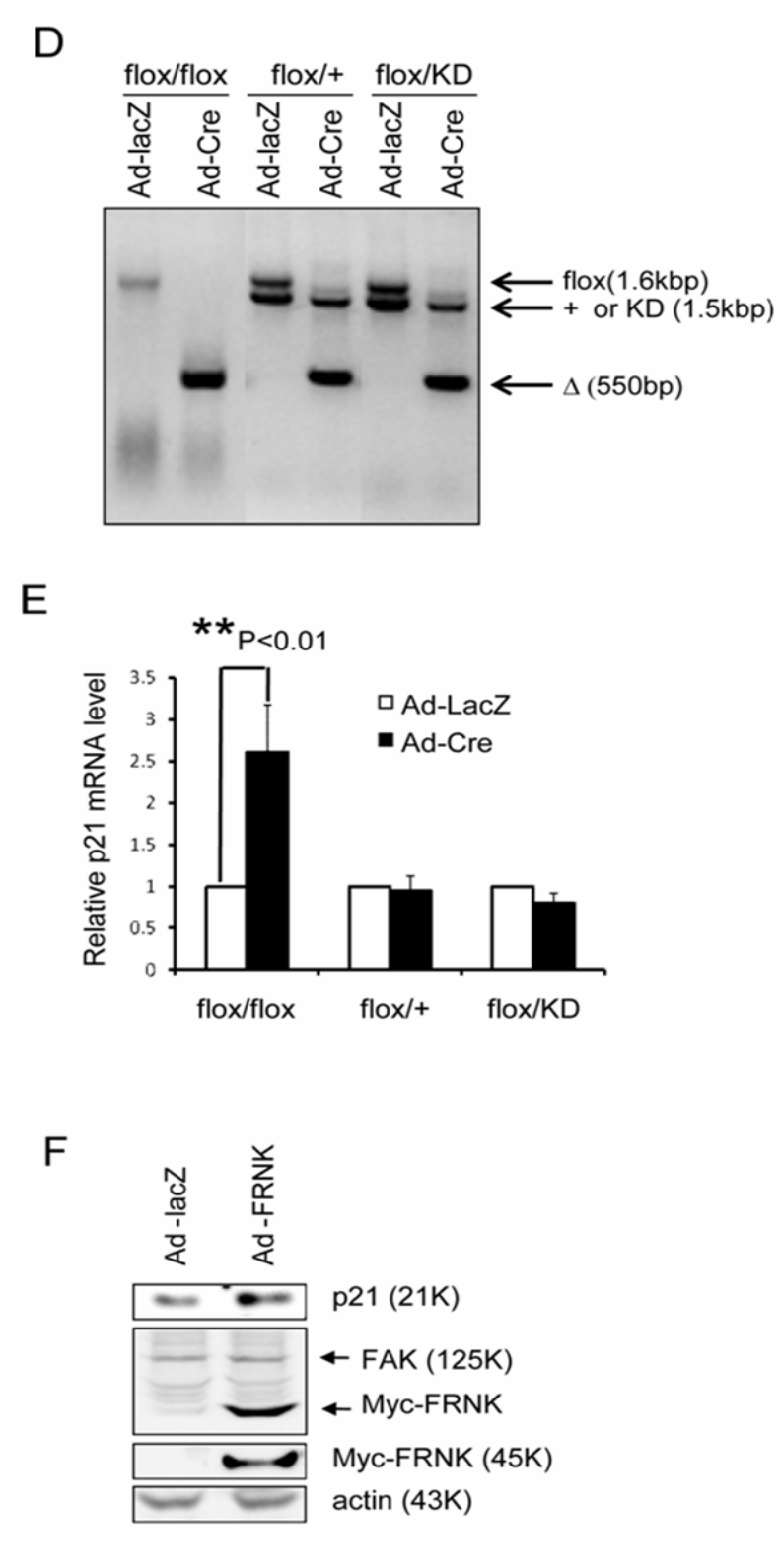


Figure 2.5 | Kinase-independent functions of FAK suppress the increased apoptosis induced by FAK deletion in vitro. ECs from FAKflox/flox, FAK+/flox, and FAKflox/KD mice were infected with Ad-Cre to delete the floxed FAK allele or Ad-lacZ as a control, as indicated. (A and B) The infected cells were then stained by Hoechst and analyzed for apoptosis using TUNEL assay. (A) Representative fields for ECs from FAKflox/flox and FAKflox/KD mice stained by Hoechst (a, d, g, and j) or TUNEL (b, e, h, and k) or merged images (c, f, i, and l). (B) Mean ± SEM from three experiments. (C) Aliquots of lysates from the infected cells were analyzed by Western blotting using various antibodies, as indicated. (D–F) The intensity of the pAkt (D), p21 (E), and p53 (F) bands was quantified from three independent experiments by densitometry. The mean ± SEM of relative intensity (normalized to lacZ-infected cells) is shown.

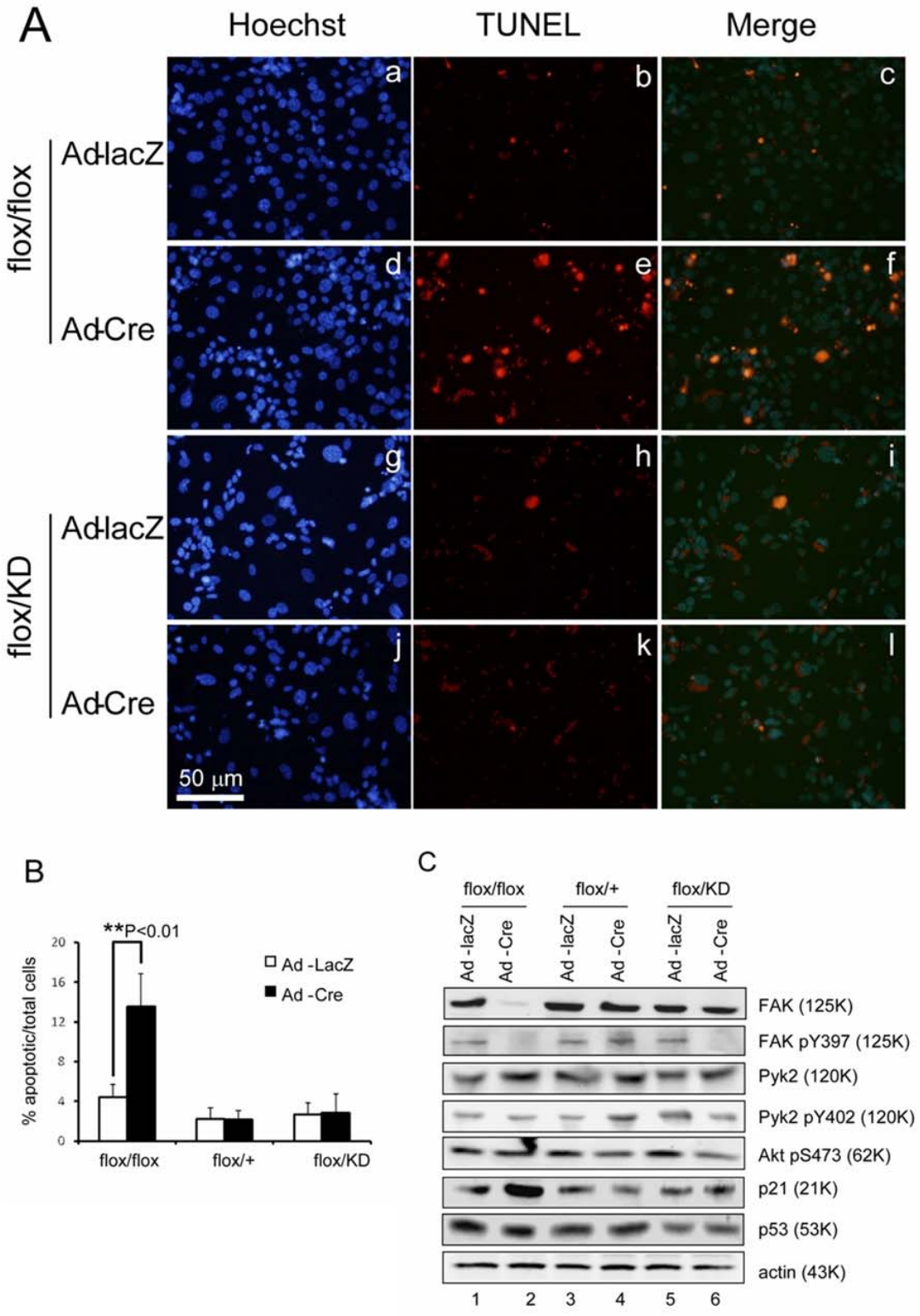
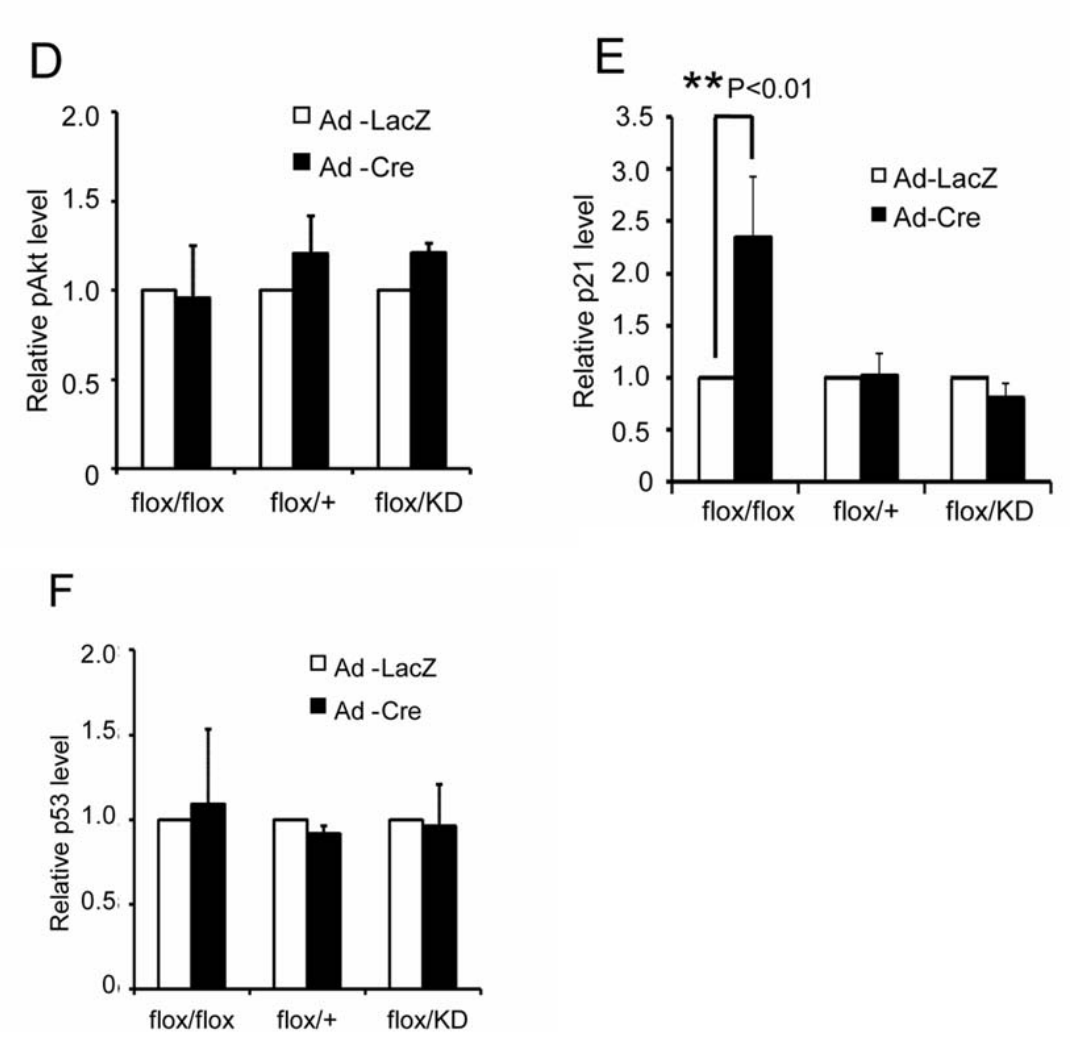

A
Hoechst
TUNEL
Merge
flox/flox
Ad-lacZ
Ad-Cre
flox/KD
Ad-lacZ
Ad-Cre
a
b
c
d
e
f
g
h
i
j
k
l
50 μm
B
**P<0.01
% apoptotic/total cells
20
16
12
8
4
0
Ad -LacZ
Ad -Cre
flox/flox
flox/+
flox/KD
C
flox/flox
flox/+
flox/KD
Ad -lacZ
Ad -Cre
Ad -lacZ
Ad -Cre
Ad -lacZ
Ad -Cre
FAK (125K)
FAK pY397 (125K)
Pyk2 (120K)
Pyk2 pY402 (120K)
Akt pS473 (62K)
p21 (21K)
p53 (53K)
actin (43K)
1
2
3
4
5
6

Figure 2.5 (Continued)



FAK. To examine the potential targets of FAK signaling that may regulate apoptosis and survival, we first analyzed the activation of Akt, which has been implicated in mediating FAK regulation of cell survival in a number of previous studies [20-22]. However, activation of Akt as measured by its phosphorylation at S473 was not changed in ECs with FAK deletion or with only expression of kinase-defective FAK compared to controls (Figures 2.5, C and D). We next examined the expression of the cyclin-dependent kinase inhibitor p21 in these cells, as the increased p21 expression upon inactivation of FAK has been shown in several previous studies in different cells [11, 23]. In contrast, we found that p21 was increased significantly upon deletion of FAK in $FAK^{flox/flox}$ ECs infected by Ad-Cre (Figure 2.5C, lanes 1 and 2). However, infection of ECs from $FAK^{flox/KD}$ or $FAK^{+/flox}$ mice by Ad-Cre did not lead to an increase in p21 expression (Figure 2.5C, lanes 3 to 6). Qualification of data from multiple experiments showed more than 2 fold increase in p21 expression level upon deletion of FAK, which was reversed in the presence of kinase-defective FAK (Figure 2.5E). Consistent with the increased p21 protein level, analysis of the mRNAs isolated from these primary ECs by quantitative RT-PCR showed an approximately 2.5-fold up-regulation of p21 mRNA upon deletion of FAK, whereas no differences were found in ECs from $FAK^{+/flox}$ or $FAK^{flox/KD}$ mice after infection of Ad-Cre (Figure 2.4E). Moreover, inhibition of FAK by expression of FAK-related nonkinase (FRNK) also increased p21 expression in primary ECs (Figure 2.4F), suggesting that this is not a secondary consequence of FAK deletion. Given the potential role of the increased p21 in apoptosis [24, 25], these results suggested that the up-regulation of p21 may contribute to increased apoptosis of ECs upon FAK deletion and that the kinase-independent functions of FAK could rescue the EC apoptosis and defective vascular development through controlling the level of p21 expression.

Lim et al. (2008) recently showed that FAK could facilitate p53 turnover in the nucleus through its FERM domain in a kinase-independent manner [11]. Thus it is conceivable that accumulation of p53 upon FAK inactivation could lead to the increased p21 expression in ECs, which could be reversed by kinase-independent functions of FAK observed in our studies. However, no difference in the expression of p53 was observed in the primary ECs upon FAK deletion or expression of the kinase-defective FAK (Figures 2.5C and 2.5F). Although we can not exclude the possibility that the activity of p53 (rather than its expression level) could be altered to regulate p21 in the primary ECs, our current data suggest potentially p53-independent mechanisms of p21 regulation by FAK in a kinase-independent manner.

2.4.4 Defective angiogenesis and dilated vessels in CFKI embryos

While the rescue of increased apoptosis upon FAK deletion by the kinase-defective FAK mutant in CFKI embryos was sufficient to support embryogenesis through E13.5, the CFKI mice exhibited various vascular defects beyond this developmental stage, leading to embryonic lethality before birth (see Table 1). These observations suggest that vascular defects other than increased apoptosis, which are not rescued by kinase-independent functions of FAK, are responsible for the embryonic lethality at E14.5 and thereafter, although they were not crucial for development through E13.5. To examine such vascular defects, we analyzed vasculature of E13.5 control and CFKI embryos as well as those surviving CFKO embryos at the same stage (about 40% of the expected Mendelian ratio). Sections from the head region of the embryos were subjected to immunohistochemical staining using anti-PECAM-1 antibody to detect blood vessels. Consistent with previous observations of a reduced angiogenesis in CFKO embryos [8, 9], fewer blood vessels were found in the sections from CFKO embryos compared to that from control embryos (Figure 2.6, panel a and b). CFKI

embryos also showed a decreased number of blood vessels (panel c). Quantification of multiple samples indicated a reduction in the density of vessels by about 50% in both CFKO and CFKI embryos (Figure 2.6B). These results suggested that the kinase-independent functions of FAK were not sufficient to rescue the angiogenesis defects upon FAK deletion.

In addition to the reduced vessel numbers, we also observed evident dilation of blood vessels in both CFKO and CFKI embryos (Figure 2.6A, panels b and c) compared to that in control embryos (panel a). Quantification of multiple samples showed an approximately 3 fold increase in the average size of the vessels in both CFKO and CFKI embryos compared to the control embryos (Figure 2.6C). The defective vessel dilations were also observed in other organs such as the spinal cord and skins of CFKO and CFKI embryos (Figures 2.6D-2.6F). Examination of the embryos by whole-mount staining of PECAM-1 also showed the enlarged vessels in CFKO embryos compared to the control embryos at E12.5 (Figure 2.7A, panels a and b). An increase in vessel size, though to a lesser extent, was also observed in the CFKI embryos (panel c). Together, these results suggest that the deficiency in the blood vessels, as exhibited by their dilation as well as reduced angiogenesis, were not rescued by the kinase-defective FAK and could be responsible for the embryonic lethality of the CFKI embryos, which were associated with signs of leaky vasculature such as multifocal hemorrhages and edema at later developmental stages.

Figure 2.6 | KD FAK is unable to rescue the reduced angiogenesis and vessel dilation caused by FAK deletion. (A) Brain sections from control, CFKO, and CFKI embryos at E13.5 were stained with anti–PECAM-1. Note the reduced density and dilated vessels (arrows) in CFKO and CFKI embryos (b and c) compared with control embryos (a). (B) Mean ± SEM of vessel numbers per field from three independent experiments. (C) Mean ± SEM of relative vessel size normalized to the value in control embryos from three independent experiments. (D) Sections from spinal cords (a–c) or skin (d–f) of control, CFKO, and CFKI embryos at E13.5 were stained with anti–PECAM-1. Vessels are marked by arrows. (E and F) Mean ± SEM of relative vessel size normalized to the value in control embryos from three independent experiments.

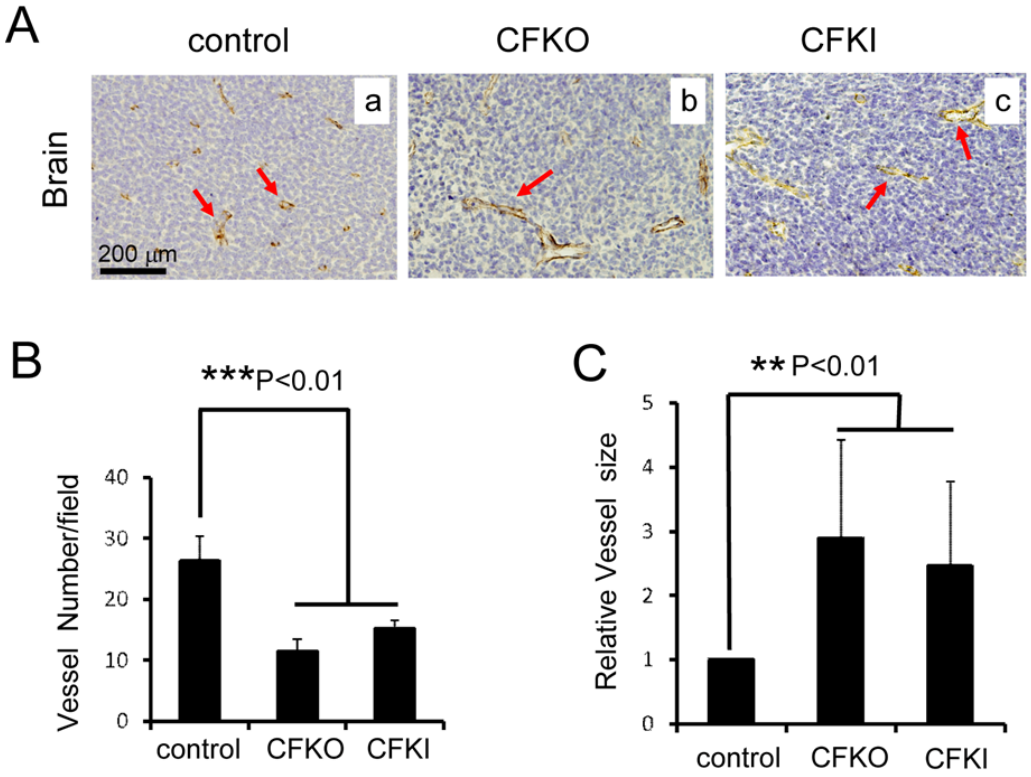
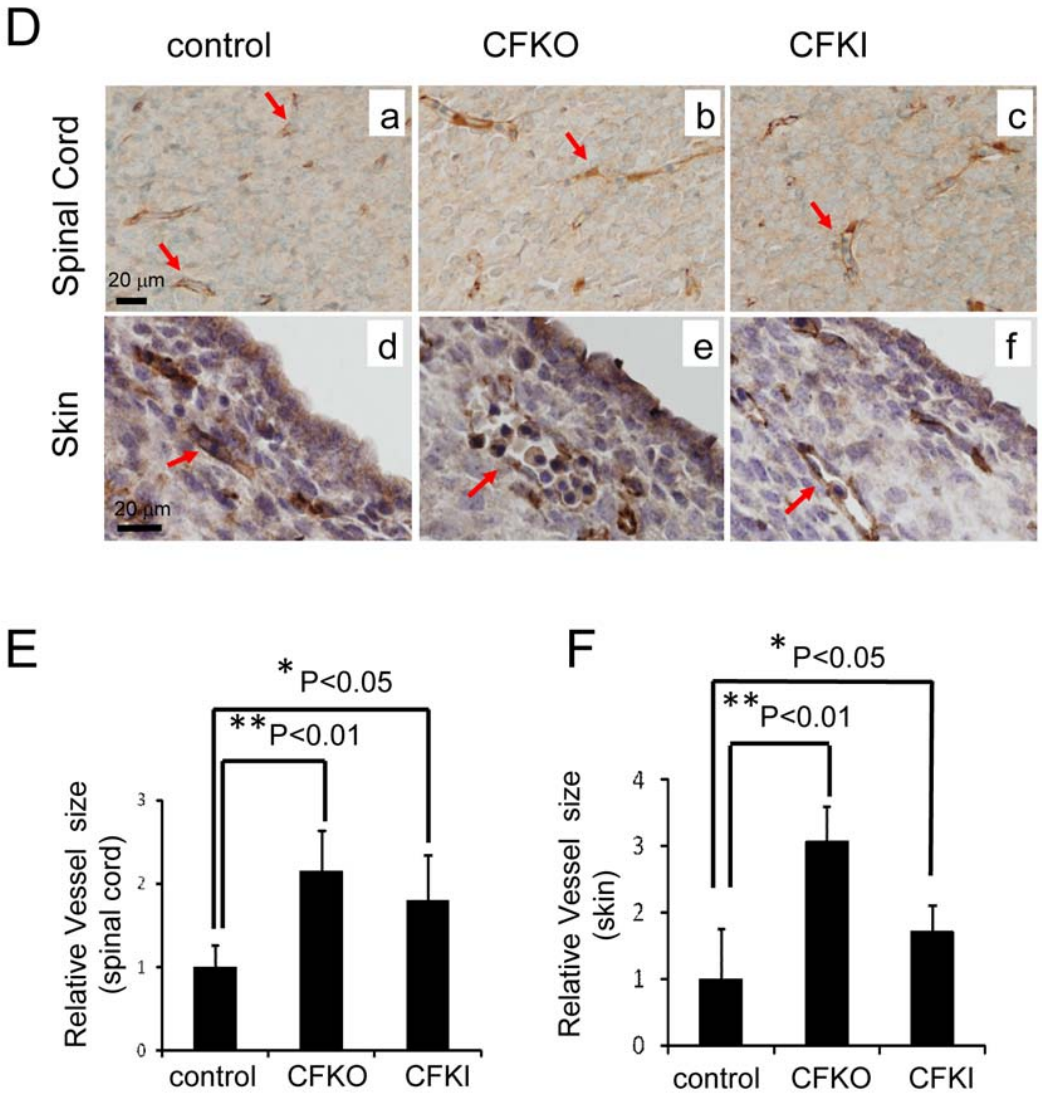

A
control
CFKO
CFKI
Brain
a
b
c
200 μm
B
***P<0.01
Vessel Number/field
40
30
20
10
0
control
CFKO
CFKI
C
** P<0.01
Relative Vessel size
5
4
3
2
1
0
control
CFKO
CFKI

Figure 2.6 (Continued)



2.4.5 FAK kinase activity is required for maintaining EC integrity and vascular endothelial cadherin (VE-cadherin) phosphorylation

To explore the potential mechanisms of the abnormal vascular integrity as reflected by dilated vessels and hemorrhages in CFKO and CFKI embryos, we examined the possible regulation of permeability of isolated primary ECs by FAK. Primary ECs from $FAK^{flox/flox}$, $FAK^{+/flox}$ and $FAK^{flox/KD}$ mice that had been infected by Ad-lacZ or Ad-Cre (see Figures 2.4D and 2.5C) were analyzed for their barrier function by measuring transport of FITC-dextran through confluent monolayer of the cell grown on transwell plates, as described previously [18]. As shown in Figure 2.8A, deletion of FAK in $FAK^{flox/flox}$ ECs by Ad-Cre infection significantly increased their permeability whereas infection of $FAK^{+/flox}$ ECs by Ad-Cre had no effect. Primary ECs with expression of only FAK KD allele (Ad-Cre infection of $FAK^{flox/KD}$ ECs) also showed increased permeability, indicating that the kinase activity of FAK was required to maintain the integrity of ECs. These results suggest that FAK regulates EC permeability and that the abnormalities in EC barrier function may contribute to the compromised integrity of the blood vessels, which, although they did not affect embryonic development through E13.5, manifested in the severe hemorrhages and edema in the later stage of embryogenesis, leading to the lethality of CFKI embryos.

VE-cadherin is a major mediator of cell-cell adhesion of ECs and is responsible for regulation of their permeability under a variety of physiological and pathological conditions [26, 27]. To understand the potential mechanisms for regulation of EC permeability by FAK, the expression and distribution of VE-cadherin was examined in $FAK^{flox/flox}$ and $FAK^{flox/KD}$ ECs that had been infected by Ad-lacZ or Ad-Cre (see Figures 2.4D and 2.5C). No apparent difference in the expression level of VE-cadherin was detected in either of the EC populations after infection with Ad-Cre (Figure 2.8B,

Figure 2.7 | Normal Whole-mount analysis of embryos and immunofluorescent staining of isolated primary ECs. (A) Superficial vasculatures in control, CFKO, and CFKI embryos at E12.5 were visualized by staining of whole mount with anti–PECAM-1. Note the apparently different size of the vessels in similar areas of the embryos (arrows). (B) Primary ECs from FAKflox/flox and FAKflox/KD mice were infected with Ad-Cre to delete the floxed FAK allele or Ad-lacZ as a control, as indicated. The cells were then subjected to immunofluorescent staining using anti-β-catenin (a-d) or anti-p120-catenin (e-h).

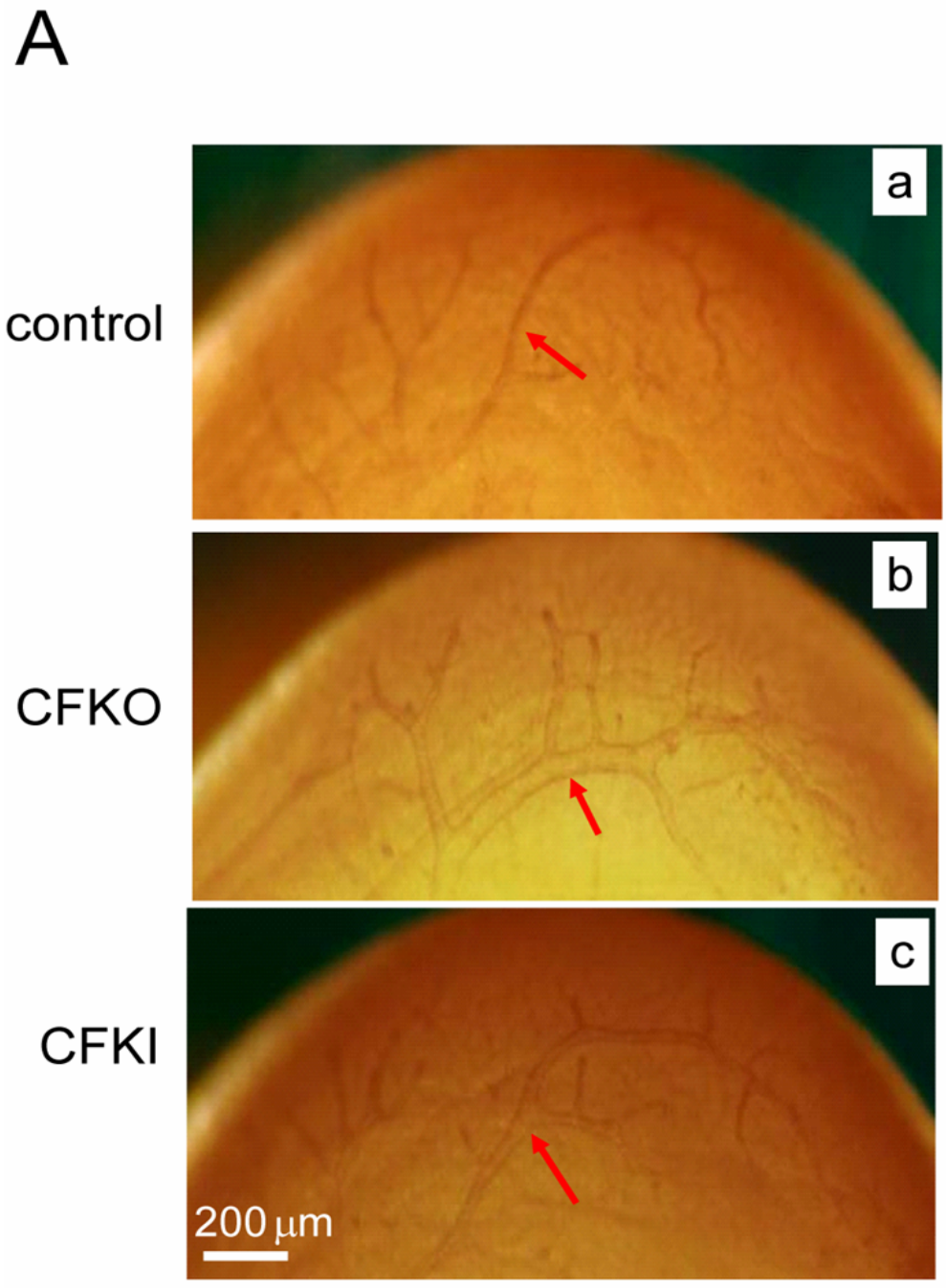

A
control
CFKO
CFKI
a
b
c
200 μm

Figure 2.7 (Continued)

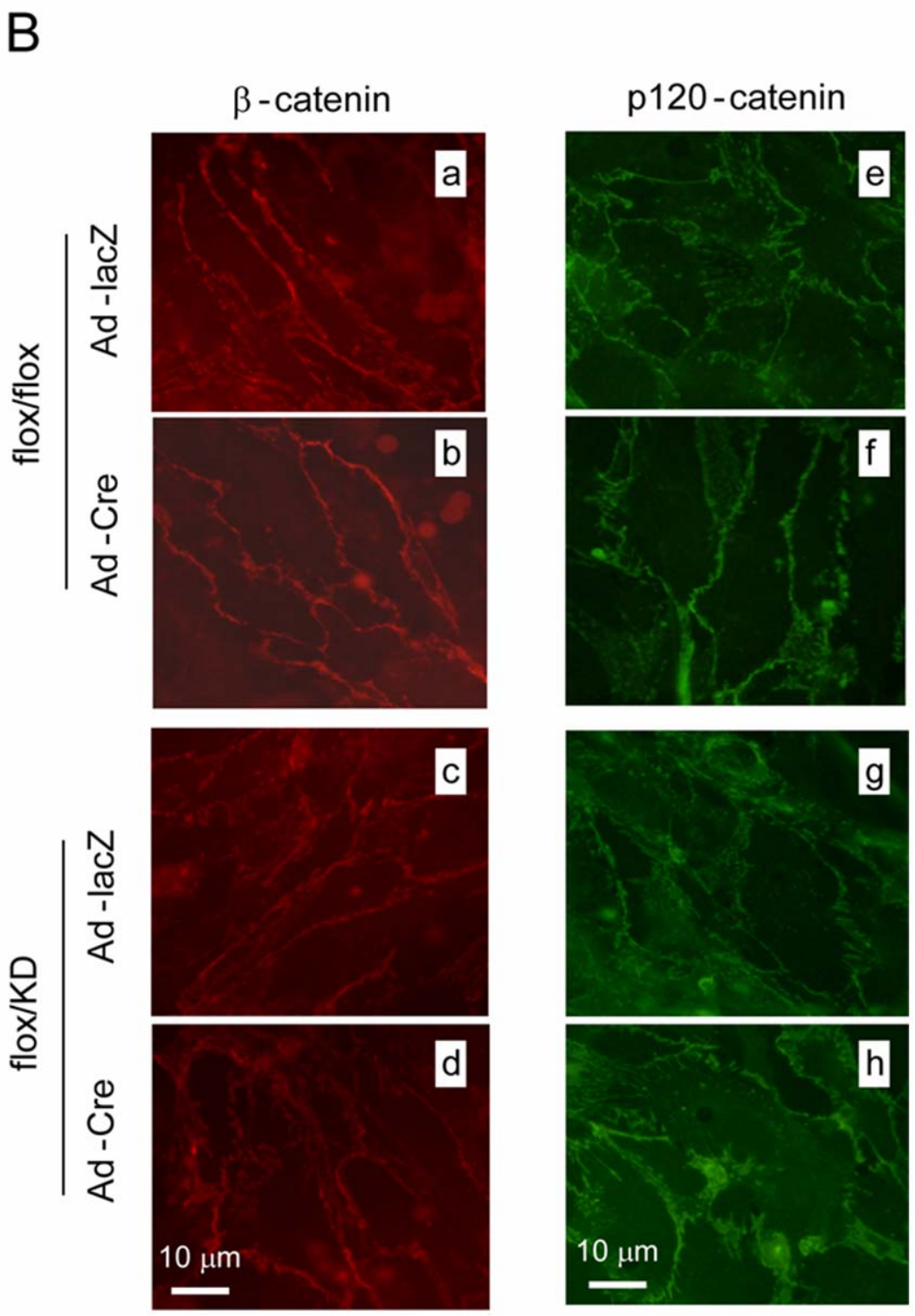


Figure 2.8 | Normal EC barrier function and distribution and Y658 phosphorylation of VE-cadherin requires FAK kinase activity. ECs from FAKflox/flox, FAK+/flox, and FAKflox/KD mice were infected with Ad-Cre (closed bars) to delete the floxed FAK allele or Ad-lacZ (open bars) as a control, as indicated. (A) The infected cells were analyzed for their barrier function using Transwell assay as described in Materials and methods. The mean ± SEM of relative EC permeability (normalized to lacZ-infected cells) is shown. (B) Representative images of immunofluorescent staining by anti–VE-cadherin for infected cells from FAKflox/flox and FAKflox/KD mice. Arrows mark the discontinuous pattern of VE-cadherin distribution in cells infected with Ad-Cre. (C) Aliquots of lysates from the infected cells were analyzed by Western blotting using various antibodies, as indicated. (D) The intensity of the VE-cadherin pY658 bands was quantified from three independent experiments by densitometry. The mean ± SEM of relative intensity (normalized to lacZ-infected cells) is shown. (E) A working model of kinase-dependent (absent in both CFKI and CFKO embryos) and -independent (present in CFKI embryos) functions of FAK in vascular development.

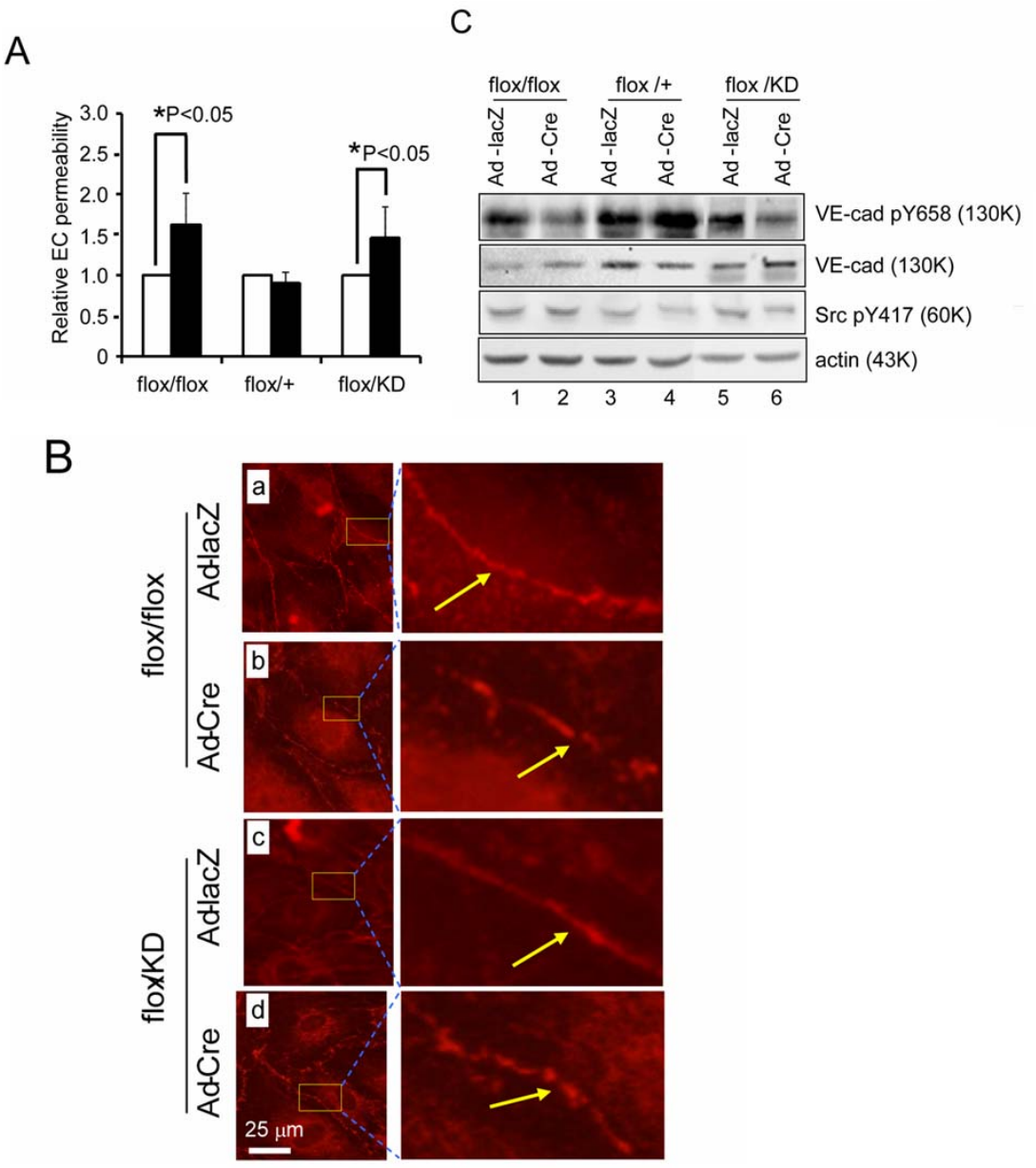
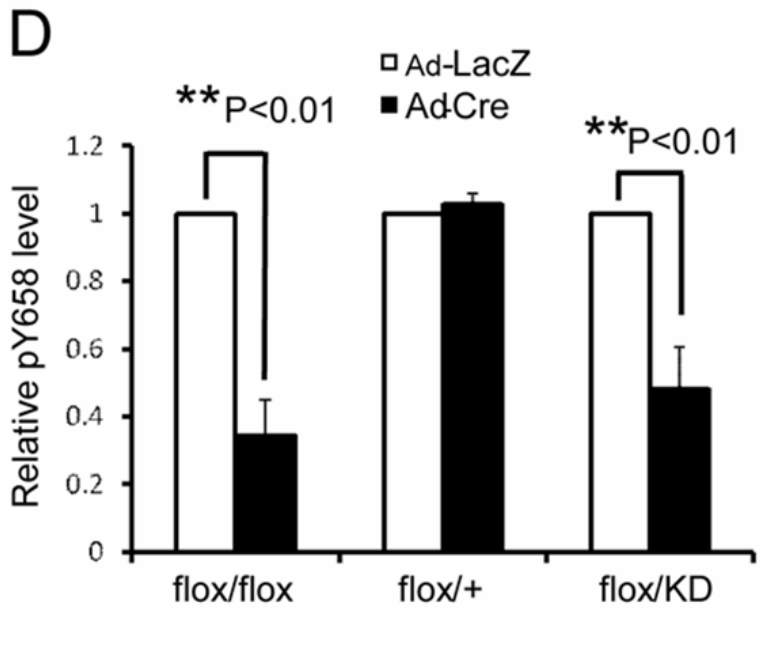
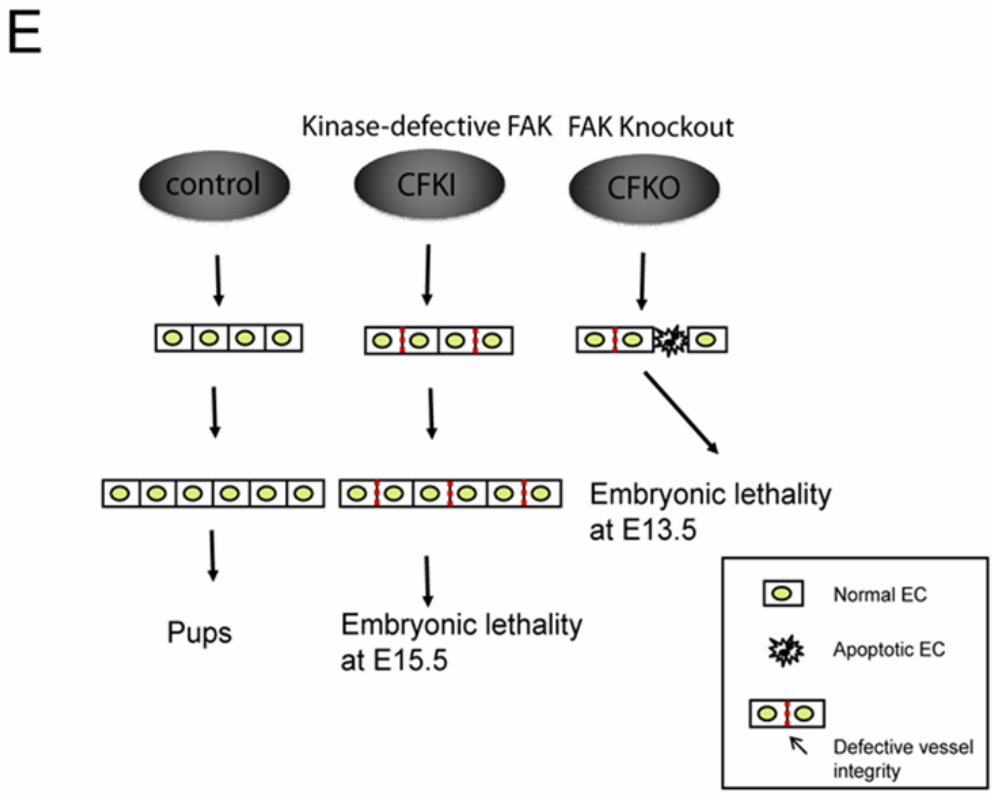

A
Relative EC permeability
3.0
2.5
2.0
1.5
1.0
0.5
0
*P<0.05
*P<0.05
flox/flox
flox/+
flox/KD
C
flox/flox
flox /+
flox /KD
Ad -lacZ
Ad -Cre
Ad -lacZ
Ad -Cre
Ad -lacZ
Ad -Cre
VE-cad pY658 (130K)
VE-cad (130K)
Src pY417 (60K)
actin (43K)
1 2 3 4 5 6
B
flox/flox
Ad-lacZ
Ad-Cre
floxKD
Ad-lacZ
Ad-Cre
a
b
c
d
25 μm

Figure 2.8 (Continued)





compare panels a and c to panels b and d), although Western blotting analysis showed a slight increase in the expression of VE-cadherin in both cells following Ad-Cre infection (see Figure 2.8C). However, significantly different pattern of staining for VE-cadherin in the plasma membrane was found after inactivation of FAK. As shown in Figure 2.8B, ECs infected by Ad-lacZ showed smooth and continuous staining of VE-cadherin on the membrane (a and c). In contrast, upon deletion of FAK in $FAK^{flox/flox}$ ECs by Ad-Cre infection, the VE-cadherin exhibited a considerably different pattern with discontinuous staining and a more zigzag pattern (Figure 2.8B, b). Similarly, a disrupted pattern of VE-cadherin on the plasma membrane was observed in $FAK^{flox/KD}$ ECs after Ad-Cre infection (Figure 2.8B, d). In contrast, no apparent differences in distribution of either β-catenin or p120-catenin were observed in these cells (Figure 2.7B). As the zigzag pattern of VE-cadherin distribution at cell–cell junctions has been correlated with increased permeability of ECs [28-30], these results suggest that FAK may affect EC integrity through its regulation of VE-cadherin in a kinase-dependent manner.

Tyrosine phosphorylation of VE-cadherin by a number of kinases has been shown to regulate its functions in cell-cell adhesion and vascular permeability in previous studies [26, 29, 31]. In particular, phosphorylation of Y658 of VE-cadherin resulted in reduced association of p120-catenin and increased EC permeability [32]. Therefore, the phosphorylation status of Y658 of VE-cadherin was examined in primary ECs from $FAK^{flox/flox}$, $FAK^{+/flox}$ and $FAK^{flox/KD}$ mice that had been infected by Ad-lacZ or Ad-Cre (see Figures 2.4D and 2.5C). To our surprise, infection of $FAK^{flox/flox}$ or $FAK^{flox/KD}$ ECs but not $FAK^{+/flox}$ ECs by Ad-Cre reduced Y658 phosphorylation rather than increased it (Figure 2.8, C and D).

Y658 of VE-cadherin is a site of phosphorylation by Src upon VEGF stimulation [32], which is also dependent on Pyk2 in the phosphorylation induced by leukocyte binding through ICAM1 [31]. Our results suggest that the basal phosphorylation of this critical residue also requires FAK kinase activity. As FAK activation and autophosphorylation at Y397 during cell adhesion has been suggested to stimulate Src activation through binding its SH2 domain [3-7], it is possible that the basal phosphorylation of Y658 is also through Src in a FAK-dependent manner. However, similar activation levels of Src were found in the primary ECs with FAK deletion or expression of only kinase-defective FAK compared to the control cells (Figure 2.8C, compare lanes 2 and 6 with lanes 1 and 5), suggesting that FAK may promote Src phosphorylation of Y658 through a scaffolding function, which requires autophosphorylation of Y397 of FAK present in wild type FAK, but not kinase-defective FAK (see Figure 2.5C), rather than by increasing Src activity per se.

Interestingly, a recent study showed that deletion of exon 15 (containing Y397) of FAK also caused late embryonic lethality with multiple vascular defects similar to those in CFKI embryos [33], suggesting that defective basal Y658 phosphorylation in VE-cadherin mediated by FAK could also be responsible in these embryos. Based on our data and the previous reports [31-33], it is possible that either increased or decreased phosphorylation of VE-cadherin at Y658 may lead to reduced EC barrier function. The former could play an essential role in physiological processes such as leukocyte transmigration in defense [31] whereas the latter could lead to defective embryonic development as observed here or potentially other pathological consequences.

2.5 Conclusions

By creating a FAK kinase-defective knockin allele in mice, we identified both kinase-independent and dependent functions of FAK important in vascular development during embryogenesis, as shown in a working model (Figure 2.8E). Deletion of FAK in ECs results in their increased apoptosis as well as more subtle defects like increased permeability, leading to severe vascular developmental defects and embryonic lethality around E13.5. The expression of kinase-defective FAK was able to rescue the increase of EC apoptosis through regulation of p21 by kinase-independent functions of FAK to allow further development of the embryos beyond this stage. However, FAK kinase activity is required for maintaining normal barrier function of ECs through proper phosphorylation of Y658 of VE-cadherin. As CFKI embryos progress in development, these subtle vascular defects led to lethality around E15.5, possibly as a result of the increased reliance of embryos on vessel integrity as they got bigger, the increased severity of defects in vessel integrity, or both.

Tyrosine kinases play essential roles in many biological processes, including vascular development, and identification of their targets have been the focus of studies over the years (for reviews see [34, 35]). Our results demonstrating a kinase-independent function of FAK in vascular development raise the possibility of such mode of actions by other tyrosine kinases in development and diseases. Therefore, future investigation on such possibilities (especially in the context of in vivo developmental or disease models) will complement the efforts in identifying key substrates of tyrosine kinases to provide a better understanding of tyrosine kinase signaling mechanisms. Future studies will also be necessary to clarify the specific FAK kinase–independent and –dependent downstream pathways in the regulation of crucial targets involved in ECs and perhaps other cell types in embryogenesis and

other biological processes. The direct demonstration of both kinase-independent and -dependent functions of FAK in vivo highlights the necessity to develop drugs that can inhibit all of FAK functions beyond the currently available kinase inhibitors for FAK ([36-38]) in the treatment of cancer and possibly other diseases.

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Chapter 3

ROLE OF PROII OF FAK IN MAMMARY TUMOR GROWTH AND METASTASIS

3.1 Abstract

Focal adhesion kinase (FAK) plays an important role in mammary tumorigenesis and metastasis. Specific deletion of FAK in mammary epithelial cells suppressed mammary tumor formation, growth and metastasis. Previous study showed that the C-terminal 2nd proline rich motif of FAK could regulate invasion of Src-transformed fibroblasts and tumor cells by regulating the expression of MT1-MMP on the plasma membrane. Here we studied the specific roles of this proline-rich motif of FAK in mammary tumor formation and development with in vivo mouse models. We generated and analyzed total knockin and mammary epithelial cell-specific and endothelial cell -specific conditional FAK knockin mouse models with mutations in this domain (designated as PA mutation). We found that homozygous mice with PA mutation did not affect mammary gland development. However, by utilizing PyVT human breast cancer mouse model, we found that all three types of knockin mice showed considerably reduced mammary tumor growth rate compared with the control mice, although the tumor appearance dates are similar between the knockin mice and control. Mammary tumor cells with the PA mutation showed reduced migration and increased cell aggregation, but comparable proliferation and survival to wild type tumor cells under subconfluent conditions. Upon confluence, however, the tumor cells with the PA mutation showed decreased "foci" formation, cell proliferation and cell survival, indicating that the second C-terminal proline-rich motif of FAK could be important for the loss of contact inhibition of mammary tumor cells. Moreover, confluent mammary tumor cells with the PA mutation also showed decreased β-

catenin expression and JNK phosphorylation, which are known to play important roles in regulating cell contact inhibition process. On the other hand, the specific knockin of this motif in the endothelial cells resulted in formation of mal-functioning blood vessels. In summary, by generating various conditional knockin mice models, we identified that FAK could promote mammary tumor growth through distinct mechanisms in different systems.

3.2 Introduction

As a critical component that integrates cell-ECM interactions, FAK has been implicated in the development and progression of breast cancers and other malignancies [1, 2]. Pathological studies suggested that a large fraction of breast cancers express elevated levels of FAK [3, 4]. Genetic analysis of primary tumor specimens and whole animal and cell culture models revealed the important roles of FAK in breast cancers: *FAK* is frequently amplified in human breast cancers [2]; FAK is required for Ras- and PI3K-dependent transformation of the mammary epithelial cells [5]; in addition, high level of FAK correlates with progression to metastasis in human breast cancers [3, 4]. Previous studies by our lab and others confirmed the critical roles of FAK in the initiation and progression of mammary tumors [5-8]. In vitro cellular function assays on these FAK-deleted mammary epithelial cells showed decreased cell proliferation and matrigel invasion, and increased anoikis, suggesting that FAK may promote mammary tumor cell survival, proliferation, and invasion [5, 6, 8]. Furthermore, analysis of protein expressions revealed that phosphorylation of p130cas and paxillin were reduced upon the depletion of FAK activity, which suggested that these molecules could be involved in these regulations mediated by FAK [5, 6]. However, due to the complexity of FAK signaling transduction, the

specific roles of a particular domain or motif of FAK in mammary tumorigenesis or progression is still unknown.

MT1-MMP is a member of MT-MMP subset of MMPs that contain additional sequences capable of anchoring on plasma membrane [9-13], which has been proposed to play a critical role in both physiology and pathology by remodeling the ECM. MT1-MMP is also over-expressed in various tumor tissues, including human colon, breast, and head and neck carcinoma [9, 14, 15], suggesting that MT1-MMP could play a critical role in tumorigenesis and tumor progression. In 2005, our lab identified a regulation of membrane expression of MT1-MMP by FAK interaction with endophilin A2 [16]. This investigation reported that v-Src-transformed cells activated a FAK-dependent phosphorylation of endophilin A2 by Src that could attenuate the endocytosis of MT1-MMP, which in turn increased cell-surface expression of MT1-MMP and cellular degradation of ECM. This regulation was found to be mediated by interactions between FAK's second C-terminal proline-rich motif (P872-P879) and endophilin A2's SH3 domain. This study suggested a regulatory mechanism of cell invasion whereby FAK promotes cell-surface presentation of MT1-MMP through its second proline-rich motif in the C-terminal, and provided a potential explanation of the roles of FAK in the formation, progression and metastasis of mammary tumors. However, since this study was carried out in the v-src transformed cell lines, and most of the experiments were done in vitro, the role of the second C-terminal proline-rich motif of FAK in vivo, i.e. in development and in tumorigenesis have not been evaluated directly.

In our study, by generating knockin mutant mice carrying mutations in the second C-terminal proline-rich motif of FAK and utilizing PyVT breast cancer mouse model, we revealed the potential roles of FAK in mammary tumor growth through regulating

cell-cell contact inhibition with this motif. Through targeting mutations specifically in endothelial cells, we also concluded that this motif of FAK may be essential for fully functioning blood vessels in mammary tumors by regulating endothelial cells.

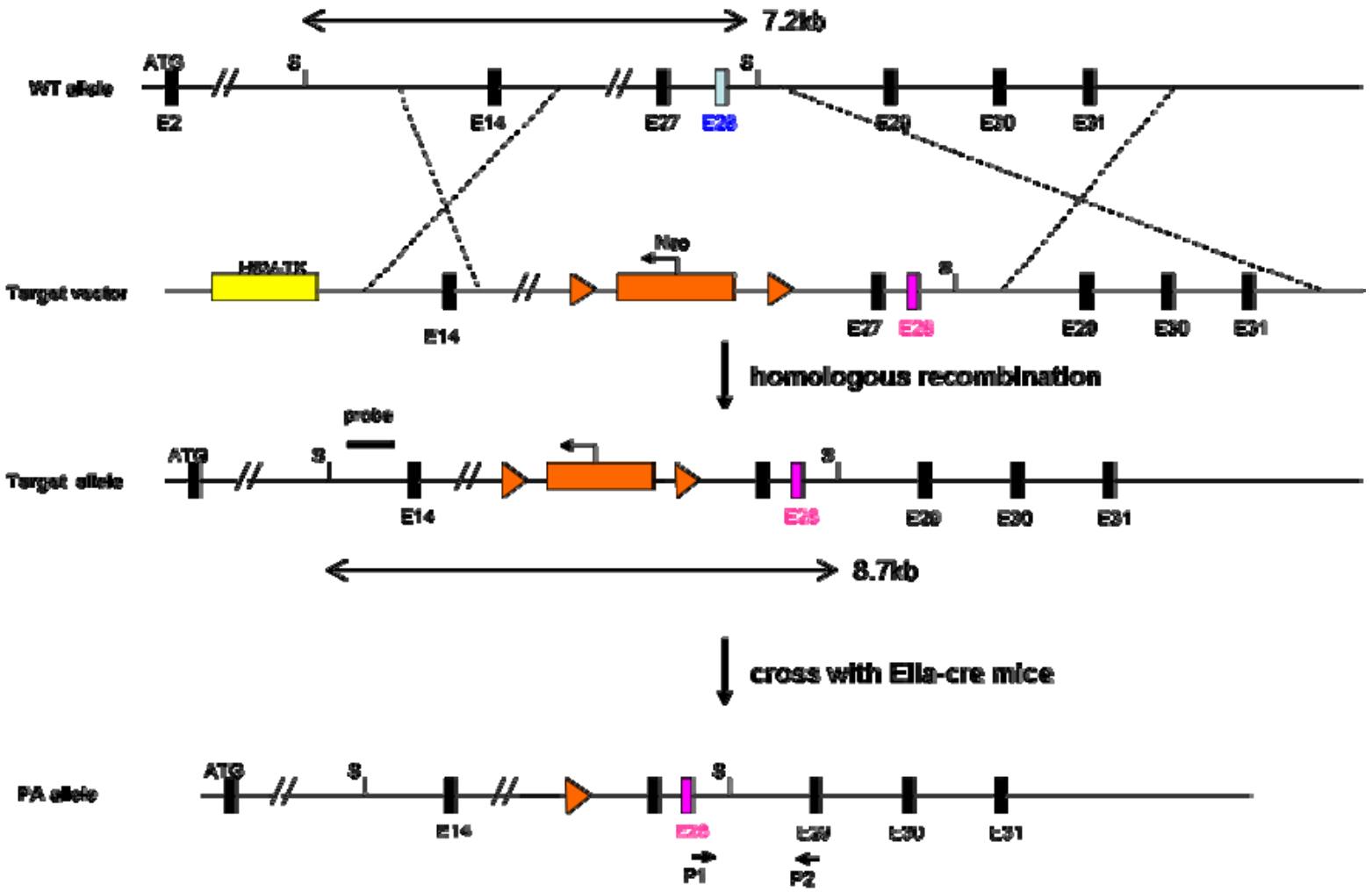
3.3 Materials and Methods

Construction of the targeting vector and generation of CFKI mice

Based on available mouse genome sequences in the Ensembl database, an isogenic 129SvJ mouse BAC genomic clone containing FAK exon 28 (where the second C-terminal proline-rich motif is encoded) and flanking sequences were obtained from BACPAC Resources Center. The presence of exon 28 in the BAC clone was verified by PCR using two pairs of primers surrounding exon 28 as well as Southern blotting. A targeting vector containing a mutated exon 28 (P872/876/879 to A) and a neo cassette was then constructed for homologous recombination (Figure 3.1).

Gene targeting in 129P2/OlaHsd- derived E14Tg2a mouse embryonic stem cells [17] was performed as described [18] with the use of ESGRO (Chemicon, Temeculah, CA). Chimeric mice were identified by coat color, and then bred to C57BL/6J mice. Transmission of the germ line was identified by PCR and confirmed by Southern blotting (Figure 3.2 left). Heterozygous targeted mice bearing PA[neo] allele (FAK+/PA[neo] mice) were obtained and then crossed with EIIa-Cre mice (Jackson Laboratory), which express Cre in the very early stage of embryogenesis [19], to delete the neomycin cassette in order to avoid its possible interference with FAK gene expression. The progenies with neomycin cassette removed (FAK+/PA; EIIa-Cre mice) were crossed with C57BL/6J mice to segregate the FAK PA allele from the heterozygote EIIa-Cre transgene. The resulting heterozygous and homozygous FAK

Figure 3.1 | Schematic of mouse FAK target vector, and genomic and targeted allele in FAK loci. Closed orange triangles represent loxP sites. The proline 872,876 and 879 to alanine mutations (i.e., PA) in the knockin allele is in exon 28 (marked by blue and pink colors, before and after mutation, respectively). Horizontal lines with arrows on both sides indicate the expected sizes of DNA bands in Southern blotting analysis of the genomic or targeted alleles as detected by the probe (thick horizontal bar) just 5' end upstream from the targeting vector. Crosses of the mice with targeted allele to EIIa-Cre mice result in the deletion of the floxed neomycin cassette to create the FAK PA knockin allele. The relevant restriction sites (ScaI [S]) and primers (P1 and P2) for PCR genotyping are indicated.


7.2kb
WT allele
ATG
S
E2
E14
E27
E28
E29
E30
E31
Target vector
HSV-TK
Neo
E14
E27
E28
E29
E30
E31
homologous recombination
probe
Target allele
ATG
S
E14
E28
E29
E30
E31
8.7kb
cross with Ella-cre mice
PA allele
ATG
S
E14
E28
E29
E30
E31
P1
P2

knockin mice (FAK+/PA mice and FAKPA/PA mice) were identified by PCR analysis and confirmed by sequencing of tail DNA.

Floxed FAK and MFCKO mice have been described previously [20, 21] MMTV-PyVT transgenic mice [22] were obtained from the mouse repository of Mouse Models of Human Cancers Consortium at National Cancer Institute, and has been described previously [6]. All mice used in this study were bred and maintained at University of Michigan under specific pathogen-free conditions in accordance with institutional guidelines.

Genotyping by PCR

Mice and embryos were genotyped by PCR analysis of genomic DNA. Isolation of genomic DNA was described previously [23]. Primers used to genotype flox and delta FAK alleles were 5'-GCTGATGTCCCAAGCTATTCC-3' and 5'-AGGGCTGGTCTGCGCTGACAGG-3', as described previously [20]. Primers used to genotype FAK PA knockin allele were P1 5'- GTCAGAAAATTAGGTATGGT–3' and P2 5' – TTTTGTATATCTGCATGACT -3' shown in Figure 3.1. PCR were performed for 30 cycles of 94^{o}C 3min, 67^{o}C (for flox allele) or 55^{o}C (for PA allele) 2 min, and 72^{o}C 4min. CreF (5'-GCAGAACCTGAAGATGTTCGCGATTA-3') and CreR (5'-TCTCCCACCGTCAGTACGTGAGATATC-3') primers were used to detect the Cre transgene, which was performed for 35 cycles of 95^{o}C 30 seconds, 60^{o}C 30 seconds and 72^{o}C 30 seconds. Mice genotyping for PyVT allele was performed, as described previously [22, 24].

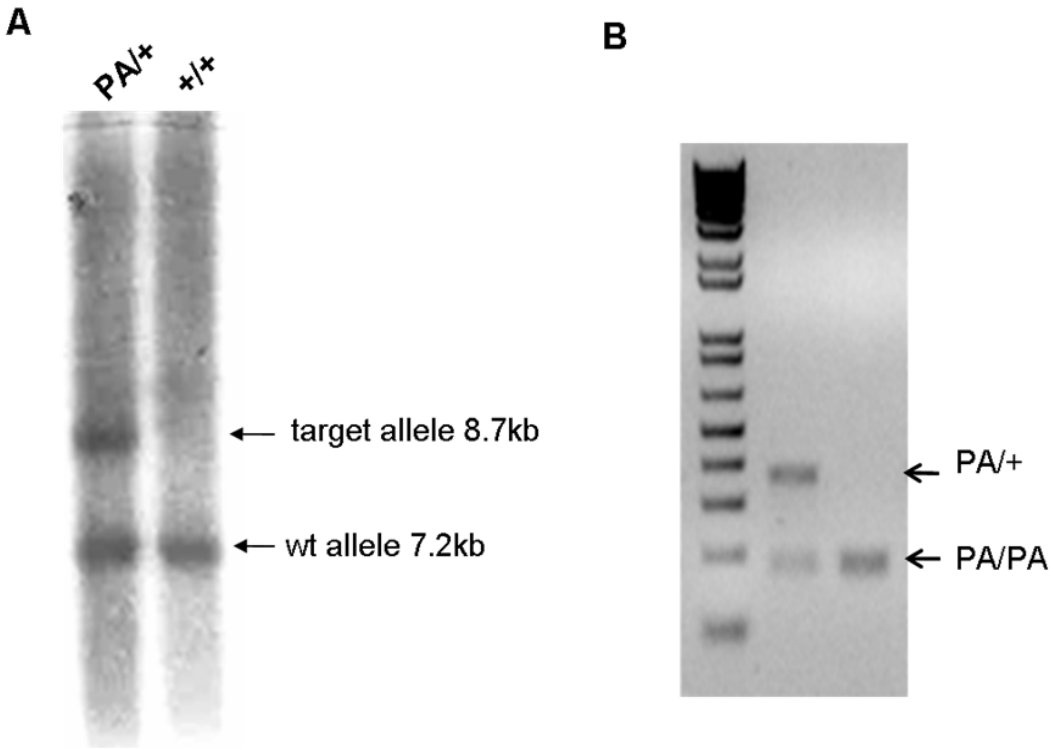


Figure 3.2 | Screening heterozygous and homozygous PA mice by southern blotting and PCR. (A) Southern blotting analysis of the tail DNA from representative mice after digestion with ScaI. (B) PCR genotyping of the tail DNA from representative mice, using primers P1 and P2 as indicated in Figure 3.1.

Mammary gland whole mounts, histology, immunohistochemistry, and immunofluorescent labeling

Mammary glands were excised, and whole mounts stained with carmine alum were analyzed, as described previously [21]. Mammary tumors or lungs were harvested from mice and subjected to analysis by histology, or immunohistochemistry labeling as described previously [21, 25]. The following antibodies were used: E-cad (1:200, Santa Cruz Biotechnology), beta-catenin (1:200, Santa Cruz Biotechnology). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole/antifade (Invitrogen)

Preparation of mammary tumor cells

Primary mammary tumor cells were prepared as previously described [6]. Briefly, after 4 to 5 weeks of tumor appearance, primary tumors or tumor transplants were removed and dissociated mechanically and enzymatically to obtain single-cell suspension. Tumor tissues were minced and dissociated in Ham's F12/DMEM (1:1, Invitrogen) supplemented with 10 mmol/L HEPES, 2% bovine serum albumin (BSA; Fraction V, Invitrogen), 5 mg/mL insulin, 0.5 mg/mL hydrocortisone, 10 ng/mL cholera toxin, 300 units/mL collagenase, and 100 units/mL hyaluronidase (all from Sigma) at 37°C for 2 to 4 h. Tumor cells were collected by centrifuging the cell suspension at 100 x g for 10 mins followed by one wash with F12/DMEM. The resulted tumor cell pellet was further digested for 5 mins in 0.05% trypsin/0.025% EDTA (Life Technologies) solution to generate a single-cell suspension. An equal volume of F12/DME-H supplemented with 5% fetal bovine serum was added to stop the digestion. The cell suspension was filtered twice through a 40-μm nylon mesh (BioDesign, Inc.). After centrifugation at 100 x g, the pellet was resuspended in F12/DMEM with a reduced calcium concentration (0.06 mmol/L, StemCell Technologies) supplemented with 5 units/mL dispase (Collaborative Biomedical

Products). To remove RBC, the pellets were treated with ammonium chloride solution. Tumor cells were prepared from tumors developed in multiple mice and pooled for the following analyses.

Western blotting

Membrane versus cytosolic proteins were extracted according to described previously [26]. Briefly, cells were lysed in ice-cold buffer A (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 250 mM sucrose, 1 mM sodium vanadate, and 1x protease inhibitor cocktail; Roche, Indianapolis, IN) using a 19-gauge needle. The samples were centrifuged at 1500 x g, 4°C for 10 min to pellet nuclei and cell debris. Membranes were pelleted from supernatants by centrifugation for 1 h at 10,000 x g in a Beckman ultracentrifuge. The resultant supernatant represented cytosolic fractions. The pellets, representing membrane fractions, were resuspended in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 250 mM sucrose, 1 mM sodium vanadate, and 1x protease inhibitor cocktail for 45 min at 4°C and then cleared by centrifugation. Total protein concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA). Antibodies used are anti-β-catenin, anti-p21, anti-p27, anti-E-cadherin and anti-actin (all from Santa Cruz Biotechnology); anti-phospho-JNK, anti-JNK and anti-phospho-p44/42 (all from Cell Signaling Biotechnology); and anti-N-cadherin (Biosource International).

Boyden chamber assay

Cell migration assays using modified 48-well chemotaxis Boyden chamber (Neuro Probe) were performed as described previously [20]. Briefly, 7.5 × 103 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).

Cell Aggregation Assay

Cell aggregation assays were performed as described earlier [27, 28]. The cells were detached in 0.2% EDTA in Hank's buffer. The degree of cell aggregation was calculated as $D = (No - Nt)/No$, where Nt is the number of remaining particles at the incubation time point t, and No is the initial number of particles corresponding to the total number of cells.

BrdU incorporation assay

Primary mammary tumor cells before confluency were serum starved for 18 h to arrest the cells in G0. Cells were then released from G0 by replating the cells in mammary tumor cell culture medium and 150 μM BrdU. After 48 hrs of growth, BrdU incorporation assays were performed using BrdU staining kit from Invitrogen according to the instruction. Briefly, labeling medium was removed from cells and cells were fixed in 70% alcohol for 15-30 mins at 4°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 mins. After 3 washes, cells were incubated with diluted denaturing solution for 30 minutes, followed by blocking solution (10 mins), primary biotinylated mouse anti-BrdU antibody (30 mins) and streptavidin-peroxidase secondary antibody (10 mins) incubation sequentially. Staining was developed with DAB reagent and counterstained with hematoxylin.

Soft agar assay (colony formation)

Soft agar assay was carried out as described previously [29]. Simply, cells were suspended in 0.3% agar medium (DMEM containing 10% FBS) and then plated on a 0.6% agar base layer at a concentration of 4×10^4 cells per 60-mm dish. The cells were incubated in a humidified atmosphere (5% CO_2) at 37°C. The number of colonies that were 50 μm or larger were counted after two weeks.

Statistical analysis

Statistical significance was evaluated by paired t test, using $P < 0.05$ as indicative of statistical significance. Kaplan-Meier tumor-free survival data were compared using the log-rank test.

3.4 Results

3.4.1 Generation of FAK mutant knockin mice

To study the role of the second C-terminal proline-rich motif of FAK in vivo, we generated a mutant FAK allele carrying three point mutations in this motif in the endogenous FAK gene (indicated as ProII hereafter). Homologous recombination was utilized to knockin the mutant FAK gene. The Pro872/876/879 to Ala mutation in the ProII of FAK was created in exon 28 of FAK genomic DNA, as described in Materials and Methods. A targeting vector containing the mutated exon 28 (P872/876/879A) and a neo cassette with flanking LoxP sequences (designated as PA[neo] allele) was then constructed and used to generate mutant mice containing the knockin mutant allele employing standard homologous recombination methods (Figure 3.1). Southern blotting analysis of DNA from targeted embryonic stem cells verified the presence of the PA[neo] allele in the cells (Figure 3.2, panel A). Heterozygous targeted mice

bearing PA[neo] allele (FAK $^{+/PA[neo]}$ mice) were obtained and then crossed with EIIa-Cre mice, which express Cre in the very early stage of embryogenesis [19], to delete the neomycin cassette in order to avoid its possible interference with FAK gene expression. The progenies with neomycin cassette removed (FAK $^{+/PA}$;EIIa-cre mice) were crossed with C57BL/6J mice to segregate the FAK PA allele from the heterozygote EIIa-Cre transgene. The resulting heterozygous and homozygous FAK knockin mice (FAK $^{+/PA}$ mice) were identified by PCR analysis (Figure 3.2, panel B), and confirmed by sequencing (Figure 3.3) of tail DNA, as described in Materials and Methods. Heterozygous FAK $^{+/PA}$ mice were then intercrossed and homozygous FAK $^{PA/PA}$ mice were found to be born at Mendelian ratio, indicating that the PA mutation of the ProII motif did not result in embryonic lethality and that this motif is not essential for embryonic development. Both male and female FAK $^{PA/PA}$ mice mice grew normally till adulthood, and they are also fertile and indistinguishable from wild type mice (data not shown), confirming that the PA mutant allele (in the endogenous gene but not over-expressed) did not lead to any severe abnormalities during either embryonic or postnatal development.

3.4.2 Mutation of the second C-terminal proline-rich motif of FAK does not impair mammary gland development, but results in much decreased tumor growth

Though the ProII motif of FAK has been shown to mediate interactions between FAK and several important downstream molecules regulating different cellular functions [30-32], interestingly, homozygous knockin PA/PA mice are found to be viable, fertile and indistinguishable from wild type mice. In previous studies, FAK has been shown to be required for mammary gland development and functions. Thus the role of the ProII motif of FAK in mammary gland development was studied in the ProII total

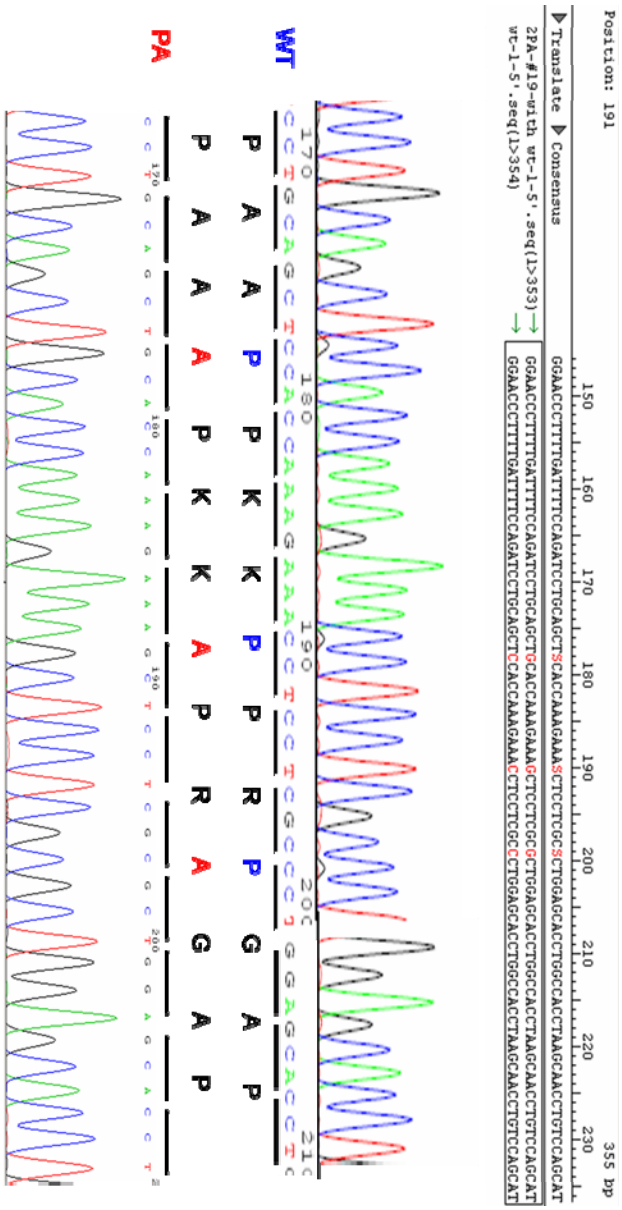


Figure 3.3 | Confirmation of PA mutations. The PCR fragments corresponding to the wild-type (WT) and PA FAK that were amplified from exon 28 were sequenced to confirm the mutations of the codons for P872/876/879 to A.

knockin mice. Extensive breeding analysis showed that female total knockin mice could give birth to normal litter size of offspring with normal Mendelian ratio and lactate normally. The mammary gland samples dissected from these mice did not show any difference in growth or differentiation from wild-type mice (data not shown). All of these data suggested that the ProII motif of FAK does not play a critical role in mammary gland development.

FAK has been implicated in the development of breast cancers. To study whether this ProII motif is important for mammary tumorigenesis, we crossed total ProII knockin mice (FAK $^{PA/PA}$) or heterozygous control mice (FAK $^{PA/+}$) with the MMTV-PyVT transgenic mice, which is a widely used mouse model that develops metastatic breast cancers induced by the PyVT oncoprotein [22]. Female mice with the genotypes FAK $^{PA/PA}$;MMTV-PyVT (designated as total knockin tumor mice) and FAK $^{PA/+}$;MMTV-PyVT (designated as control tumor mice) of 50% FVB background were established and mammary tumor development in these mice was monitored by physical palpation. Palpable mammary tumors were detected in half of both total knockin and control mice by approximately the age of 11 weeks ($T_{1/2}$ =11 weeks), and there was no statistical difference between these two groups (Figure 3.4, panel A). Consistent with these results, whole-mount staining of mammary glands from total knockin and control mice at the age of 6 and 8 weeks did not show significant difference in hyperplasia (Figure 3.4, panel B). These data indicated that mutations in the ProII motif of FAK did not result in the changes of mammary tumorigenesis driven by PyVT congene.

Next we analyzed whether the mutations of the ProII motif of FAK could affect tumor growth and lung metastasis. The average tumor sizes of the mammary tumors at different stages after tumor appearance in total knockin and control mice were

measured twice a week. Interestingly, the total knockin mice showed a much slower growth rate compared with the control mice (Figure 3.5). At 8 weeks after the initial detection of primary tumors, lung metastases were detected in almost all of the control tumor mice, but only about 35% of total knockin tumor mice are metastasis positive (Figure 3.6, panel A). By quantification, the number of metastatic nodules in the lung sections was greatly reduced compared with the controls (Figure 3.6, panel B). It should be noted that the much reduced tumor growth rate may contribute to, or even accounted for the decreased number of lung colonies observed in the total knockin mice. Similar analyses of the tumor growth and lung metastasis were performed with 100% FVB background tumor mice. Consistently, tumorigenesis of the total knockin tumor mice was similar to the controls (Figure 3.7, panel A), but the tumor growth rate was still lower than that of the controls, though to a much lesser degree (Figure 3.7, panel B). Interestingly, the lung metastases analyzed by section staining still indicated a significant reduction for the knockin tumor mice (Figure 3.8), though the difference in tumor growth between knockin and control mice was quite small. This suggested that the ProII motif of FAK could regulate the metastasis of mammary tumors to lungs independently of the regulation of tumor growth. However, more detailed studies are still necessary to show a direct effect of the mutation of this motif of FAK on the suppression of metastasis.

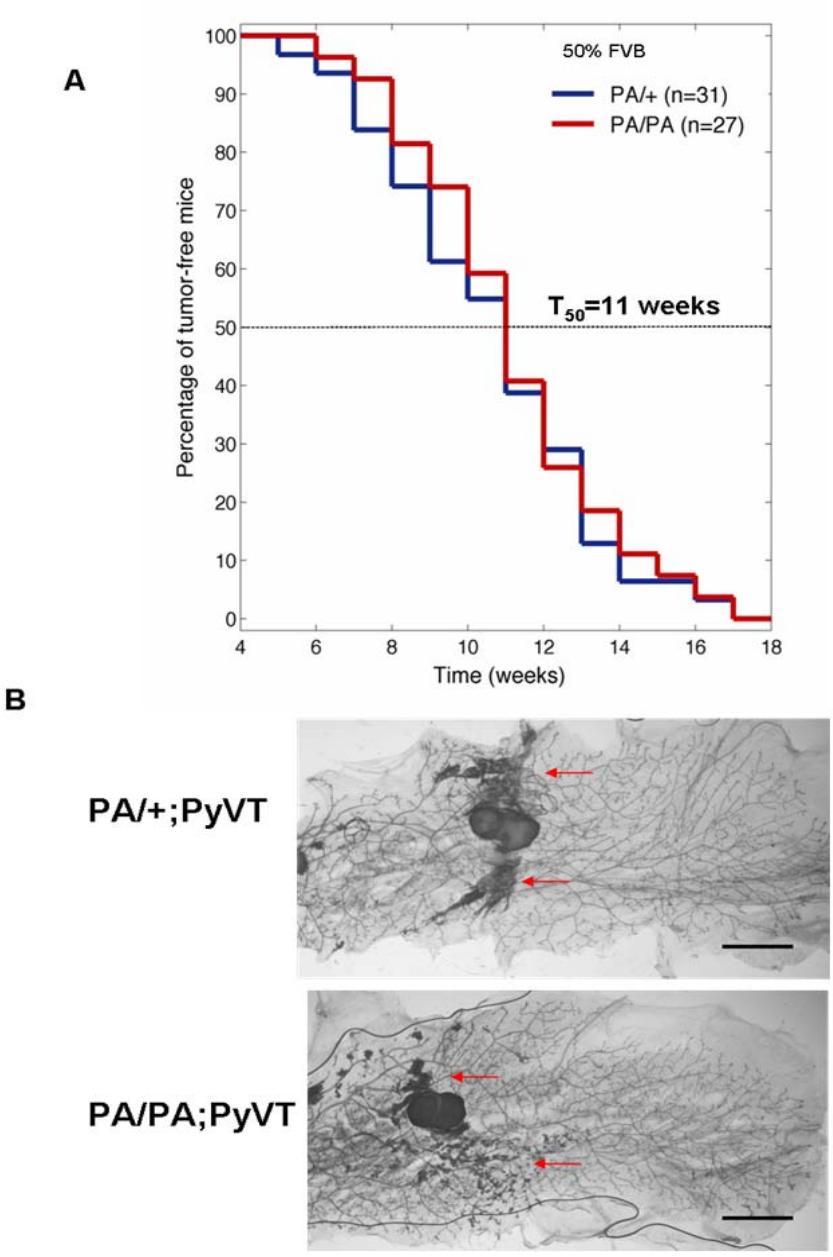


Figure 3.4 | PA mutations of FAK do not interfere with tumor initiation in PyVT breast cancer model. (A) Kaplan-Meier analysis of mammary tumor development in the control PA/+ (n = 31), and total knockin PA/PA (n = 27) mice. Control versus total knockin, P >0.05 by the log-rank test. (B) Representative mammary gland whole mounts from 6-wk-old control (top) and total knockin (bottom) mice. The arrow marks hyperplasic nodules. Scale bars, 5 mm.

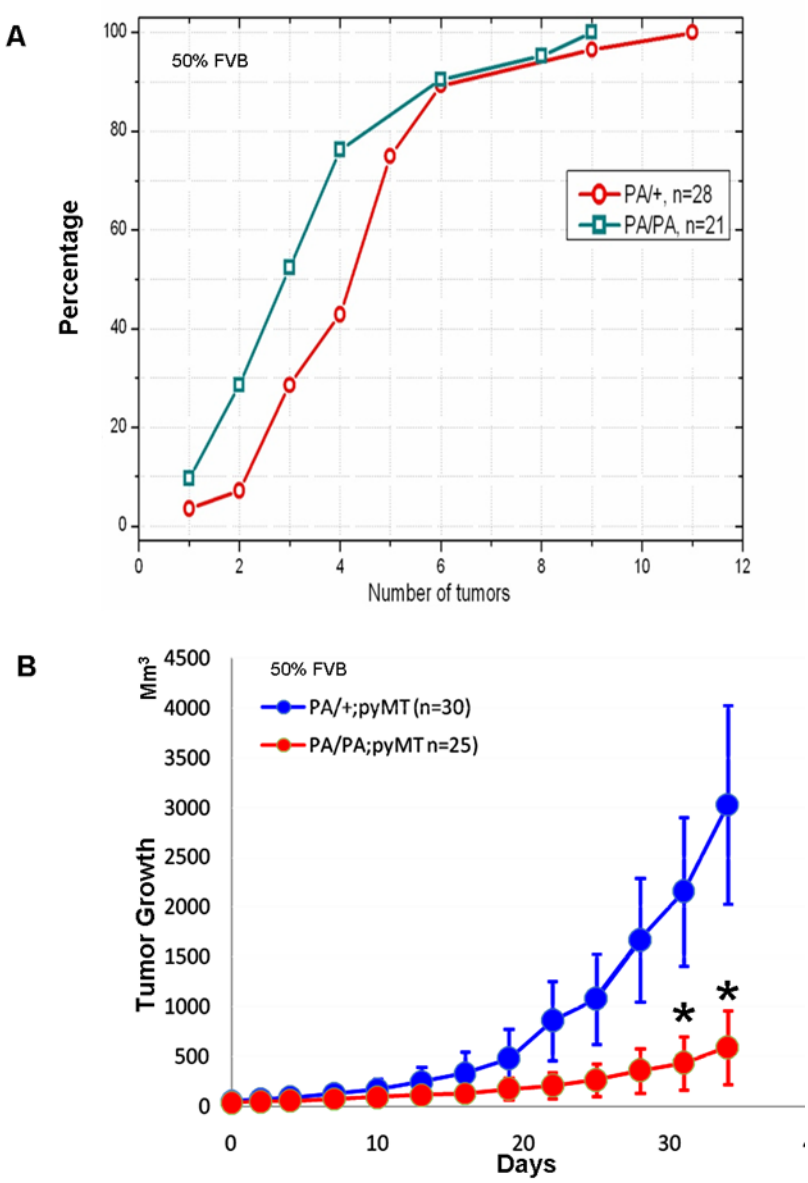


Figure 3.5 | Mutations of the second C-terminal proline-rich motif of FAK led to fewer tumor formations and decreased tumor growth. (A) Percentage of mice with palpable mammary tumors of indicated number per mouse at 8 weeks after tumor appearance. Total knockin versus control, $P < 0.05$. (B) Mean cumulative mammary tumor volume (±SD) for each genotype at indicated times after primary tumor appearance were plotted and analyzed. *, $P < 0.05$.

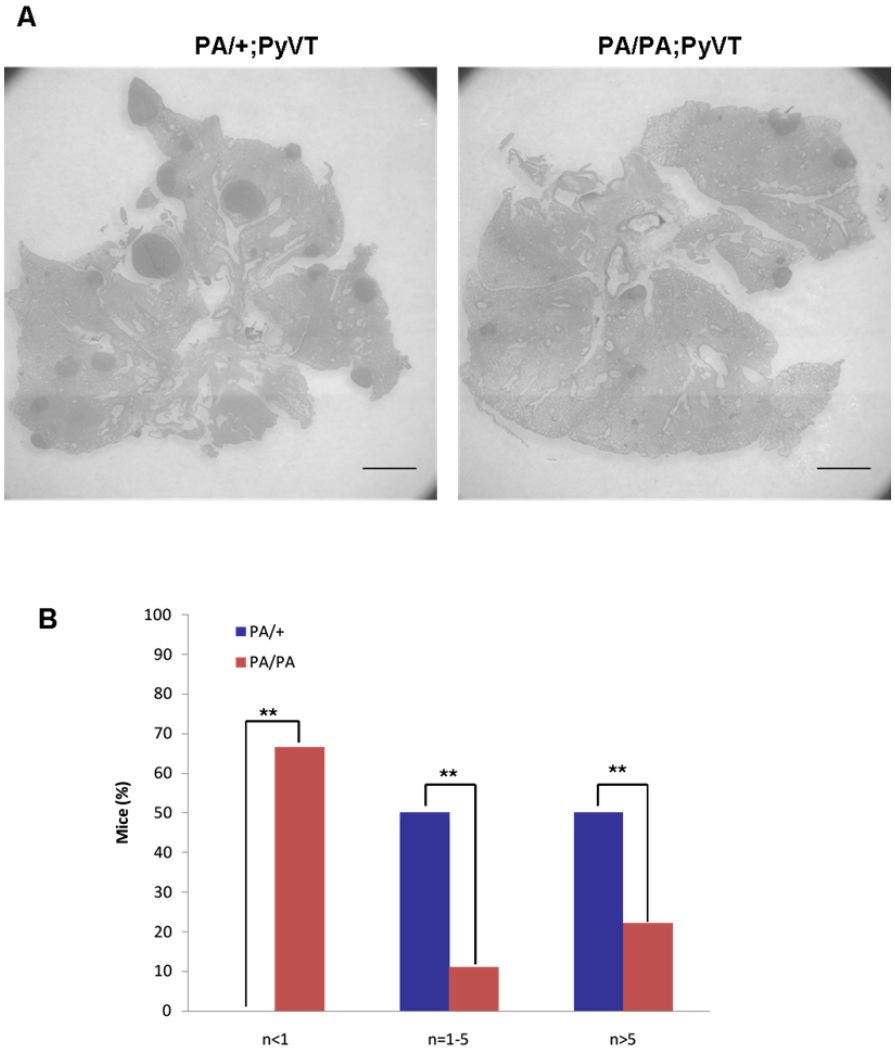


Figure 3.6 | Analysis of lung metastasis in FAK total knockin tumor mice. (A) representative lung sections (control, left and total knockin, right) were prepared at 8 wk after the primary mammary tumor onset and stained with H&E. Scale bars, 2 mm. (B) percentages of mice of the indicated genotype with <1, 1-5,or >5 metastases per lung section. The micrometastatic nodules were quantified under a microscope. **, $P < 0.01$.

3.4.3 Intrinsic PA mutation in the mammary epithelia cells can lead to reduced tumor growth and lung metastasis

In the total knockin tumor mice, all cells and tissues contain the mutated ProII motif in *FAK* gene. In order to figure out whether intrinsic PA mutation in the mammary tumor cells will lead to decreased tumor growth and lung metastasis, mammary epithelial tumor cells from either FAK $^{PA/PA}$ total knockin tumor mice or control FAK $^{PA/+}$ mice of 50% FVB background were isolated at the same days after tumor appearance, and transplanted into the pre-cleared mammary gland fat pads of NOD SCID mice. Tumor growth was monitored at regular intervals after transplantation. As shown in Figure 3.9, the tumors grown from the FAK $^{PA/PA}$ tumor cells were much smaller than those from the control tumor cells. And transplantation with either $5x10^4$ or $2x10^5$ cells generated the same conclusion. The transplantation experiments revealed that the PA mutation in the mammary epithelial cell itself could lead to reduced tumor growth. Lung metastasis was also checked in the recipient mice after the primary tumors reached similar sizes for two groups. Though not much metastases were seen in either group, there was still a reduction for the knockin group (data not shown). This result also confirmed that mutations in ProII motif could lead to defective metastasis of mammary tumor cells to lung, which is independent from their effects on the tumor growth.

To better clarify and confirm the intrinsic effects of the ProII motif mutation in tumor growth, we generated mice carrying a mammary epithelial cell-specific mutation of FAK (designated as MFCKI) by crossing MaEC(mammary epithelial cell)-specific FAK conditional knockout mammary tumor mice (designated as MFCKO tumor mice with FAK $^{f/f}$;MMTV-Cre;PyVT genotype) [6] with total

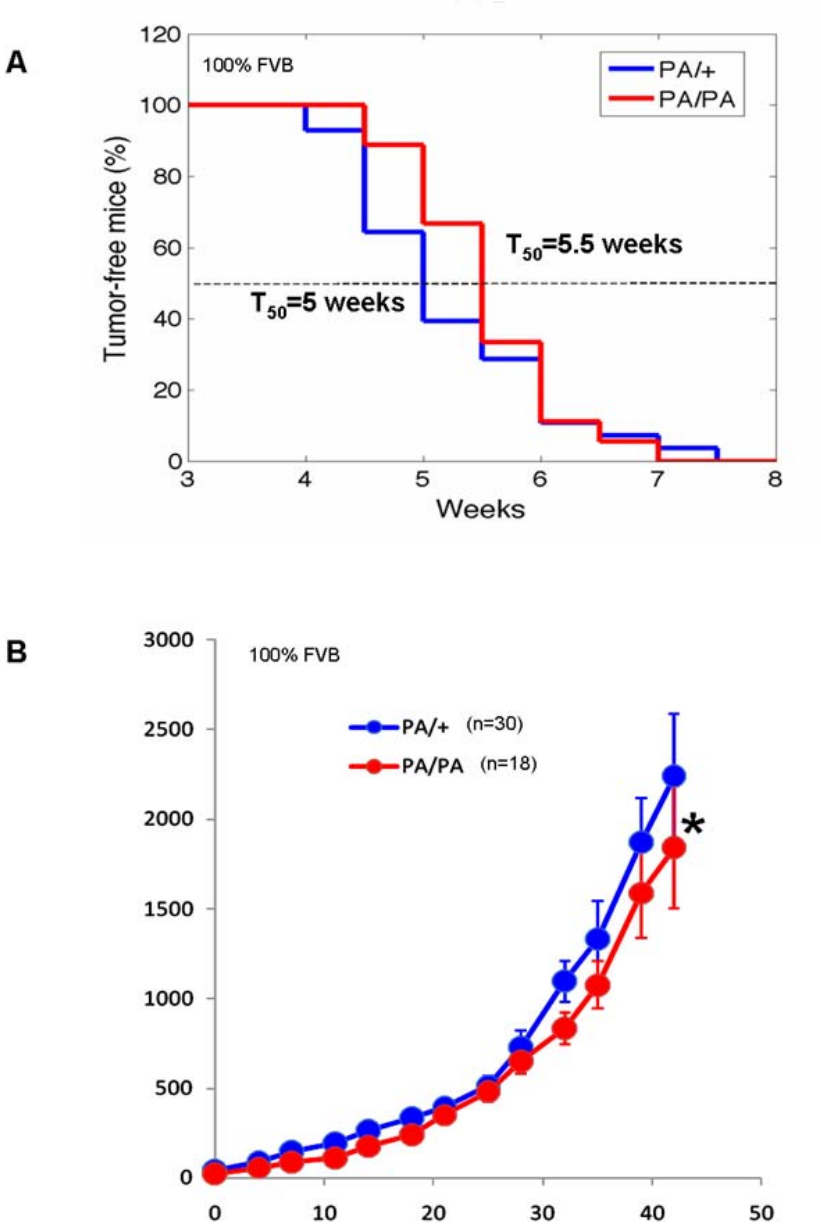


Figure 3.7 | Tumorigenesis and tumor growth in PA total knockin mice with 100%FVB background. (A) Kaplan-Meier analysis of mammary tumor development in control PA/+ (n = 30), and total knockin PA/PA (n = 20) mice. Control versus total knockin, P >0.05 by the log-rank test.(B) mean cumulative mammary tumor volume (±SD) for each genotype at indicated times after primary tumor appearance were plotted and analyzed. *, P < 0.05

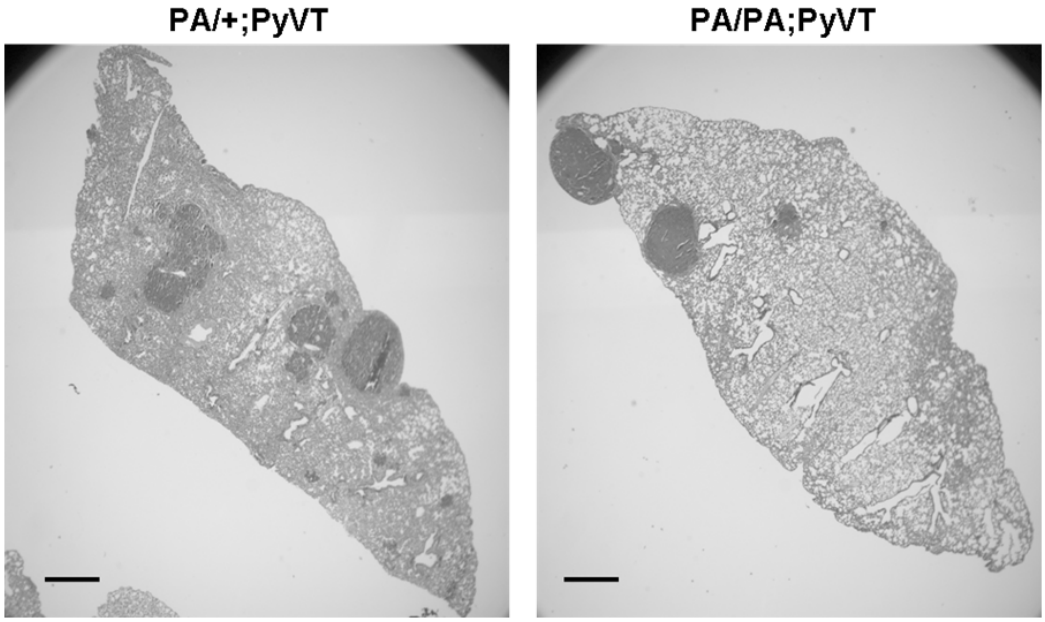


Figure 3.8 | Analysis of lung metastasis in total PA knockin tumor mice with 100% FVB background. Representative lung sections (control, left and total knockin, right) were prepared at 8 wk after the primary mammary tumor onset and subjected to H&E staining. Scale bars, 1 mm.

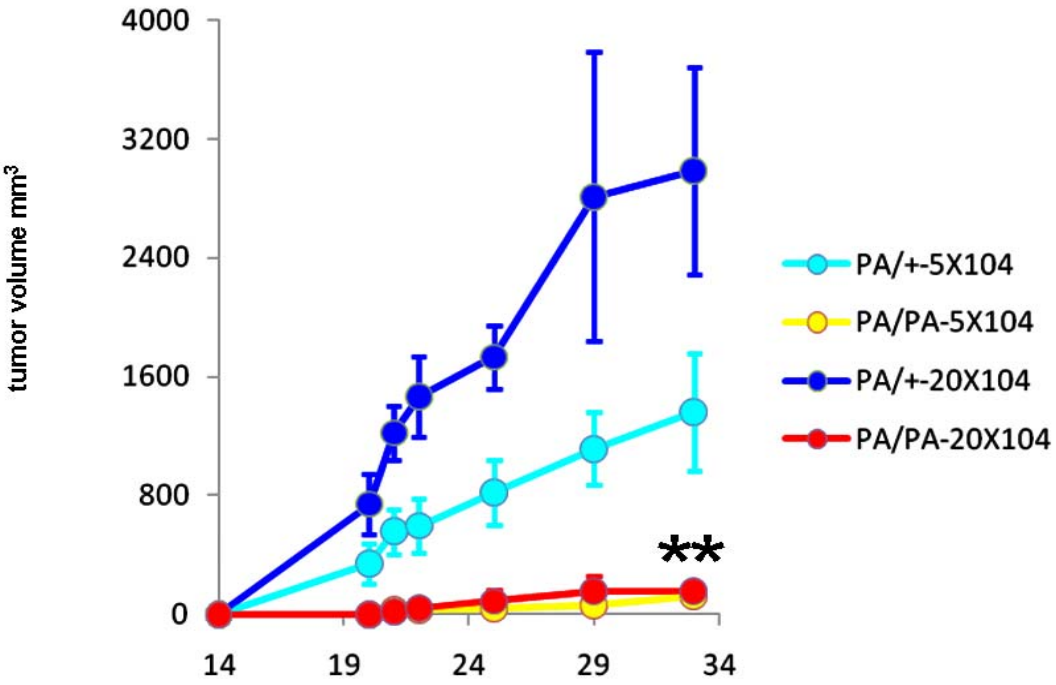


Figure 3.9 | Decreased tumor growth from PA knockin mammary epithelial tumor cells. Tumor growth curves resulted from tumor cells isolated from control PA/+;PyVT and knockin PA/PA;PyVT mice of 50% FVB background. Either $5X10^4$ or $20X10^4$ cells were used for transplantation.**, $P<0.01$.

FAK $^{PA/PA}$ mice. These MFCKI mice, with genotype as FAK $^{PA/f}$;MMTV-cre;PyVT, express FAK PA mutated protein only in mammary epithelial cells from the knockin PA mutant allele (flox allele is converted to deleted allele by MMTV-cre), but contain functional FAK (i.e. the flox allele) in other cells. We found that, similar to the total knockin tumor mice, there was no difference in tumor-free interval between conditional knockin tumor mice and the controls (Figure 3.10, panel A). However, tumor volume measurement revealed that tumor growth in the MFCKI tumor mice was significantly reduced by the mutation compared with the control tumor mice (FAK $^{PA/f}$;PyVT) without MMTV-Cre expression (Figure 3.10, panel B). Furthermore, lung metastasis was also reduced in MFCKI tumor mice compared to control mice (Figure 3.11). Together with data from transplantation experiments, we concluded that the mutations of FAK ProII motif in the mammary epithelial cells alone can lead to defective tumor growth and metastasis. However, we could not exclude that the PA mutation in the ProII of FAK in other cells and tissues could also contribute to the reduced mammary tumor growth and lung metastasis observed in total knockin tumor mice.

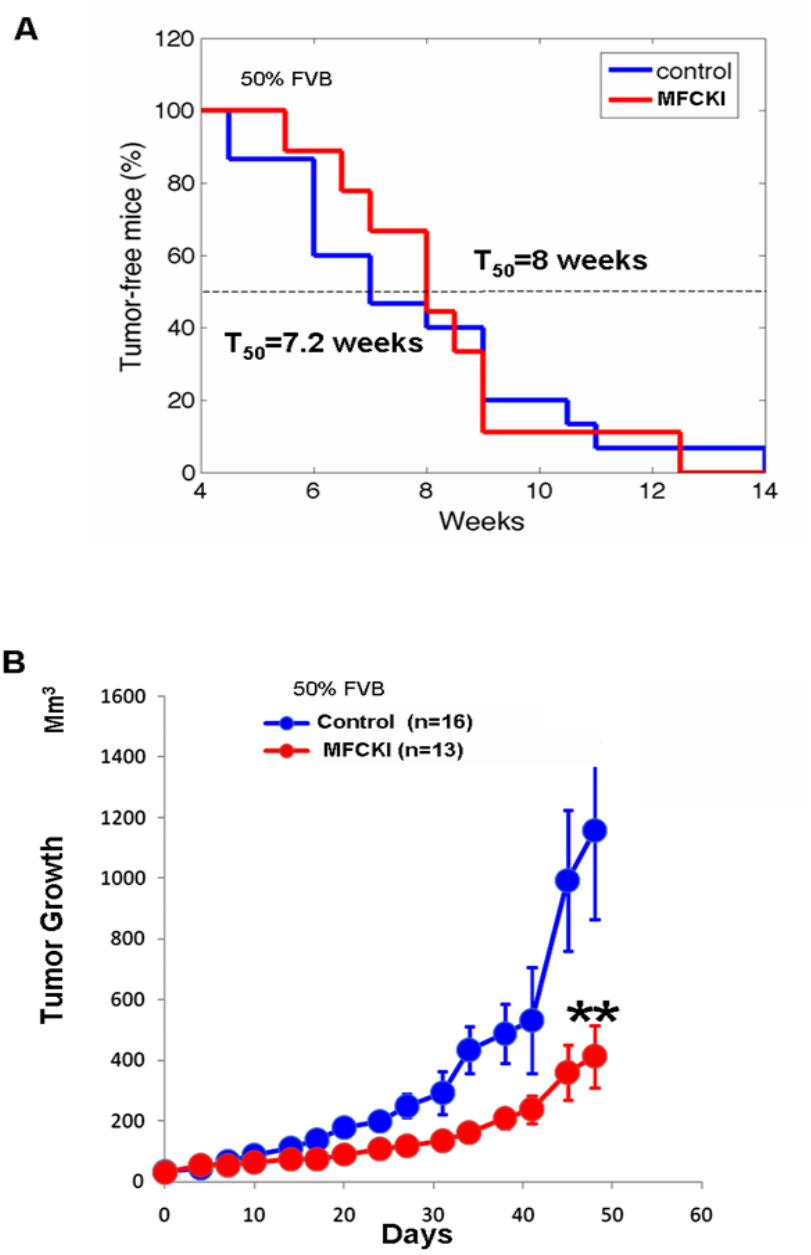


Figure 3.10 │PA mutations of FAK in mammary epithelial cells alone resulted in slowed tumor growth. (A) Kaplan-Meier analysis of mammary tumor development in control (n = 16), and MFCKI (n = 13) mice. Control versus MFCKI, P >0.05 by the log-rank test.(B) mean cumulative mammary tumor volume (±SD) for each genotype at indicated times after primary tumor appearance were plotted and analyzed. **, P < 0.01.

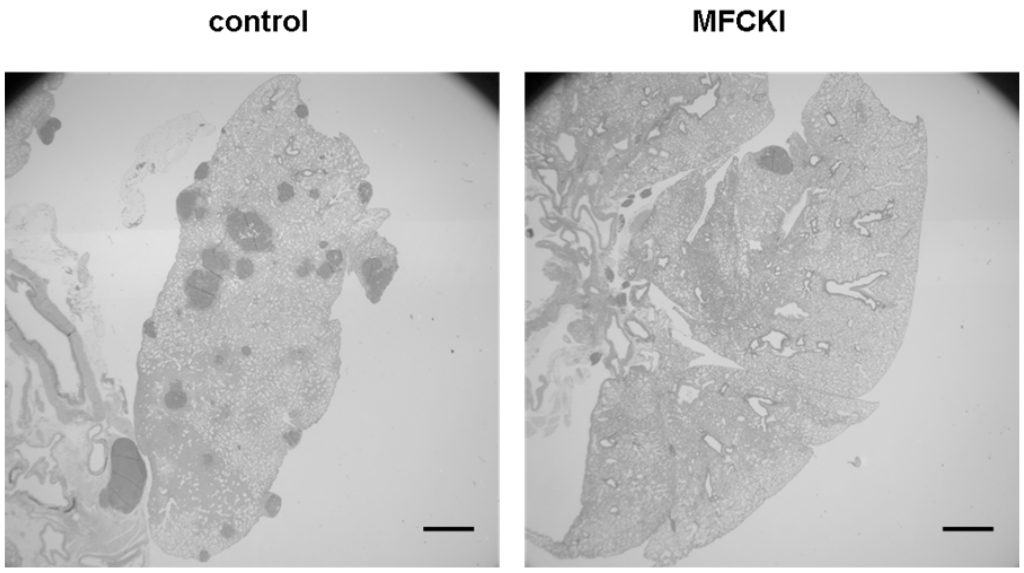


Figure 3.11 │Analysis of lung metastasis in mammary epithelial specific knockin tumor mice. Representative lung sections (control, left and MFCKI, right) were prepared at 8 wk after the primary mammary tumor onset and subjected to H&E staining. Scale bars, 1 mm.

3.4.4 PA mutation resulted in decreased cell migration and increased cell aggregation

To investigate the mechanism involved in the reduced tumor growth in the knockin mice and the roles of the ProII motif of FAK in tumor progression, mammary tumor epithelial cells were isolated from either control (FAK $^{PA/+}$; PyVT) or knockin (FAK $^{PA/PA}$;PyVT) tumor mice and analyzed in vitro using different assays as described in Materials and Methods [6].Cell proliferation indicated by BrdU incorporation assay (Figure 3.12, panel A and B) and cell survival with or without treatments determined by ELISA analysis (Figure 3.13, panel A) both showed negative results between

PA/PA;PyVT cells and control PA/+;PyVT cells. These results indicated that the reduced tumor growth in the FAK $^{PA/PA}$ tumor mice was not due to defective tumor cell proliferation, or increased apoptosis of the mutated tumor epithelial cells. Moreover, we did not detect any difference in anchorage independent growth (Figure 3.13, panel B) or colony formation in soft agar (Figure 3.14) for tumor cells from total knockin mice compared to control cells. These results suggested that PA mutation in the ProII motif of FAK did not affect proliferation and survival of the mammary epithelial cells, including anchorage independent growth (anoikis). Therefore, the reduced tumor growth in the knockin tumor mice was likely caused by changes in other cellular properties rather than defective cell proliferation or cell survival.

Cell migration was checked using Boyden chambers, and the results showed that FAK $^{PA/PA}$ mammary tumor cells (PA/PA;PyVT) moved much slower towards FN than the control tumor cells (PA/+;PyVT), as shown in Figure 3.15 panel A and B. However, the motility towards serum was not different between these two cells (data not shown), suggesting that the defective cell motility of the knockin tumor cells was substrate specific. When trypsin was used to treat the attached mammary epithelial cells, the FAK PA/PA mammary tumor cells rounded up and detached much more slowly than the controls, indicating that FAK PA/PA mammary tumor cells formed tighter cell-cell and cell-ECM adhesions. In order to examine cell adhesion more carefully, cell aggregation assay was performed as previously described [27, 28]. As shown in Figure 3.16, panel A, FAK $^{PA/PA}$ tumor cells formed more aggregates compared to control tumor cells. Moreover, much larger aggregates were formed by FAK $^{PA/PA}$ tumor cells after overnight incubation in suspension (Figure 3.16, panel B), indicating that the ProII motif of FAK could regulate cell-cell adhesion and that the PA mutation of this motif could result in tighter cell adhesion and increased cell aggregation.

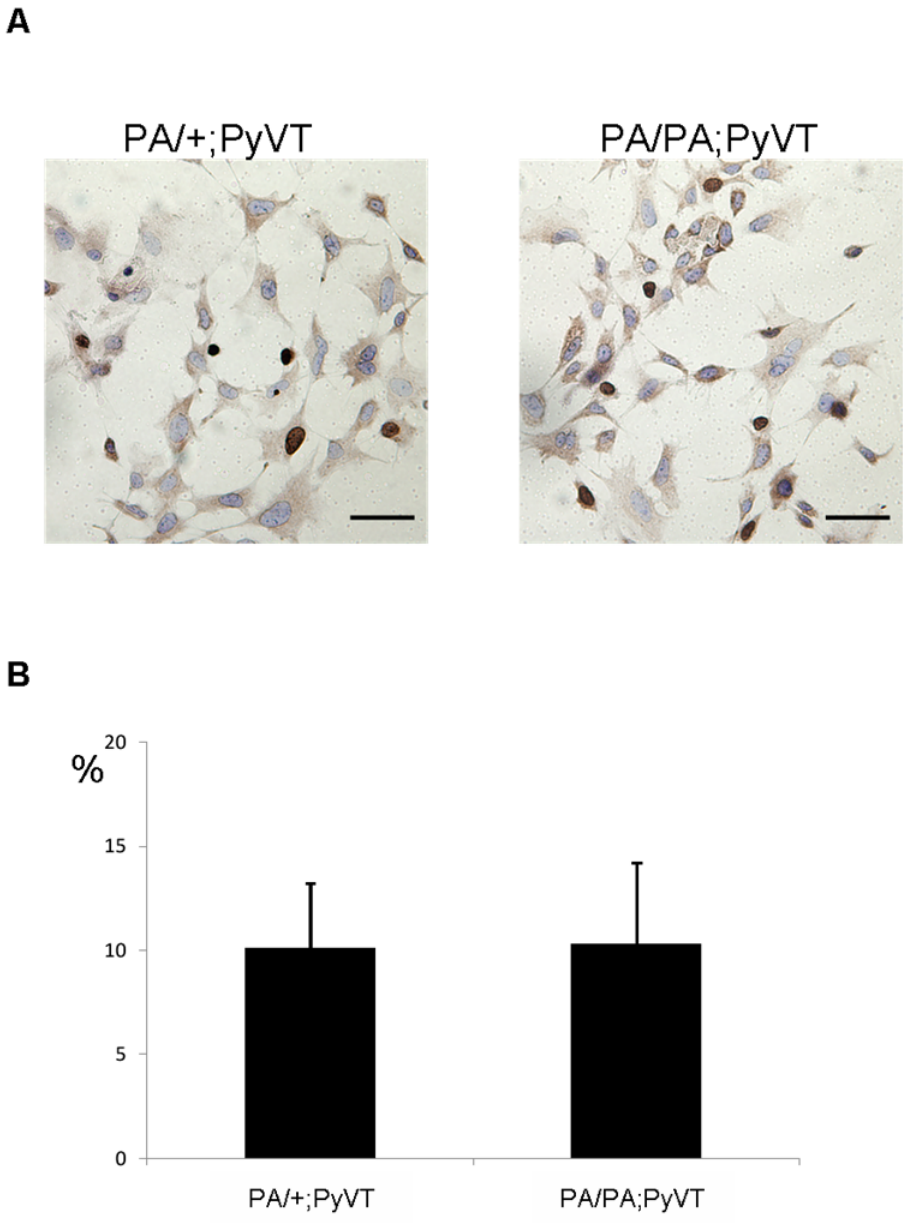


Figure 3.12 | Comparable proliferation of the isolated primary mammary epithelial tumor cell from control and total knockin mice. Primary mammary tumor cells from control and knockin tumor mice were measured proliferation by BrdU incorporation assay as described in Materials and methods. (A) representative pictures. Scale bars, 20 μm. (B) quantification. P>0.05.

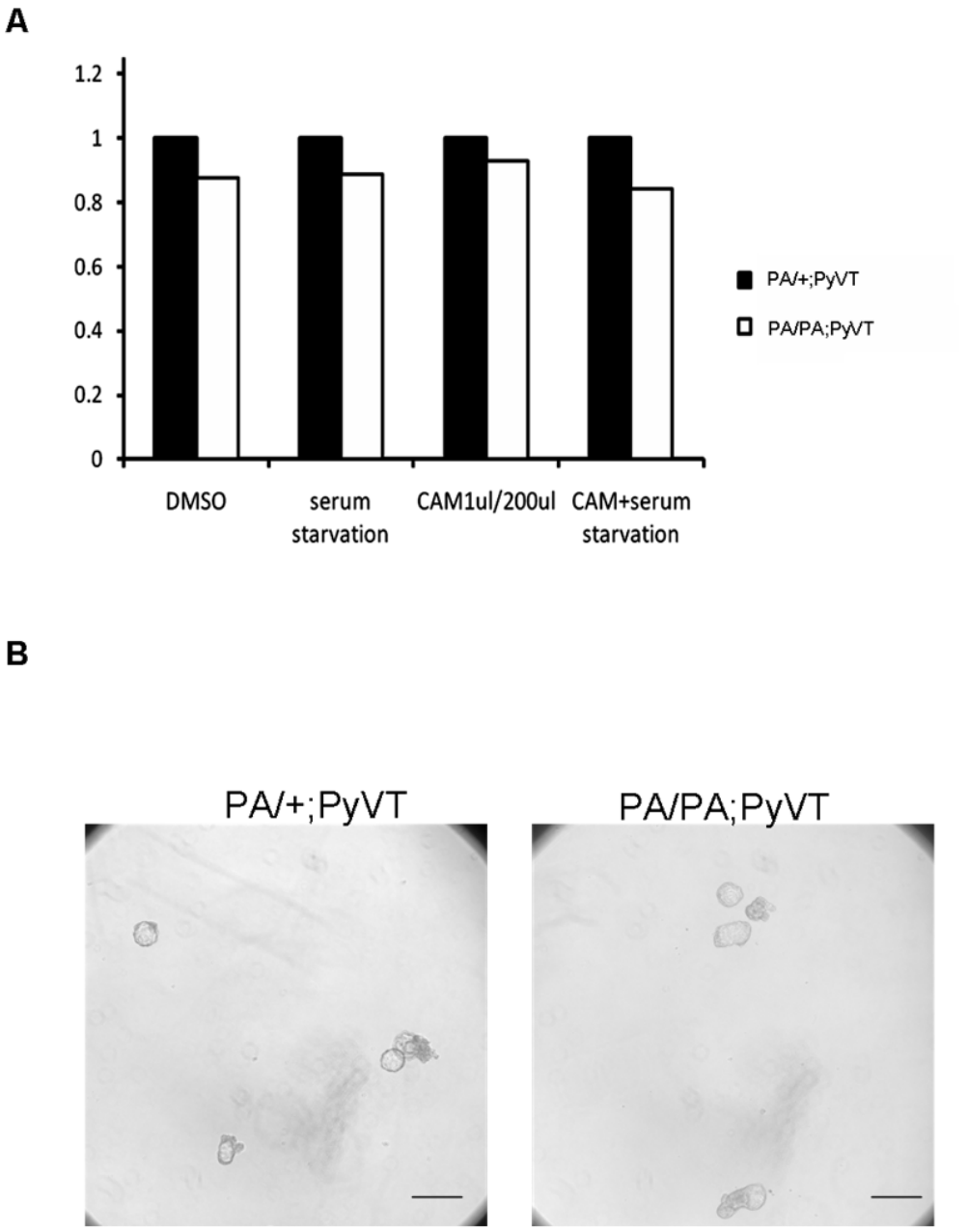


Figure 3.13 | PA mutated mammary epithelial tumor cells did not have increased apoptosis and anoikis. Mammary tumor cells from control and knockin mice were measured for apoptosis using ELISA assay (A) and anoikis assay (B), as described in Materials and methods. Scale bars, 50μm.

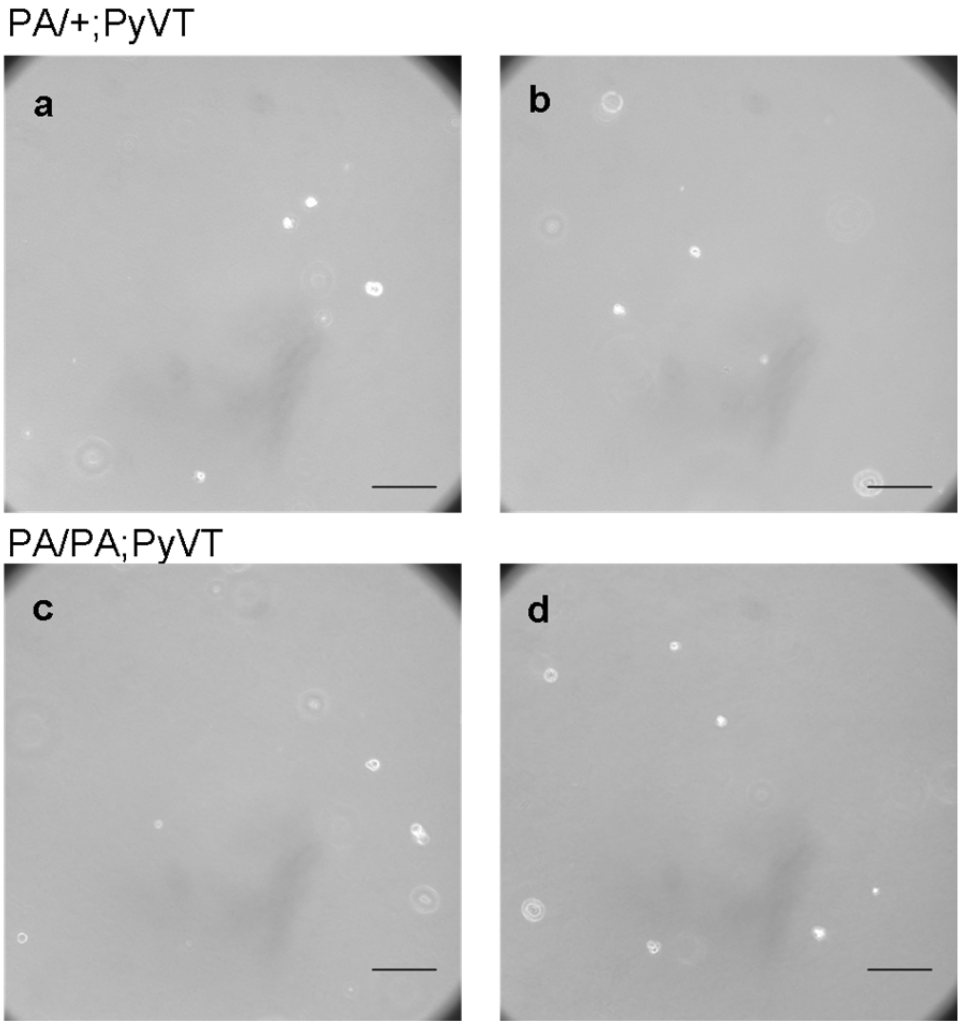


Figure 3.14 | Colony formation analysis of PA mutated mammary epithelial tumor cells. Colony formation in soft agar was measured on mammary tumor cells from control and knockin mice. Representative pictures were shown for control (a and b) and knockin cells (c and d). Scale bars, 50μm.

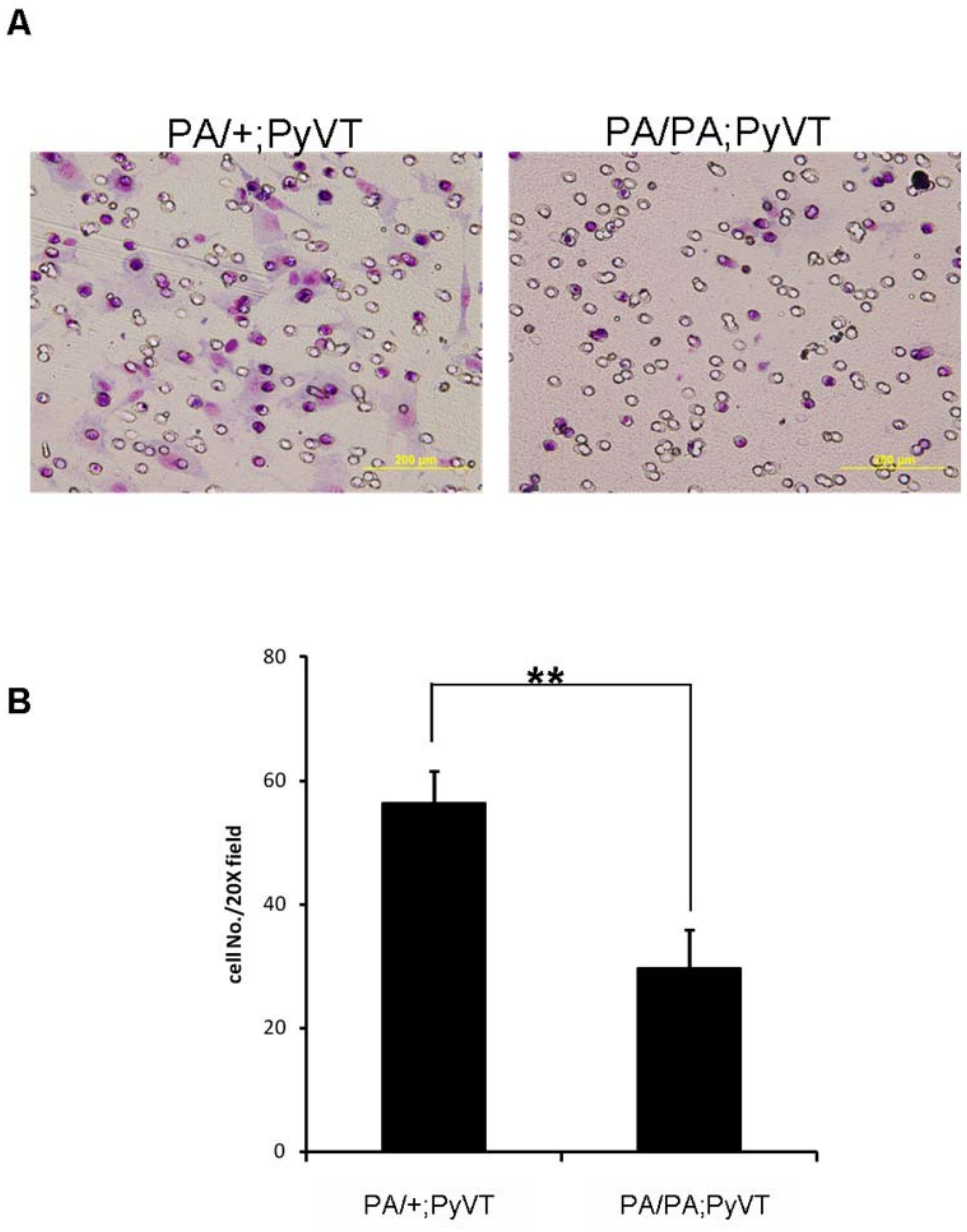


Figure 3.15 | Mammary epithelial cells with PA mutations have defective motility. Primary mammary tumor cells were isolated from control and knockin tumor mice and cell migration assay were examined by Boyden chamber assay. (A) Representative picture of control cells (left) and knockin cells (right). (B) quantification. **, P<0.01.

3.4.5 ProII motif mediates contact inhibition regulated by FAK activation

As described above, cell proliferation and cell survival were unaffected by PA mutation in FAK when cells were analyzed before they reaching confluence (Figure 3.12 and 3.13, panel A). After getting confluent, mammary epithelial tumor cells from the control FAK $^{PA/+}$;PyVT mice started to grow "foci" with cells piling up, which is common for epithelial tumor cells and known to be caused by the loss of contact inhibition. We noticed, however, this process was much delayed for the mammary epithelial tumor cells from FAK $^{PA/PA}$;PyVT mice. As shown in Figure 3.17, at 4 days after confluence, several foci could be seen for the PA/+ cells, but none for the PA/PA cells. Such foci did not start to form from the PA/PA tumor cells until day 7, and most of the cells still stayed in a confluent monolayer. For the PA/+ cells, more and bigger foci could be found at this time. Cell numbers were also counted at the same period of time after cell reaching confluence (also the same time after plating cells), and PA/PA cells were much fewer than PA/+ (data not shown), indicating the cell proliferation after cell getting confluence was decreased by PA mutation when the cells were confluent and contacting each others.

The increased contact inhibition for PA/PA cells was also confirmed when the confluent cells were suspended and re-plated on dishes. One day after re-plating, PA/+ cells adhere and grow well as shown in Figure 3.18, panel a, however, PA/PA tumor cells showed rounded-up shape and less adhesive to the dish surface (Figure 3.18, panel b). These rounded cells were Trypan blue staining free, suggesting they were viable cells. More importantly, two days after the re-plating, PA/PA tumor cells seemed to "recover" and started to grow "normally" and got confluent at similar time with PA/+ tumor cells (Figure 3.18, panel c and d).

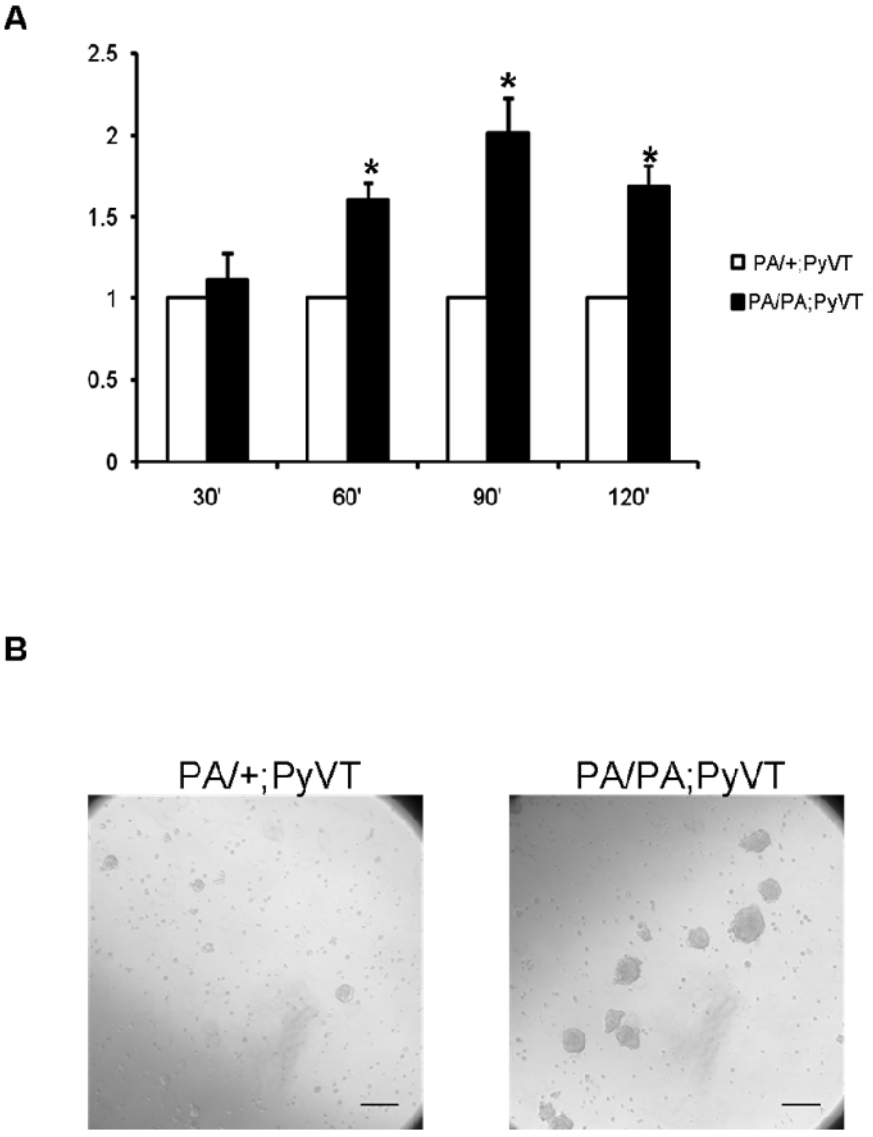


Figure 3.16 | Enhanced cell-cell aggregation in mammary epithelial tumor cells from knockin mice. Primary mammary tumor cells were isolated from control and knockin tumor mice and cell aggregation assay were performed as described in material and methods. (A) number of cell aggregated at different time point after incubation. *, $P<0.05$. (B) Representative picture of cell aggregates after overnight incubation. control cells (left) and knockin cells (right). Scale bars, 50μm.

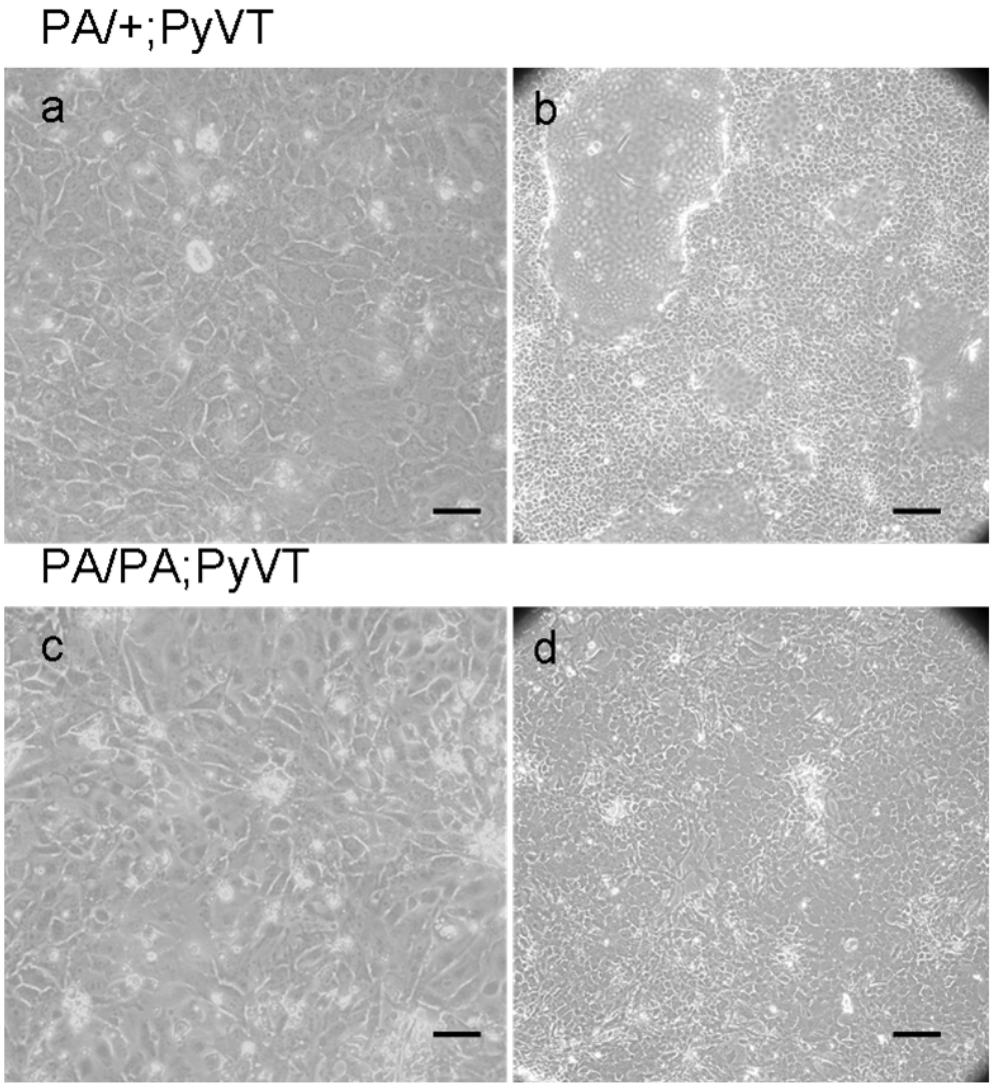


Figure 3.17 | Mammary epithelial tumor cells from knockin mice have more cell-cell contact inhibition. (a and c)Primary mammary epithelial tumor cells were isolated from control mice (a) and knockin mice (c), and were cultured to confluence, designated as day 0.Scale bars, 20μm. (b and d) Representative pictures of control cells (b) or knockin cells (d) at 4 days after confluence. Scale bars, 50μm.

3.4.6 FAK regulates cell contact inhibition through Wnt and MAPK signaling pathways

To explore the potential mechanisms through which FAK regulates the cell contact inhibition of the mammary epithelial tumor cells, cell lysates were prepared from the cultured tumor cells and analyzed for the activation status of FAK downstream targets and signaling molecules known to be important for contact inhibition. We first analyzed the expression level of β-catenin, which was shown to be important for the regulation of cell contact inhibition [33-35]. As shown in Figure 3.19, after cells reached confluence, β-catenin expression was decreased in PA/PA tumor cells compared with PA/+ tumor cells, both in cytoplasm and the plasma membrane fractions. As expected, cyclin D1, a target of β-catenin, was also found to be decreased in PA/PA tumor cells. Furthermore, the expression of E-cadherin on cell membrane was increased with PA mutation. p120- catenin, another important associated regulator for E-cad was not different between control and knockin tumor cells. We also examined the activation of JNK (C-jun N terminal kinase) in the confluent cells, which was shown to be another important regulator of cell contact inhibition [36]. In PA/PA tumor cells, p-JNK was decreased compared to PA/+ tumor cells, but the expression level of JNK were the same for both. However, the activity of p44/42, another MAP kinase, was not affected by PA mutation. More interestingly, the changes of β-catenin and E-cadherin expression and JNK activation found in confluent cells were not seen in non-confluent cells (Figure 3.20), confirming that these changes were associated with cell density and could be potential mechanisms for regulating cell contact inhibition. P21 and P27, which were known to be critical regulators of cell contact inhibition, were not changed upon PA mutation in the ProII motif of FAK (Figure 3.19), suggesting an alternative signaling pathway could be involved.

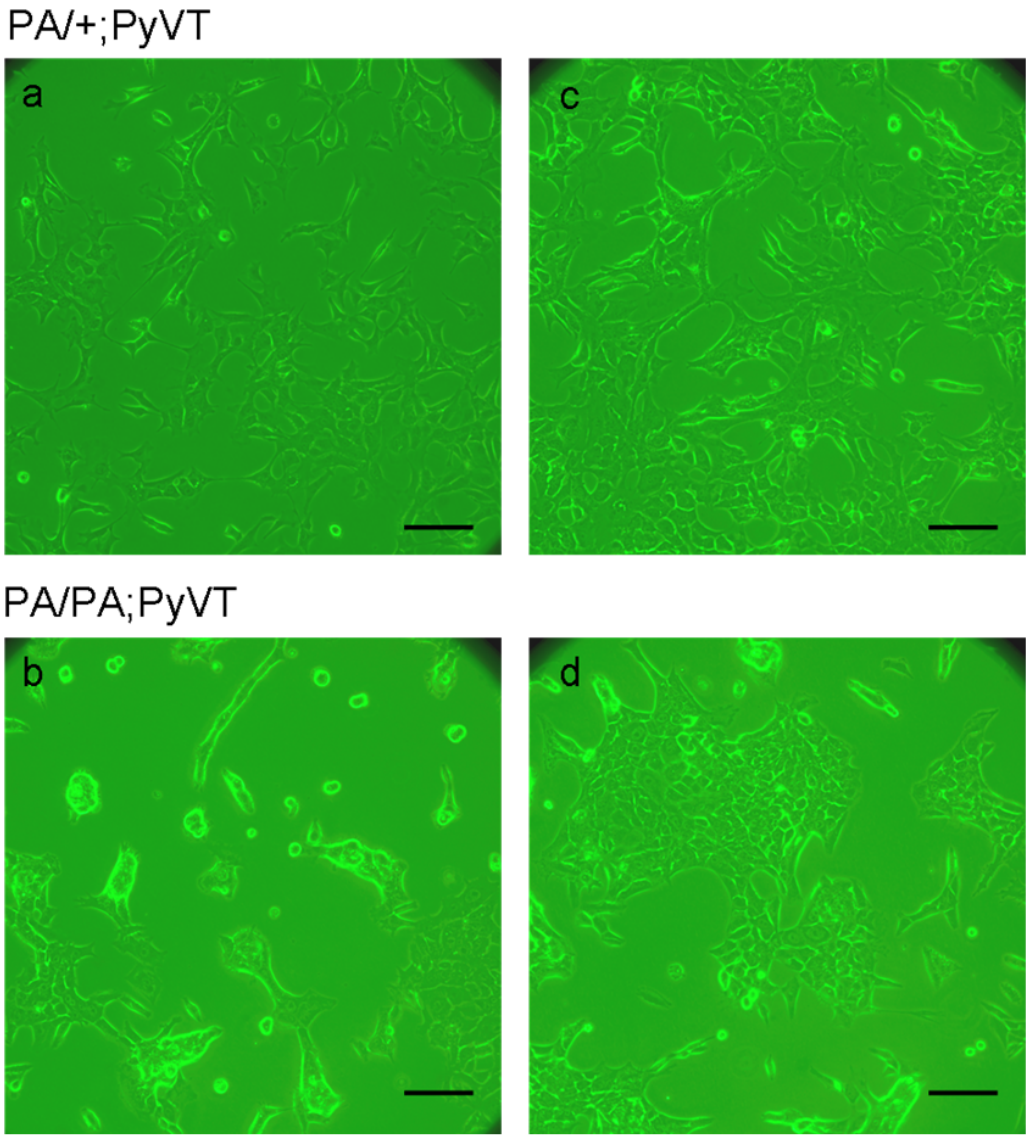


Figure 3.18 | PA mutations of FAK in mammary epithelial tumor cells resulted in cell growth arrest after replating. Mammary epithelial tumor cells from control mice (a and c) and knockin mice (b and d) were replated after confluence. Representative pictures were shown at 1 day (a and b) and 2 days (c and d) after replating. Scale bars, 20μm.

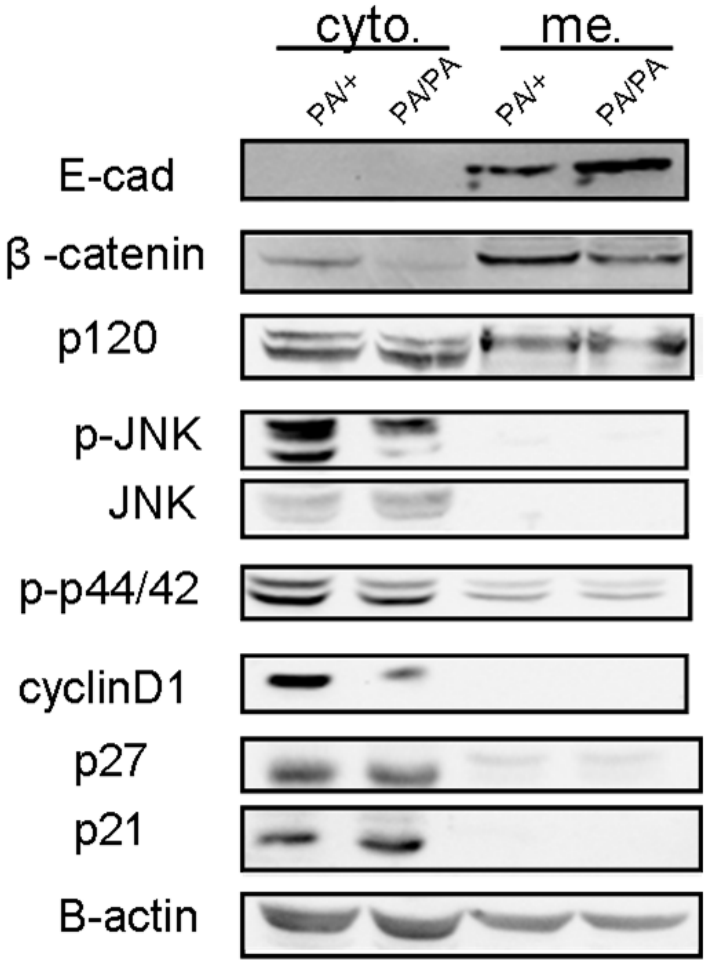


Figure 3.19 │ Effects of FAK PA mutations on signaling pathways regulating contact inhibition upon confluence in the isolated primary mammary epithelial tumor cells. After cells reaching confluence, cell lysates of primary mammary epithelial tumor cells from control and knockin mice were analyzed by Western blotting with various antibodies as indicated.

Consistently, in situ staining of E-cad and β-catenin in mammary tumors confirmed the changes of these two proteins in the PA knockin tumors. As indicated in Figure 3.21, more E-cadherin but less β-catenin were seen in the tumors from the ProII

mutated mice, especially in the core regions of tumors, where cells are more packed and adherent tighter with each other. These results may correspond with those changes seen only with the confluent cells, but not the subconfluent cells.

3.4.7 PA mutation in endothelial cells also contribute to the impaired tumor growth

FAK plays an important role in angiogenesis, both in embryonic development and in adults [20, 37, 38]. Previously, our lab has shown that deletion of FAK in the endothelial cells led to reduced tumor growth. Re-expression of FAK could rescue the tumor growth, while kinase-defective FAK, Y397F and S732A mutant could not ([39] and data unpublished). This suggested that FAK played important roles in tumor angiogenesis and the tumor growth would be impaired without FAK expression or activity in the endothelial cells.

In order to determine whether the PA mutation in the ProII motif of FAK in endothelial cells affect the tumor angiogenesis and the tumor growth, we generated endothelial cell (EC)-specific FAK knockin mammary tumor mice (designated as ECKI) with genotype of FAK $^{PA/f}$;Tie2Cre;PyVT, by crossing FAK $^{f/+}$;Tie2Cre mice [20] with FAK $^{PA/PA}$;PyVT mice. In these mice, the PA mutations only occur in the endothelial cells by deletion of the floxed allele by Tie2Cre. Since no phenotypes were found in mice development (during both embryogenesis and adulthood) with the PA mutation in endothelial cells, the effects of the PA mutation in tumor angiogenesis could be examined specifically. Tumor appearance and tumor size of these mice were monitored as described before, and FAK $^{PA/f}$; PyVT and FAK $^{f/+}$; Tie2Cre; PyVT mice served as control. As seen in the total PA knockin mice and mammary epithelial cell specific knockin mice, the tumorigenesis in the endothelial cell specific knockin mice

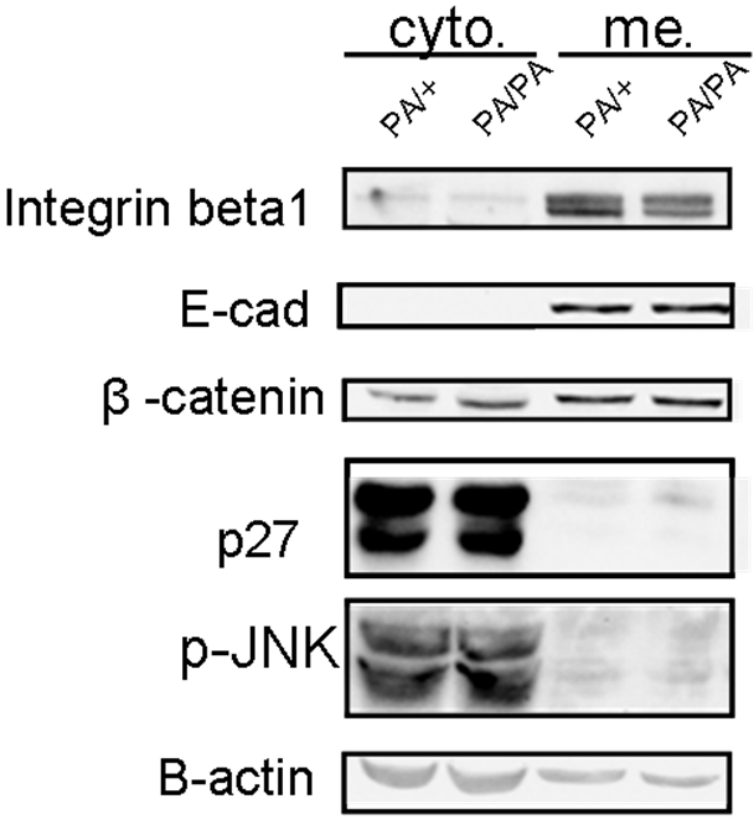


Figure 3.20 | Analysis of contact inhibition regulating pathways in knockin tumor cells before reaching confluence. Cell lysates of control mammary epithelial tumor cells and knockin cells, without reaching confluence, were analyzed by Western blotting with various antibodies as indicated.

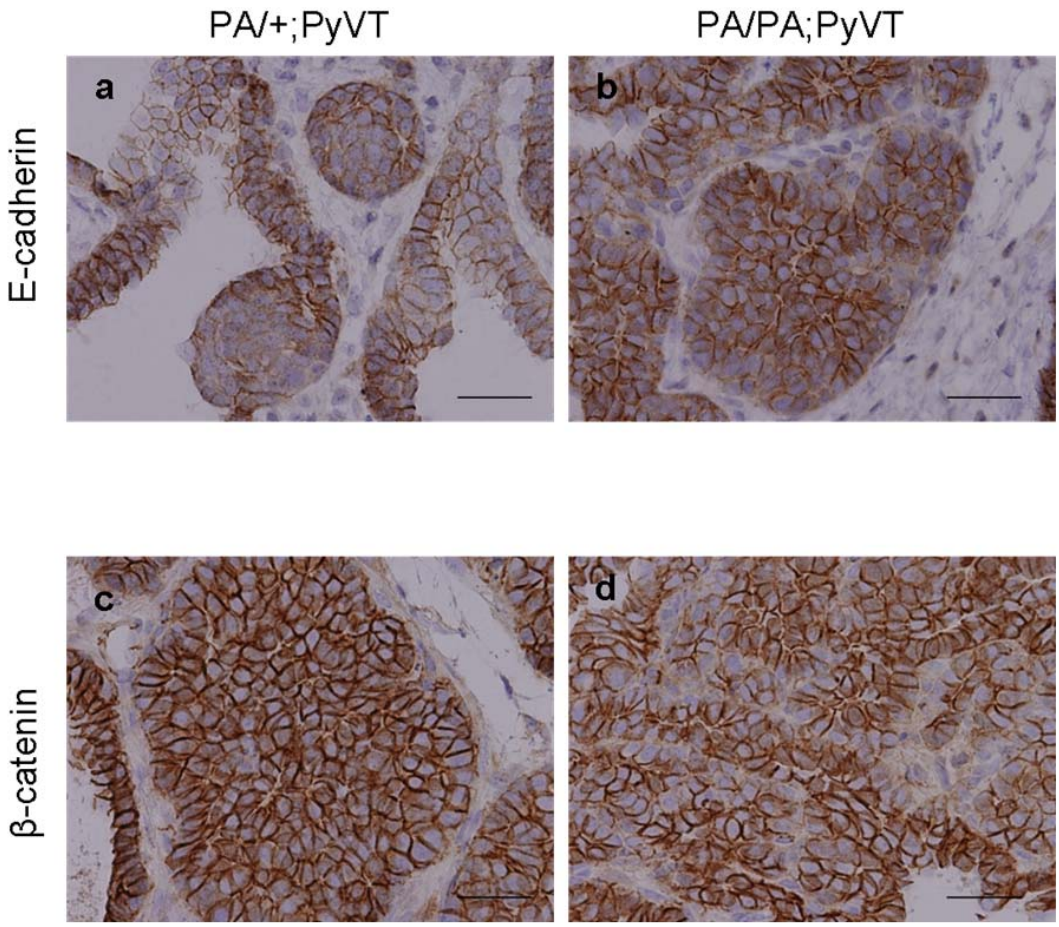


Figure 3.21 | Analyses of protein expression in FAK PA knockin tumor mice. Sections from the primary tumors of the control (a, c) and knockin (b, d) mice were analyzed by immunohistochemistry using antibodies against E-cadherin (a, b) or β-catenin (c, d). Scale bars, 50 μm.

was not affected compared with the controls (data not shown), indicating that the PA mutation in the ProII motif of FAK in endothelial cells did not affect cell transformation and tumor formation either. Interestingly, the tumors grown from the ECKI mice were also found to be much smaller than those from the controls along with tumor progression. In addition, metastases to lungs were also reduced (Figure 3.22, panel A and B). These results suggested hat efficient tumor growth also requires proper functions of the ProII motif of FAK in endothelial cells.

In order to investigate possible difference in tumor angiogenesis, tumor samples were collected from both ECKI and control tumor mice, and subjected to PECAM-1 staining, an endothelial cell marker, to examine blood vessel density and morphology. Examination of the blood vessels showed that the number and density of the blood vessels were not changed in the ECKI tumors from the controls (Figure 3.23), indicating that blood vessel formation in tumors was not altered by PA mutation in the ProII motif of FAK. However, detailed examinations of the tumor sections from the ECKI tumor mice revealed that subtle changes in tumor vessels could be involved. As shown in Figure 3.24, massive necrosis areas could be found in the ECKI tumors, even though there were sufficient blood vessels formed around or within those areas. In addition, primary endothelial cells isolated from either the control or the ECKI tumor cells did not show any differences in cell proliferation, survival and migration (data now shown). Together, these results suggested that some “subtle” defects of the endothelial cells may be involved in regulating blood barrier functions and the ProII motif of FAK could play a critical role in those processes.

3.5 Discussion

FAK has been shown to play critical roles in mammary gland development and functions [21, 40]. By specifically deleting FAK in the mammary epithelial cells, our

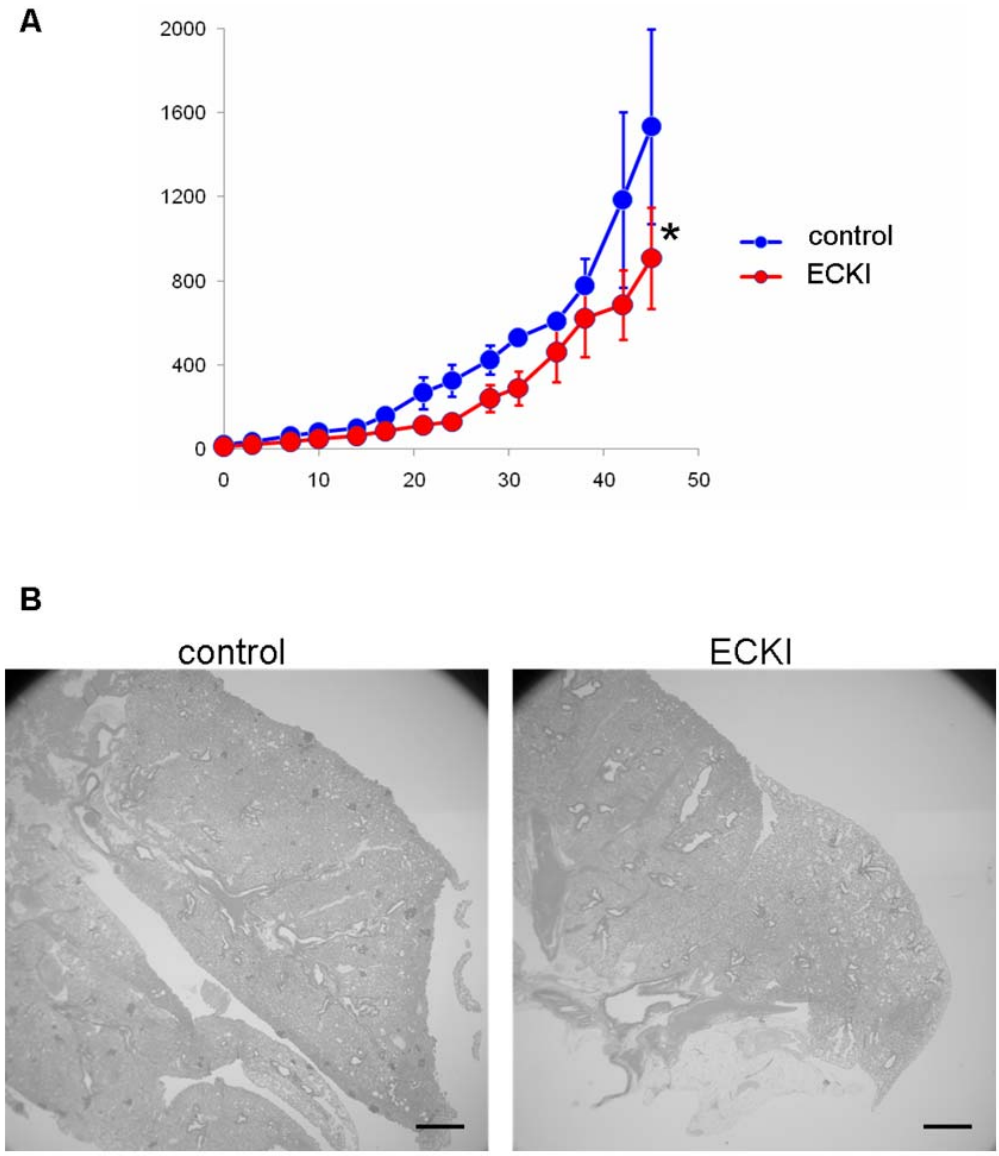


Figure 3.22 | Tumor growth and lung metastasis were defected in EC-specific knockin mice. (A) mean cumulative mammary tumor volume (±SD) for each genotype at indicated times after primary tumor appearance were plotted and analyzed. *, $P < 0.05$.(B) Representative lung sections (control, left and ECKI, right) were prepared at 8 wk after the primary mammary tumor onset and subjected to H&E staining. Scale bars, 1 mm.

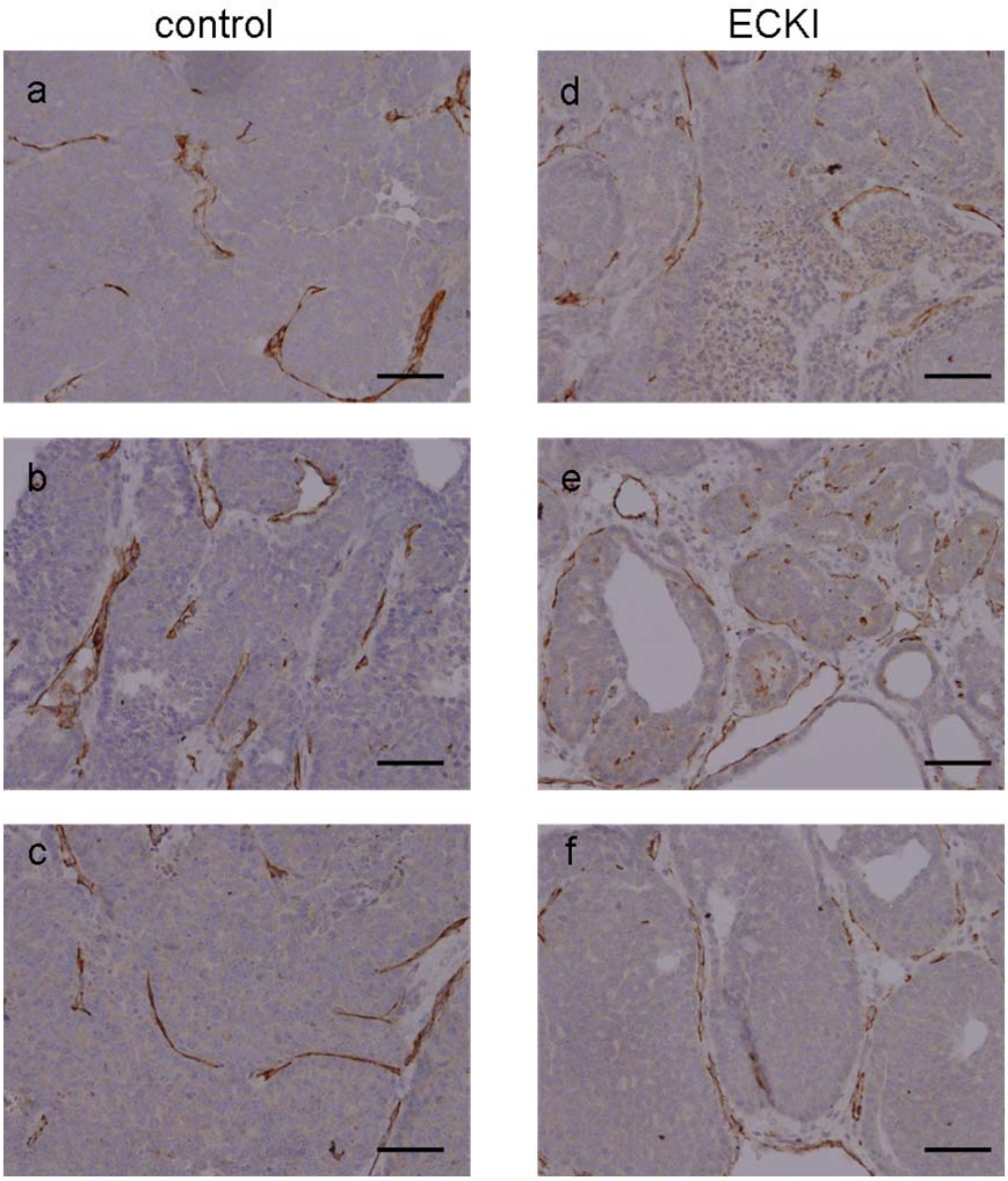


Figure 3.23 | Analyses of tumor angiogenesis in FAK PA knockin tumor mice. Sections from the primary tumors of the control (a-c) and ECKI (d-f) mice were analyzed by immunohistochemistry using antibodies against PECAM-1. Scale bars, 50 μm.

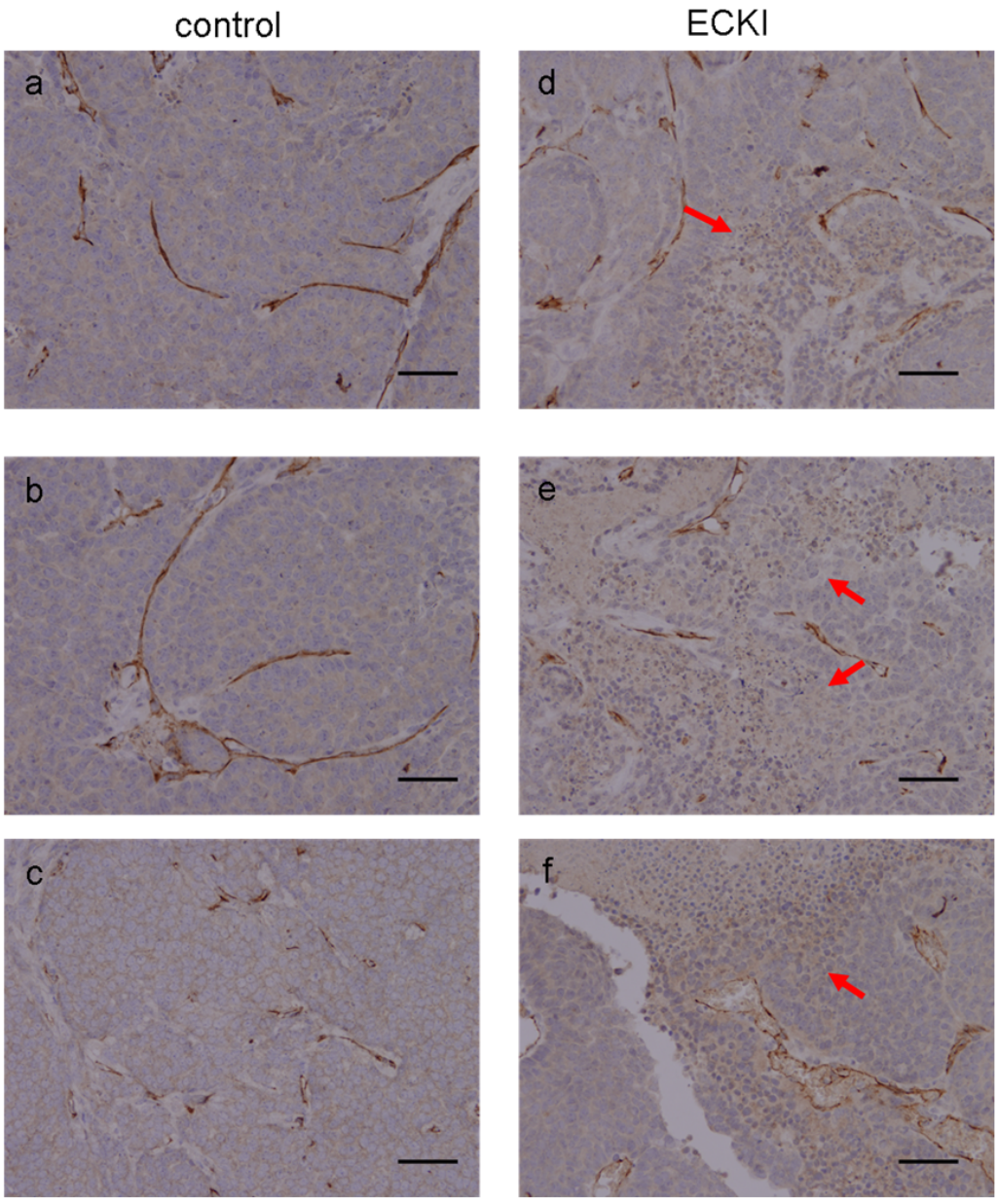


Figure 3.24 | Massive necrotic areas were found in ECKI mice even with presence of tumor blood vessels. Sections from the primary tumors of the control (a-c) and ECKI (d-f) mice were analyzed by immunohistochemistry using antibodies against PECAM-1. Scale bars, 50 μm. Necrotic areas were indicated by arrows.

lab showed previously that FAK deficiency caused decreased mammary epithelial proliferation during pregnancy, leading to lobulo-alveolar hypoplasia. Furthermore, loss of FAK also resulted in an inhibition of secretory differentiation in the mammary epithelial cells [21, 40]. The second C-terminal proline-rich motif of FAK could interact with several SH3 domain containing proteins, including p130cas, ASAP,GRAF and endoII [16, 30, 31, 41]. By binding to these molecules, this motif functions to regulate cell migration and invasion [16, 41-44] . However, all of studies were carried out in cultured cells. By generating ProII knockin mice, the physiological roles for this motif were clarified in vivo in the present study. In contrast FAK deletion in mammary epithelial cells, expression of FAK mutant with PA mutation in the second C-terminal proline-rich motif allowed mammary gland to develop normally. This result indicated that FAK regulation of mammary epithelial cell proliferation and cell cycle progression does not require its second C-terminal proline-rich motif.

As a key mediator of signal transduction from ECM and a point of convergence of integrin and growth factor signaling, FAK has been suggested to play a role in breast cancer [4-8, 45, 46]. Targeted disruption of FAK in mammary tumor cells, shown by our lab and others, can suppress the tumor initiation and block the progression of hyperplasia tissues to full carcinomas in PyMT mouse models [18, 19, 72, 73]. Though the second C-terminal proline-rich motif of FAK was not essential for mammary gland development, mutations in this motif did lead to defective mammary tumor growth and metastasis. Therefore the tumor specific function of this motif may make it a potentially excellent target for drug development targeting mammary tumors, which will not affect the normal functions of mammary glands. The reduced development of mammary tumors was shown in both total knockin mice and mammary epithelial specific knockin mice. This indicated that mutations of this motif

in the mammary epithelial cells alone could decrease the tumor growth and metastasis. In order to figure out the mechanisms involved, various cellular functions were analyzed for mammary tumor cells from the knockin mice and controls. Previous researches have shown that deletion of FAK in mammary epithelial cells could result in defects of cell proliferation and cell survival, which could contribute to the hampered tumor growth and metastases. However, mammary epithelial cell proliferating and cell apoptosis were comparable in the mutant and control tumor cells. On the other hand, cell aggregation of the mammary epithelial cells carrying knockin mutations was significantly enhanced. Cell aggregation reflects the degree of cell-cell adhesion, which was believed to be responsible for a variety of dynamic processes, including cell locomotion and differentiation. Indeed, cell migration of the mammary epithelial cells from knockin mice was reduced in comparison to control cells, which may be due to the increased cell-cell adhesion and aggregation.

When moving and proliferating cells touch and form contacts with each other, they promote the formation of adherent junctions (AJs) and the cessation of cell movement and proliferation. This phenomenon has been recognized as “contact inhibition of cell movement and proliferation”, and the capability for cells to judiciously regulate movement and proliferation responses in the context of cell-cell contact is a fundamental requirement for the organization and maintenance of specialized tissues in multi-cellular organisms. Transformed cells or tumor cells lose contact inhibition of cell movement and proliferation, resulting in abnormal cell proliferation, cell invasion and metastasis[47]. Interestingly, tumor cells isolated from the knockin mice showed decreased contact inhibition compared to control cells. Further analysis revealed that there were increased E-cadherin associated with cell membrane and decreased expression of β-catenin and phosphorylation of JNK in the

knockin cells upon confluence. Though there were a large body of evidence for the pivotal roles of cadherins, wnt signaling pathway and MAP kinases in the regulation of cell contact inhibition [33-35, 47-49], there are no reports on the mechanisms regulating these signaling pathways. Our study for the first time indicated that FAK could be an upstream signaling molecule in the regulation of contact inhibition and the second C-terminal proline-rich of FAK may be essential for this function. In tumor cells, elevated expression and activity of FAK may interfere with the accumulation of E-cadherin on cell membrane to enhance its affiliation with wnt signaling, and increase the MAP kinases activities as well. In addition to the phosphorylated tyrosine 925, the second C-terminal proline-rich motif of FAK has been suggested to bind to Grb2 [50]. Previous studies have shown that Grb2 is required for β-catenin- wnt signaling, and double mutations in the SH3 domains of Grb2 blocked Wnt-mediated signaling [51]. The expression of FAK in HEK293 cells strongly synergized with wnt signaling, and it was completely abrogated by the dominant negative, double SH3 domain mutant Grb2, suggesting that Grb2 lies downstream of FAK mediating wnt signaling activation. Recent studies also identified jnk/c-jun as downstream of Grb2 [51]. The synergy between Grb2 and Wnt3a is blocked by the JNK inhibitor SP600125, and by the dominant negative c-jun, but not by an ERK inhibitor or by dominant negative Ras. All of these indicated that JNK is downstream of a FAK-Grb2 pathway, and it also suggested that FAK could coordinate wnt and MAPK signaling pathways, and the second C-terminal proline-rich motif of FAK could play an essential role in this coordination through its binding to Grb2. Of course, in addition to the second proline-rich motif, other domains of FAK could also be essential for this regulation and more detailed analysis would be necessary to explore the mechanisms involved.

In addition to the roles of FAK in mammary epithelial cells, FAK has been suggested to be an important regulator of tumor angiogenesis switch [52-58]. Based on both in vitro and in vivo studies, when FAK was depleted from the endothelial cells, there would be less angiogenesis in the tumors, resulting in slower tumor growth and less lung metastases [38, 39]. The autophosphorylation and the kinase activity of FAK have been shown to be essential in this process [39]. An intact vascular network is critical for the growth and survival of solid tumors, by delivery of oxygen and other nutrients and facilitating the development of metastatic disease, and the switch from an avascular to the vascular phenotype is a key event for the development of solid tumor bigger than 2mm of diameter. Hence treatment targeting tumor angiogenesis has become a promising and efficient method for cancers treatment [59-64]. Though there are a large body of researches regarding the role of FAK in tumor angiogenesis [52-58], the direct evidence for FAK in blood vessel formation and functioning in tumors is missing. Through generating endothelial cell specific knockin mice model with PyVT oncogene over-expression, we confirmed the critical role of FAK in tumor growth and metastasis by regulating endothelial cell behaviors. In these conditional knockin tumor mice, we found that the second C-terminal proline-rich motif of FAK in endothelial cells was indispensable for tumor development and progression. Interestingly, detailed studies revealed that the number and density of blood vessels in tumor were not affected in the conditional knockin mice, instead, the functions of those blood vessels seemed to be damaged, indicated by increased necrosis areas in the knockin tumors. Mechanistic studies showed that there were no defects in proliferation, survival and migration for these mutated endothelial cells, but in addition to these “obvious” endothelial cell defects, “subtle” changes of endothelial cells could also lead to defective blood vessel functions. One example is tumor-vascular disrupting agents (tumor-VDAs), which acts as tubulin-binding agents and

selectively target tumor endothelial cells by disrupting the actin cytoskeleton [65-69]. The resulting cytoskeleton rearrangements and selective disruption of the molecular engagement of the junctional protein vascular endothelial-cadherin result in rapid changes in endothelial cell shape and increases vascular permeability followed by blood flow inhibition and vasoconstriction [68, 70-76], which could ultimately results in hemorrhagic necrosis of murine tumors, particularly within the core of the tumor. Mutations in the second C-terminal proline-rich motif of FAK could regulate the functions of endothelial cells via these "subtle" changes, but still lead to efficient decreases of tumor growth and metastases. In this sense, this motif of FAK could be a potential target as drug development with fewer side-effects but powerful efficiency in tumor treatments.

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Chapter 4

CONCLUSIONS AND PROSPECTS

4.1 The role of FAK in the regulation of apoptosis and EC barrier functions

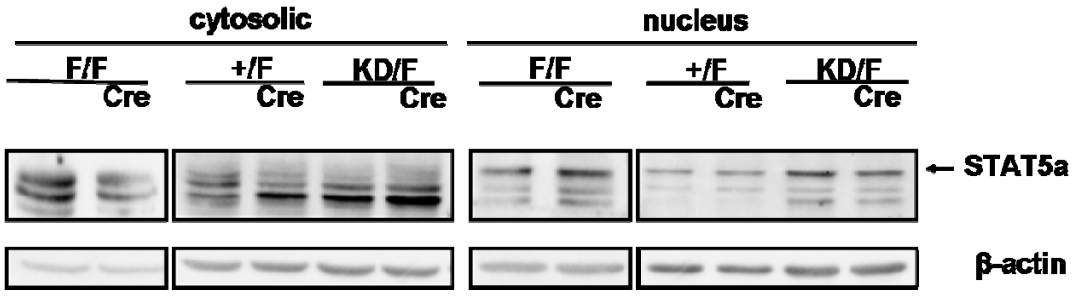
As a critical signal transducer, FAK has been implicated in the regulation of many cellular functions in various cell types [1-6]. In particular, the role of FAK in regulating cell survival / apoptosis has been extensively studied in the past two decades [3, 7-15], and the kinase activity of FAK has been considered to be essential for maintaining cell survival in most of the studies. However, since most of these studies were carried out in vitro utilizing cell lines and over-expression of kinase-defective FAK, the in vivo role of FAK, particularly that of its kinase activity, in cell survival / apoptosis remains unclear.

In my research on kinase-defective FAK knock-in mice, as described in Chapter 2, I characterized the role of FAK in the survival of ECs through both in vivo and in vitro studies, and revealed that FAK functions to protect ECs from apoptosis in independent of its kinase activity. In addition, the increased apoptosis induced by FAK deletion, which was rescued by the kinase-defective FAK mutant knock-in allele, was found to be mediated by the deregulation of p21 expression, but not the reduced Akt activation. These results are distinctive and interesting, but the mechanisms involved have not been fully elucidated. A recent study focusing on the function of nuclear FAK revealed that FAK could regulate the degradation of p53 in a FERM domain dependent manner [16]. Through this mechanism, the authors showed that in p21-null fibroblasts, FAK could regulate cell proliferation and cell survival in a kinase activity-independent manner. However, in my study on the primary endothelia cells, deficiency of FAK did not lead to significant increase of p53, indicating that the

elevated apoptosis upon FAK deletion involves mechanisms other than the p53 degradation. Furthermore, preliminary data revealed that the translocation of STAT5 to the nucleus was regulated by FAK in primary ECs and fibroblast cells. As shown in Figure 4.1A, when FAK was deleted in primary ECs, the expression level of STAT5a in the nucleus was dramatically increased compared to the control, but the STAT5a in the cytoplasm was unchanged upon FAK deletion. Interestingly, such increase of STAT5a in the nucleus was not observed when there was kinase-defective FAK allele present in the cells, suggesting that the kinase activity of FAK is not essential for this regulation. Similar results were also demonstrated in fibroblast cells (Figure 4.1B). Moreover, as shown in Figure 4.2, FAK has bee suggested to regulate the translocalization of STAT5a to nucleus by direct binding to it, though the exact binding domain / site has not been identified yet. Therefore, the translocation of STAT5a to the cell nucleus could account for the increased p21 expression and the resulted EC apoptosis.

STAT5 belongs to the Signal Transducers and Activators of Transcription (STATs) family, which are transcription factors that play critical roles in cytokines, hormones and growth factor-mediated intracellular signal transductions [17-20]. STAT proteins are initially present in inactive forms in the cytoplasm. In the general model for the activation of the Janus Kinase (JAK) / STAT pathway, the activation cascade starts with the activation of receptor-bound JAKs following ligand stimulation and receptor dimerization. STATs are then phosphorylated at tyrosine residues, and subsequently form homo- or heterodimers. Dimerized STAT proteins immediately enter the nucleus and bind to specific DNA sequences in the promoter regions of various genes, resulting in gene activation or repression [17-20]. There are seven members in the mammalian STATs family (STAT1, 2, 3, 4, 5a, 5b and 6), and STAT5

A



B

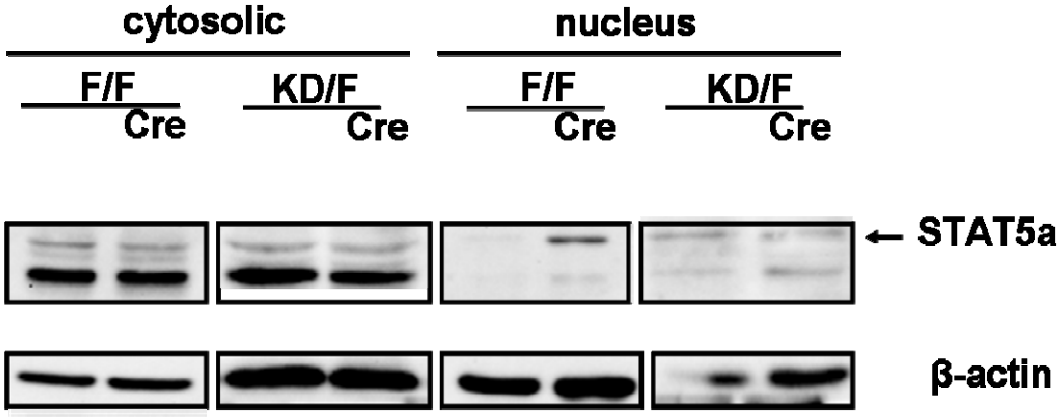


Figure 4.1 | FAK regulates translocalization of STAT5a to cell nucleus. Primary EC (A) or fibroblasts were isolated from either F/F, +/F or KD/F mice and infected with/without Cre adenovirus. After twice infection, cytosolic and nucleus lysates were made as previously described and subjected to western blotting with STAT5 and β-actin antibodies. Arrows indicate STAT5a.

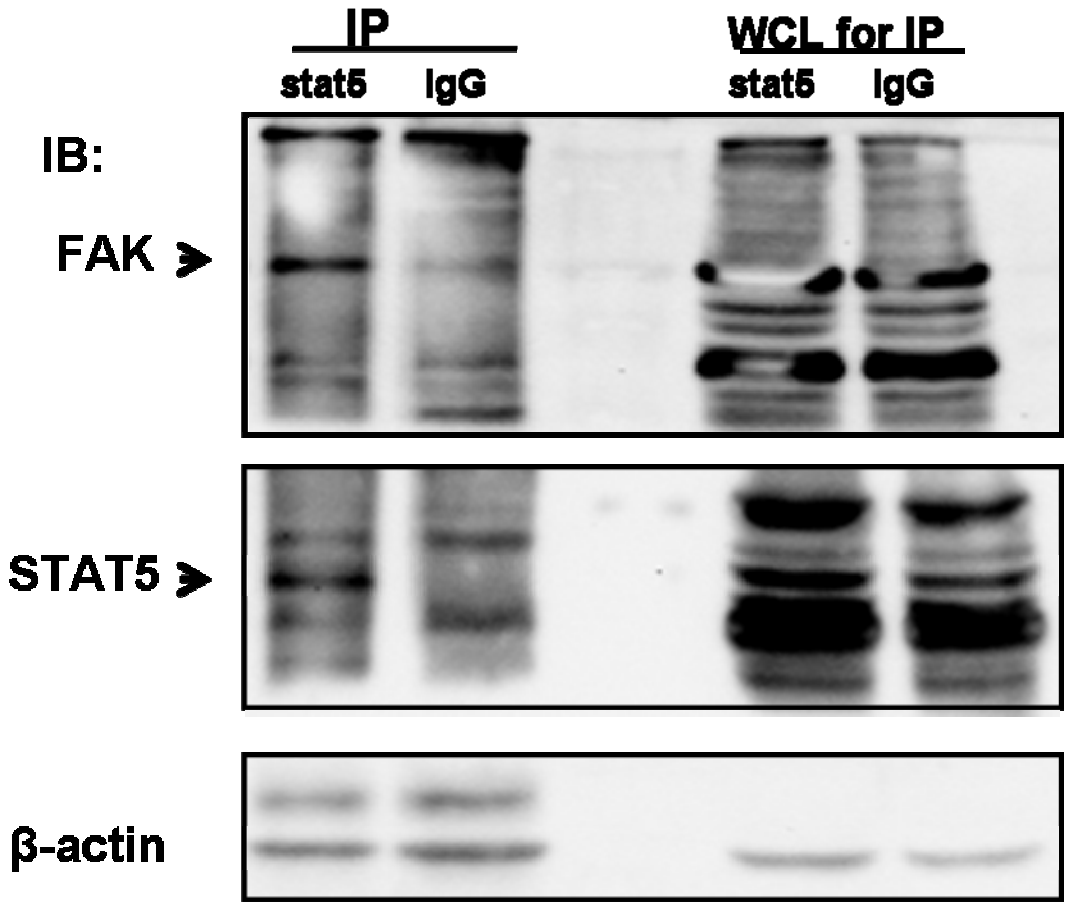


Figure 4.2 | FAK directly binds to STAT5. Cell lysates from immortalized mouse embryonic fibroblasts (MEF) were immunoprecipitated with STAT5 antibody, with rabbit IgG as control, followed by immunoblotting with FAK antibody to check binding between endogenous FAK and STAT5. Whole cell lysates for each immunoprecipitation was blotted with FAK, STAT5 and β-actin as control.

was found to be highly expressed in hematopoietic and ECs. STAT5 has been shown to regulate several cellular functions in different cell types, especially cell proliferation and cell survival [21-25]. In particular, one research showed that, after IL-3 treatment, hematopoietic cells expressing a constitutively active STAT5 mutant underwent apoptosis or differentiation followed by cell death [26]. In these cells, mRNA expression of some growth inhibitory genes such as CIS, JAB/SOCS-1/SSI-1, and P21 $^{WAF/Cip1}$ was highly induced. This suggested that in hematopoietic cells, sustained STAT5 activation preferentially induces apoptosis. However, the role of STAT5 in regulating cell survival is still controversial since contradicting results have been reported as well. It is possible that STAT5 could have different or even opposite roles in the regulation of cell survival depending on the cell types and the experiment conditions. Therefore, it remains to be verified whether the regulation of STAT5 is responsible for the increased apoptosis in primary ECs upon FAK deletion. If that is proven to be the case, the detailed mechanisms of STAT5a translocation to the cell nucleus by FAK also need to be further clarified.

In addition to the cell apoptosis, I also explored the role of FAK in angiogenesis and barrier maintenance of the EC as described in Chapter 2. I found that when FAK was deleted from the ECs, the phosphorylation of VE-cadherin at tyrosine 658 was significantly reduced. As a result, the expression pattern of VE-cadherin on cell membrane became disorganized and the integrity of blood vessels was impaired, leading to decreased blood vessel formation [27], massive hemorrhages and edema, and eventually embryonic death. It was also found that this regulation by FAK required its kinase activity. However, detailed mechanisms for the essential role of FAK in cell-cell adhesion and blood vessel integrity are not fully understood yet. There have been previous studies on the role of tyrosine phosphorylation of VE-

cadherin in cell-cell adhesion and permeability [28-33]. However, most of these studies reported a role of VE-cadherin phosphorylation contradictory to our results in primary ECs as described in Chapter 2. For example, in CHO cells, elevated tyrosine 658 phosphorylation of transfected VE-cadherin, via either tyrosine kinase activation or phosphatase inactivation, has been associated with inhibited cell barrier function [33]. TNF induced endothelial permeability of human umbilical vein ECs (HUVECs) was also shown to be due to the tyrosine phosphorylation of VE-cadherin [28]. Furthermore, tyrosine 658 phosphorylation of VE-cadherin was shown to be dependent on Src upon VEGF stimulation [33]; in leukocyte transendothelial migration (TEM) induced by ICAM-1 engagement, both Src and Pyk2 were required for phosphorylation of VE-cadherin at tyrosine 658 and 731[29]. It is noteworthy that these studies utilized exogenous expression of VE-cadherin or cell lines, and the basal level of tyrosine phosphorylation of VE-cadherin was not evaluated. Although our study proposed an alternative role for phosphorylation of VE-cadherin in the EC barrier functions, the exact biochemical mechanism by which FAK regulates tyrosine phosphorylation of VE-cadherin remains elusive. One possibility is that Src is involved in this regulation as shown in previous researches [29, 33]. Since Src activity was not affected by FAK depletion (see Chapter2), FAK may promote Src-dependent phosphorylation of VE-cadherin through a scaffolding function. FAK could bring Src adjacent to VE-cadherin by binding to Src through the autophosphorylated of Y397 rather than directly regulating its activity. FAK itself could also account for the tyrosine phosphorylation of VE-cadherin. Previous studies showed that treatment on bovine pulmonary artery endothelial cells (BPAECs) with H_2O_2 increased tyrosine phosphorylation of FAK and VE-cadherin; however, over-expression of FRNK attenuated H_2O_2-mediated tyrosine phosphorylation of FAK and VE-cadherin [4]. This suggested that FAK could phosphorylate VE-cadherin at tyrosine 658 directly, but not

through Src. Several experiments could be carried out to test these hypotheses. For example, co-immunoprecipitation assay can be performed to investigate whether there is direct binding between FAK and VE-cadherin in primary ECs (which has been demonstrated in S1P-challenged human pulmonary artery endothelial cell [34]). And in vitro kinase assay can be used to confirm whether VE-cadherin could be phosphorylated by FAK alone. In addition, it is also possible that other molecules are responsible for the phosphorylation of VE-cadherin. For example, vascular endothelial protein tyrosine phosphatase (VE-PTP) has been shown to play important roles in regulating VE-cadherin phosphorylation and EC barrier functions [35] . In that case, FAK could either act as a scaffolding protein or its kinase activity of FAK is required for the functions of those molecules.

4.2 Functions of FAK in cell contact inhibition and tumor angiogenesis

In Chapter 3, I characterized the role of the second C-terminal proline-rich motif of FAK in mammary tumor growth and metastasis. Cellular functional assays revealed that the FAK mutant of this motif enhanced cell contact inhibition only upon cell confluence, including cell growth arrest and slowed cell migration. Furthermore, I observed increased E-cadherin on cell membrane, and decreased β-catenin and p-JNK. All of these could be related to or responsible for the elevated cell contact inhibition [1, 36-39]. However, whether these changes are primary or secondary to the deletion of FAK and how FAK regulates cell contact inhibition through these molecules are still not clear.

One possibility is that the expression level of E-cadherin on epithelial cell surface could be the primary effect after FAK deletion. E-cadherin has been implicated in contact inhibition and recognized as a tumor suppressor [37, 40, 41]. The elusive nature of E-cadherin in the regulation of contact inhibition is likely due to the fact that

E-cadherin can control cell proliferation by different mechanisms, through either β-catenin dependent [42] or independent regulation [36, 37, 43]. Several studies have shown that FAK could regulate EMT process by down-regulating membrane-bound E-cadherin, which involves Src induced de-organization and/or endocytosis of E-cadherin [44, 45]. The second C-terminal proline-rich motif of FAK could be indispensable for this role in addition to the tyrosine phosphorylations, which have been demonstrated to be essential for this process [44]. However as shown in Chapter 3, the expression level of β-catenin decreased in both cytoplasm / nucleus and membrane-bound compartments, indicating that the change was unlikely due to the translocalization of β-catenin resulting from increased E-cadherin on cell membrane. Therefore, β-catenin could be a direct target of FAK. Under normal conditions, the cytoplasmic pool of β-catenin is regulated by proteolytic degradation to remain low. The proteolysis of β-catenin is initiated by binding to the tumor suppressor APC (adenomatous polyposis coli), GSK-3β (glycogen synthase kinase-3β) and axin / conduction [46] and finally mediated by proteasome system [47]. In the absence of the Wnt/wg signal, phosphorylation of specific serine residues on β-catenin leads to its ubiquitination, degradation, and removal from the cytoplasm [48]. In osteocytes, FAK could promote β-catenin stabilization in response to PFF via activation of the PI3K/Akt pathway. Administration of FAK inhibitor abolished this effect [49]. In mammary tumor epithelial cells, FAK could also be important in regulating β-catenin stability which is dependent on the second C-terminal proline-rich motif. Integrin signalling has been related to the Wnt signalling pathways by several investigations [50-54]. For example, in HEK293 cells, the integration of the β-catenin-dependent Wnt pathway with integrin/FAK signalling has been shown to be through the adaptor molecule Grb2 [54]. Grb2 is an adaptor associated with FAK and is believed to mediate MAPK signaling pathway downstream of FAK [55, 56]. Additionally,

mutations of the SH3 of Grb2 blocked Wnt signaling transduction [54]. Interestingly, Ohmori et al. demonstrated that Grb2 could bind to the second C-terminal proline-rich motif of FAK via its SH3 domain in addition to the binding between Grb2 and phosphorylated tyrosine 925 of FAK [57]. Therefore, PA mutations in this motif of FAK could have disturbed its binding to Grb2, and resulted in deregulation of the Wnt / β-catenin signaling transduction and the cell contact inhibition. Co-immonoprecipitation and rescue experiments would be helpful to elucidate such a hypothesis.

In addition to Grb2, several other molecules such as GRAF, ASAP1, amphiphysin and endophilin A2, have been reported to bind to FAK at the second C-terminal proline-rich motif [58-62]. Through binding to FAK, these molecules could mediate various cell functions such as cell proliferation, migration and invasion [58-62]. Therefore, decreased migration and enhanced cell contact inhibition of the FAK mutated mammary epithelial cells could be due to the disrupted binding between FAK and any of these molecules. To clarify their roles, it is thus necessary to confirm their bindings with FAK in the mammary tumor epithelial cells and investigate any changes in their downstream pathways that could be involved.

4.3 The second C-terminal proline-rich motif of FAK and tumor angiogenesis

In Chapter 3, I generated the EC specific PA knock-in mice mediated by Tie2-Cre and studied the blood vessel formation in mammary tumors by crossing the conditional knock-in mice with human breast cancer model mice with PyVT overexpression. I found no differences in the number and density of the tumor blood vessels, nor any defects in the proliferation, survival and migration of the ECs. However, in vivo examinations revealed massive necrosis in the tumors, suggesting that there still could be other defects in ECs expressing PA mutant FAK. Experiments on cell proliferation

and survival of the ECs were performed in vitro, but such examinations (BrdU and TUNEL assays) should have been carried out in vivo for better clarification. In addition, subtle vascular impairment, other than defective cell proliferation and survival, could also lead to defective blood vessel formation and reduced tumor growth. Such subtle defects could possibly be defective cell-cell adhesion between the ECs, which would result in increased vascular leakage and more infiltration of immune cells. Another possibility is that the mutations of the second C-terminal proline-rich motif led to changes in the EC behaviors only under pathological conditions, and the negative results generated from in vitro experiments could be due to the lack of proper stimulations. Impaired responses of the ECs to angiogenesis inducing factors such as bFGF, VEGF and TGF-alpha could also account for the formation of malfunctioning blood vessels in mammary tumors. Therefore, proper experiment conditions could be critical for the exploration of the mechanisms involved in defective blood vessel formation in knock-in tumor mice.

4.4 The differential roles of FAK kinase activity and the second C-terminal proline-rich motif in other systems

In Chapter 2 and Chapter 3, I studied the roles of the kinase activity of FAK in embryonic angiogenesis and the roles of the second C-terminal proline-rich motif of FAK in mammary tumor growth and metastasis, utilizing kinase-defective knock-in and PA knock-in mice respectively. However, the roles of the kinase activity or the second C-terminal proline-rich motif of FAK in other cell types / systems remains unknown. Such investigations could be done using similar strategies by creating tissue-specific knock-in mice using different Cre transgenic mice.

In addition to the indispensable role of FAK in cardiovascular system during embryonic development, utilizing inducible Cre expression in ECs (End-SCL-Cre-

ER^T), researchers have discovered that FAK was also important for tumor and wound-healing associated angiogenesis during adulthood [63]. Although ATP-competitive inhibitor was used to suggest that the kinase activity was essential for FAK functions, no direct evidence has been presented for the role of kinase activity of FAK in adulthood angiogenesis. Moreover, this study was compromised by the compensatory up-regulation of Pyk2 expression. By taking advantage of the endogenous expression of kinase-defective allele of FAK, we could possibly study the kinase function of FAK in the adult ECs without interfering the expression or activity of Pyk2. Primary ECs containing kinase-defective FAK could be isolated from mice; therefore the results would be more accurate for the investigation of its roles in ECs in vivo. Similarly, in neuronal system, FAK has been proven to play an important role in axonal proliferation and outgrowth [3, 64]. In one of these reports, researchers found that FAK regulated netrin-1-induced neurite outgrowth and attractive growth cone turning by binding and phosphorylating netrin receptor DCC (Deleted in Colorectal Cancer), and, interestingly by expressing kinase-defective FAK in FAK null fibroblast, the DCC phosphorylation was shown not to require the kinase activity of FAK [3]. This could be further confirmed in kinase-defective FAK knock-in mice and this signaling pathway could become one more regulation mediated by FAK in a kinase-independent manner.

As to the mammary tumor study in the PA knock-in mice, the tumor growth analysis in either total or tissue specific knock-in mice indicated that FAK in multiple cells / tissues could contribute to mammary tumor growth. In other words, mutations of FAK in systems other than the mammary epithelial cells and ECs could also lead to reduced tumor growth. These systems could include but not limited to immune system and stroma cells. Further studies with more tissue specific knock-in mice (for example,

CD19-Cre for B-cell, LCK-cre for T-cell, and Fsp-cre for stromal fibroblasts specific deletion) could provide more reliable information, and these studies would help in efforts to design drugs more specific and potent for mammary tumor treatment.

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