RalA Function in Dermal Fibroblasts Is Required for the Progression of Squamous Cell Carcinoma of the Skin

Adam G. Sowalsky\textsuperscript{1,2}, Addy Alt-Holland\textsuperscript{3}, Yulia Shamis\textsuperscript{1}, Jonathan A. Garlick\textsuperscript{4}, and Larry A. Feig\textsuperscript{1,2}

Abstract

A large body of evidence has shown that stromal cells play a significant role in determining the fate of neighboring tumor cells through the secretion of various cytokines. How cytokine secretion by stromal cells is regulated in this context is poorly understood. In this study, we used a bioengineered human tissue model of skin squamous cell carcinoma progression to reveal that RalA function in dermal fibroblasts is required for tumor progression of neighboring neoplastic keratinocytes. This conclusion is based on the observations that suppression of RalA expression in dermal fibroblasts blocked tumorigenic keratinocytes from invading into the dermal compartment of engineered tissues and suppressed more advanced tumor progression after these tissues were transplanted onto the dorsum of mice. RalA executes this tumor-promoting function of dermal fibroblasts, at least in part, by mediating hepatocyte growth factor (HGF) secretion through its effector proteins, the Sec5 and Exo84 subunits of the exocyst complex. These findings reveal a new level of HGF regulation and highlight the RalA signaling cascade in dermal fibroblasts as a potential anticancer target. Cancer Res; 71(3): 758–67. ©2010 AACR.

Introduction

Solid tumors consist of oncogenically transformed cells embedded in a tissue microenvironment containing a multitude of additional cell types including fibroblasts, immune cells, and endothelial cells. Recent studies have established that these stromal cells surrounding cancer cells are key mediators in the process of tumor progression (for review, see ref. 1). Furthermore, as the tumor progresses, the surrounding tissue evolves as well in ways that support tumor progression (2). For example, immune cells are recruited to the growing tumor mass and “cancer-associated fibroblasts” with novel properties appear. These stromal constituents secrete factors that act either directly on tumor cells or indirectly, for example, by promoting angiogenesis.

Hepatocyte growth factor (scatter factor, HGF) is a multifunctional cytokine that is secreted by fibroblasts to promote the maintenance of neighboring epithelial cells (3). It acts on epithelial cells through the c-Met receptor to upregulate genes involved in the epithelial-to-mesenchymal transition, a process that is important during development and tissue repair. When deregulated, it can also contribute to early steps in tumorigenesis (4). There is growing evidence that tumor cells can activate stromal cells to stimulate the secretion of HGF, promoting their own tumorigenicity (5). Understanding how stromal cells regulate the secretion of tumorigenic factors such as HGF may reveal new strategies to suppress progression of tumor cells to malignant states by targeting stromal cells.

Ral GTPases, RalA and RalB, are best known for their roles as downstream targets of Ras GTPases (6). Ras binds to and promotes the activation of Ral-specific nucleotide exchange factors (Ral-GEF), which subsequently activate RalA and/or RalB. The resulting active GTP-bound Ral proteins have the capacity to regulate many cellular functions by binding to and altering the activities of a set of effector proteins, including the Sec5 and Exo84 subunits of the exocyst complex (7–10), the CDC42 GTPase-activating protein RalBP-1 (11–13), and the transcription factor ZONAB (14).

Although RalA and RalB are quite similar (>85% identity) and have the potential to activate the same effectors, they actually play remarkably distinct roles in cells, most likely because of distinct subcellular localizations (15, 16) and differences in effector binding efficiency (15). For example, RalA, but not RalB, promotes the delivery of E-cadherin to the basolateral membrane of polarized epithelial cells through the exocyst subunit Exo84 (15), RalA, but not RalB, also uses the exocyst to promote early steps in cytokinesis (17) as well as cell polarity in neurons (18). Endocytosis of AMPA receptors in neurons that induces LTD, an important form of synaptic plasticity, is regulated specifically by RalA through RalBP1 (19). Finally, RalA promotes insulin exocytosis from islet beta cells through the exocyst (20). RalB also has distinct functions,
such as the ability to activate the TBK1 kinase through the exocyst subunit Sec5 to mount an innate immune response (21). It also differs from RalA in its ability to promote the completion of cytokinesis (17).

As downstream effectors of Ras, Ral proteins have been intensively investigated in cancer cells for their contributions to Ras-induced tumorigenesis (22). As expected from their differing normal functions described earlier, RalA and RalB can also play distinct roles in mediating carcinogenesis. For example, RalB activation of TBK-1 through the Sec5 subunit of the exocyst is important for tumor cells to avoid apoptosis (21) whereas RalA function through the exocyst is involved in promoting anchorage-independent growth (22) via integrin-dependent exocytosis of lipid rafts (23). Moreover, RalB seems to be more critical than RalA for metastasis in tail-vein injection assays, although the effecter involved has not been revealed (24). Also, knockdown of RalA, but not RalB, blocked Ral-GEF–induced tumorigenesis in primary epithelial cells (16). Moreover, enhanced RalA and RalB activity correlates with tumorigenicity of pancreatic cells better than enhanced Erk activity (24). Finally, the tumor suppressor PP2A inhibits RalA activation by dephosphorylating its C-terminus (25), implying that a super-active RalA may participate in cancers associated with the loss of PP2A such as those of the lung, breast, and colon.

Interestingly, Ral proteins can also play inhibitory roles in tumorigenesis. For example, knockdown of RalB actually increases Ral-GEF–induced tumorigenesis in primary epithelial cells (16). Moreover, RalA through RalBP-1 suppresses tumor progression via inhibition of translation of the antiapoptotic protein FLIP(s) (26). Finally, we recently reported that RalA, through Exo84, inhibits tumor progression in oncogenic Ras–expressing keratinocytes by promoting delivery of the suppressor of cell invasion E-cadherin to the plasma membrane (27). These examples highlight the fact that Ral GTPases can play distinct functions in different cell types and even a single cell type depending upon which effectors are activated.

In this study, we used a bioengineered human tissue model of Ras-induced squamous cell carcinoma (SCC) to find a new tumor-promoting function for RalA in stromal fibroblasts. RalA, through the exocyst, promotes fibroblast secretion of HGF that is required for tumor progression.

Materials and Methods

Cell lines

HaCaT-II-4, HaCaT-II-4-H-2K4-Ecad, HaCaT-II-4-sh-RalA, MSCC-1-Inv-1, and human foreskin fibroblast (HFF) cells were maintained as previously described (27). Cell lines were tested and authenticated by their ability to generate three-dimensional skin tissues in vitro.

Three-dimensional cell culture

Bioengineered human skin tissues were prepared as previously described (28). Briefly, normal or manipulated human foreskin fibroblasts were mixed with bovine type I collagen (2.5 × 10⁶ cells/mL) and the gels were allowed to contract for 7 days in deep-well polycarbonate tissue culture inserts (Organogenesis). A total of 5 × 10⁵ epithelial cells of the different keratinocyte cell lines mentioned earlier were seeded on top of the contracted collagen gels and grown submerged for 3 days in low-calcium epidermal growth medium. Cultures were then maintained for an additional 2 days in normal calcium epidermal growth medium and subsequently raised to grow at an air–liquid interface for 7 days.

Tissue transplantation

All animal experiments were carried out according to a protocol approved by the Tufts University Institutional Animal Care and Use Committee. Briefly, for each cell line tested, a 1.3-cm dorsal skin section was removed from at least 5 athymic nude mice (Taconic Labs). Bioengineered tissues were grown as described earlier and transplanted onto fascia at the site of skin excision. Bandages were removed after 2 weeks, and mice were sacrificed 4 weeks after transplantation. Skin tumors were excised, fixed in formalin, and processed according to common methods.

Quantification of cell invasion

For each tissue generated, epithelial cells that had invaded into the underlying matrix were counted and averaged from approximately 150 microscope fields (=2 serial sections from ≥2 different depths). Averages shown represent 3 or more independent experiments ± SD. P values, where shown, are calculated by the Mann–Whitney test.

Quantification of tumor volume

Two weeks after transplantation, tumors were photographed every 3 to 4 days. Tumor volume is calculated by the formula

\[ V = \frac{L \times W \times H}{2} \]

Quantification of tumor differentiation

Tumor sections were analyzed by hematoxylin and eosin (H&E) staining as well as K1/K10 immunohistochemistry. The total and differentiated areas of the tumors were measured using Spot Advanced software (Diagnostic Instruments). The ratio of differentiated areas and total tumor area was calculated using the formula

\[ R = \frac{T_D}{T_A}, \]

where \( R \) is the \( K_D/K_A \) positive area and \( T_A \) is the total area of the tissue-containing tumor cells.

RNA interference

Lentiviral particles expressing shRNA against RalA, RalB, Exo84, Sec5, and RalBP-1 were previously described (27). Lentiviral vectors overexpressing shRNA against HGF were purchased from Sigma (TRCN clones 3307, 3309, and 3310) and packaged into lentiviral particles as previously described (27).

ELISA

Reagents for human-specific HGF and interleukin (IL)-6 ELISA were purchased from R&D Systems. A total of 4 × 10⁶ fibroblasts were seeded onto 100-mm tissue culture plates and grown in a 10 mL solution for 2 days. Media was assayed in duplicate from 3 or more independent samples. The HGF
ELISA recognizes both the uncleaved and mature forms of HGF (29).

**Data analysis**

Data represented in graphs were analyzed by the Mann–Whitney or Student t tests, using GraphPad Prism 4.0 for Windows.

**Results**

**Suppression of RalA, but not RalB, expression in dermal fibroblasts blocks tumor progression of neighboring keratinocytes by increasing their E-cadherin expression**

To test the importance of stromal fibroblast function in the progression of cutaneous SCC, we used a well-characterized bioengineered human tissue model that mimics early steps of malignant disease, the loss of cell–cell adhesion, and the acquisition of invasive behavior (30). This organotypic system uses immortalized human keratinocytes (HaCaT cells) grown on top of collagen gels populated with HFFs expressing sh-Scram, sh-RalA, or sh-RalB. Tissue sections were stained with H&E. Bar, 100 μm. Invading cells in representative images from 3 or more independent experiments are indicated with arrows. C, invading E-cadherin-suppressed cells were quantified from more than 100 microscope fields in more than 20 sections from multiple experiments. *, P < 0.01 for invasion of II-4-H-2KΔ-Ecad keratinocytes in tissues composed of sh-RalA HFFs versus sh-Scram HFFs, using Mann–Whitney test. IB, immunoblotting.

To investigate how Ral proteins in dermal fibroblasts influence the progression of skin SCC in tissues, RalA or RalB expression was reduced (~90%) in dermal fibroblasts by stable expression of either RalA (sh-RalA) or RalB (sh-RalB) shRNA (Fig. 1A). These cells along with scrambled control shRNA (sh-Scram) expressing fibroblasts were used to populate the dermal compartment of engineered skin equivalents that contained II-4-H-2KΔ-Ecad keratinocytes in the epidermal compartment. Analysis of these tissues showed that depletion of RalA in the dermal fibroblasts blocked the invasion of keratinocytes as seen by the uninterrupted (or intact) basement membrane interface between the surface epithelium and the underlying fibroblast-populated collagen gel (Fig. 1B, middle). This was in contrast to the invasive pattern seen when keratinocytes were grown on control, fibroblast-populated collagen gels (Fig. 1B, left). In contrast, comparable knockdown of RalB in fibroblasts (Fig. 1B, right) did not block tumor cells invasion. Quantification of invading cells in multiple tissue sections from independent experiments revealed that in tissues populated with RalA knockdown fibroblasts, invasion of II-4-H-2KΔ-Ecad keratinocytes was reduced by ~95% (Fig. 1C). A second sequence to knockdown RalA in fibroblasts was also
tested (Supplementary Fig. 1A), with similar effects on invasion by II-4-H-2Kd-Ecad cells (Supplementary Fig. 1B, middle). As the invasive phenotype of II-4-H-2Kd-Ecad cells has been previously shown to be dependent upon decreased E-cadherin function (32), we analyzed E-cadherin protein expression in these tissues by immunofluorescence. In contrast to tissues with sh-Scram and sh-RalB dermal fibroblasts (Fig. 2A, left and right), in which weak E-cadherin staining of invasive II-4-H-2Kd-Ecad keratinocytes was observed, RalA knockdown in dermal fibroblasts led to strong keratinocyte staining consistent with elevated E-cadherin protein in noninvasive II-4-H-2Kd-Ecad keratinocytes (Fig. 2A, middle) and a more normal-ized tissue architecture (Fig. 1B, middle). This conclusion was confirmed by immunoblotting using the epidermal layer that was peeled from the underlying connective tissue and homogenized (Fig. 2B). Analysis of mRNA levels showed that knockdown of RalA in fibroblasts led to strong keratinocyte staining consistent with elevated E-cadherin protein in noninvasive II-4-H-2Kd-Ecad keratinocytes (Fig. 2A, middle) and a more normalized tissue architecture (Fig. 1B, middle). This conclusion was confirmed by immunoblotting using the epidermal layer that was peeled from the underlying connective tissue and homogenized (Fig. 2B). Analysis of mRNA levels showed that knockdown of RalA in fibroblasts led to an increase in E-cadherin gene expression in neighboring II-4-H-2Kd-Ecad keratinocytes. However, RalA knockdown in dermal fibroblasts led to a vital tumor-promoting function in the early stages of squamous carcinoma progression by contributing to the downregulation of E-cadherin gene in neighboring keratinocytes.

In contrast to this novel tumor-promoting role for RalA in dermal fibroblasts, we recently showed that RalA plays an opposite, tumor-suppressing function in Ras-expressing keratinocytes, as modest inhibition of RalA expression induced an invasive phenotype by reducing E-cadherin stability (27). Thus, we investigated which function of RalA (its tumor-suppressing function in keratinocytes or its tumor-promoting function in dermal fibroblasts) is dominant in this system by engineering tissues with RalA suppressed in both cell types. We observed that the invasive phenotype associated with RalA depletion in II-4 cells (Fig. 3A, compare left and middle) was blocked when RalA was also depleted in the dermal fibroblasts (Fig. 3A, right). sh-RalA fibroblasts also increased E-cadherin levels in neighboring RalA knockdown keratinocytes assayed by immunoblotting of isolated epithelium (Fig. 3C). Thus, the tumor-promoting function of RalA in dermal fibroblasts is dominant in this bioengineered model of skin SCC. Quantification of data is shown in Fig. 3D.

The antitumor properties of RalA knockdown fibroblasts was tested further by populating the epidermal layer of tissues with more advanced tumor cells: an E-cadherin-suppressed, oral SCC cell line (MSCC-1-Inv-1) isolated from a lymph node metastasis (35). Despite having a different and more potent oncogenic background than the genetically defined II-4-H-2Kd-Ecad keratinocytes, the invasive property of these cells was also sensitive to RalA depletion in fibroblasts. MSCC-1-Inv-1 cells grown above sh-Scram fibroblasts showed a robust invasive phenotype (Supplementary Fig. 2A, left), whereas their invasive properties were repressed ~90% when grown above sh-RalA fibroblasts (Supplementary Fig. 2A, right).
above RalA depleted fibroblasts (Supplementary Fig. 2A, right; Supplementary Fig. 2B). As before, inhibition of tumor progression was associated with an increase in E-cadherin levels in tumor cells (Supplementary Fig. 2C).

Finally, to test whether RalA knockdown fibroblasts affect later stages of SCC progression in an in vivo microenvironment, bioengineered tissues were transplanted to the dorsum of nude mice. Four weeks after grafting, tissues with II-4-H-2Kd-Ecad cells grown in combination with sh-Scram control fibroblasts formed large, exophytic tumors with raised, irregular borders (Fig. 4A, top). H&E staining of tumor sections revealed sheets of pleiomorphic cells, with regions of highly infiltrative tumor cells that invaded throughout the underlying stroma (Fig. 4A, top). Ki-67 staining revealed proliferative cells throughout the tumor mass (Fig. 4C, top). Staining for cytokeratins 1 and 10, markers of keratinocyte differentiation, were absent, indicating a poor degree of differentiation and a high-grade tumor cell behavior in the majority (~65%) of tumor area (Fig. 4C, top; Fig. 4D).

In contrast, grafting of tissues constructed with II-4-H-2Kd-Ecad cells grown in combination with RalA knockdown fibroblasts yielded tumors that grew to less than one fifth the size of those formed with tissues composed of control fibroblasts (Fig. 4A, bottom; and Fig. 4B). Analysis of H&E staining revealed well-demarcated tumor islands that showed well-defined borders with the adjacent stroma in tumors derived from tissues engineered with RalA knockdown fibroblasts (Fig. 4A, bottom). These cells also showed a more organized tissue architecture and absence of cellular pleomorphism. Ki-67 staining showed less cell proliferation in these tumors, of which most were confined to the periphery of the tumor islands (Fig. 4C, bottom). Finally, staining for the differentiation marker cytokeratin 1 was found in a significantly larger number of tumor cells in tissues containing RalA knockdown fibroblasts than control fibroblasts, consistent with the presence of more differentiated tumor cells and a low-grade tumor cell behavior (Fig. 4D). Overall, these findings show that tissues composed of RalA knockdown fibroblasts...
generated smaller tumors that contained fewer poorly differentiated tumor cells with a highly aggressive phenotype than tissues that contained control fibroblasts. Thus, RalA function in dermal fibroblasts is necessary for full tumor progression to occur in overlying and adjacent keratinocytes with tumorigenic potential.

**Suppression of exocyst subunits Sec5 and Exo84 in dermal fibroblasts mimics the tumor-blocking effects of RalA knockdown in these cells**

To understand how RalA in dermal fibroblasts promotes the invasive properties of adjacent II-4-H-2K4-Ecad keratinocytes in the epidermal layer, we tested for RalA effectors whose knockdown could mimic the effect of sh-RalA HFF cells. Each of the three most investigated RalA effectors, Exo84, Sec5, and RalBP-1 were individually depleted in fibroblasts (Fig. 5A). Then, each was used to populate the dermal compartment of bioengineered tissues containing II-4-H-2K4-Ecad keratinocytes in the epithelial layer. RalBP-1 knockdown had no detectable effect on tumor cell invasion (Fig. 5B, bottom left) when compared with control tissues (Fig. 5B, top left). In contrast, Sec5- or Exo84-depleted fibroblasts each yielded partial inhibition of invasion (Fig. 5B, top middle and right, and Fig. 5C). When both Sec5 and Exo84 were knocked down in dermal fibroblasts (Fig. 5A), inhibition of invasion (Fig. 5B, bottom middle; Fig. 5C) was comparable with that seen when RalA was suppressed (Fig. 1C). As expected, sh-Exo84/Sec5 HFF cells increased E-cadherin expression at cell–cell junctions in neighboring II-4-H-2K4-Ecad keratinocytes (Fig. 5D) through an increase in E-cadherin mRNA caused by a decrease in Snail and Slug transcription factors (Supplementary Fig. 3A–C). These findings imply that RalA in fibroblasts influences tumor progression, at least in part, through the function of both of its exocyst effectors Sec5 and Exo84.

**Suppression of RalA or its effectors Sec5 and Exo84 in dermal fibroblasts blocks tumor progression by inhibiting HGF secretion**

The exocyst is part of the secretion machinery of the cell (36), suggesting that RalA may influence tumor progression in neighboring keratinocytes with neoplastic potential through the secretion of one or more soluble factor(s). Dermal fibroblasts secrete HGF that influences neighboring keratinocytes (37). For example, HGF downregulates E-cadherin gene expression in keratinocytes as part of a wound-healing process (3). To test whether altered HGF secretion was mediating the effect of RalA knockdown fibroblasts on tumor cell invasion into the underlying matrix, HGF levels were measured by

---

**Figure 4. Suppression of RalA expression in dermal fibroblasts suppresses the growth of E-cadherin-suppressed, Ras-induced tumors in vivo.** A, tissues composed of E-cadherin-suppressed II-4-H-2K4-Ecad cells and HFFs expressing either sh-Scram or sh-RalA were transplanted to the dorsa of immunocompromised mice and tumors were analyzed after 4 weeks. Representative images are shown of H&E staining from tumor sections at 4x and 20x magnifications. Bar, 100 μm. B, tumor volume (L x W x H) measured at the indicated time points following the removal of bandages after tissue transplantation. *, P < 0.05 comparing tumors harboring sh-Scram versus sh-RalA fibroblasts using the Student's t test at days 7, 10, and 14. n = 5 for each cohort. C, representative Ki-67 and K1/K10 immunohistochemistry images of serial sections from the tumors shown in A. Bar, 100 μm. D, percentage of area containing K1/K10-positive, differentiated tumor cells in each group of tumors. *, P < 0.05 comparing tumors harboring sh-Scram HFFs versus sh-RalA HFFs, using the Student's t test.
ELISA from growth media of fibroblasts. In sh-RalA fibroblast cultures, HGF levels were approximately 4-fold lower than those from control fibroblasts (Fig. 6A). In addition, the same RalA effector proteins in fibroblasts that we found contribute to tumor progression in neighboring epithelial cells (see Fig. 5) were also found to be involved in the regulation of HGF secretion. In particular, knockdown of both the Sec5 and Exo84 subunits of the exocyst in HFF cells, but not RalBP1, was required to mimic the effect of RalA depletion on HGF secretion in monolayer cell cultures (Fig. 6A, left).

Most studies have shown that HGF secretion is regulated at the level of gene expression (37). However, this was not the case here, wherein no significant change in HGF precursor mRNA was detected in RalA knockdown fibroblasts (Fig. 6A, right). This is consistent with the known function of the exocyst as a stimulator of the process of protein secretion. However, in this system, RalA does not regulate all protein secretion, as RalA depletion in HFF cells did not suppress the secretion of cytokine IL-6 (Supplementary Fig. 4), nor does it suppress contraction of the collagen gel (Supplementary Fig. 5), which occurs through the secretion of collagenases (38).

We then confirmed that the decrease in HGF secretion by RalA knockdown fibroblasts is responsible, at least in part, for the inhibition of tumor progression by directly reducing HGF secretion to the same degree via stable expression of HGF shRNA in fibroblasts (Fig. 6B). A cell line was chosen that generated culture media with HGF levels similar to that found in RalA-depleted fibroblasts (compare Fig. 6B, left, with Fig. 6A, left). When these HGF knockdown fibroblasts were used to populate the dermis of engineered tissues (Fig. 6C, top), E-cadherin expression in II-4-H-2Kd-Ecad keratinocytes increased (Fig. 6C, bottom) and their invasive properties decreased to...
levels comparable with those found in the tissues populated with RalA-depleted fibroblasts (compare Fig. 6D, right with Fig. 1C). Specificity of shRNA against HGF in reducing the invasive properties of II-4-H-2Kd-Ecad keratinocytes was confirmed by supplementing growth media with recombinant human HGF (rh-HGF; Fig. 6D). Supplementation of tissues harboring sh-HGF fibroblasts with rh-HGF to produce phosphorylation of c-Met, the HGF receptor, in II-4-H-2Kd-Ecad keratinocytes decreased E-cadherin levels (left) and increased invasion (right) to levels comparable with tissues grown with sh-Scram fibroblasts. Thus, RalA and the exocyst function in dermal fibroblasts to mediate the secretion of HGF, which is necessary for tumor cell invasion in this bioengineered model of skin SCC.

Discussion

This study reveals a key role for the RalA GTPase in how stromal cells support carcinoma development. Using a bioen-
engineered human tissue model of the early steps in skin SCC, we show that RalA in dermal fibroblasts promotes tumor progression in epithelial cells of the neighboring epidermis through its regulation of HGF secretion. This conclusion is based on the observation that shRNA-mediated suppression of RalA, but not RalB, expression in dermal fibroblasts blocked tumorigenic keratinocytes from invading into the dermal layer of engineered tissues. Strikingly, it also prevented metastatic oral SCC cells from invading in this system despite their having a far more transformed oncogenic background.

Knockdown of RalA expression in dermal fibroblasts also suppressed more advanced tumor progression after these tissues were transplanted onto the dorsa of mice. Suppression of tumor progression in vivo is striking in light of the fact that once the engineered human tissues are transplanted, normal mouse stromal cells are likely to participate in promoting tumor progression. Knockdown of RalA in fibroblasts suppressed HGF secretion by approximately 4-fold. This degree of HGF inhibition was significant because tumor progression was also blocked when HGF secretion was inhibited to a similar degree by expression of HGF shRNA. Moreover, we found that tumor suppression induced by RalA depletion in fibroblasts was associated with changes in neighboring keratinocytes that are known to occur when HGF levels are reduced, such as an increase in the expression of E-cadherin and a decrease in the expression of Snail and Slug, transcription factors that suppress E-cadherin RNA levels.

HGF plays an important role in dermal fibroblast support of epidermal function. Its secretion by fibroblasts is enhanced during specific stages of development and in response to wounding (3). In these cases and in most other examples of increased HGF release, regulation occurs at the level of increased gene expression (39). In contrast, we show here that HGF mRNA levels are not altered in RalA knockdown fibroblasts, suggesting that regulation of cytokine secretion also occurs at the level of the secretion process in dermal fibroblasts. This is consistent with our results implying that RalA functions through Sec5 and Exo84, the two Ral effectors that are subunits of the exocyst, a regulator of the process of exocytosis and cellular secretion (36). Thus, this study reveals a new level of HGF regulation in stromal fibroblasts that is mediated by RalA, Exo84, and Sec5.

The role of RalA in regulating HGF secretion in fibroblasts may have additional effects on tumor progression, as HGF is also known to promote angiogenesis (40). Thus, future studies using experimental model systems that detect the contribution of angiogenesis to tumor progression may be revealing. Moreover, HGF is known to support tumor progression in other tumor systems such as breast cancer and colon cancer (41, 42), suggesting that RalA in stromal cells may play a tumor-supporting role in many types of cancer. Finally, although the addition of HGF to the media could restore the invasive properties of tumorigenic keratinocytes grown in tissues populated with HGF knockdown dermal fibroblasts, it did not do the same in comparable tissues populated with RalA knockdown fibroblasts. Thus, although HGF secretion induced by RalA in dermal fibroblast RalA is required for SCC tumor progression, it is not sufficient. Experiments are underway to identify the missing RalA-induced, SCC-promoting factor(s).

The tumor-promoting role of RalA in stromal fibroblasts found in this study dramatically contrasts with the tumor-suppressing function of RalA in Ras-expressing keratinocytes found in our previous study that used this same engineered human tissue model of SCC (27). In that study, RalA functioned as a tumor suppressor through its ability to stimulate E-cadherin delivery to the plasma membrane. One possible explanation for these two opposing roles for RalA stems from results from our previous study showing that RalA functioned as a tumor suppressor in Ras-expressing keratinocytes through Exo84, but not Sec5, whereas in this study, RalA functions as a tumor promoter in fibroblasts through both exocyst subunits. Thus, RalA plays opposing roles in two cell types that populate skin tissue by functioning through different sets of effectors.

Interestingly, when tissues were engineered with RalA suppressed in both fibroblasts and Ras-expressing keratinocytes, no invading keratinocytes were observed. This indicates that the tumor-suppressing effect of knocking down RalA in fibroblasts was dominant over the tumor-promoting effect of knocking down RalA in Ras-expressing keratinocytes. In a previous study on mice with functional inactivation of the gene for the Ral activator RalGDS (43), induction of SCC of the skin by the application of tumor promoters that lead to Ras activation was severely suppressed (43). It was assumed that this phenotype was a consequence of the effects of RalGDS loss in keratinocytes. However, the experiments described in this study suggest that this suppression of tumor progression may have also been due to depletion of RalGDS in dermal fibroblasts.

Finally, a variety of previous studies have highlighted how RalA signaling in tumor cells may be an attractive anticancer target (44). Our study suggests that targeting the RalA/exocyst signaling cascade that regulates HGF secretion in stromal cells may complement this approach, with the benefit that, unlike tumor cells, fibroblasts are genetically stable with a low propensity for drug resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Y. Szwec-Levin, T. Des Rochers, C. Romhanka, H. Hatch, A. Maoine, X. Tian, and S. Dong for excellent technical assistance. We thank N. Fusseneg for HaCaT cells and their derivatives and F. Watt for the H-2Kd-Ecad vectors.

Grant Support

This work was supported by grants to L.A. Feig from NIGMS and to J.A. Garlick from NIDCR. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 28, 2010; revised November 24, 2010; accepted November 29, 2010. Published OnlineFirst December 15, 2010.
References

RaLA Function in Dermal Fibroblasts Is Required for the Progression of Squamous Cell Carcinoma of the Skin

Adam G. Sowalsky, Addy Alt-Holland, Yulia Shamis, et al.