Mechanotransduction Pathways Promoting Tumor Progression Are Activated in Invasive Human Squamous Cell Carcinoma

S. Jan Ibbetson,* Natasha T. Pyne,‡ Anthony N. Pollard,§ Michael F. Olson,∥ and Michael S. Samuel*‡∥

From the Division of Surgical Pathology* and the Tumour Microenvironment Laboratory, SA Pathology, Adelaide, Australia; the Beatson Institute for Cancer Research,∥ Bearsden, United Kingdom; and the School of Molecular and Biomedical Science,‡ University of Adelaide, Adelaide, Australia

Cutaneous squamous cell carcinomas (SCCs) are commonly diagnosed skin cancers that may progress to invasiveness in the absence of early intervention. Using a murine model of SCC, we have previously demonstrated that activation of the Rho-associated kinase (ROCK) signaling pathway promotes rapid progression of pre-neoplastic lesions to invasive SCC. Herein we demonstrate that in human cutaneous SCC, ROCK signaling is increasingly up-regulated with tumor progression in both tumor cells and cells of the tumor microenvironment and is accompanied by key tumor-promoting changes in the extracellular matrix protein composition. The mechanotransduction pathway mediated by integrin signaling through FAK, GSK3β, and the transcriptional coactivator β-catenin is also progressively activated in human cutaneous SCC. Our observations indicate that ROCK activation is a tumor promoter in human cutaneous SCC and acts via mechanotransduction of signals to β-catenin. Our experiments raise the possibility that inhibition of ROCK signaling could be a useful therapeutic approach to halt cutaneous SCC progression by reducing the signal flux through this pathway to physiologic levels, thereby normalizing the extracellular matrix composition. (Am J Pathol 2013, 183: 930–937; http://dx.doi.org/10.1016/j.ajpath.2013.05.014)

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Accepted for publication May 14, 2013.
Address correspondence to Michael S. Samuel, Ph.D., Tumour Microenvironment Laboratory, Adelaide, SA 5000, Australia. E-mail: michael.samuel@adelaide.edu.au.

Cutaneous squamous cell carcinomas (SCCs) often arise as a result of sun damage and are among the most frequently diagnosed cancers.1 Approximately 3.5 million new non-melanoma skin cancer lesions, of which SCCs account for up to 50%, are diagnosed in the United States each year, and the incidence is increasing rapidly.2 Although early lesions are readily treated by complete excision, SCCs left untreated pose a substantial risk of invasion and metastasis.3 Patients in whom SCCs have been resected are at a lifelong increased risk of recurrence, and vigilant surveillance is necessary.4 Noninvasive therapeutic interventions are therefore needed, and these can be achieved only by gaining understanding of the underlying mechanisms that govern progression of SCC.

Using a multistage carcinogenesis murine model of cutaneous SCC,5 we have previously demonstrated that activation of the Rho-associated kinase (ROCK) signaling pathway promotes tumor progression via a mechanism that involves mechanosensation and integrin-mediated signaling. Inhibiting this cytoskeletal regulatory pathway by means of topical application of a ROCK inhibitor dramatically slowed tumor progression and halted conversion from benign to malignant disease.6 Progression of the disease in this model was characterized by increased collagen deposition within the extracellular matrix (ECM), which increased tissue stiffness. Increased tissue stiffness acted to promote cell proliferation within the tumor via a mechanism involving integrin-mediated signaling through the focal adhesion kinase (FAK) and stabilization of the pro-tumorigenic transcriptional coactivator β-catenin.6

The tumor microenvironment is increasingly the subject of vigorous research as our understanding of its profound influence on the tumor phenotype deepens. Much recent

Supported by The Royal Adelaide Hospital Research Fund (M.S.S., S.J.I.), the National Health and Medical Research Council (M.S.S.), the Australian Research Council (M.S.S.), and Cancer Research UK (M.F.O.).
work has focused on the microenvironment, not simply as the medium for tumor-promoting inflammation but as an integral part of the tumor that determines many major characteristics of the disease. As such, the concept that the extracellular tumor microenvironment could serve as a diagnostic and prognostic tool as well as a therapeutic target continues to gain support. We therefore wanted to determine whether changes within the ECM are brought about by ROCK signaling and whether mechanotransduction pathways that promote tumor progression are engaged in the disease in humans. Inasmuch as the ROCK signaling pathway is tractable and numerous inhibitors are already available (reviewed by Rath and Olson\textsuperscript{7}), we reasoned that it may prove a useful target for therapy. In this study, we demonstrate that ROCK is progressively activated during human SCC disease progression and results in increased ECM deposition of not only collagen but also fibronectin and peristin. Mechanotransduction signaling pathways downstream of ECM-integrin interactions are activated in human SCC and correlate with the stabilization of β-catenin and progression to invasive cancer.

Materials and Methods

Mice

We have previously described the generation and characterization of K14-ROCK:ER and K14-KD:ER mice.\textsuperscript{6,8} All procedures were performed under appropriate licenses and with the oversight of the institutional animal ethics committee constituted according to the Animal Welfare Act 1985 of South Australia.

In Vivo Epidermal Activation of ROCK Signaling Pathway

4-Hydroxytamoxifen (4-HT; Sigma-Aldrich, St. Louis, MO), 1 mg, in 20 μL dimethyl sulfoxide was applied to shaved dorsal skin once daily for 5 consecutive days. At 24 hours after the final application, mice were humanely sacrificed, and the dorsal skin was fixed in formalin overnight at 4°C and processed for histologic analysis.

Using Second Harmonic Generation Microscopy to Image Collagen Fibers

Histologic samples were imaged by second harmonic generation (SHG) microscopy using a 20 × 1.0 NA water immersion objective on an upright fixed-stage two-photon laser scanning microscope system (Carl Zeiss AG, Oberkochen, Germany). The excitation source was a Ti:Sapphire femtosecond laser cavity (Mai Tai; Newport Corp., Irvine, CA) coupled to an LSM 710 scan module (Carl Zeiss AG). An excitation wavelength of 890 nm was used to collect the second harmonic signal (435 ± 20 nm) from collagen. Signal was acquired from three separate areas measuring 320 × 320 μm\textsuperscript{2} across each sample. Immunofluorescence images were acquired concurrently with SHG data.

Histologic Analysis and Immunohistochemistry

Histologic analysis and immunohistochemistry (IHC) were performed as previously described.\textsuperscript{6} Antigen retrieval buffer, method, and antibody dilutions used are given in Table 1. Histology slides were imaged using a NanoZoomer Digital Pathology slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) and Digital Slide Server software (Slidepath; Leica Microsystems). Immunofluorescence and SHG images were acquired simultaneously using an LSM 710 two-photon excitation microscope (Carl Zeiss AG, Oberkochen, Germany). IgG isotype control antibodies were incubated with sections from each paraffin block used in the study to verify antigen specificity of the monoclonal antibodies used (representative wide field images of sections of invasive tumors are shown in Supplemental Figure S1). Secondary-only control procedures were performed to verify that none of the secondary antibodies recognized antigens within the tissues analyzed (data not shown). Conditions were the same as those used for the appropriate primary antibodies, as given in Table 1.

Quantification of SHG Signal from Collagen or Immunofluorescence

ImageJ was used to calculate percentage area covered by SHG signal per image, after conversion to a binary image based on a single manually determined threshold value applied across all images. Results were expressed as medians, ranges, and quartiles across all data sets for each histologic type.

Positive Pixel Analysis of IHC Stains

Analysis of IHC stains was performed using ImageScope software (Aperio, Vista, CA). Positive pixel data are expressed as the percentage of dianibenidine-positive pixels relative to total pixel number of the hematoxylin counterstain.

Statistical Analysis

Box-and-whisker plots were used to show medians and quartiles. \( P \) values were calculated using one-way analysis of variance, and the Mann-Whitney post hoc test was used to compare the spread of values. In all cases, \( P < 0.05 \) was assessed as significant.

Results

ROCK Signaling Is Activated in Human Cutaneous SCCs

To determine whether ROCK signaling is activated in human cutaneous SCC, we analyzed 22 separate primary
human SCCs for ROCK expression levels using a validated antibody that recognizes both ROCK1 and ROCK2 and assessed ROCK activation by determining the levels of direct ROCK-mediated myosin phosphatase targeting subunit (MYPT) phosphorylation at Thr696. Relatively unaffected skin at the margins of each resection exhibited low ROCK expression and limited MYPT phosphorylation. In contrast, hyperproliferative skin and invasive regions of SCCs exhibited substantially higher ROCK expression and MYPT phosphorylation as determined by IHC staining (Figure 1A) followed by positive pixel analysis (Figure 1B). These results indicated that ROCK is up-regulated but also highly activated in human cutaneous SCC in a manner similar to our previously reported observations in a murine model of cutaneous SCC.

ROCK Activation Correlates with Increased Deposition of ECM Proteins in Human Cutaneous SCC

We have previously demonstrated that activation of the ROCK signaling pathway within the murine epidermis caused a threefold increase in dermal collagen. This causal relationship between activation of ROCK and the production of collagen was also observed in a mouse model of cutaneous SCC. Therefore, we sought to determine whether the activation of ROCK signaling observed in human SCC samples was accompanied by increased collagen deposition, which would similarly suggest a cause-effect relationship. Using SHG microscopy, we imaged regions corresponding to relatively unaffected skin at resection margins, hyperproliferative skin, or invasive SCC, representing stages of disease progression. These analyses were performed on 22

Table 1

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<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Antigen Retrieval</th>
<th>Boiling Time (min)</th>
<th>Dilution</th>
<th>Incubation</th>
<th>Time (h)</th>
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<tr>
<td>ROCK1</td>
<td>Chemicon</td>
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<tr>
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<td>Millipore</td>
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<td>1:100</td>
<td>4</td>
</tr>
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<td>CK17</td>
<td>Sigma-Aldrich</td>
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<td>1:500</td>
<td>4</td>
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<tr>
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<td>p(Ser473)Akt</td>
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<td>NA</td>
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<td>1:50</td>
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EDTA, ethylenediaminetetraacetic acid; IF, immunofluorescence; NA, not applicable.
separate human cutaneous SCCs (Figure 2A). Area coverage analysis revealed that activation of ROCK, as evidenced by phosphorylation of MYPT and increased collagen deposition within the ECM that surrounds tumors, correlated with tumor progression (Figure 2B). ROCK activation was also observed in cells within the microenvironment of invasive SCCs that did not express cytokeratin (CK) 14 (Figure 2), indicating that ROCK activation in nontumor, nonepidermal cells in proximity to the tumor may contribute to the lesion.

Although collagen is a major component of the ECM, other proteins such as fibronectin and periostin have structural and functional roles in maintaining tissue density and regulating cell signaling pathways (reviewed by Schwarz-bauer and DeSimone10 and Gritsenko et al11). To determine whether increased collagen deposition we observed during tumor progression was mirrored by other ECM components, we analyzed fibronectin and periostin expression in the human cutaneous SCC collection. Immuno-fluorescence followed by area coverage analysis revealed that both fibronectin (Figure 3, A and B) and periostin (Figure 4, A and B) were up-regulated during progression from normal to invasive cutaneous SCC suggesting that, like collagen, fibronectin and periostin deposition may also be regulated downstream of ROCK activation.

Epidermal ROCK Activation in a Murine Model Increases Fibronectin and Periostin Deposition within the Dermis

To determine whether increased ROCK signaling could be responsible for the greater fibronectin and periostin deposition in progressive cutaneous human SCC, we used a mouse model (K14-ROCK:ER) that we had previously generated in which ROCK may be conditionally activated.6,8 On ROCK activation within the epidermis of this model, we observed increased fibronectin (Figure 5A) and periostin (Figure 5B) expression in the dermis compared with that observed in a control strain of mice expressing a kinase-dead version of ROCK (K14-KD:ER). These observations strongly suggested that ROCK signaling not only regulates collagen but also other components of the ECM such as fibronectin and periostin. Considered together, our observations that collagen,6 fibronectin (Figure 3), and periostin (Figure 4) are up-regulated in human SCC reveal...
a role for ROCK in regulating ECM composition during cutaneous SCC progression.

Human Cutaneous SCCs Exhibit Progressive Activation of Integrin-Mediated Mechanotransduction Pathways

In the murine cutaneous SCC model, tumor progression is influenced by increased ECM density resulting from high levels of collagen deposition around the tumor by increased ROCK signaling. Increased ECM density stimulates outside-in signaling via the integrin signaling pathway through FAK\textsuperscript{12,13}, resulting in stabilization of the major mechanotranscription target β-catenin by a mechanism involving phosphoinositide 3-kinase, Akt, and inhibition of GSK3β\textsuperscript{6}. β-Catenin, a well-known regulator of self-renewal within the skin,\textsuperscript{14} is phosphorylated on Ser37 and Thr41 by GSK3β\textsuperscript{15} to be targeted for proteasomal degradation. To determine whether activation of the integrin-mediated mechanotransduction pathway is a feature of human cutaneous SCCs, we performed immunofluorescence analysis on resected SCCs to assess the level of Tyr397 auto-phosphorylated active FAK within hyperproliferative and invasive tumors compared with margin skin. Our results indicated a stepwise increase in FAK activation during progression from relatively unaffected margin skin to hyperproliferative skin and invasive SCC (Figure 6). The increase is particularly dramatic in invasive SCC, with CK17-positive tumor cells invading individually through the ECM without maintaining cell-cell contact with other tumor cells exhibiting the highest levels of p-Tyr397-FAK. Collectively invading cells, which invade as a group while maintaining cell-cell contact with other invading tumor cells, also exhibited increased levels of active FAK, although these levels were lower than those observed in

![Figure 4](image_url) Periostin deposition in invasive cutaneous SCC. A: Representative images of immunofluorescence analysis of periostin (green) in representative samples of resected margin skin, hyperproliferative lesions, and invasive cutaneous SCC. Sections have been counterstained for CK1/6 (both in red) to permit visualization of skin cells. Scale bars: 100 μm. B: Results of area coverage analysis of periostin show the level of this protein observed per histotype in 22 cutaneous SCC lesions. Box-and-whisker plots show range, median and quartiles. P values were calculated using the U test. HP, hyperproliferative lesions. **P < 0.01.

![Figure 5](image_url) Intradermal fibronectin and periostin expression in a murine model of ROCK pathway activation. In all panels, the surface of the epidermis is delineated by a white line to aid interpretation because the intensity of CK6 staining (red) is low in all cases except ROCK-activated skin, as previously reported.\textsuperscript{6} Scale bars: 100 μm. Immunofluorescence analysis of fibronectin (green) and CK1/6 (red) (A) and periostin and CK1/6 (red) (B) in vehicle-treated or ROCK-activated (+4HT)-treated K14-ROCK:ER and K14-KD:ER skin. DNA staining was via DAPI (blue).
individually invading cells. Elevated FAK activation in invasive SCC was accompanied by increased nuclear localization of active β-catenin, which lacks GSK3β target site phosphorylation (Figure 7A). In agreement with these observations, inactivation of GSK3β, determined via immunofluorescence analysis specific for inactive pSer9-GSK3β, was observed in analogous regions of invasive cutaneous SCC (Figure 7B), particularly evident in individually invading cells but also present in collectively invading cells. It therefore seems that Akt is activated by FAK downstream of integrin signaling, leading to GSK3β inactivation by phosphorylation at Ser9 and consequent stabilization of β-catenin. Considered together, these results strongly suggest that the mechanotransduction pathway activated by increased ECM protein deposition and stiffness is a feature of cutaneous SCCs, activated to a greater degree

**Figure 6** FAK activation status in cutaneous SCC. Representative images of immunofluorescence analysis of autophosphorylated, active FAK (p-Tyr397-FAK, green in merged images and white in grayscale images) in representative samples of resected margin skin, hyperproliferative lesions, and invasive cutaneous SCC. Sections were counterstained for CK14 (red) to enable visualization of skin cells. The panels labeled Mag are enlargements of regions delineated by boxes in the images of invasive SCC. **Magenta arrowheads** indicate highly contracted invasive cells positive for CK14 and that express high levels of pFAK; **white arrowheads**, tumor cells exhibiting collective invasion and moderately elevated levels of pFAK. Scale bars: 100 μm (pFAK panels); 20 μm (Mag panel).

**Figure 7** Active β-catenin (ABC; not phosphorylated at the GSK3β target sites Ser37 and Ser41) and inactive GSK3β (phosphorylated at Ser9) in cutaneous SCC. **A**: Representative images of immunofluorescence analysis of active β-catenin (white in grayscale images and green in merged images) in representative samples of patient-derived hyperproliferative lesions and invasive cutaneous SCC. Merge panels include counterstaining for CK17 (red) to enable visualization of skin cells. **Magenta arrowheads** indicate nuclear localization of active β-catenin. **B**: Representative images of immunofluorescence analysis of inactive pSer9-GSK3β (white in grayscale images and green in merged images) in representative samples of patient-derived hyperproliferative lesions and invasive cutaneous SCC. Merge panels include counterstaining for CK14 (red) to enable visualization of skin cells. **Magenta arrowheads** indicate elevated levels of pSer9-GSK3β in individually invading skin cells; **white arrowheads**, moderately elevated levels of pSer9-GSK3β in collectively invading skin cells. Scale bars: 100 μm.
as tumors progress from benign to invasive forms, and may drive tumor progression.

Discussion

The tumor microenvironment comprises a cellular component that includes cells such as fibroblasts and immune cells and a noncellular component, the ECM, both of which change substantially with disease progression. Cancer-associated fibroblasts and immune cells exhibit modified characteristics relative to their counterparts in normal tissues and may promote or oppose tumor progression under various circumstances in ways that are only beginning to be understood (reviewed by Allen and Jones16 and Keely17). These cells are also important in generating and remodeling the ECM, which is composed of a variety of polysaccharides and fibrillar proteins, several of which may act as tumor promoters.

Collagen, fibronectin, and periostin are major components of ECM. While acting as a scaffold upon which cells adhere and migrate, these proteins also maintain tissue rigidity through organization and cross-linking of their fibers. Elevated tissue density caused by increased production and high-order organization of ECM components is a known risk of cancer in organs such as the breast.18 ECM proteins are thought to influence tumor progression in two ways. Increased deposition and cross-linking of ECM proteins can directly promote tumor progression via mechanical force-induced clustering of signaling receptors such as integrins19 or function as ligands by directly binding receptors such as the discoidin domain—containing family of receptor tyrosine kinases,20 members of the mannose receptor family such as the urokinase plasminogen activator receptor—associated protein21 and others. Although regulation of integrin signaling is a key function of direct ECM-cell interactions,17 ECM proteins can also bind and modulate the activity of signaling molecules such as bone morphogenetic protein 1,22 which can then stimulate tissue resident fibroblasts to secrete pro-tumorigenic factors.23 The resulting increased signaling through molecules including FAK, Akt, LIM kinase, and extracellular signal-regulated kinase thereby promotes tumor progression by inducing processes such as cell proliferation, epithelial-mesenchymal transition, and invasion.

Our previous studies6,24 have indicated that ROCK signaling is not only an effector pathway downstream of changes in the ECM but also promotes collagen production within the dermis, resulting in increased tissue density, activation of an integrin-mediated signaling cascade, epidermal hyperproliferation, and tumor promotion in a murine model of ROCK activation. Our present results reveal that, consistent with our previous observations in the murine model, the increase in ROCK activation observed in progressive human cutaneous SCC stages is correlated with increased collagen deposition in the ECM. In addition, not only collagen but other ECM components such as fibronectin and periostin are also strongly up-regulated in progressive stages of cutaneous SCC. Our murine model expressing activated ROCK also exhibited increased fibronectin and periostin within the dermis, which suggests that ROCK activation results in the increased production of these ECM proteins. We observed that nonepidermal cells of the tumor microenvironment also frequently exhibited activated ROCK, which suggests that activation of ROCK may have a role in remodeling not only the extracellular but also the cellular components of the tumor microenvironment to promote tumor progression. These observations are consistent with a recent report that overexpression of miR-511-3p, a micro RNA that targets ROCK2, in a subset of tumor-associated macrophages inhibits tumor growth.25

Further work is needed to identify the mechanisms by which ECM remodeling occurs downstream of ROCK signaling. However, these mechanisms likely involve a combination of changes in transcription and protein synthesis, and mechanical forces exerted on the ECM by the cytoskeletons of tumor cells and cells of the microenvironment, because ROCK was progressively activated in both of these cell types. Indeed, we have previously shown in inhibitor-based studies that the increased collagen deposition on ROCK activation was dependent on its ability to regulate the actomyosin cytoskeleton through LIM kinase and myosin adenosine 5′-triphosphatase.6 We therefore propose that the ROCK signaling pathway has an important function in generating a tumor-promoting ECM by regulating the deposition and remodeling of fibrillar ECM proteins.

The best-described transcriptional changes downstream of ROCK are mediated by the serum response factor, a widely expressed transcription factor that regulates expression of a large number of target genes encoding proteins that have roles in regulating the actin cytoskeleton (reviewed by Miano et al26). Serum response factor target genes influence many cellular processes that are dependent on the actin cytoskeleton, although no definitive link with ECM remodeling has thus far been demonstrated. However, the ROCK pathway also engages in cross-talk with many other signaling pathways including the c-Jun N-terminal kinase27 and NF-κB28,29 pathways, the Wnt planar cell polarity pathway (reviewed by Schlessinger et al30), and the canonical Wnt signaling pathway,6,24 many of which mediate changes in gene transcription. It is therefore possible that transcription changes downstream of ROCK linked to one or more of these signaling pathways in cells of the tumor or the tumor microenvironment may mediate ECM regulation.

Together with our previous report that ROCK pathway activation promotes tumor progression by increasing ECM stiffness, the present research points to the potential usefulness of ROCK inhibitors as therapeutic agents against cutaneous SCC. Our murine studies demonstrated that at least one of these inhibitors, Y-27632, was readily absorbed through the skin when applied using the appropriate vehicle and was effective in substantially slowing tumor progression by acting locally.6 It is therefore possible to speculate that
topical delivery of ROCK inhibitor in patients with SCC could be useful for targeted inhibition of ROCK signaling within the tumor and its microenvironment, thereby minimizing the risk of deleterious effects associated with systemic administration.

Acknowledgments

We thank Prof. Angel Lopez for critically reading the manuscript; members of the Division of Tissue Pathology within the Surgical Pathology directorate of SA Pathology, who retrieved and sectioned the human SCC blocks; and the RAH Research Fund, which provided financial support for the purchase of the microscopy equipment used in this research.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.05.014

References