Cell Cycle 12:20, 3272–3285; October 15, 2013; © 2013 Landes Bioscience

Focal adhesion complex proteins in epidermis and squamous cell carcinoma

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Keywords: integrin, focal adhesion, skin, squamous cell carcinoma, extracellular matrix

Abbreviations: cSCC, cutaneous squamous cell carcinoma; DMBA-TPA, 7,12-dimethylbenz(a)anthracene 12-O-tetradecanoylphorbol-13-acetate; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ERK, extracellular signal regulated kinase; FA, focal adhesion; FAK, focal adhesion kinase; GRPR, gastrin releasing peptide receptor; GSK3β, glycogen synthase kinase 3 beta; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; IGF1R, insulin like growth factor receptor 1; ILK, integrin linked kinase; K5, Keratin 5; K14, Keratin 14; KS, Kindler syndrome; MAPK, mitogen activated protein kinase; MBP, myelin basic protein; NFκB, nuclear factor kappa light chain enhancer of activated B cells; OSCC, oral squamous cell carcinoma; PI3K, phosphoinositide 3 kinase; PKB, protein kinase B; PKC, protein kinase C; PLCγ, phospholipase C gamma; PTEN, phosphatase and tensin homolog; SH3, Src homology 3; TGFβRII, transforming growth factor β receptor II; UV, ultraviolet radiation; VEGFR2, vascular endothelial growth factor receptor 2

Focal adhesions (FAs) are large, integrin-containing, multi-protein assemblies spanning the plasma membrane that link the cellular cytoskeleton to surrounding extracellular matrix. They play critical roles in adhesion and cell signaling and are major regulators of epithelial homeostasis, tissue response to injury, and tumorigenesis. Most integrin subunits and their associated FA proteins are expressed in skin, and murine genetic models have provided insight into the functional roles of FAs in normal and neoplastic epidermis. Here, we discuss the roles of these proteins in normal epidermal proliferation, adhesion, wound healing, and cancer. While many downstream signaling mechanisms remain unclear, the critically important roles of FAs are highlighted by the development of therapeutics targeting FAs for human cancer.

Introduction

The discovery of focal adhesions in the 1970s as highly conserved signal integrators that physically link the extracellular matrix (ECM) and the cytoskeleton suggested that these large protein complexes may be functionally necessary for tissue structure and the multicellularity of organisms.[1] Integrins, which function as αβ heterodimers, are catalytically inactive receptors within focal adhesions that directly bind ECM ligands to initiate downstream signaling responses. There is a large number of integrin subunits (18 α subunits and 8 β subunits) and αβ heterodimers (24 total), all of which have some redundancy in ligand binding. Despite this, many individual integrin subunits are necessary for organismal viability.[2-6] Relevant mouse models have revealed that certain integrin subunits or focal adhesion proteins are necessary for embryonic development, while others are required only for development and homeostasis of certain tissue types. This is very apparent in skin, where loss of specific FA proteins can lead to defects in adhesion, wound healing and proliferation.

In pathological conditions such as squamous cell carcinoma, microenvironmental changes cause disorganization of the epidermis, degradation of the basement membrane, overexpression of specific integrin subunits and altered secretion and cleavage of ECM components. These microenvironmental changes lead to altered focal adhesion formation and downstream signaling, which has been shown to enhance the ability of tumor cells to proliferate, invade and metastasize. Functional studies defining the roles of specific focal adhesion complex proteins in both normal and tumor tissues has led to a better understanding of how individual members of these complexes can be targeted therapeutically. In this review, we summarize the current understanding of the functional roles for specific integrin subunits and FA complex proteins in development, skin homeostasis, and relevant in vivo squamous cell carcinoma models. This review will also highlight some of the key FA proteins that have become the recent focus of targeted therapeutics.

Squamous Cell Carcinoma: Current Therapy

Cutaneous SCC is a generally under-appreciated public health concern, as non-melanoma skin malignancies cases are typically excluded from national cancer registries. The incidence of cSCC

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Submitted: 08/13/2013; Revised: 09/03/2013; Accepted: 09/05/2013
http://dx.doi.org/10.4161/cc.26385

in the US is now estimated to be over 700 000 new cases/year, with a 4–12.5% risk of metastasis.[7-9] Although this metastasis risk is lower than many other malignancies, the large burden of disease is such that in most regions of the US, total deaths due to cSCC may be as common as those from melanoma.[10] While surgical excision is very effective treatment for local tumors, therapeutic options for disseminated disease are limited with few proven effective treatments. Clinical treatment regimens based on traditional chemotherapeutic agents, including cisplatin, bleomycin, doxorubicin, and fluoropyrimidines, radiation therapy, or newer targeted biologics including EGFR or general tyrosine kinases inhibitors do not result in long-term remissions in most cases.[11-13] New therapeutic agents are needed to improve treatment outcomes for unresectable cSCC.

cSCC is primarily the consequence of chronic UV photodamage resulting in loss of p53 function, followed by activation of EGFR and its downstream pathways including Ras MAPK, PI3K/Akt, PLCγ/PKC, and Src kinases. Oncogenic signaling through these pathways in skin, as well as other epithelial tissues, is frequently associated with upregulation of integrin proteins. As activation of effector cascades initiating at integrin-containing focal adhesions appears to be necessary for the full malignant potential of some epithelial tumors, targeting integrins at the relatively accessible plasma membrane is an attractive option that may have clinical utility.[14]

Focal Adhesion Complex and Hemidesmosome Structure in Skin

Integrins are delivered to the cellular membrane as inactive, bent heterodimers. These heterodimers are initially partially activated through binding of cytoplasmic proteins (primarily Talin and Kindlin) to the intracellular integrin tails ("inside-out" signaling). Subsequent binding to extracellular matrix ligands ("outside-in" signaling) further extends the heterodimer and generates the fully active receptor. These integrin receptors lack intrinsic catalytic activity and execute their signaling and structural roles through recruitment of other proteins to adhesion complexes at the plasma membrane (**Fig. 1**).

Integrin heterodimers are key components of 2 distinct types of adhesion complexes: FA complexes, which link the actin cytoskeleton to the ECM, and hemidesmosomes, which structurally link intermediate filaments to the ECM. Through these complexes, integrin receptors play both a structural role mediating physical attachment of epithelial cells to underlying basement membrane, and also a signaling role promoting cellular proliferation and migration. This signaling response is evident in skin tissue, where basal epidermal cells assemble on the ECM-rich basement membrane, and are given cues to proliferate. As these proliferative progenitors lose their self-renewal capacity, they detach from the basement membrane, stratify into a 5–10 cell thick epidermis and terminally differentiate, forming the epidermal barrier of human skin.[15]

Hemidesmosomes play a major structural role in the epidermis but have also been shown to activate several intracellular signaling pathways, including Rac1, RhoA, and Akt signaling.[16] Unlike focal adhesions, hemidesmosomes are not frequently recycled and therefore serve to maintain keratinocyte anchorage to the basement membrane. Loss of the only hemidesmosomal integrin heterodimer, α6β4, leads to severe epidermal adhesion defects.[17,18] Integrin α6β4 and its ligand, laminin-332, are also both required for squamous cell carcinoma formation in relevant epidermal in vivo models.[19] This has been nicely reviewed by MP Marinkovich and will not be a major focus of this review.[16] Because of the frequently severe skin blistering seen in patients lacking a number of different extracellular and intracellular hemidesmosomal proteins, targeting hemidesmosomal components was initially not considered to be a viable therapeutic strategy. However, it was later discovered that a specific domain of laminin-332, G45, is present only in tumor tissue and promotes tumor formation and progression.[20] Blocking antibodies against G45 were shown in a pre-clinical model to be effective against SCC tumor formation through blockade of PI3K and ERK signaling, but to have no effect on normal skin homeostasis.[20] This is one of the few examples of targeting specific ECM ligands for cancer therapy, but highlights the need for a deeper understanding of how the role of adhesion signaling and specific ECM ligands differ between various homeostatic and pathologic states.

In contrast to hemidesmosomes, FA complexes are dynamic adhesions that frequently assemble and disassemble, particularly during cell migration. In tissue culture, stimulation of FA complex formation using a variety of ECM ligands leads to engagement of many different signaling pathways, including PI3K/Akt activation, Rac1-mediated cytoskeleton re-organization, and NFκB pathway activation. However, the degree to which these pathways are activated upon integrin ligation and their relative functional importance in either 3-dimensional culture or in vivo tissue remains unclear. The remainder of this review will focus on the roles of specific FA complex proteins in both normal skin and epidermal squamous cell carcinoma.

Focal Adhesion Integrins

Much effort has been focused on defining roles for individual integrin subunits in epidermal homeostasis. As integrin signaling requires an intact, structurally correct basement membrane zone lacking in traditional tissue culture, much of this work has employed mouse genetic models. Efforts have been made to generate knockout mice or conditional knockout mice for each of the 26 integrin subunits. Out of these 26 subunits, 18 are expressed at a detectable level in human or mouse skin, while the others are leukocyte-specific. Knockout mice have been generated for all of these 18 subunits, but many of these mice experience embryonic lethality, and the skin-specific null phenotypes have not been determined (**Table 1**). Regardless, the available knockout mice have provided valuable insight into the roles of integrins in epidermal proliferation, hair follicle formation and turnover, wound healing, and susceptibility to squamous cell carcinoma. Many of these phenotypes have been nicely reviewed by Janes et al. and Margadant et al.[21,22] In the next section, we provide a brief overview of these phenotypes, and discuss newly discovered roles for epidermal integrins not summarized elsewhere.

β1 integrin and its phosphorylation

Severe phenotypes are seen in β1-null mouse skin. Loss of β1 during development leads to severe epidermal defects, including skin blistering and hair loss.[23,24] There are differences in these phenotypes depending on the promoter used for Cre-mediated recombination. K5-Cre-induced β1 deletion leads to differentiation defects, skin thickening, and mouse death at approximately 6 weeks after birth, potentially due to hypoproliferation in the esophagus.[23] K14-Cre-induced β1 deletion results in normal differentiation, but significant epidermal hypoproliferation and perinatal death within days after birth.[24] Despite these severe developmental defects, β1 loss in adult mouse skin has no apparent deleterious phenotype.[14,25] Using gene expression profiling and network topology analysis, integrin β1 was identified as a key oncogenic hub in a human skin graft model of squamous cell carcinoma.[14] Subsequently, antibody-mediated blockade of integrin β1 both prevented tumor formation and slowed tumor progression, with no deleterious effects on normal human skin tissue or overall mouse health (**Fig. 1B**).[14]

Binding of Talin to β1 cytoplasmic tails disrupts a salt bridge between the α and β cytoplasmic tails, helping to separate the tails and enhance integrin binding affinity to ECM ligands. This is thought to be the first step in "inside-out" integrin activation. With subsequent integrin activation and clustering, Src phosphorylation of β1 tyrosines in the cytoplasmic NPxY motifs is thought to reduce binding of the adaptor proteins Talin and Kindlin.[26] This is consistent with the rounded morphology and loss of adhesion seen in v-Src-transformed cells, which have high levels of β1 tyrosine phosphorylation.[27] In addition, focal adhesion kinase (FAK), which plays a role in promoting oncogenic transformation, is activated in response to β1 integrin phosphorylation (**Fig. 1**).[28]

Complicating this understanding, determined largely from cell culture systems, are in vivo studies suggesting

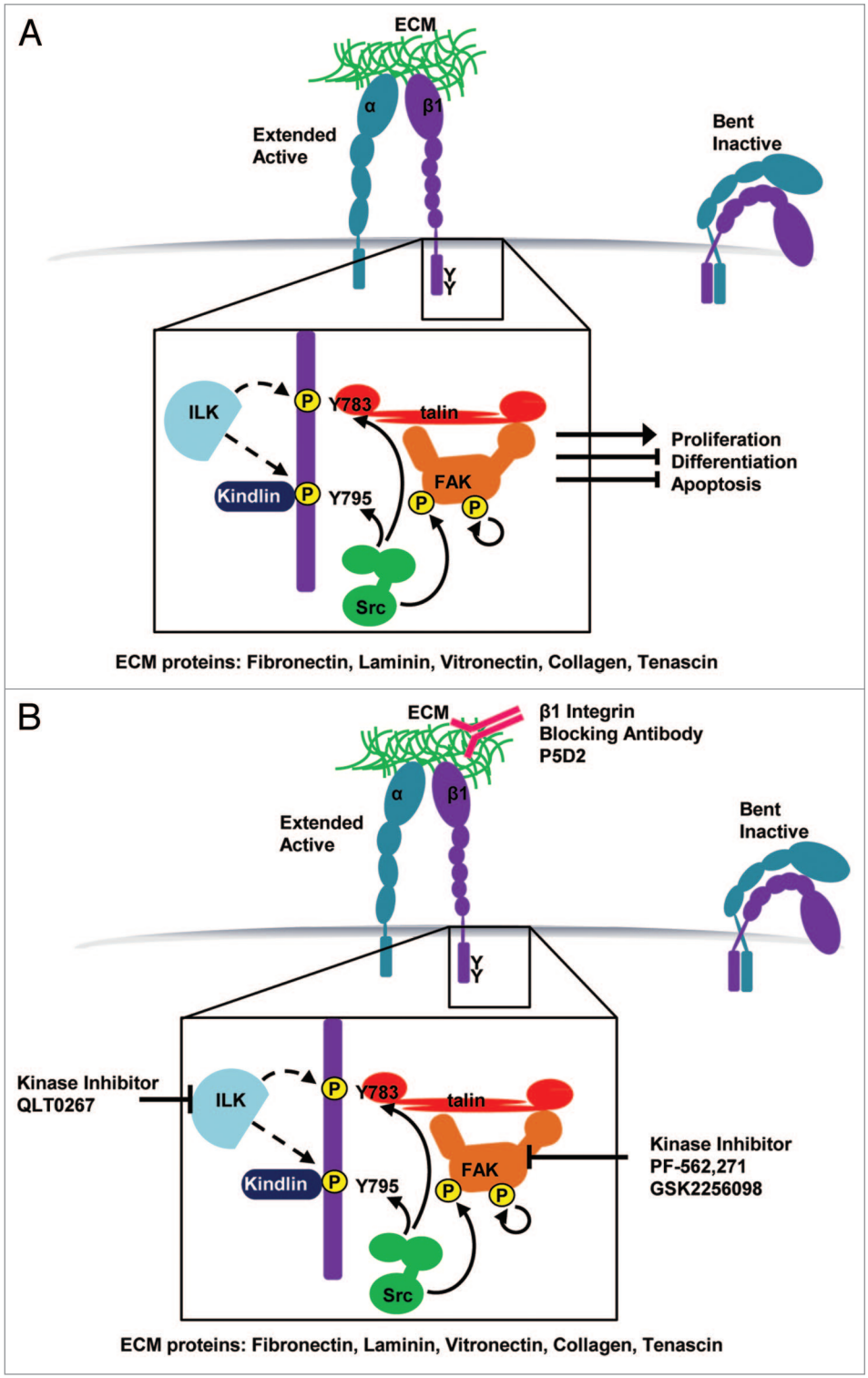

Figure 1. Depiction of focal adhesion structure, key phosphorylation events and therapeutics targeting individual focal adhesion proteins for treatment of cSCC. (**A**) In normal basal keratinocytes, integrin binding to the ECM initiates Talin binding to the membrane proximal NPxY motif (Y783) and Kindlin binding to the membrane distal NPxY motif (Y795). FAK is recruited to the adhesion and undergoes auto-phosphorylation at Y397. Src kinase phosphorylates both NPxY tyrosines on the β1 integrin tail and phosphorylates active FAK at Y925. It remains controversial whether ILK phosphorylates β1 integrin at these same sites. This adhesion assembly and phosphorylation sequence ultimately promote cell cycle progression and inhibit differentiation and apoptosis programs. (**B**) Three current strategies in development for treatment of cSCC are blocking β1 integrin with a P5D2 blocking antibody, inhibiting ILK kinase activity using QLT0267, and inhibiting FAK kinase activity using PF-562,271 or GSK2256098.

Table 1. Functional roles for focal adhesion-associated integrins in both mouse development and mouse skin

Gene	Knockout mouse phenotype	Conditional skin knockout phenotype (K5 or K14 promoter)	Role in SCC
Integrin α1	-viable, fertile and no apparent abnormalities[130]	N/A	N/A
Integrin α2	-Healthy, viable, fertile[41] -No change in re-epithelialization or BM deposition, but increase in neoangiogenesis during wound healing[42,44]	N/A	-K14-HPV mice crossed with α2-null mice shows decreased lymph node metastases and tumor formation[45]
Integrin α3	-Survive until birth, but die shortly after due to kidney and lung defects[33] -Minor blistering of the epidermis, but normal stratification[33]	-Disorganized BM[34,131] -Blistering at epidermal-dermal junction[34,131] -Spatial and temporal differentiation is intact[34,131] -Hair loss and impaired hair follicle growth[35] -Enhanced re-epithelialization during wound healing[36] -Enhanced epidermal turnover[38]	-Significantly reduced papilloma formation upon DMBA-TPA treatment of mice lacking α3 in the epidermis[38] -SCCs that form are more poorly differentiated[38]
Integrin α4 (VCAM-1)	-Required for formation of umbilical cord and placenta during development[3] -Mostly embryonic lethal E8.5, but a small number of viable, fertile mice[3]	N/A	N/A
Integrin α5	-Mesodermal defects and embryonic death at E10–11[132]	N/A	N/A
Integrin α6	-Embryonic lethal at E14.5-E18.5[18] -Skin blistering similar to epidermolysis bullosa[18] -Normal differentiation and stratification of the epidermis[18,131]	-mild hyperproliferation, blistering and inflammation upon tamoxifen-induced deletion in epidermis[133]	See integrin β4
Integrin α7	-Viable, fertile mice[134] -Muscular dystrophy[135] -Defective axonal elongation[135]	N/A	N/A
Integrin α8	-Death immediately after birth likely due to renal deficiencies[136]	N/A	N/A
Integrin α9	-Normal at birth, but die at day 6–12 due to respiratory failure[137] -Edema and lymphocyte infiltration into chest wall[137]	-Poor re-epithelization during wound healing[39]	N/A
Integrin α10	-viable, fertile[138] -stunted growth of long bones[138]	N/A	N/A
Integrin α11	-Viable, fertile[139] -Dwarfism and defective tooth movement[139]	N/A	N/A
Integrin αv	-Mostly embryonic lethal at E9.5, but 20% of mice born alive[5] -Defects in placental function[5] -Intracerebral and intestinal hemorrhage[5] -Cleft palates[5]	N/A	N/A
Integrin β1	-Embryonic lethal[2,4] -Die immediately after attaching to uterine epithelia and invading the stroma, around E5[2,4]	-Severe hair loss[23,24] -Reduced numbers of hemidesmosomes[23,24] -Disruption in BM and blistering[23,24] -K14-Cre model shows normal spatial and temporal differentiation, but K5-Cre model shows enhanced differentiation[23,24] -K14-Cre model shows epidermal thinning, but K5-Cre model shows epidermal thickening[23,24] -Poor re-epithelialization during wound healing[140] -K14-CreER 4-OHT excision in adult epidermis has no apparent phenotype[25]	-Activating mutation T1881β1 stimulated conversion of papillomas to SCCs upon DMBA-TPA[141] -Blocking antibodies against integrin β1 block tumor formation and progression in a human tissue graft model of SCC[14]

Table 1. Functional roles for focal adhesion-associated integrins in both mouse development and mouse skin (continued)

Integrin β3	-Enhanced re-epithelialization during wound healing[142]	N/A	N/A
Integrin β4	-Die shortly after birth due to respiratory and intestinal failure and skin fragility[17] -Skin blistering defects similar to epidermolysis bullosa[17] -Normal stratification of the epidermis[17]	-Loss of hemidesmosomes, skin blistering, but normal differentiation and proliferation[131,143]	-β4 knockout or blocking antibodies prevented Ras-driven tumorigenesis in human tissue graft model of SCC[19]
Integrin β5	-Viable, fertile and no apparent abnormalities[43]	N/A	N/A
Integrin β6	-Hair loss[144] -Inflammation of skin and lungs[144]	-Retarded hair follicle regression after depilation[40] -Enhanced keratinocyte proliferation[40]	N/A
Integrin β8	-65% die at midgestation due to insufficient vasculogenesis[6] -35% die shortly after birth due to intracerebral hemorrhage[6] -Leaky brain capillaries and endothelial hyperplasia[6]	N/A	N/A

that the tyrosine residue itself, and not its phosphorylation, is most important for β1 function.[29] Mutation of tyrosine to alanine in either the membrane proximal NPxY motif (Y783, Talin binding motif) or the membrane distal NPxY motif (Y795, kindlin binding motif) results in embryonic lethality.[30] However, mutation of either of these residues to phenylalanines, which contains the aromatic ring but is unable to be phosphorylated, results in viable, fertile mice with no apparent abnormalities.[29,30]

Mice with keratinocyte-restricted expression of both Y783A and Y795A (YY/AA) using the K5-Cre promoter phenocopy mice with keratinocyte-restricted deletion of β1.[30] These mice experience impaired hair follicle morphogenesis, abnormal skin pigmentation, skin blistering, and thickened epidermis.[30] In vitro, keratinocytes with the YY/AA mutation have no β1 integrin activation, and decreased expression of other integrin subunits, including β4, α6, and α2.[30] Mice with keratinocyte-restricted expression of either Y783A or Y795A have much less severe epidermal defects.[31] These individual mutations lead to patchy hair loss but normal proliferation, epidermal adhesion, and hemidesmosome localization.[31] In vitro, keratinocytes containing the Y783A mutation experience adhesion and spreading defects and rapid terminal differentiation, implying that binding of Talin to β1 inhibits keratinocyte differentiation.[31] Mice containing both Y783F and Y795F mutations (YY/FF mice) develop normally and have normal skin.[32] However, these mice are less susceptible to DMBA-TPA induced skin tumorigenesis.[32] Mutation of each residue alone does not have any effect on susceptibility to tumor formation.[32] Although Talin1, Talin2, Kindlin1, and Kindlin2 preferentially bind to wild-type β1 over YY/FF β1 in vitro, binding to the mutants is only reduced by approximately 50%.[32] This indicates that the YY/FF mouse may have hypomorphic β1 activity, which is sufficient to block tumor formation but not to affect normal skin homeostasis.

Other focal adhesion integrins

Integrin α3-null mice exhibit skin blistering and basement membrane disorganization as well as kidney and lung defects that lead to lethality shortly after birth.[33] Mice with epidermis-specific ablation of integrin α3 exhibit the same skin blistering defect and additional hair follicle and wound healing abnormalities.[34-36] Previously, human skin disease had not been associated with mutations in integrin α3 or any other FA integrin. Recently, however, homozygous mutations in the integrin α3 gene were described in 3 patients with skin blistering disease.[37] These 3 mutations are different, but are all predicted to lead to loss of integrin α3 function.[37] All 3 of these patients died within 2 years of birth due to infection or multi-organ failure associated with reduced kidney and lung barrier function, similar to the phenotype seen in integrin α3-null mice.[33,37] It was also recently shown that mice with a skin-specific deletion of integrin α3 have significantly reduced susceptibility to tumor formation upon DMBA-TPA treatment.[38] The authors show that this reduced susceptibility to tumor development is the result of increased epidermal turnover seen in mouse epidermis lacking α3, leading to increased differentiation and shedding of the cells that accumulate mutations upon carcinogen treatment.[38] Despite this reduced tumor formation, the squamous cell carcinomas that do form in mouse skin lacking α3 show reduced differentiation, a marker for increased malignancy, suggesting that integrin α3 plays dual roles in tumor formation and progression.[38]

As summarized in **Table 1** , several other integrins are utilized in specific epidermal contexts. For instance, integrin α9 plays a crucial role in enhancing keratinocyte migration and proliferation during wound healing.[39] Loss of β6 in mouse epidermis leads to retarded hair growth after depilation and enhanced keratinocyte proliferation.[40] Integrin α2 and β5 are not essential for mouse epidermis, since corresponding knockout or conditional knockout mice have no apparent skin abnormalities.[41-44] However, integrin α2 was shown to play a key role in HPV-driven SCC tumorigenesis and metastasis.[45] K14-HPV16/ITGA2$^{-/-}$ mice had reduced lymph node metastases in comparison to K14-HPV16/ITGA2$^{+/+}$ mice.[45] In addition, SCC cell lines developed from tumors in the K14-HPV16/ITGA2$^{-/-}$ mice had reduced tumor growth and increased tumor latency compared with SCC lines

derived from K14-HPV16/ITGA2$^{+/+}$ mice.[45] While it remains to be seen whether this metastasis phenotype is microenvironment-dependent, this study indicates that targeting integrin α2 may be a viable therapeutic target for HPV-driven SCC.

Downstream of integrins, dozens of proteins participate in FA dynamics. However, it remains unclear which different integrin heterodimers form complexes with different FA proteins, and which integrin functions are mediated by each FA complex protein. The remainder of this review will cover the roles of FA complex proteins in epidermal morphogenesis, hair follicles, wound healing, and squamous cell carcinoma, with an emphasis on insights gained from transgenic mouse models. We will focus on focal adhesion kinase (FAK), integrin-linked kinase (ILK), and Kindlin, because of their well-characterized epidermal roles.

Focal Adhesion Kinase (FAK)

Focal adhesion kinase (FAK) in normal skin

Focal adhesion kinase (FAK) has been shown to be both a signaling kinase and an adaptor protein that helps link integrin adhesion complexes to the actin cytoskeleton.[46,47] While FAK is only indirectly associated with β integrin cytoplasmic domains through binding to Paxillin and Talin, it is rapidly recruited to focal adhesions and auto-phosphorylated upon cellular adhesion to ECM proteins (**Fig. 1**). This auto-phosphorylation can lead to recruitment and activation of a variety of downstream signaling proteins.[46,47]

FAK is required for mouse development, since FAK-null mice die during embryogenesis at about E8.5, with mesodermal defects.[48,49] This phenotype is highly similar to the phenotype of the fibronectin-knockout mouse, which also shows specific defects in mesoderm development.[50] This suggests that FAK is essential for focal adhesions involving the fibronectin-binding integrins, including α4β1, α5β1, αIIbβ3, αvβ3, αvβ6, and αvβ8. Autophosphorylation is required for FAK function in vitro; however, mice lacking the autophosphorylation site of FAK have a slightly different phenotype than FAK knockout mice.[51] Mice lacking exon 15 of FAK, which contains the Y397 autophosphorylation site, proceed through embryonic development until E12.5, 5 days longer than FAK-null mice.[51] These mutant mice display hemorrhage, edema, and vascular remodeling defects at E12.5.[51] While it remains clear that this autophosphorylation plays an essential role in development, this study highlights the fact that FAK likely plays an important scaffolding role as well.

Skin-specific deletion of FAK leads to hair cycle irregularities, sebaceous gland hypoplasia and slight epidermal thinning.[52] Isolated keratinocytes from these mice undergo apoptosis in culture, potentially due to inability to adhere to tissue culture plastic.[52] Furthermore, loss of FAK in the epidermis seems to have no effect on wound healing.[52] The phenotypes of FAK loss are not nearly as striking as the phenotype of β1 loss in the epidermis, indicating that FAK is only responsible for mediating a fraction of β1 integrin function in skin.[23,24]

Focal adhesion kinase (FAK) in squamous cell carcinoma

FAK expression and activity is elevated in multiple epithelial cancers, including squamous cell carcinoma. In a mouse model of SCC driven by loss of TGFβRII in the mouse epidermis, enhanced integrin-FAK-Src signaling and keratinocyte migration was observed.[53] Further, loss of only one FAK allele significantly reduces papilloma formation upon DMBA-TPA treatment.[54] Loss of both alleles prevents papilloma progression to SCC.[55] FAK was shown to be necessary for phosphorylation of ERK downstream of Ras in cultured cells, and loss of FAK reduced migration of keratinocytes in vitro.[54,55] Use of a FAK kinase inhibitor, PF-562,271, also blocked tumor cell migration, anchorage-independent growth, and SCC xenograft growth (**Fig. 1B**).[56] This inhibition of FAK activity correlated with a decrease in phosphorylation of Src at tyrosine 416.[56] Skin-specific loss of FAK also prevented phorbol ester-induced skin carcinogenesis, potentially due to prevention of β-catenin-induced stem cell mobilization in the bulge of the hair follicle.[57] Inhibition of Src, a kinase that intricately associates with FAK at FA complexes also showed the same effect on preventing stem cell mobilization.[57]

In other types of carcinomas, FAK signaling has been intricately associated with PI3K/Akt signaling, providing a potential mechanism by which FAK acts to promote tumor growth and progression to malignancy. In a neuroblastoma tumor model, treatment with Y15, a FAK inhibitor, or with a FAK siRNA blocks gastrin-releasing peptide receptor (GRP-R)-mediated tumor growth and metastasis to the liver.[58] This loss of FAK activity correlates with decreased phosphorylation of both Akt and ERK 1/2.[58] Similarly, in melanoma, FAK interaction with the insulin-like growth factor receptor-1, IGF-1R, is necessary for downstream phosphorylation of Akt.[59] Abrogation of the FAK/IGF-1R interaction led to melanoma regression in an orthotopic mouse model.[59] Furthermore, in glioblastoma, FAK activity is dependent on PI3K/Akt activation, since knockdown of either the PIK3CA or PIK3R1 gene reduces FAK phosphorylation and tumor cell-invasive properties.[60] PI3K/Akt signaling is upregulated in the vast majority of HNSCC cases due to direct mutation of PIK3CA or PTEN, or increased signaling flux due to other oncogenic mutations.[61,62] The potential interplay between PI3K/Akt and FAK signaling in SCC thus merits further investigation.

While FAK clearly plays a pro-tumorigenic role, loss of FAK in SCC also results in increased resistance to radiation therapy.[63] This resistance appeared dependent on p53-mediated induction of p21.[63] In many contexts, FAK has been shown to both bind p53 and mediate its degradation.[64-66] While further studies are required to verify this phenomenon in an orthotopic in vivo context, this result suggests that the viability of FAK as a therapeutic target may depend on p53 status.

Integrin Linked Kinase (ILK)

Integrin-linked kinase (ILK) in normal skin

Integrin-linked kinase (ILK) was discovered as a β1-integrin-interacting protein using a 2-hybrid screen.[67] ILK was further shown to phosphorylate the β1 NPxY cytoplasmic motifs in vitro.[67] It has been proposed in recent literature that ILK acts as a pseudo-kinase, because structural studies of its presumed kinase domain do not predict activity, and kinase-active or kinase-dead

Table 2. Functional roles for intracellular focal adhesion proteins in mouse development

Gene	Knockout mouse phenotype
α-Actinin-4	-Die at several months of age due to glomerular disease and proteinuria[91]
FAK	-Embryonic lethal E8.5[48,49] -Defect in mesodermal development[48,49] -Able to implant and initiate gastrulation[48,49]
FLNA (Filamin A)	-Embryonic lethal E13.5–14.5[93] -Cardiac and vascular failure, and impaired brain development[93]
ILK	-Die shortly after implantation, E8.5[71,72] -Fail to form epiblast[71,72] -Mice with kinase-dead ILK mutants (S343A) or kinase hyperactive ILK mutants (S343D) have no developmental or skin defects[71,72] -Mice with mutation in ATP-binding site of kinase domain (K220A or K220M) die shortly after birth due to kidney defects, but have normal skin[71,72]
KIND-1 (Kindlin-1)	-Skin atrophy[84] -Intestinal dysfunction resulting in perinatal lethality[84]
KIND-2 (Kindlin-2)	-Peri-implantation lethality at E7[83] -Detachment of endoderm and epiblast from basement membrane[83]
Migfilin	-Viable, fertile and no apparent abnormalities[95]
Cas (p130Cas)	Embryonic lethality at E11.5–12.5[96] -Poor development of heart and blood vessel dilation[96]
Paxillin	-Embryonic lethality at E9.5[99] -Disrupted development of mesodermal structures[99]
PINCH1	-Embryonic lethality at E6.5 or E9.5[100,101] -Disorganized egg cylinder, and significant cell death[100,101] -Abnormal epiblast polarity, and impaired cavitation into basement membrane[100,101]
PINCH2	-Viable, fertile with no apparent phenotype[102]
Talin	-Embryonic lethality at E8.5–9.5[145] -Disorganization of embryos at gastrulation[145] -Defective embryonic ectoderm[145]
Tensin	-Normal at birth, but develop kidney abnormalities that lead to lethality[106]
Tensin-3	-Growth retardation and postnatal lethality in 1/3 of mice that is associated with impaired development of the intestine, lung and bone[107]
Vinculin	-Embryonic lethality around E8–10[146] -Attenuated cranial nerve, spinal nerve and heart development[146] -Defective ectoderm development[146]
Zyxin	-Viable, fertile and no apparent abnormalities[110]

mutants of ILK were shown to block protein–protein interactions.[68,69] However, integrin-linked kinase has been shown to directly phosphorylate GSK-3 in vitro and modulate its activity in cultured cells.[70] Furthermore, kinase-active, but not kinase-deficient ILK can phosphorylate PKB/Akt on serine-473.[70] The phenotypes of knockout and knock-in mice have provided more insight into ILK's potential kinase role in vivo. ILK-knockout mice exhibit embryonic lethality at the peri-implantation stage due to epiblast failure.[71] Surprisingly, when endogenous ILK is replaced with either a kinase-dead ILK (S343A), or a hyperactive ILK (S343D), mice are normal, viable, and fertile, with no apparent skin abnormalities and normal phosphorylation of Akt and GSK3β.[72] However, mice containing K220A or K220M mutations in ILK's ATP binding site die due to kidney failure but do not exhibit the severe embryonic lethality seen in ILK-null mice.[72] Neither of these mutations altered phosphorylation of MBP, Akt, or GSK3β, but did reveal diminished binding to the FA adapters β- and α-parvin, which could be mediating this phenotype.[72]

Epidermal targeted ILK loss results in epidermal detachment, blister formation, and extension of proliferating keratinocytes to suprabasal layers.[73,74] These mice also exhibit hair loss, attributed to the inability of proliferating progenitor cells in the follicle to migrate and replenish the hair matrix required for hair cycling.[73] In culture, ILK-null keratinocytes have a severe migration defect and de-stabilized lamellipodia.[73,74] These defects in cellular spreading and movement can be rescued through re-expression of active Rac1 or RhoG.[74] Surprisingly, this skin phenotype does not appear dependent on either ILK's presumed kinase activity or ATP-binding, since mice expressing S343A, S343D, K220A, or K220M mutant ILK display normal skin.[72] ILK loss in the stem cells of the mouse hair follicle bulge using a K15-Cre promoter revealed a novel role for ILK in regenerating the epidermis after injury.[75] Recently, a role was described for ILK in mediating keratinocyte phagocytosis, which could explain the abnormal pigmentation also seen in ILK-deficient epidermis. ILK-deficient keratinocytes are unable to engulf fluorescent microspheres in response to keratinocyte growth factor (KGF), similar to β1-null

keratinocytes.[76] It remains unclear exactly how ILK regulates downstream signaling pathways to mediate skin development. It is possible that ILK does have true kinase activity, but that this kinase activity is not essential for mouse development and can be compensated for through other pathways. It is also possible that the phosphorylation of substrates seen in culture is largely artifactual, and that ILK serves primarily as a scaffold protein in vivo.

ILK in squamous cell carcinoma

ILK's role in tumorigenesis has not been extensively explored in relevant epidermal SCC mouse models. However, there are a few studies that suggest a role for ILK in epithelial cancer. ILK expression was shown to be elevated in ~70% of lung SCC cases. This expression is associated with both reduced E-cadherin expression and poor patient prognosis.[77] In oral, keratinocyte-derived SCC, ILK protein expression is increased in approximately 90% of primary samples and 100% of tumor metastases, with expression strongly correlated with enhanced tumor invasion and metastasis, higher tumor grade, poor outcome, and increased risk of recurrence.[78] ILK expression also correlates with increased expression of EMT markers, such as Snail and N-cadherin.[78] In vitro, SCCHN cell lines respond to ILK kinase inhibition (using QLT0267) by undergoing cell cycle arrest and apoptosis, associated with a decrease in Akt phosphorylation (**Fig. 1B**).[79] These data are highly suggestive that ILK plays a key role in tumor malignancy, and that targeting ILK's kinase activity may be an effective therapeutic strategy. Perhaps ILK kinase activity plays a more important role during tumorigenesis than normal skin homeostasis. However, further studies using relevant in vivo models are required.

Kindlin in Skin

Kindler syndrome (KS) is an autosomal recessive disease characterized by a variety of skin phenotypes, including: blistering, sensitivity to UV, abnormal pigmentation, and skin fragility.[80] Linkage analysis of a group of Panamanian patients in 2003 led to the discovery of the gene responsible for this syndrome, KIND1, which encodes for the protein Kindlin-1.[81] Many additional mutations in the KIND1 gene have been described that lead to loss of protein expression or function.[82] There are three Kindlin isoforms—Kindlin-1, Kindlin-2, and Kindlin-3—that have tissue-specific expression, which can explain why Kindler syndrome results only from mutations in KIND1. Kindlin-1 is expressed exclusively in epithelial tissue, Kindlin-3 is expressed exclusively in hematopoietic lineages, and Kindlin-2 is expressed ubiquitously. Loss of KIND2 in mice leads to peri-implantation lethality, presumably because Kindlin-2 is absolutely required for Talin-induced activation of integrin signaling.[83] As expected, loss of KIND1 in mice leads to some of the defects seen in KS patients, including skin atrophy.[84] However, this phenotype is associated with severe intestinal dysfunction, leading to perinatal lethality in these mice.[84] Ulcerative colitis is a common feature in a fraction of KS patients, but it does not lead to death in humans so early in life. This indicates that either there are significant differences between Kindlin-1 function in mouse and human colons, or that perhaps the mutations seen in KS patients do not result in complete loss of KIND1 activity.

Epidermis deficient in Kindlin-1 displays defects in basal keratinocyte polarity and proliferation, and enhanced apoptosis.[85] Keratinocytes lacking Kindlin-1 also show reduced proliferation and adhesion in culture, and undirected cellular motility and plasma membrane protrusion.[85] More thorough analysis of FA complexes revealed that Kindlin-1 is phosphorylated, and that it co-immunoprecipitates with both Kindlin-2 and Migfilin in the skin.[85,86] However, Kindlin-1 in keratinocytes is not required for KIND2 or FBLIM1 (Migfilin) gene expression or protein localization.[86] Furthermore, it was shown that Kindlin-1 forms a complex with β1 integrin, α-actinin, Migfilin, and FAK and regulates cell shape and migration by controlling lamellipodia formation.[87] Kindlin-1 expression is required for activation of motility-related proteins, including Rac1, RhoA, Cdc42, p21-activated kinase 1 (PAK1), LIM kinase, and Cofilin.[87] KS primary keratinocytes, or keratinocytes lacking Kindlin-1, show reduced clonogenicity and upregulation of cell senescence markers, potentially explaining the skin fragility seen in these patients.[88] Kindlin-1 and Kindlin-2 were shown to have some overlapping functions in skin; however, loss of Kindlin-2 alone in cultured keratinocytes led to reduction of cell motility and formation of cell–cell adhesions.[89] Irradiation of keratinocytes led to loss of Kindlin-2 protein expression, which could explain why patients with KS experience enhanced UV sensitivity.[89]

The function of Kindlin-1 or -2 has not been directly assessed in mouse models of SCC; however, there is a higher incidence of skin cancer in patients affected with KS.[90] Given the relative fitness disadvantage of nontumorigenic Kindlin-1 mutant cells, this increased cancer susceptibility seems somewhat surprising. However, keratinocytes lacking Kindlin-1 show reduced epithelial features and signs of epithelial–mesenchymal transition, including reduced α6β4, E-cadherin, and collagen XVII expression, and increased levels of mesenchymal markers vimentin and fibronectin, along with increased secretion of MMPs and pro-inflammatory cytokines.[90] This suggests an anti-tumor and anti-invasive role for Kindlin-1 in normal keratinocytes, though in vivo studies are required to verify this possibility.

Other Focal Adhesion Proteins

Many other scaffolds, adaptor and signaling proteins exist within Focal adhesions and are also essential for mouse viability. Most of these proteins have no known functional role in skin in vivo, as conditional knockout mice have not been generated. Here we review what is known about the role of additional FA proteins in keratinocytes or squamous cell carcinoma cell lines.

α-actinin is a cytoskeletal protein that binds directly to actin to link integrins to the cellular cytoskeleton. This protein exists in 4 isoforms that are restricted to specific cell types. α-actinin-2 and -3 are muscle-specific, while α-actinin-1 and -4 are expressed more ubiquitously. ACTN1 (the gene encoding α-actinin-1) has not been knocked out in a mouse, and there is no information on any role for α-actinin-1 in keratinocytes. However, the ACTN4-knockout mouse has been created and exhibits lung and kidney

failure, leading to death several months after birth.[91] In culture, knockdown of ACTN4 in keratinocytes leads to loss of polarity, decreased directional migration, and decreased activity of cofilin, an actin remodeling protein.[92] Additionally, the focal contact area in these cells is increased and the hemidesmosome proteins in these cells are mislocalized.[92] This could indicate that skin lacking ACTN4 may have a structural or wound healing defect, but in vivo data are needed to verify this.

Filamin A is another actin binding protein that participates in organization of the actin cytoskeleton. The FLNA-knockout mouse is embryonic lethal due to cardiac failure and defects in blood vessel formation.[93] Filamin A has been shown to be required for calcium-induced keratinocyte differentiation in culture, though the mechanism by which this occurs is unknown.[94] Migfilin, also known as Filamin binding LIM protein 1, also binds to Filamin A and plays a role in the actin cytoskeleton. However, the FBLIM1 (gene that encodes Migfilin) knockout mouse is normal with no noticeable defect in skin or tissue morphology.[95] Primary keratinocytes isolated from Migfilin deficient mice exhibited a migration defect in culture which appeared to be independent of Filamin binding.[95]

P130cas, encoded by the BCAR1 gene, is a direct substrate of FAK and is involved in cellular migration, survival, and invasion. The BCAR1-knockout mouse experiences embryonic lethality due to cardiac defects and improper blood vessel formation, similar to the phenotype seen in the FLNA knockout mouse.[96] Nothing is known about the role of this protein in skin. However, in lung squamous cell carcinoma, phosphorylation of p130cas is associated with increased invasiveness, and knockdown of p130cas could reverse the increased invasion induced by a mutation in the EphA2 gene.[97] This suggests a possible role for this protein in SCC invasion.

Paxillin is a signal adaptor protein that is recruited to the β1 integrin cytoplasmic domain following attachment of SCC cells to type IV collagen.[98] Paxillin may also play a role in SCC invasion, since overexpression of this protein stimulated in vitro invasive properties of an SCC cell line.[98] There is not any in vivo data on the role of this protein in skin or SCC, since the PXN-knockout mouse has disrupted development of mesodermal structures, leading to embryonic lethality at E9.5.[99]

PINCH1 and PINCH2 are LIM domain-containing proteins that bind directly to ILK.[100] The PINCH1-knockout mouse exhibits embryonic lethality between E6.5–E9.5, with abnormal epiblast polarity and impaired cavitation, similar to the ILK-null mouse.[100,101] The PINCH2-knockout mouse, however, is viable and fertile, with no apparent phenotype.[102] Both expressed at high levels in mouse skin, but their functional roles are unknown.[103]

Several additional secreted glycoproteins have been reported to bind to and activate integrin αv heterodimers. These proteins comprise the SIBLING (small integrin binding ligand N-linked glycoprotein) family, which consists of proteins such as dentin sialophosphoprotein (DSPP), bone sialoprotein (BSP), and osteopontin (OPN). These proteins play major roles in mineral deposition in the bone and have been shown to bind and activate matrix metalloproteases. Though it is not known whether these proteins play a role in skin homeostasis, their expression is highly upregulated in clinical cases of OSCC.[104] Furthermore, DSPP and OPN are expressed at histologically negative margins of OSCCs, and their expression predicts recurrence following tumor resection.[105]

Tensin is a FA protein that is phosphorylated upon integrin activation or upon transformation by oncogenes such as v-Src. Tensin also binds and cross-links actin filaments. Two additional Tensin isoforms, Tensin-2 and Tensin-3, were discovered more recently. The Tensin knockout mouse is normal at birth, but develops kidney abnormalities.[106] Some Tensin-3-knockout mice exhibit growth retardation and postnatal lethality, which is associated with incomplete development of the small intestine, lung, and bone.[107] Tensin-3 has been shown to be required for tumorigenesis and metastasis in a number of different cell lines, though its role in squamous cell carcinoma specifically has not been determined.[108]

Finally, Zyxin is an adaptor protein containing both LIM domains and SH3 domains. Zyxin has a punctate staining in the cytoplasm of keratinocytes within the normal epidermis; however, this staining re-localizes the the edge of migrating keratinocyte sheets during wound healing.[109] The Zyxin knockout mouse is normal, which could potentially be due to functional redundancy between the Zyxin family members LPP or TRIP6.[110]

Therapeutic Targeting

Inhibitors targeting integrin activation and the focal adhesion kinases FAK and ILK are in various stages of development, with clinical trials currently ongoing for integrin inhibitors, integrin blocking antibodies, and FAK inhibitors. Although in vitro data support the use of ILK kinase inhibitors for therapy, no clinical trials have been started for those compounds.

Cilengitide (EMD 121974) is an RGD-based peptide targeted against αvβ3 and αvβ5 integrins, which are upregulated on blood vessels during tumor angiogenesis.[111] In preclinical studies, this drug showed induction of apoptosis of angiogenic endothelial cells and additional direct anti-tumor activity.[112] While preclinical models showed promise, only a fraction of glioblastoma patients respond to therapy, and there is variability in response of patients with other types of tumors to the drug.[112] Treatment of patients with squamous cell carcinomas of the head and neck with cilengitide resulted in partial response or stable disease for all patients tested, and randomized phase II clinical trials are currently in progress.[113] More recently, it was shown that low concentrations of this inhibitor actually stimulate tumor growth by promoting VEGFR-2 trafficking to the endothelial cell surface.[114] It is therefore possible that dose of the drug is highly important in tumor response, which could account for some of the variability seen thus far.

CNTO-95 (intetumumab) is a fully humanized anti-αv-integrin monoclonal antibody that was also developed to target angiogenic blood vessels and some primary tumors, but broadly binds to all αv heterodimers.[115] The expression of αv integrins has been shown to be essential for survival of melanoma cells in 3-dimensional cultures, and thus most of the clinical trials

Table 3. Functional roles for intracellular focal adhesion proteins in mouse skin

Gene	Conditional skin knockout phenotype (K5 or K14 promoter)	Role in SCC
FAK	-Hair cycle irregularities[52] -Loss of proliferation in sebaceous gland[52] -Slight epidermal thinning, but no change in wound healing[52]	-FAK+/– mice show reduced papilloma formation, but no change in conversion of papillomas to SCCs upon DMBA-TPA treatment[54] -K14-CreER 4-OHT induced deletion of FAK in the epidermis both prevented tumor formation and stopped tumor progression upon DMBA-TPA treatment[55]
ILK	-Epidermal blistering[73] -Movement of proliferative cells to suprabasal layers[73] -Hair loss[73]	N/A

for this antibody have been for stage IV melanoma patients.[116] This therapy was well tolerated in phase I trials and has shown variable success in phase II trials.[117-119] There is a trend toward improved survival, but it is not yet significant, and studies with larger patient cohorts may be necessary.

In preclinical studies, inhibition of FAK kinase activity showed promising anti-tumor activity for a variety of different types of malignancies. A specific inhibitor, PF-00562271, has shown safety and some efficacy in phase I trials for advanced solid tumors (including head and neck tumors).[120] Phase I trials are currently ongoing for a second FAK kinase inhibitor, GSK2256098.

Another potential strategy for targeting integrin activation in cancer is to target specific extracellular matrix ligands. Secretion of proteases during tumorigenesis can lead to cleavage of ECM components to generate new ligands with distinct structure and binding affinity for specific integrin heterodimers. Cleavage of type IV collagen into 2 epitopes, HU177 and HUIV26, occurs in the extracellular matrix surrounding melanoma tumors. This collagen cleavage exposes additional integrin-binding motifs within these epitopes that enhance signaling of αvβ3 within angiogenic blood vessels.[121] Blocking antibodies against these epitopes have shown anti-angiogenic, anti-tumor and anti-metastasis efficacy.[122] Additionally, increased shedding of HU177 is observed in melanoma patient sera and has been shown to correlate with poor prognosis and disease progression.[123,124] More work is required to determine if additional epitopes are released, and if this differs between tumor types.

Conclusions and Future Directions

It has become increasingly clear that FA complexes anchoring epidermal keratinocytes to the basement membrane are essential for many aspects of skin homeostasis, including basal keratinocyte proliferation, differentiation, barrier function, migration and wound healing, and hair follicle morphogenesis. Many of the individual FA complex proteins, however, are not absolutely required for skin, but play key roles during epithelial cell carcinogenesis. It is possible to exploit this biological information to target FA complexes from outside the cell, using blocking antibodies, RGD-based peptides, or small-molecule inhibitors to block integrin activation.[125] However, a better understanding of individual integrin subunit roles in both normal skin and SCC is required for development of effective therapy. Maximizing the medical relevance of these studies will require functional experiments in native 3-dimensional environments, as traditional 2-dimensional experiments utilizing cultured cells results in different responses that are not seen in vivo.[126] While functional roles for some integrin subunits, FAK, ILK, and Kindlin have been established in mouse epidermis, there exist many other FA complex proteins with no known in vivo functional role in skin. Loss of most of these proteins results in embryonic lethality (**Table 2**), and additional studies using conditional knockout strategies or human organotypic skin reconstructs are required to determine the roles these proteins play in skin homeostasis.

Generation of such a large number of integrin-knockout mice is expensive, time-consuming, and can lead to misleading conclusions due to strain-specific defects (as shown in the case of β1 integrin). Furthermore, there are many structural differences between mouse and human skin.[127] More recently, organotypic skin substitutes have been developed that contain intact and structurally correct human basement membrane and human epidermal and dermal cells.[128] These epidermal cells can be genetically engineered to overexpress or knockdown expression of multiple genes of interest. This type of experimental platform can greatly accelerate research on the functional roles of integrins and FA proteins in human skin and SCC. This tissue can also be xenografted onto mice, allowing for the study of human skin and SCC in vivo.[129] This type of experimental platform is a potentially useful approach for translating the developmental and biological insight we have gained from mouse models into therapy for human disease. While clinical trials for integrin antagonists or blocking antibodies, and FAK kinase inhibitors are ongoing, there remains much to learn regarding integrin signaling in tissue, and how it changes in disease conditions. A better understanding of both integrin signaling and the different roles of each FA kinase protein in vivo will hopefully lead to the development of more effective cancer therapies (**Table 3**).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

TWR is supported by a grant from the NIH/NCI (RO1 CA163566). EKD is supported by an NIH/NIAMS training grant (5-T32-AR0007465-30). We would like to thank Dr Sarah Millar at the University of Pennsylvania for critical pre-submission manuscript review.

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