TUMORIGENESIS AND NEOPLASTIC PROGRESSION

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,[†] Centre for Cancer Biology, 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

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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) 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, GSK3 β , and the transcription 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>)

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.^{3,4} 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

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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 Olson⁷), 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 periostin. 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.^{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 μ L 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

$320 \times 320 \mu\text{m}^2$ 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.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 diaminobenzidine-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 ROCK2⁶ and assessed ROCK activation by determining the levels of direct ROCK-mediated myosin phosphatase targeting subunit (MYPT) phosphorylation at Thr696.⁹ 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.⁶

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.⁶ 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

Figure 1 ROCK expression and activation in human cutaneous SCC. **A:** Representative images of IHC staining (brown) of ROCK and pThr696-MYPT in normal skin and invasive cutaneous SCC. Sections were counterstained with hematoxylin. Scale bars: 200 μ m. **B:** Positive pixel analysis on images of IHC staining for ROCK and pMYPT in the regions indicated. Data were derived from 22 separate invasive cutaneous SCC lesions. *P* values were derived by applying the Mann-Whitney post hoc test as described in *Materials and Methods*. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Table 1 Experimental Conditions for IHC and Immunofluorescence Analysis of Tissues

Antibody	Supplier	Antigen Retrieval		Dilution		Incubation	
		Buffer	Boiling Time (min)	IHC	IF	Temperature ($^{\circ}$ C)	Time (h)
ROCK1	Chemicon	10 mmol/L Citrate (pH 6)	15	1:100	NA	4	18
p(Thr696) MYPT	Millipore	10 mmol/L Citrate (pH 6)	20	1:100	1:100	4	18
CK17	Sigma-Aldrich	10 mmol/L Citrate (pH 6)	15	NA	1:200	4	18
CK14	Novocastra	10 mmol/L Citrate (pH 6)	20	NA	1:500	4	18
CK1	Covance	10 mmol/L Citrate (pH 6)	20	NA	1:500	4	18
CK6	Covance	10 mmol/L Citrate (pH 6)	20	NA	1:500	4	18
Fibronectin	BD Biosciences	10 mmol/L Citrate (pH 6)	20	NA	1:2000	4	18
Periostin	Sigma-Aldrich	10 mmol/L Citrate (pH 6)	20	NA	1:500	4	18
p(Ser473)Akt	Cell Signaling Technologies	10 mmol/L Tris-Cl (pH 8); 1 mmol/L EDTA	20	NA	1:50	4	18
AlexaFluor secondary antibodies	Invitrogen	NA	NA	NA	1:200	25	1
Active β -catenin	Millipore	10 mmol/L Citrate (pH 6)	20	NA	1:100	4	18
p(Tyr397)FAK	Invitrogen	10 mmol/L Tris-Cl (pH 9)	10	NA	1:50	4	18
p(Ser9)GSK3 β	Cell Signaling Technologies	10 mmol/L Citrate (pH 6)	10	NA	1:50	4	18

EDTA, ethylenediaminetetraacetic acid; IF, immunofluorescence; NA, not applicable.

Figure 2 ROCK activation and collagen expression in invasive cutaneous SCC. **A:** Representative images of immunofluorescence analysis of pMYPT (green) and SHG analysis of collagen (magenta) 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. Scale bars: 100 μ m. **Arrowheads** (also shown magnified in **insets**) show pMYPT staining in non-skin cells (negative for CK14) within the tumor microenvironment of invasive SCC. **B:** Results of area coverage analysis of pMYPT and collagen show the amount of pMYPT and collagen 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* < 0.05, ***P* < 0.01, and ****P* < 0.001.

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 Schwarzbauer and DeSimone¹⁰ and Gritsenko et al¹¹). To determine whether the 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. Immunofluorescence 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

Figure 3 Fibronectin expression in invasive cutaneous SCC. **A:** Representative images of immunofluorescence analysis of fibronectin (green) in representative samples of resected margin skin, hyperproliferative lesions, and invasive cutaneous SCC. Sections have been counterstained for CK1 and CK6 (CK1/6) (both in red) to enable visualization of skin cells. Scale bars: 100 μ m. **B:** Results of area coverage analysis of fibronectin 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* < 0.05.

Figure 4 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.

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,^{12,13} resulting in stabilization of the major mechanotranscription target β -catenin by a mechanism involving phosphoinositide 3-kinase, Akt, and inhibition of GSK3 β .⁶ β -Catenin, a well-known regulator of self-renewal within the skin,¹⁴ is phosphorylated on Ser37 and Thr41 by GSK3 β 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 5 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.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 [4-hydroxytamoxifen (4HT)-treated] K14-ROCK:ER and K14-KD:ER skin. DNA staining was via DAPI (blue).

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 μ m (pFAK panels); 20 μ m (Mag panel).

individually invading cells. Elevated FAK activation in invasive SCC was accompanied by increased nuclear localization of active β -catenin, which lacks GSK3 β —target site phosphorylation (Figure 7A). In agreement with these observations, inactivation of GSK3 β , determined via immunofluorescence analysis specific for inactive pSer9-GSK3 β , 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 GSK3 β 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 7 Active β -catenin (ABC; not phosphorylated at the GSK3 β target sites Ser37 and Ser41) and inactive GSK3 β (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-GSK3 β (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-GSK3 β in individually invading skin cells; **white arrowheads**, moderately elevated levels of pSer9-GSK3 β in collectively invading skin cells. Scale bars: 100 μ m.

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 studies^{6,24} 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- κ B^{28,29} 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.

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Supplemental Data

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References

1. Australian Institute of Health and Welfare and Australasian Association of Cancer Registries. Cancer in Australia 2010: an overview. Cancer series No. 60. Cat. No. CAN 56. Canberra, Australia; 2010.
2. Cancer Facts and Figures 2012. Atlanta, GA, American Cancer Society; 2012
3. Clayman GL, Lee JJ, Holsinger FC, Zhou X, Duvic M, El-Naggar AK, Prieto VG, Altamirano E, Tucker SL, Strom SS, Kripke ML, Lippman SM: Mortality risk from squamous cell skin cancer. *J Clin Oncol* 2005, 23:759–765
4. Glass AG, Hoover RN: The emerging epidemic of melanoma and squamous cell skin cancer. *JAMA* 1989, 262:2097–2100
5. Hennings H, Glick AB, Lowry DT, Krsmanovic LS, Sly LM, Yuspa SH: FVB/N mice: an inbred strain sensitive to the chemical induction of squamous cell carcinomas in the skin. *Carcinogenesis* 1993, 14:2353–2358
6. Samuel MS, Lopez JI, McGhee EJ, Croft DR, Strachan D, Timpson P, Munro J, Schröder E, Zhou J, Brunton VG, Barker N, Clevers H, Sansom OJ, Anderson KI, Weaver VM, Olson MF: Actomyosin-mediated cellular tension drives increased tissue stiffness and β -catenin activation to induce epidermal hyperplasia and tumor growth. *Cancer Cell* 2011, 19:776–791
7. Rath N, Olson MF: Rho-associated kinases in tumorigenesis: reconsidering ROCK inhibition for cancer therapy. *EMBO Rep* 2012, 13:900–908
8. Samuel MS, Munro J, Bryson S, Forrow S, Stevenson D, Olson MF: Tissue selective expression of conditionally-regulated ROCK by gene targeting to a defined locus. *Genesis* 2009, 47:440–446
9. Feng J, Ito M, Ichikawa K, Isaka N, Nishikawa M, Hartshorne DJ, Nakano T: Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J Biol Chem* 1999, 274:37385–37390
10. Schwarzbauer JE, DeSimone DW: Fibronectins, their fibrillogenesis, and in vivo functions. *Cold Spring Harb Perspect Biol* 2011, 3:a005041
11. Gritsenko PG, Ilina O, Friedl P: Interstitial guidance of cancer invasion. *J Pathol* 2012, 226:185–199
12. Egeblad M, Rasch MG, Weaver VM: Dynamic interplay between the collagen scaffold and tumor evolution. *Curr Opin Cell Biol* 2010, 22:697–706
13. Zhao J, Guan JL: Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev* 2009, 28:35–49
14. Malanchi I, Peinado H, Kassen D, Hussenet T, Metzger D, Chambon P, Huber M, Hohl D, Cano A, Birchmeier W, Huelsken J: Cutaneous cancer stem cell maintenance is dependent on beta-catenin signalling. *Nature* 2008, 452:650–653
15. Aberle H, Bauer A, Stappert J, Kispert A, Kemler R: beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J* 1997, 16:3797–3804
16. Allen M, Louise Jones J: Jekyll and Hyde: the role of the microenvironment on the progression of cancer. *J Pathol* 2011, 223:162–176
17. Keely PJ: Mechanisms by which the extracellular matrix and integrin signaling act to regulate the switch between tumor suppression and tumor promotion. *J Mammary Gland Biol Neoplasia* 2011, 16:205–219
18. Boyd NF, Martin LJ, Stone J, Greenberg C, Minkin S, Yaffe MJ: Mammographic densities as a marker of human breast cancer risk and their use in chemoprevention. *Curr Oncol Rep* 2001, 3:314–321
19. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, Weaver VM: Tensional homeostasis and the malignant phenotype. *Cancer Cell* 2005, 8:241–254
20. Vogel W, Gish GD, Alves F, Pawson T: The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol Cell* 1997, 1:13–23
21. Behrendt N, Jensen ON, Engelholm LH, Mørtz E, Mann M, Danø K: A urokinase receptor-associated protein with specific collagen binding properties. *J Biol Chem* 2000, 275:1993–2002
22. Huang G, Zhang Y, Kim B, Ge G, Annis DS, Mosher DF, Greenspan DS: Fibronectin binds and enhances the activity of bone morphogenetic protein 1. *J Biol Chem* 2009, 284:25879–25888
23. Yang S, Pham LK, Liao CP, Frenkel B, Reddi AH, Roy-Burman P: A novel bone morphogenetic protein signaling in heterotypic cell interactions in prostate cancer. *Cancer Res* 2008, 68:198–205
24. Samuel MS, Olson MF: Actomyosin contractility: force power drives tumor growth. *Cell Cycle* 2011, 10:3409–3410
25. Squadrito ML, Pucci F, Magri L, Moi D, Gilfillan GD, Ranghetti A, Casazza A, Mazzone M, Lyle R, Naldini L, De Palma M: miR-511-3p modulates genetic programs of tumor-associated macrophages. *Cell Rep* 2012, 1:141–154
26. Miano JM, Long X, Fujiwara K: Serum response factor: master regulator of the actin cytoskeleton and contractile apparatus. *Am J Physiol Cell Physiol* 2007, 292:C70–C81
27. Teramoto H, Crespo P, Coso OA, Igishi T, Xu N, Gutkind JS: The small GTP-binding protein rho activates c-Jun N-terminal kinases/stress-activated protein kinases in human kidney 293T cells: evidence for a Pak-independent signaling pathway. *J Biol Chem* 1996, 271:25731–25734
28. Anwar KN, Fazal F, Malik AB, Rahman A: RhoA/Rho-associated kinase pathway selectively regulates thrombin-induced intercellular adhesion molecule-1 expression in endothelial cells via activation of I kappa B kinase beta and phosphorylation of RelA/p65. *J Immunol* 2004, 173:6965–6972
29. Benitah SA, Valerón PF, Lacal JC: ROCK and nuclear factor-kappaB-dependent activation of cyclooxygenase-2 by Rho GTPases: effects on tumor growth and therapeutic consequences. *Mol Biol Cell* 2003, 14:3041–3054
30. Schlessinger K, Hall A, Tolwinski N: Wnt signaling pathways meet Rho GTPases. *Genes Dev* 2009, 23:265–277