Snail1 controls epithelial–mesenchymal lineage commitment in focal adhesion kinase–null embryonic cells

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Mouse embryonic cells isolated from focal adhesion kinase (FAK)–null animals at embryonic day 7.5 display multiple defects in focal adhesion remodeling, microtubule dynamics, mechanotransduction, proliferation, directional motility, and invasion. To date, the ability of FAK to modulate cell function has been ascribed largely to its control of posttranscriptional signaling cascades in this embryonic cell population. In this paper, we demonstrate that FAK unexpectedly exerts control over an epithelial–mesenchymal transition (EMT) program that commits embryonic FAK-null cells to an epithelial status highlighted by the expression of E-cadherin, desmoplakin, and cytokeratins. FAK rescue reestablished the mesenchymal characteristics of FAK-null embryonic cells to generate committed mouse embryonic fibroblasts via an extracellular signal–related kinase– and Akt-dependent signaling cascade that triggered Snail1 gene expression and Snail1 protein stabilization. These findings indentify FAK as a novel regulator of Snail1-dependent EMT in embryonic cells and suggest that multiple defects in $FAK^{-/-}$ cell behavior can be attributed to an inappropriate commitment of these cells to an epithelial, rather than fibroblastic, phenotype.

Introduction

FAK-null embryonic cells isolated from embryonic day (E) 7.5–8.5 mice, classically termed as $FAK^{-/-}$ mouse embryonic fibroblasts (MEFs), display multiple defects in cytoskeletal organization, adhesivity, motility, and invasive activity (Webb et al., 2004; Ezratty et al., 2005; McLean et al., 2005; Mitra et al., 2005; Liu et al., 2007; Serrels et al., 2007; Lim et al., 2008b; Parsons et al., 2008; Tomar and Schlaepfer, 2009). To date, efforts to assign mechanistic functions to FAK have largely focused on the ability of the nonreceptor tyrosine kinase to modulate fibroblast function by controlling posttranscriptional signal transduction cascades (Parsons, 2003; Webb et al., 2004; Ezratty et al., 2005; McLean et al., 2005; Mitra et al., 2005; Serrels et al., 2007; Lim et al., 2008b; Tomar and Schlaepfer, 2009). The possibility, however, that FAK exerts upstream transcriptional control over lineage commitment of FAK-deficient embryonic cells has not been considered. Here, we demonstrate that $FAK^{-/-}$ embryonic cells fail to express classical mesenchymal cell markers and unexpectedly adopt an epithelial cell phenotype characterized by E-cadherin, desmoplakin, and cytokeratin expression. The inability of $FAK^{-/-}$ cells to display mesenchymal cell characteristics is linked directly to the dysregulated expression of Snail1, a developmentally regulated transcriptional repressor that controls cell epithelial–mesenchymal transition (EMT) programs (Peinado et al., 2007; Thiery et al., 2009). After FAK reexpression, $FAK^{-/-}$ embryonic cells activate Snail1 mRNA transcription, stabilize Snail1 protein levels, and repress epithelial cell markers while activating the cellular machinery that is characteristic of normal mesenchyme. These findings not

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Abbreviations used in this paper: CAM, chorioallantoic membrane; EMT, epithelial–mesenchymal transition; ERK, extracellular signal–related kinase; KO, knockout; MEF, mouse embryonic fibroblast; MMTV-PyMT, mouse mammary tumor virus–polyoma middle T antigen; PI3K, phosphatidylinositol 3-kinase.

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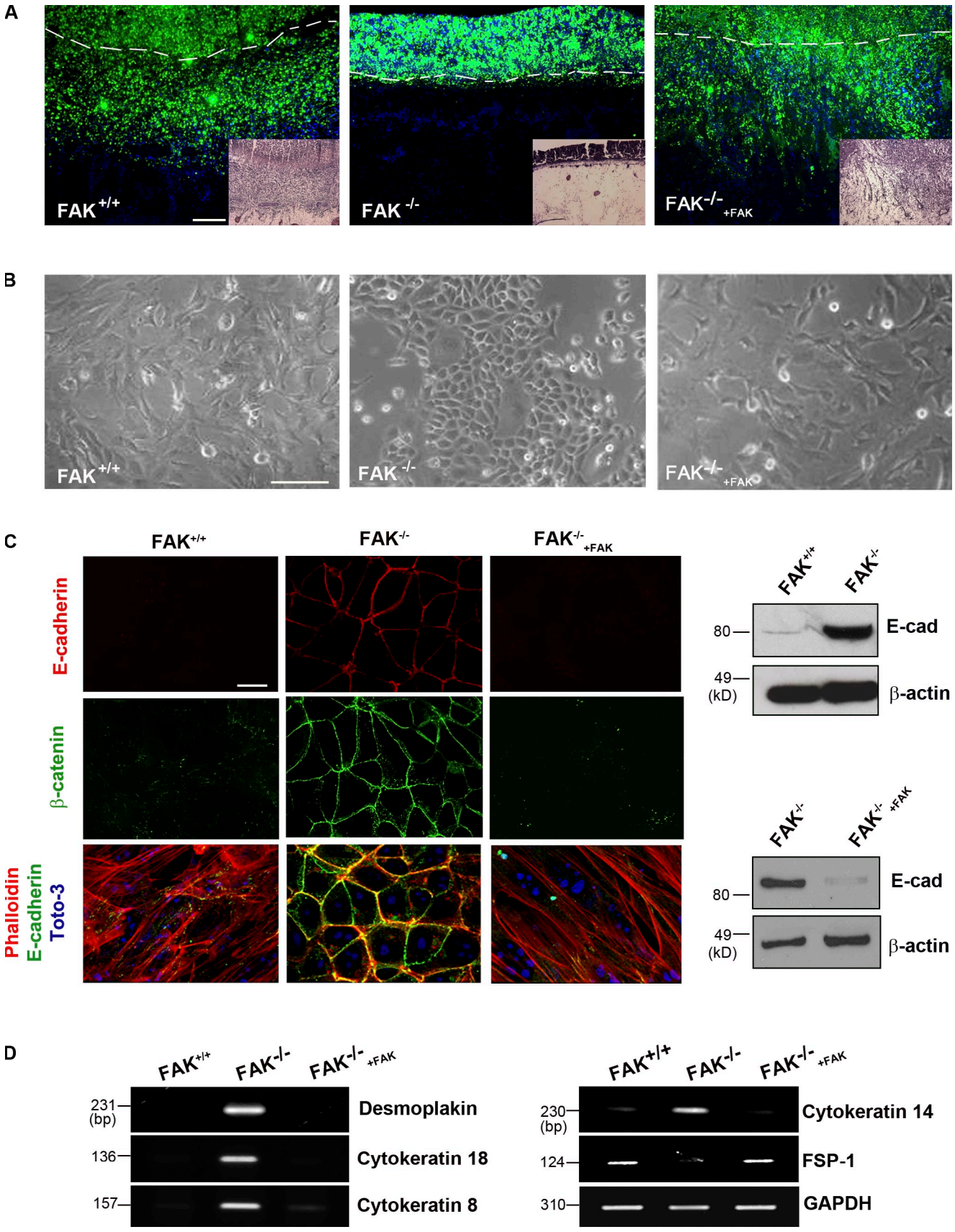

Figure 1. **FAK regulates the mesenchymal phenotype of MEFs.** (A) CAM invasion by nanobeads (green) labeled *FAK*$^{+/+}$, *FAK*$^{-/-}$, and FAK-rescued *FAK*$^{-/-}$ (*FAK*$^{-/-}$$_{+FAK}$) embryonic cells. Cell invasion was visualized by fluorescence microscopy of CAM cross sections that were prepared after a 2-d incubation period. The CAM surface is marked by dashed lines. Quantification of invasion and the efficiency of FAK rescue are shown in Fig. S1. Hematoxylin and

only identify FAK as a novel regulator of Snail1-dependent EMT but also suggest that the multiple phenotypic defects categorized previously in $FAK^{-/-}$ embryonic cells (i.e., FAK-null MEFs) arise as a consequence of the inappropriate commitment of these cells to an epithelial, rather than mesenchymal, lineage.

Results and discussion

Immortalized FAK-null cells isolated from tissue explants E7.5 $FAK^{-/-}$ mice display multiple defects in motile, proteolytic, and invasive activity in vitro (Parsons, 2003; Webb et al., 2004; Ezratty et al., 2005; McLean et al., 2005; Mitra et al., 2005; Serrels et al., 2007; Lim et al., 2008b; Tomar and Schlaepfer, 2009). Consistent with these observations, whereas $FAK^{+/+}$ MEFs cultured atop the chorioallantoic membrane (CAM) of 11-d-old chicks rapidly infiltrate underlying stromal tissues, FAK-null cells are noninvasive (Fig. 1 A and Fig. S1; Ota et al., 2009). In contrast, $FAK^{-/-}$ embryonic cells engineered to stably express FAK regain invasive potential to a degree similar, if not identical, to that observed in the $FAK^{+/+}$ control population (Fig. 1 A and Fig. S1). Although these results support potential roles for FAK and downstream signal transduction cascades in regulating motility and invasion (Parsons, 2003; Webb et al., 2004; Ezratty et al., 2005; McLean et al., 2005; Mitra et al., 2005; Liu et al., 2007; Serrels et al., 2007; Lim et al., 2008b; Tomar and Schlaepfer, 2009), $FAK^{-/-}$ embryonic cells cultured in vitro adopt an epithelioid morphology relative to $FAK^{+/+}$ cells, suggestive of a partial defect in EMT (Fig. 1 B). Indeed, unlike $FAK^{+/+}$ MEFs, $FAK^{-/-}$ embryonic cells obtained from three independent sources (see Materials and methods) unexpectedly assemble E-cadherin– and β-catenin–positive junctional complexes and organize their actin cytoskeleton into an epithelial-like cortical pattern (Fig. 1 C and Fig. S1; Halbleib and Nelson, 2006). In keeping with a potential defect in EMT that locks $FAK^{-/-}$ embryonic cells in an epithelial cell-like phenotype, FAK-null cells also express a variety of epithelial cell markers, including desmoplakin as well as cytokeratins 8, 14, and 18, in tandem with a complete loss of expression of the fibroblast-specific marker FSP-1 (Fig. 1 D and Fig. S1; Bragulla and Homberger, 2009; Zeisberg and Neilson, 2009). Importantly, FAK-null embryonic cells adopt a classical fibroblastic phenotype after FAK reexpression that is accompanied by a loss of desmoplakin and cytokeratin expression as well as the up-regulation of FSP-1 (Fig. 1, B–D; and Fig. S1).

In vivo, transitions between epithelial and mesenchymal phenotypes are orchestrated by a family of zinc finger transcriptional repressors, including Snail1, Snail2, ZEB1, and ZEB2, as well as the basic helix-loop-helix factors Twist1 or Twist2 (Peinado et al., 2007; Thiery et al., 2009). As assessed by RT-PCR and real-time PCR, FAK-null embryonic cells display significantly decreased levels of Snail1 and Twist2 transcripts relative to $FAK^{+/+}$ cells (Fig. 2 A). Expression of these transcripts is restored to normal levels after FAK rescue (Fig. 2 A). Transcriptional profiling of $FAK^{-/-}$ embryonic cells, $FAK^{+/+}$ MEFs, and FAK-rescued $FAK^{-/-}$ cells not only confirms changes in Snail1 and Twist2 but also reveals significant alterations in a range of epithelial and mesenchymal markers as well as EMT-inducing molecules, including HMGA2, Foxc2, and Notch1, as well as multiple motility/invasion-promoting factors (e.g., Pak1, Itg5, and Myl9/Mic2; Fig. 2 B; Grum-Schwensen et al., 2005; Thuault et al., 2006; Mani et al., 2007; Peinado et al., 2007; Ansieau et al., 2008; Coniglio et al., 2008; Sahlgren et al., 2008; Medjkane et al., 2009). In each case, the increased expression of epithelial markers and the coupled decreased expression of mesenchymal markers or EMT-inducing factors in $FAK^{-/-}$ embryonic cells are reversed fully after rescue with a wild-type FAK expression vector (Fig. 2 B). However, with the exception of Snail1, none of these EMT-associated factors are known to play required roles in the mesodermal programs associated with early development (Peinado et al., 2007; Thiery et al., 2009). As Snail1 mRNA and protein levels can be regulated in a discoordinate fashion, and nuclear rather than cytosolic Snail1 protein expression plays a required role in mediating EMT (Zhou et al., 2004; Yook et al., 2005, 2006; MacPherson et al., 2010), the ability of FAK to regulate nuclear Snail1 protein levels in wild-type and $FAK^{-/-}$ cells was assessed. Indeed, whereas MEFs isolated from $FAK^{+/+}$ mice at E7.5 express readily detectable levels of nuclear Snail1 protein as well as FSP-1, $FAK^{-/-}$ embryonic cells fail to express either Snail1 or FSP-1 protein until such time that FAK expression is reconstituted (Fig. 2 C).

Although these data demonstrate that FAK is able to control Snail1 expression in embryonic cells cultured in vitro, immortalized $FAK^{+/+}$ MEFs and $FAK^{-/-}$ embryonic cells are commonly generated in a $p53^{-/-}$ background, as FAK-deleted MEFs harboring wild-type p53 alleles display marked defects in proliferative potential (Lim et al., 2008a). In this regard, it remains possible that the p53-null status of $FAK^{+/+}$ or $FAK^{-/-}$ MEFs is permissive for the introduction of spontaneous mutations that impact Snail1 regulation (Tilghman et al., 2005; Kim et al., 2011b). Interestingly, however, $FAK^{-/-}$ mouse embryos in a p53 wild-type background display lethal defects in mesodermal cell function at early developmental stages that bear resemblance to those reported in $Snail1^{-/-}$ mice (Furuta et al., 1995; Ilić et al., 1995a; Murray and Gridley, 2006; Lomelí et al., 2009), raising the possibility that FAK regulates Snail1 expression in vivo. As such, embryos were isolated from $FAK^{+/+}$/$p53^{+/+}$ and $FAK^{-/-}$/$p53^{+/+}$ mice at E7.5 (i.e., the stage in which phenotypic abnormalities first emerge in $FAK^{-/-}$ mice and immortalized cells are harvested from tissue explants of the wild-type and null mice [Ilić et al., 1995a]), and Snail1 protein levels were assessed by Western blotting. Although Snail1 protein is expressed in both $FAK^{+/+}$ and FAK heterozygous mice, only trace levels are detected in FAK-null embryos at this time

eosin–stained cross sections of CAMs are shown as insets. Results are representative of three experiments performed. (B) Phase-contrast images of $FAK^{+/+}$, $FAK^{-/-}$, and FAK-rescued null cells. (C) Confocal laser micrographs of E-cadherin (E-cad)–, β-catenin–, and phalloidin-stained cells (nuclei are stained with Toto-3). E-Cadherin protein levels in $FAK^{+/+}$, $FAK^{-/-}$, and FAK-rescued null embryonic cells by Western blot analysis. (D) RT-PCR analysis of desmoplakin and cytokeratins 18, 8, and 14 as well as FSP-1 mRNA levels in $FAK^{+/+}$, $FAK^{-/-}$, and FAK-rescued null MEFs. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Bars: (A and B) 100 µm; (C) 20 µm.

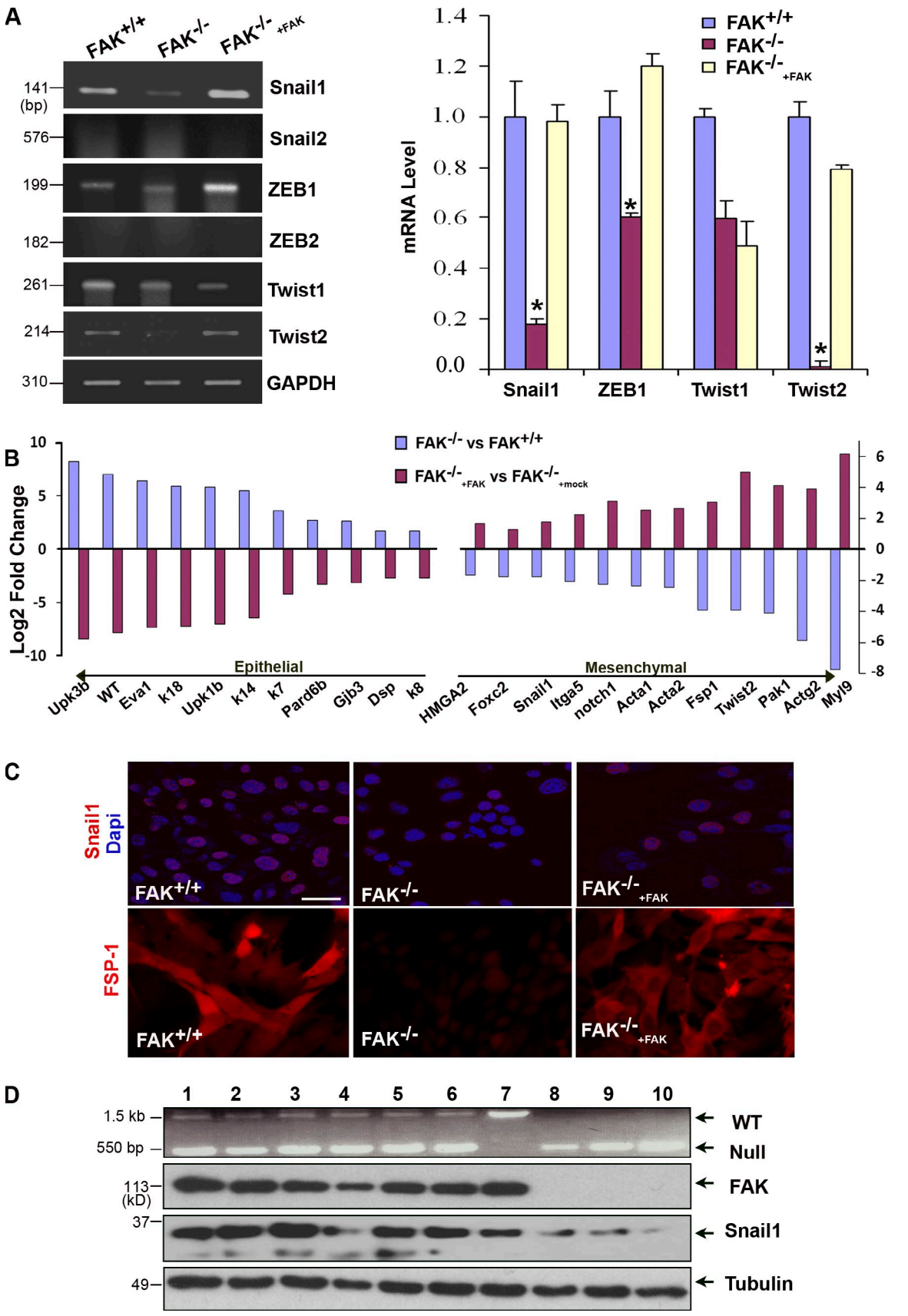

Figure 2. **FAK-dependent expression of Snail1.** (A) RT-PCR and real-time PCR analysis of Snail1, Snail2/Slug, ZEB1, ZEB2, Twist1, and Twist2 mRNA levels in *FAK*$^{+/+}$ MEFs and *FAK*$^{-/-}$ or FAK-rescued embryonic cells. Results are expressed as the means ± SEM (n = 3). *, P < 0.01. (B) mRNA was isolated from *FAK*$^{+/+}$, *FAK*$^{-/-}$, and FAK-rescued *FAK*$^{-/-}$ cells and hybridized to mouse 430 2.0 cDNA microarrays. Results are representative of a single experiment

point (Fig. 2 D). Though Snail1 expression levels increase to wild-type levels in a delayed fashion by E8.5 in *FAK*$^{-/-}$ embryos (unpublished data), these data demonstrate that FAK exerts temporal control over the induction of Snail1 expression in vivo. Although defects in mesodermal development in mouse embryos and the retention of epithelial cell-like characteristics in harvested embryonic cells are consistent with the delayed induction of Snail1 in *FAK*$^{-/-}$ mice (Carver et al., 2001), committed fibroblasts are epigenetically stable and are not known to adopt epithelial phenotypes. Indeed, when FAK or Snail1 expression is silenced in adult fibroblasts, the cells are unable to adopt an epithelial phenotype in long-term culture (Fig. S2). Hence, the FAK-Snail1 EMT program is restricted to embryonic states when epithelial–fibroblast commitment is first initiated.

FAK has not previously been implicated as a direct-acting modulator of Snail1 expression, but the nonreceptor tyrosine kinase is known to regulate Akt and MAPK signal transduction pathways (Slack-Davis et al., 2003; Mitra and Schlaepfer, 2006), either of which could potentially impact Snail1 expression at the transcriptional or posttranslational levels (Zhou et al., 2004; Julien et al., 2007; Peinado et al., 2007; Rowe et al., 2009). Indeed, although the addition of serum to *FAK*$^{+/+}$ MEFs triggers robust Akt and ERK activation as well as Snail1 mRNA expression, *FAK*$^{-/-}$ embryonic cells fail to activate either of these signal transduction cascades or induce Snail1 expression unless FAK expression is reconstituted (Fig. 3, A and B). A direct role for the phosphatidylinositol 3-kinase (PI3K)/Akt axis and extracellular signal–related kinase (ERK) in Snail1 expression is confirmed by the fact that Snail1 mRNA levels in *FAK*$^{+/+}$ MEFs are significantly inhibited in the presence of the PI3K inhibitor LY294002 or the ERK inhibitor PD98059 (Fig. 3 C). Similarly, after FAK reconstitution in *FAK*$^{-/-}$ embryonic cells, Snail1 promoter activity is increased threefold in a LY294002- or PD98059-sensitive fashion (Fig. 3 D). Independent of the ability of FAK to regulate Snail1 mRNA expression, Snail1 protein half-life is tightly controlled in a multistep process involving GSK3-β–dependent phosphorylation, β-TrCP–directed ubiquitination, and proteasomal destruction (Zhou et al., 2004; Yook et al., 2005, 2006). As the PI3K/Akt axis can negatively regulate GSK3-β–dependent Snail1 phosphorylation and, hence, stabilize Snail1 levels (Barberà et al., 2004; Zhou et al., 2004; Rowe et al., 2009), the ability of FAK to control Snail1 protein turnover was determined. When *FAK*$^{+/+}$ MEFs or *FAK*$^{-/-}$ embryonic null cells were transduced with an epitope-tagged Snail1 expression vector, steady-state levels of exogenous Snail1 protein are stabilized in wild-type MEFs relative to *FAK*$^{-/-}$ cells (Fig. 3 E). Furthermore, Snail1 half-life in *FAK*$^{-/-}$ embryonic cells increases to control levels after FAK rescue (Fig. 3 E). Finally, although Snail1 transcription is regulated by both ERK and Akt, Snail1 protein half-life falls under the control of PI3K-dependent activity alone in both *FAK*$^{+/+}$ MEFs as well as FAK-rescued *FAK*$^{-/-}$ embryonic cells (Fig. 3 E). In contrast to wild-type FAK, FAK constructs harboring mutations at Y397F, P712A, or Y925F are unable to rescue Snail1 expression in *FAK*$^{-/-}$ cells (Fig. S3). As FAK autophosphorylation at Y397 and its subsequent association with Src family kinases controls the phosphorylation of downstream signaling molecules, including p130cas at p712/715 and Grb2 at Y925 (Mitra et al., 2005), these data demonstrate that each of these FAK-initiated signal transduction pathways plays critical roles in regulating Snail1 expression and activity.

To finally determine the degree to which Snail1 participates in FAK-dependent EMT, FAK-null embryonic cells were transiently transfected with a FAK expression vector in the absence or presence of siRNAs directed against Snail1. As shown in Fig. 4 A, clusters of *FAK*$^{-/-}$ embryonic cells electroporated with a wild-type FAK expression vector down-regulate E-cadherin expression and assume a fibroblastoid phenotype. In contrast, *FAK*$^{-/-}$ cells engineered to express FAK in the presence of either of two Snail1-specific siRNAs maintain E-cadherin staining as well as epithelial-like cell–cell adhesive interactions (Fig. 4 A and Fig. S3). Transient expression of FAK in *FAK*$^{-/-}$ embryonic cells similarly induces the down-regulation of cytokeratins 8 and 18 mRNA levels via a process that is blocked by siRNAs directed against Snail1 (Fig. 4 B). Finally, consistent with a required role for Snail1 in mediating FAK-dependent EMT, transient expression of Snail1 in *FAK*$^{-/-}$ embryonic cells represses E-cadherin expression and induces cell invasion in vivo to a degree similar to that observed after wild-type FAK reexpression (Fig. 4, A and C). Hence, FAK exerts global control over the embryonic cell phenotype by inducing both the loss of epithelial characteristics and the adoption of mesenchymal cell properties.

Relative to the *FAK*$^{+/+}$ MEFs isolated from tissue explants of E7.5 mice, *FAK*$^{-/-}$ cells—termed *FAK*$^{-/-}$ MEFs by previous convention—have been shown to display a multiplicity of defects in the regulation of Rho GTPase activity, focal adhesion remodeling, microtubule dynamics, mechanotransduction, directional motility, and invasion that are each reversed after rescue with wild-type FAK (Webb et al., 2004; Ezratty et al., 2005; Mitra et al., 2005; Serrels et al., 2007; Lim et al., 2008b; Tomar and Schlaepfer, 2009). Implicit in the characterization of FAK's functional contribution to these pathways has been the assumption that *FAK*$^{+/+}$ and *FAK*$^{-/-}$ cells isolated from embryonic mouse tissues are both fibroblastic in terms of lineage commitment. Instead, we find that FAK-null MEFs are more closely aligned to epithelial cells in terms of both patterns of gene expression and behavior. As *FAK*$^{-/-}$ MEFs have been reported to up-regulate expression of the FAK-related proline-rich kinase Pyk2 as a compensatory mechanism to control adhesion and motility (Lim et al., 2008b), we considered the possibility that elevated Pyk2 levels might interfere with Snail1-dependent EMT programs. However,

involving four paired samples (i.e., *FAK*$^{+/+}$, *FAK*$^{-/-}$, FAK-rescued *FAK*$^{-/-}$, and mock-rescued *FAK*$^{-/-}$ embryonic cells with a subset of epithelial- and mesenchymal-associated transcripts identified). Dsp, desmoplakin. (C) *FAK*$^{+/+}$ MEFs, *FAK*$^{-/-}$, and FAK-rescued embryonic cells were cultured in vitro, fixed, and stained with anti-Snail1 monoclonal antibody or anti–FSP-1. Nuclei were visualized with Toto-3 (blue). Bar, 100 µm. (D, top) E7.5 embryos from *FAK*$^{+/-}$/*FAK*$^{+/-}$ matings were isolated and genotyped to identify *FAK*$^{+/+}$ (lane 7), *FAK*$^{+/-}$ (lanes 1–6), and *FAK*$^{-/-}$ (lanes 8–10) embryos. Protein extracts from the embryos were examined for FAK and Snail1 protein levels by Western blot analysis. Tubulin levels were used as a loading control. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; WT, wild type.

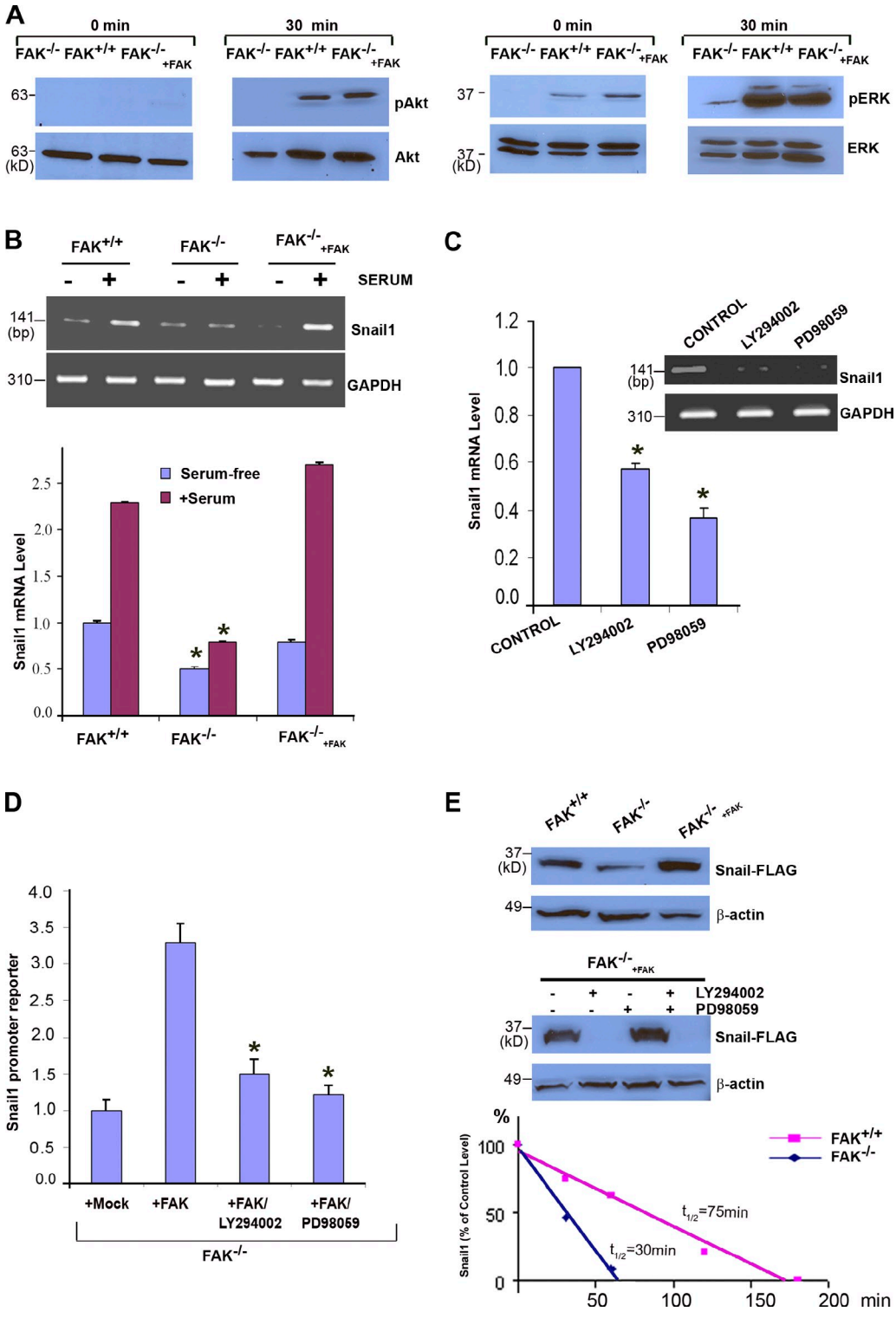

Figure 3. **FAK-dependent regulation of Snail1 expression via PI3K and MAPK signaling pathways.** (A) Serum-starved *FAK*$^{+/+}$, *FAK*$^{-/-}$, or FAK-rescued cells were treated with 10% serum for 0 or 30 min. Cell lysates were prepared, and total as well as phospho-Akt and total as well as phospho-ERK levels were determined by Western blot analysis. (B) Snail1 mRNA levels in serum-starved and serum-treated *FAK*$^{+/+}$ MEFs or *FAK*$^{-/-}$ and FAK-rescued *FAK*$^{-/-}$ embryonic cells are shown after a 2-h culture period. (C) Snail1 mRNA levels were determined in wild-type MEFs treated with 20 µM LY294002 or 20 µM PD98509 for 24 h as determined by RT-PCR (inset) and quantified by real-time PCR. (D) Reporter gene assay of Snail1 promoter activity in mock or FAK-transfected *FAK*$^{-/-}$ cells treated with 20 µM LY294002 or 20 µM PD98509 for 48 h. (E) *FAK*$^{+/+}$ MEFs or *FAK*$^{-/-}$ and FAK-rescued *FAK*$^{-/-}$ embryonic cells were transfected with a FLAG-tagged Snail1 expression vector in the absence or presence of LY294002, PD98059, or LYS94002 and PD98059 in combination. After a 4-h culture period, epitope-tagged Snail1 levels were determined by Western blotting using the anti-FLAG monoclonal antibody. Cycloheximide chase assays of *FAK*$^{+/+}$ MEFs or *FAK*$^{-/-}$ cells transfected with FLAG-Snail1 were performed by treating cells with cycloheximide for 0–180 min. Snail1 levels at each time point were determined by Western blotting using the anti-FLAG antibody and quantified as the percentage of the Snail1 levels at time 0. β-Actin was used as the loading control. Results are expressed as the means ± SEM (n = 3). *, P < 0.01. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

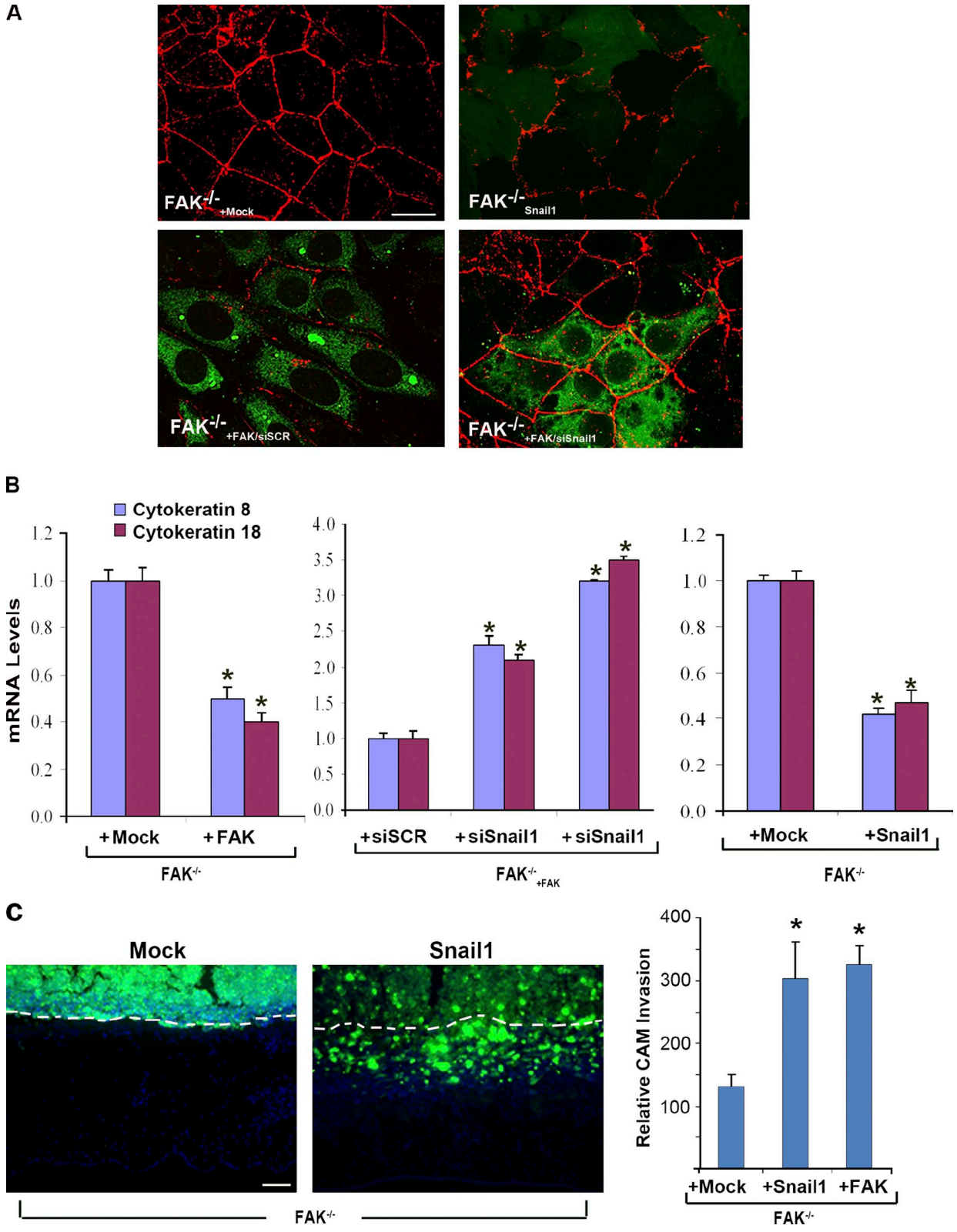

Figure 4. **Snail1 directs FAK-dependent EMT in *FAK*$^{-/-}$ embryonic cells.** (A) FAK-null embryonic cells were transfected with either a mock control vector, a FAK-GFP expression vector in combination with a scrambled siRNA (siSCR) control or Snail1-specific siRNAs, or a Snail1 expression vector coexpressing GFP. After a 5-d culture period, MEFs were stained for E-cadherin (red) with GFP visualized by fluorescence microscopy. Results are representative of three or more experiments. (B) *FAK*$^{-/-}$ embryonic cells were transiently transfected with mock or FAK expression vectors alone or in combination with control or two Snail1-specific siRNAs. 5 d after transfection, GFP-positive cells were sorted by flow cytometry, and cytokeratins 8 and 18 mRNA levels were determined by real-time PCR. (C) *FAK*$^{-/-}$ embryonic cells were transduced with a mock, Snail1, or wild-type FAK adenoviral expression vector labeled with fluorescent nanobeads (green) and cultured atop the live chick CAM for 3 d. The CAM surface is marked by dashed lines, and CAM invasion was quantified as described in Materials and methods. Results are expressed as the means ± SEM (n = 3). *, $P < 0.01$. Bars, 100 µm.

after Pyk2 silencing, *FAK*$^{-/-}$ embryonic cells continue to express E-cadherin and maintain cell–cell junctional complexes (unpublished data). Thus, FAK appears to orchestrate the expression of a cohort of EMT-associated gene products, independently of Pyk2 activity, with Snail1 serving as a dominant effector of mesenchymal cell transformation in FAK-deficient embryonic cells. Consistent with these findings, *FAK*$^{-/-}$ embryos likewise displayed defects in Snail1 expression despite the fact that elevated Pyk2 levels have not been detected in embryonic tissues of FAK conditional knockout (KO) mice (with the exception of osteoblasts [Weis et al., 2008]). Together, these data indicate that embryonic cells isolated from FAK-null mice have not yet undergone a full, FAK-dependent EMT program and consequently retain epithelial-like characteristics. The fact that both *FAK*$^{-/-}$- and *Snail1*$^{-/-}$-null embryos display similar defects in mesoderm development and die between E8.5 and 9.5 (Ilić et al., 1995a; Shen et al., 2005; Murray and Gridley, 2006; Lomelí et al., 2009; Tomar and Schlaepfer, 2009) supports a model wherein FAK and Snail1 play interacting roles in controlling developmentally linked EMT programs. It should be noted, however, that the precise roles played by FAK or Snail1 during developmental EMT in vivo remain controversial, as *FAK*$^{-/-}$ as well as *Snail1*$^{-/-}$ embryos can form mesodermal tissues (Furuta et al., 1995; Ilić et al., 1995a,b; Murray and Gridley, 2006; Thiery et al., 2009). Furthermore, we find that defects in Snail1 expression in FAK-null embryos are transient as opposed to the more permanent loss observed in *FAK*$^{-/-}$ embryonic cells maintained in vitro. Nevertheless, mesoderm function is severely compromised in both KO lines, a finding consistent with significant, if not lethal, consequences for normal developmental programs. These findings should not be misconstrued to suggest that Snail1 exerts each of its effects on the *FAK*$^{-/-}$ phenotype in a direct-acting fashion or, for that matter, that all *FAK*$^{-/-}$ embryonic cell defects are Snail1 dependent. For example, MEFs isolated from mice harboring an inactivating mutation in ZEB1, a downstream target of Snail1 (Guaita et al., 2002), also express E-cadherin (Liu et al., 2008) and likely require reclassification. *FAK*$^{-/-}$ embryonic cells also display comprehensive shifts in their transcriptome, affecting >1,000 genes across functional programs that extend beyond EMT itself (Fig. S3 E). The precise subset of FAK-regulated genes that fall under Snail1 control alone will require further study, but the current results highlight unexpected roles for the FAK-Snail1 axis in developmental EMT. Though recent studies demonstrate that loss of p53 function can also increase Snail1 expression and activity (Kim et al., 2011a,b), the p53-null status of standard *FAK*$^{-/-}$ embryonic cell lines is not sufficient to bypass the apparent requirement for FAK-dependent Snail1 transcription.

From their first description in 1995, *FAK*$^{-/-}$ MEFs isolated from mouse embryos have attracted particular attention as a consequence of the multiplicity of phenotypic defects observed in these cells, particularly with regard to cell adhesion, motility, invasion, survival, and proliferation (Webb et al., 2004; Ezratty et al., 2005; McLean et al., 2005; Liu et al., 2007; Lim et al., 2008a,b; Tomar and Schlaepfer, 2009). Given the ability of all defects to be reversed by reconstituting FAK expression, it has long been assumed that FAK rescued cell function by mechanisms restricted to the recoupling of aborted signal transduction cascades. Although in part true, functional comparisons between *FAK*$^{+/+}$ MEFs isolated from E7.5 mouse embryonic tissues and *FAK*$^{-/-}$ embryonic cells are invariably overlaid by the dramatic phenotypic and genotypic alterations associated with mesodermal versus epithelial commitment. Hence, our findings not only indicate that functional characterizations of *FAK*$^{-/-}$ embryonic cells need be revisited but also that the epithelial/mesodermal status of MEFs isolated from transgenic mouse embryos must be documented carefully, especially when harvested from embryonic tissues at time points associated with EMT or mesenchymal–epithelial transition. Finally, independent of a requisite reappraisal of the previously ascribed roles played by FAK in cells of embryonic origin (Webb et al., 2004; Ezratty et al., 2005; Mitra et al., 2005; Serrels et al., 2007; Lim et al., 2008a,b; Tomar and Schlaepfer, 2009), increases in FAK as well as Snail1 activity have also been linked to pathological states ranging from fibrosis to cancer (Mitra and Schlaepfer, 2006; Lahlou et al., 2007; Peinado et al., 2007; Parsons et al., 2008; Provenzano et al., 2008; Pylayeva et al., 2009; Rowe et al., 2011). Indeed, preliminary experiments indicate that FAK can regulate Snail1 expression in mammary carcinoma cells generated in mouse mammary tumor virus–polyoma middle T antigen (MMTV-PyMT) mice (Fig. S3; Luo et al., 2009). As such, the findings presented here support a model wherein FAK activity may serve to control developmental as well as pathological EMT programs by recruiting Snail1 and its downstream effectors

Materials and methods

Antibodies and reagents

Antibodies directed against the following molecules were obtained commercially: E-cadherin, β-catenin (BD), FSP-1 (Abcam), Akt, Akt phospho-Ser473, ERK, ERK phospho-Tyr204 (Cell Signaling Technology), FLAG, and β-actin (Sigma-Aldrich). Anti-Snail1 monoclonal antibodies were generated and characterized as previously described (Rowe et al., 2009). FLAG-tagged Snail1 expression vector, GFP-FAK expression vector, and a mouse Snail1 promoter reporter construct were used as previously described (Li et al., 2002; Yook et al., 2005; Peinado et al., 2007). Snail1 siRNA oligonucleotides were described previously (Yook et al., 2006) or purchased from Invitrogen.

Cell culture and transfection

FAK$^{+/+}$ MEFs, *FAK*$^{-/-}$ MEFs, and FAK-rescued *FAK*$^{-/-}$ were isolated as previously described (Ilić et al., 1995a) and obtained from either the Ilić laboratory, Schlaepfer laboratory, or American Type Culture Collection. In brief, E7.5 embryos were dissected in phosphate-buffered saline and incubated with trypsin and EDTA at 37°C, and the cells were plated in DME supplemented with 10% FCS. Cultures were genotyped to determine *FAK*$^{+/+}$ and *FAK*$^{-/-}$ cells. FAK-rescued cells were generated by stable transfection of *FAK*$^{-/-}$ cells with pcDNA3.1-FAK as described previously (Sieg et al., 1999). Cells were transfected transiently with transfection reagent (Nucleotransfector MEF2 [Lonza]; Lipofectamine [Invitrogen]) according to the manufacturers' instructions (Lim et al., 2008b).

Mice

All mice were maintained on a C57BL6 background. FAK heterozygote mice were crossed, and embryos were isolated on E7.5 and genotyped by PCR analysis of genomic DNA as previously described (Shen et al., 2005). Primers flanking exon 3 of mouse FAK gene (5′-GCTGATGTCCCAAGCTATTCC-3′ and 5′-AGGGCTGGTCTGCGCTGACAGG-3′) were used for genotyping, which yielded 1.5-kb and 550-bp products for wild-type and null alleles, respectively. All animal experiments were in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and approved by the University of Michigan Institutional Review Board.

Immunofluorescence

Snail1 immunocytochemistry was performed as described previously (Rowe et al., 2009). Cells were fixed in 4% paraformaldehyde, permeabilized

with 1% sodium dodecyl sulfate, denatured with 6 M urea and 0.1% glycine, pH 3.5, for 10 min, blocked with 3% goat serum, and incubated with the monoclonal antibody 173EC2 (at 1:1,000) overnight followed by detection with an Alexa Fluor 488–labeled anti–mouse secondary antibody (Invitrogen). For E-cadherin, β-catenin, or FSP-1 staining, cells were paraformaldehyde fixed and permeabilized with Triton X-100. After incubation with primary antibodies, antigen–antibody complexes were detected with either Alexa Fluor 594– or Alexa Fluor 488–labeled secondary antibodies (Invitrogen). Confocal images of cells were acquired on a confocal microscope (FV500) using a 100× water immersion lens with a 1.20 numerical aperture using FluoView software (all obtained from Olympus). Phase-contrast images were acquired with an inverted microscope (DM-ILB; Leica) with a 20× objective and 0.40 numerical aperture, and CAM images were acquired on a microscope (DM-LB) with a 20× objective and 0.50 numerical aperture (Leica).

Chick CAM invasion assays

Cell invasion by *FAK*$^{+/+}$ MEFs or *FAK*$^{-/-}$ embryonic cells in vivo was assessed using 11-d-old chick embryos in which an artificial air sac was created (Rowe et al., 2009). Wild-type, null, or FAK-rescued embryonic cells were labeled with 45-nm-diam carboxylate microspheres (Fluoresbrite; Polysciences) for 30 min. 10^5 cells were inoculated atop the CAM for 2 d, and the CAM was removed at the end of the incubation period. Tissues were fixed overnight in 4% paraformaldehyde, and after an overnight incubation in 30% sucrose, CAM tissue was frozen in the optimum cutting temperature compound, and cross sections were prepared for fluorescence microscopy and hematoxylin and eosin staining. Invasion was quantified as a function of cell-associated fluorescence localized beneath the CAM surface (ImageQuant version 5.2; Molecular Dynamics, Inc.; Rowe et al., 2009).

RT-PCR and real-time PCR

Total RNA was isolated using TRIZOL reagent (Invitrogen). Reverse transcription was performed with 1 µg of total RNA and oligodeoxythymidylic acid primers by using the First Strand Synthesis kit (Invitrogen). Quantitative PCR was performed using the SYBR green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. Gene-specific primers are listed in Table S1.

Transcriptional profiling

Total RNA was isolated from *FAK*$^{+/+}$ MEFs or *FAK*$^{-/-}$ embryonic cells and then labeled and hybridized to mouse 430 2.0 cDNA microarrays (Affymetrix). Results from at least three experiments were analyzed by the University of Michigan Microarray Core. Differentially expressed probe sets were determined using a minimum fold change of 2.0. Gene ontology analysis was performed to identify biological processes transcriptionally regulated by FAK (Rowe et al., 2009).

Reporter gene assay

Snail1 promoter activity was determined by transiently transfecting 5×10^5 *FAK*$^{+/+}$ or *FAK*$^{-/-}$ MEF embryonic cells, 500 ng Snail1 promoter reporter construct, and 20 ng thymidine kinase-*Renilla reniformis* luciferase construct (Promega) as a control for transfection efficiency and either 500 ng wild-type FAK expression vector or a mock expression vector. After a 16-h culture period, cells were treated with LY294002 or PD98059 for 48 h as described in the legend of Fig. 3 D. Luciferase activities were measured using the Dual-Luciferase Reporter Assay kit (Promega). Results represent the means ± SEM of at least three independent experiments performed in triplicate samples.

Snail1 steady-state and half-life determinations

For determinations of Snail1 half-life, *FAK*$^{+/+}$ MEFs or *FAK*$^{-/-}$ embryonic cells were transfected with a FLAG-tagged Snail1 expression vector and, 48 h later, treated with 50 µg/ml cycloheximide. Cells were then harvested and lysed for Western blotting at the time points indicated in the legend of Fig. 3 E. The levels of FLAG-tagged Snail1 were quantified by densitometry.

Statistical analysis

$P < 0.01$ (indicated by an asterisk in the figures) was determined using Student's *t* test.

Online supplemental material

Fig. S1 provides quantitative analyses of CAM invasion and FAK levels after FAK rescue as well as associated changes in the expression of epithelial and mesenchymal markers by either quantitative PCR or immunoblotting. Fig. S2 characterizes the differential effect of FAK silencing in MEFs versus adult fibroblasts as well as the ability of FAK or Snail1 silencing to alter the expression of epithelial or mesenchymal markers by quantitative PCR or immunoblotting. Fig. S3 characterizes the effect of various FAK mutants on Snail1 expression, the utility of siRNAs directed against Snail1 to silence Snail1 protein expression, the role of FAK in regulating Snail1 expression in MMTV-pyMT carcinoma cells, and gene ontology analysis of FAK-deleted embryonic cells. Table S1 contains the primer sequences used for RT-PCR. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201105103/DC1.

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References

Ansieau, S., J. Bastid, A. Doreau, A.P. Morel, B.P. Bouchet, C. Thomas, F. Fauvet, I. Puisieux, C. Doglioni, S. Piccinin, et al. 2008. Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. *Cancer Cell*. 14:79–89. http://dx.doi.org/10.1016/j.ccr.2008.06.005

Barberà, M.J., I. Puig, D. Domínguez, S. Julien-Grille, S. Guaita-Esteruelas, S. Peiró, J. Baulida, C. Francí, S. Dedhar, L. Larue, and A. García de Herreros. 2004. Regulation of Snail transcription during epithelial to mesenchymal transition of tumor cells. *Oncogene*. 23:7345–7354. http://dx.doi.org/10.1038/sj.onc.1207990

Bragulla, H.H., and D.G. Homberger. 2009. Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. *J. Anat.* 214:516–559. http://dx.doi.org/10.1111/j.1469-7580.2009.01066.x

Carver, E.A., R. Jiang, Y. Lan, K.F. Oram, and T. Gridley. 2001. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol. Cell. Biol.* 21:8184–8188. http://dx.doi.org/10.1128/MCB.21.23.8184-8188.2001

Coniglio, S.J., S. Zavarella, and M.H. Symons. 2008. Pak1 and Pak2 mediate tumor cell invasion through distinct signaling mechanisms. *Mol. Cell. Biol.* 28:4162–4172. http://dx.doi.org/10.1128/MCB.01532-07

Ezratty, E.J., M.A. Partridge, and G.G. Gundersen. 2005. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat. Cell Biol.* 7:581–590. http://dx.doi.org/10.1038/ncb1262

Furuta, Y., D. Ilić, S. Kanazawa, N. Takeda, T. Yamamoto, and S. Aizawa. 1995. Mesodermal defect in late phase of gastrulation by a targeted mutation of focal adhesion kinase, FAK. *Oncogene*. 11:1989–1995.

Grum-Schwensen, B., J. Klingelhofer, C.H. Berg, C. El-Naaman, M. Grigorian, E. Lukanidin, and N. Ambartsumian. 2005. Suppression of tumor development and metastasis formation in mice lacking the S100A4(mts1) gene. *Cancer Res.* 65:3772–3780. http://dx.doi.org/10.1158/0008-5472.CAN-04-4510

Guaita, S., I. Puig, C. Franci, M. Garrido, D. Dominguez, E. Batlle, E. Sancho, S. Dedhar, A.G. De Herreros, and J. Baulida. 2002. Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by MUC1 repression and ZEB1 expression. *J. Biol. Chem.* 277:39209–39216. http://dx.doi.org/10.1074/jbc.M206400200

Halbleib, J.M., and W.J. Nelson. 2006. Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev.* 20:3199–3214. http://dx.doi.org/10.1101/gad.1486806

Ilić, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, and T. Yamamoto. 1995a. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*. 377:539–544. http://dx.doi.org/10.1038/377539a0

Ilić, D., Y. Furuta, T. Suda, T. Atsumi, J. Fujimoto, Y. Ikawa, T. Yamamoto, and S. Aizawa. 1995b. Focal adhesion kinase is not essential for *in vitro* and *in vivo* differentiation of ES cells. *Biochem. Biophys. Res. Commun.* 209:300–309. http://dx.doi.org/10.1006/bbrc.1995.1503

Julien, S., I. Puig, E. Caretti, J. Bonaventure, L. Nelles, F. van Roy, C. Dargemont, A.G. de Herreros, A. Bellacosa, and L. Larue. 2007. Activation of NF-kappaB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. *Oncogene*. 26:7445–7456. http://dx.doi.org/10.1038/sj.onc.1210546

Kim, N.H., H.S. Kim, N.G. Kim, I. Lee, H.S. Choi, X.Y. Li, S.E. Kang, S.Y. Cha, J.K. Ryu, J.M. Na, et al. 2011a. p53 and MicroRNA-34 are suppressors of canonical Wnt signaling. *Sci. Signal.* 4:ra71. http://dx.doi.org/10.1126/scisignal.2001744

Kim, N.H., H.S. Kim, X.-Y. Li, I. Lee, H.-S. Choi, S.E. Kang, S.Y. Cha, J.K. Ryu, D. Yoon, E.R. Fearon, et al. 2011b. A p53/miRNA-34 axis regulates

Snail1-dependent cancer cell epithelial–mesenchymal transition. *J. Cell Biol.* 195: 417–433. http://dx.doi.org/10.1083/jcb.201103097

Lahlou, H., V. Sanguin-Gendreau, D. Zuo, R.D. Cardiff, G.W. McLean, M.C. Frame, and W.J. Muller. 2007. Mammary epithelial-specific disruption of the focal adhesion kinase blocks mammary tumor progression. *Proc. Natl. Acad. Sci. USA*. 104:20302–20307. http://dx.doi.org/10.1073/pnas.0710091104

Li, S., P. Butler, Y. Wang, Y. Hu, D.C. Han, S. Usami, J.L. Guan, and S. Chien. 2002. The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells. *Proc. Natl. Acad. Sci. USA*. 99:3546–3551. http://dx.doi.org/10.1073/pnas.052018099

Lim, S.T., X.L. Chen, Y. Lim, D.A. Hanson, T.T. Vo, K. Howerton, N. Larocque, S.J. Fisher, D.D. Schlaepfer, and D. Ilić. 2008a. Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. *Mol. Cell*. 29:9–22. http://dx.doi.org/10.1016/j.molcel.2007.11.031

Lim, Y., S.T. Lim, A. Tomar, M. Gardel, J.A. Bernard-Trifilo, X.L. Chen, S.A. Uryu, R. Canete-Soler, J. Zhai, H. Lin, et al. 2008b. PyK2 and FAK connections to p190Rho guanine nucleotide exchange factor regulate RhoA activity, focal adhesion formation, and cell motility. *J. Cell Biol.* 180: 187–203. http://dx.doi.org/10.1083/jcb.200708194

Liu, S., S.W. Xu, L. Kennedy, D. Pala, Y. Chen, M. Eastwood, D.E. Carter, C.M. Black, D.J. Abraham, and A. Leask. 2007. FAK is required for TGFbeta-induced JNK phosphorylation in fibroblasts: implications for acquisition of a matrix-remodeling phenotype. *Mol. Biol. Cell*. 18:2169–2178. http://dx.doi.org/10.1091/mbc.E06-12-1121

Liu, Y., S. El-Naggar, D.S. Darling, Y. Higashi, and D.C. Dean. 2008. Zeb1 links epithelial-mesenchymal transition and cellular senescence. *Development*. 135:579–588. http://dx.doi.org/10.1242/dev.007047

Lomelí, H., C. Starling, and T. Gridley. 2009. Epiblast-specific Snai1 deletion results in embryonic lethality due to multiple vascular defects. *BMC Res Notes*. 2:22. http://dx.doi.org/10.1186/1756-0500-2-22

Luo, M., H. Fan, T. Nagy, H. Wei, C. Wang, S. Liu, M.S. Wicha, and J.L. Guan. 2009. Mammary epithelial-specific ablation of the focal adhesion kinase suppresses mammary tumorigenesis by affecting mammary cancer stem/progenitor cells. *Cancer Res.* 69:466–474. http://dx.doi.org/10.1158/0008-5472.CAN-08-3078

MacPherson, M.R., P. Molina, S. Souchelnytskyi, C. Wernstedt, J. Martin-Pérez, F. Portillo, and A. Cano. 2010. Phosphorylation of serine 11 and serine 92 as new positive regulators of human Snail1 function: potential involvement of casein kinase-2 and the cAMP-activated kinase protein kinase A. *Mol. Biol. Cell*. 21:244–253. http://dx.doi.org/10.1091/mbc.E09-06-0504

Mani, S.A., J. Yang, M. Brooks, G. Schwaninger, A. Zhou, N. Miura, J.L. Kutok, K. Hartwell, A.L. Richardson, and R.A. Weinberg. 2007. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc. Natl. Acad. Sci. USA*. 104:10069–10074. http://dx.doi.org/10.1073/pnas.0703900104

McLean, G.W., N.O. Carragher, E. Avizienyte, J. Evans, V.G. Brunton, and M.C. Frame. 2005. The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. *Nat. Rev. Cancer*. 5:505–515. http://dx.doi.org/10.1038/nrc1647

Medjkane, S., C. Perez-Sanchez, C. Gaggioli, E. Sahai, and R. Treisman. 2009. Myocardin-related transcription factors and SRF are required for cytoskeletal dynamics and experimental metastasis. *Nat. Cell Biol.* 11:257–268. http://dx.doi.org/10.1038/ncb1833

Mitra, S.K., and D.D. Schlaepfer. 2006. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr. Opin. Cell Biol.* 18:516–523. http://dx.doi.org/10.1016/j.ceb.2006.08.011

Mitra, S.K., D.A. Hanson, and D.D. Schlaepfer. 2005. Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* 6:56–68. http://dx.doi.org/10.1038/nrm1549

Murray, S.A., and T. Gridley. 2006. Snail family genes are required for left-right asymmetry determination, but not neural crest formation, in mice. *Proc. Natl. Acad. Sci. USA*. 103:10300–10304. http://dx.doi.org/10.1073/pnas.0602234103

Ota, I., X.Y. Li, Y. Hu, and S.J. Weiss. 2009. Induction of a MT1-MMP and MT2-MMP-dependent basement membrane transmigration program in cancer cells by Snail1. *Proc. Natl. Acad. Sci. USA*. 106:20318–20323. http://dx.doi.org/10.1073/pnas.0910962106

Parsons, J.T. 2003. Focal adhesion kinase: the first ten years. *J. Cell Sci.* 116:1409–1416. http://dx.doi.org/10.1242/jcs.00373

Parsons, J.T., J. Slack-Davis, R. Tilghman, and W.G. Roberts. 2008. Focal adhesion kinase: targeting adhesion signaling pathways for therapeutic intervention. *Clin. Cancer Res.* 14:627–632. http://dx.doi.org/10.1158/1078-0432.CCR-07-2220

Peinado, H., D. Olmeda, and A. Cano. 2007. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat. Rev. Cancer*. 7:415–428. http://dx.doi.org/10.1038/nrc2131

Provenzano, P.P., D.R. Inman, K.W. Eliceiri, H.E. Beggs, and P.J. Keely. 2008. Mammary epithelial-specific disruption of focal adhesion kinase retards tumor formation and metastasis in a transgenic mouse model of human breast cancer. *Am. J. Pathol.* 173:1551–1565. http://dx.doi.org/10.2353/ajpath.2008.080308

Pylayeva, Y., K.M. Gillen, W. Gerald, H.E. Beggs, L.F. Reichardt, and F.G. Giancotti. 2009. Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling. *J. Clin. Invest.* 119:252–266.

Rowe, R.G., X.Y. Li, Y. Hu, T.L. Saunders, I. Virtanen, A. Garcia de Herreros, K.F. Becker, S. Ingvarsen, L.H. Engelholm, G.T. Bommer, et al. 2009. Mesenchymal cells reactivate Snail1 expression to drive three-dimensional invasion programs. *J. Cell Biol.* 184:399–408. http://dx.doi.org/10.1083/jcb.200810113

Rowe, R.G., Y. Lin, R. Shimizu-Hirota, S. Hanada, E.G. Neilson, J.K. Greenson, and S.J. Weiss. 2011. Hepatocyte-derived Snail1 propagates liver fibrosis progression. *Mol. Cell. Biol.* 31:2392–2403. http://dx.doi.org/10.1128/MCB.01218-10

Sahlgren, C., M.V. Gustafsson, S. Jin, L. Poellinger, and U. Lendahl. 2008. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc. Natl. Acad. Sci. USA*. 105:6392–6397. http://dx.doi.org/10.1073/pnas.0802047105

Serrels, B., A. Serrels, V.G. Brunton, M. Holt, G.W. McLean, C.H. Gray, G.E. Jones, and M.C. Frame. 2007. Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. *Nat. Cell Biol.* 9:1046–1056. http://dx.doi.org/10.1038/ncb1626

Shen, T.L., A.Y. Park, A. Alcaraz, X. Peng, I. Jang, P. Koni, R.A. Flavell, H. Gu, and J.L. Guan. 2005. Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. *J. Cell Biol.* 169:941–952. http://dx.doi.org/10.1083/jcb.200411155

Sieg, D.J., C.R. Hauck, and D.D. Schlaepfer. 1999. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J. Cell Sci.* 112:2677–2691.

Slack-Davis, J.K., S.T. Eblen, M. Zecevic, S.A. Boerner, A. Tarcsafalvi, H.B. Diaz, M.S. Marshall, M.J. Weber, J.T. Parsons, and A.D. Catling. 2003. PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. *J. Cell Biol.* 162:281–291. http://dx.doi.org/10.1083/jcb.200212141

Thiery, J.P., H. Acloque, R.Y. Huang, and M.A. Nieto. 2009. Epithelial-mesenchymal transitions in development and disease. *Cell*. 139:871–890. http://dx.doi.org/10.1016/j.cell.2009.11.007

Thuault, S., U. Valcourt, M. Petersen, G. Manfioletti, C.H. Heldin, and A. Moustakas. 2006. Transforming growth factor-β employs HMGA2 to elicit epithelial–mesenchymal transition. *J. Cell Biol.* 174:175–183. http://dx.doi.org/10.1083/jcb.200512110

Tilghman, R.W., J.K. Slack-Davis, N. Sergina, K.H. Martin, M. Iwanicki, E.D. Hershey, H.E. Beggs, L.F. Reichardt, and J.T. Parsons. 2005. Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells. *J. Cell Sci.* 118:2613–2623. http://dx.doi.org/10.1242/jcs.02380

Tomar, A., and D.D. Schlaepfer. 2009. Focal adhesion kinase: switching between GAPs and GEFs in the regulation of cell motility. *Curr. Opin. Cell Biol.* 21:676–683. http://dx.doi.org/10.1016/j.ceb.2009.05.006

Webb, D.J., K. Donais, L.A. Whitmore, S.M. Thomas, C.E. Turner, J.T. Parsons, and A.F. Horwitz. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat. Cell Biol.* 6:154–161. http://dx.doi.org/10.1038/ncb1094

Weis, S.M., S.T. Lim, K.M. Lutu-Fuga, L.A. Barnes, X.L. Chen, J.R. Göthert, T.L. Shen, J.L. Guan, D.D. Schlaepfer, and D.A. Cheresh. 2008. Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK. *J. Cell Biol.* 181:43–50. http://dx.doi.org/10.1083/jcb.200710038

Yook, J.I., X.Y. Li, I. Ota, E.R. Fearon, and S.J. Weiss. 2005. Wnt-dependent regulation of the E-cadherin repressor snail. *J. Biol. Chem.* 280:11740–11748. http://dx.doi.org/10.1074/jbc.M413878200

Yook, J.I., X.Y. Li, I. Ota, C. Hu, H.S. Kim, N.H. Kim, S.Y. Cha, J.K. Ryu, Y.J. Choi, J. Kim, et al. 2006. A Wnt-Axin2-GSK3beta cascade regulates Snail1 activity in breast cancer cells. *Nat. Cell Biol.* 8:1398–1406. http://dx.doi.org/10.1038/ncb1508

Zeisberg, M., and E.G. Neilson. 2009. Biomarkers for epithelial-mesenchymal transitions. *J. Clin. Invest.* 119:1429–1437. http://dx.doi.org/10.1172/JCI36183

Zhou, B.P., J. Deng, W. Xia, J. Xu, Y.M. Li, M. Gunduz, and M.C. Hung. 2004. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat. Cell Biol.* 6:931–940. http://dx.doi.org/10.1038/ncb1173