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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Snail in Fibroblast Activation in Cancer

A Thesis submitted in partial satisfaction of the requirements for the degree of
Master of Science

in

Biology

by

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2012

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2012

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ABSTRACT OF THE THESIS

The Role of Snail in Fibroblast Activation in Cancer

by

Nimesh Rajakumar

Master of Science in Biology

University of California, San Diego, 2012

Professor Shyni Vaghese, Chair
Professor Douglass Forbes, Co-Chair

Cancer has classically been understood as the malignant growth of cells caused by the gradual acquisition of mutations that allow these cells to avoid normal checks on cell growth, division, and death. However, new findings are showing that

the tumor microenvironment also plays a crucial role in the promotion of cancer growth by regulating factors such as angiogenesis, the immune response, and the extracellular matrix. One of the key mediators of these factors is the fibroblast. In the presence of tumors, fibroblasts gain an activated phenotype, which allows them to stimulate angiogenesis, the immune response, and ECM remodeling. In addition, many carcinomas express the transcription factor Snail, and our lab has shown that noncancerous Snail transgenic keratinocytes are sufficient to activate dermal fibroblasts. Considering these factors, it is possible that Snail-expressing cancer cells cause the activation of surrounding fibroblasts, which allows the fibroblast to create a microenvironment conducive to tumor growth. To test this hypothesis, various cancer cells were screened for Snail expression by RT-PCR, Western blot, and immunofluorescence. Conditioned media from cell cultures was then added to fibroblasts, which were assayed for activation by their ability to contract collagen, proliferate, and express the activation marker α -SMA to test whether fibroblast activation could be correlated with Snail expression. Snail expression could not be correlated with collagen contraction, but it could be correlated with proliferation, suggesting that Snail expression could be a factor in activation but not be entirely sufficient for total activation.

Introduction

Tumor growth has classically been understood as the malignant growth of cancer cells due to gradual acquisitions of genetic mutations that allow these cells to avoid normal checks on cell growth, division, and death (Hanahan 2011). As such, many cancer therapies work by attempting to directly destroy cancer cells by methods such as boosting immune recognition of cancer cells, introducing oncolytic vectors to these cells, and gene therapy (Cross 2006). However, the past decade of cancer research has shown that in addition to the intrinsic properties of cancer cells, the properties of the surrounding microenvironment of a tumor also play a crucial role in tumor growth and progression (Hanahan 2011).

Tumor growth seems to be favored by the creation of a tumor stroma that creates an environment conducive to tumor growth. Characteristics of this environment include increased activity of endothelial cells promoting angiogenesis to deliver nutrients to tumors; misregulation of pericytes that normally maintain vascular integrity; and the mounting of an immune response that promotes angiogenic, epithelial, and stromal growth in its attempt to heal what it perceives as a wound (Hanahan 2011).

The common factor involved in many cases of the cancer-associated remodeling of stromal cells is the fibroblast. Fibroblasts are involved in the maintenance of the extracellular matrix (ECM), the major component of stroma, and play a critical role in wound-healing in the skin. It has been found that cancer-associated fibroblasts remain perpetually activated in the tumor stroma, leading to excessive deposition of extracellular matrix around a tumor, a condition that can lead

to organ fibrosis (Kalluri 2006). Since the stroma plays such a crucial role in tumor progression, elucidating the mechanism by which fibroblasts become activated around cancer cells could lead to additional therapeutic targets in the treatment of cancer.

Skin was determined to be the most appropriate model system to study the relationship between fibroblasts and cancer due to the high frequency of skin cancer and the prevalence of fibroblasts in the skin. In addition, fibroblasts are compartmentalized from the other layers of skin, making them easy to extract, and depending on the condition of the skin, the constituent fibroblasts may be in either their inactivated state or their activated state.

Fibroblasts activation plays a crucial role in wound-healing

The two major layers of the skin are the epidermis, which is the outermost layer, and the dermis, which lies just below the epidermis. The epidermis is primarily composed of epithelial cells called keratinocytes, which function to provide a barrier against the outside environment and respond to pathogenic invasion by releasing compounds that activate specific arms of the immune system. The dermis, on the other hand, is mostly composed of the ECM, which interacts with cells to influence adhesion, proliferation, and migration. The dermis is where fibroblasts in the skin are located.

Depending on the state of the tissue they are located in, fibroblasts can take the form of one of three states. In normal healthy tissues, fibroblasts remain in a relatively quiescent state. These fibroblasts function to maintain the homeostasis of the ECM by either depositing ECM constituents, like collagen and fibronectin, or by secreting ECM-degrading proteins, like matrix metalloproteinases (MMPs) (Kalluri

2006). However, in the presence of a wound fibroblasts take on an activated state. In early wounds, fibroblasts change their morphology and increase their rate of ECM deposition. These fibroblasts are termed proto-myofibroblasts. In the later stages of wound-healing, proto-myofibroblasts take on more smooth muscle cell-like features and are subsequently termed differentiated myofibroblasts (Tomasek 2002).

The specific localization of fibroblasts in the dermis is what allows fibroblasts to play their role in wound healing. In healthy tissue, ECM serves to protect its resident cells from the tensile stresses of the tissue. When a wound creates an opening in the skin, this protection is gone, and the subsequent increase in extracellular tension on fibroblasts causes them to change their morphology and express stress fibers (Hinz 2001). The resulting proto-myofibroblasts move into the wound to deposit ECM. The new ECM forms the granulation tissue of the wound, providing an environment conducive to cell growth to aid in the repair of the wound (Tomasek 2002).

Additionally, proto-myofibroblasts come into contact with a flood of cytokines and growth factors released by the damaged cells at the site of the wound. Some of these factors, such as TGF- β 1, stimulate proto-myofibroblasts to express α -smooth muscle actin and become differentiated myofibroblasts. The accumulation of stress fibers and actin elements give myofibroblasts the ability to generate a contractile force that allows them to constrict the wound and eventually close it up (Vaughan 2000).

Under normal circumstances, once a wound is healed, the number of myofibroblasts decreases, possibly by reverting back to a resting phenotype or through apoptosis. However, in abnormal cases myofibroblasts will remain active

even after wound healing has completed. One result of this is an excessive accumulation of ECM around the site of the former wound, a condition known as fibrosis (Kalluri 2006). Fibrosis can cause disruption of the tissue architecture and function and eventually lead to organ failure. The resultant increase in interstitial fluid pressure can also hinder macromolecule and oxygen diffusion, leading to cell necrosis. In addition, the continued contractile forces exerted by the myofibroblasts results in a condition of permanent contraction of the scar tissue called contracture (Tomasek 2002).

Curiously, numerous clinical observations have shown that the development of cancer is frequently found in tissues that have suffered chronic inflammatory disease (Schafer 2008). These observations lead physician Harold Dvorak to the idea that “tumors are wounds that do not heal” (Dvorak 1986). Many of the events that occur in wound healing also occur around the sites of tumors, albeit in a far less controlled manner. Large depositions of ECM, a sustained inflammatory response, and angiogenesis are all events that not only occur in the presence of tumors as well as wound-healing, but also occur at much higher and more sustained levels in their tumor counterparts (Schafer 2008).

Cancer acts like a wound that does not heal

The presence of large numbers of fibroblasts and myofibroblasts, as seen in wound-healing, in particular, is a hallmark of epithelial cancers, known as carcinomas (Schafer 2008) and plays a role in their growth, maintenance, and invasiveness. The composition of the tumor stroma actually resembles the granulation tissue of healing skin wounds (Dvorak 1986). Additionally myofibroblasts have been shown to remodel

the ECM in such a way to enable the invasive properties of squamous cell carcinomas (Gaggioli 2007).

Fibroblasts found in the stroma of cancer cells are referred to as cancer-associated fibroblasts (CAFs), and like fibroblasts involved in wound healing, they acquire modified functional characteristics and phenotypes, including deposition of ECM and expression of α -smooth muscle actin (Kalluri 2006). CAFs have been shown to play crucial roles in every step of cancer growth from initiation to progression to metastasis.

In terms of initiation, a series of studies comparing the effects of normal fibroblasts to those of CAFs have shown that CAFs are sufficient to induce tumorigenesis in epithelial cells. When immortalized prostate epithelial cells were grafted onto mice in combination with either normal fibroblasts or CAFs, tumors formed only in the mice with CAFs (Olumi 1999).

In terms of tumor progression, human breast cancer cells showed increased growth when coinjected with CAFs into mice compared to coinjection with normal fibroblasts (Orimo 2005). Furthermore, activated fibroblasts increase their deposition of MMPs, which can cleave the extracellular domains off membrane proteins required for adhesion (Lochter 1997) or off protease-activated receptors (PARs), leading to cancer cell invasiveness (Boire 2005).

However, it is still unclear why CAFs appear at the sites of tumors. On the one hand pro-fibrotic growth factors, like TGF- β and fibroblast growth factor 2 (Elenbaas 2001), released by cancer cells could be inducing local CAF invasion. On the other hand CAF invasion could be a response similar to that found in wound-healing in an attempt to contain the cancer (Kalluri 2006).

Another issue is determining the source of CAFs. One suggestion is that epithelial-to-mesenchymal transition (EMT), in which epithelial cells lose cell-cell contacts and acquire mesenchymal properties, occurs in epithelial cells surrounding the cancer cells (Kalluri 2003). It has already been shown that up to 30% of activated fibroblasts in kidney fibrosis originate from EMT of tubular epithelial cells (Iwano 2002). Since such a phenomenon is already well-established as a source of fibroblasts in tissue fibrosis, it is possible that this could happen in cancers as well considering the similarities between the two diseases. However, the mechanism by which this occurs has yet to be determined.

The transcription factor Snail could link cancer and fibroblast activation

One common factor between EMT and many types of carcinomas is the expression of the transcription factor Snail (Du 2010). Snail is expressed in a wide range of carcinomas (Becker 2007) and has a well-established role as a mediator of EMT during embryogenesis (Nieto 2002). As such, Snail could prove to be a vital mediator between carcinomas and their CAFs.

Snail was originally discovered as a transcriptional repressor that is necessary for the proper formation of the mesoderm during embryonic development (Alberga 1991; LaBonne 2000). It carries out this process by repressing transcription of neuroectodermal genes, thereby forming the mesoderm by, at least in part, excluding alternative cell fates (Nieto 2002). Further research has shown that in addition to Snail, Snail homologues also play critical roles in large-scale cell movements, such as the formation of the neural crest (Nieto 1994).

Snail's role in promoting cell movement has recently been expanded to EMT in general. Snail induces EMT by repressing E-cadherin transcription, which mediates

the cell-cell adhesion necessary to form epithelial tissues (Cano 2000). Additionally, Snail expression is associated with a gain of mesenchymal characteristics, allowing the former epithelial cells greater motility within the tissue. E-cadherin-negative mesodermal cells, for example, originate from E-cadherin-positive epithelial cells during mesoderm formation (Damjanov 1986).

The expression of Snail in many carcinomas has also been inversely correlated with E-cadherin expression; the resulting loss of cell-cell contacts and potential increased gain in motility suggests not only a mechanism by which carcinomas become invasive (Batlle 2000) but also a source of CAFs (Kalluri 2006). However, other studies have shown that Snail expression can recapitulate early features of metastasis without inducing a complete EMT (Du 2010). Instead, invasiveness is caused by the recruitment and activity of macrophages and $\gamma\delta$ T cells, which prime the skin for the formation and metastasis of tumors (Du 2010). Indeed, there is a lack of definitive evidence of carcinoma cells undergoing EMT *in vivo* (Thompson 2005).

However, our lab has shown that Snail-transgenic mouse skin, where keratinocytes perpetually overexpress Snail, has activated fibroblasts in the dermis. It was then shown that conditioned media collected from Snail-transgenic keratinocyte cultures increase α -smooth muscle actin and connective tissue growth factor expression in fibroblasts, signaling their activation. Additionally, lineage tracing showed that these activated fibroblasts did not originate from the epidermis, ruling out the possibility that they appeared from an EMT. This suggests that Snail-expressing keratinocytes secrete a compound in the epidermis that makes its way to the dermis to activate fibroblasts.

Snail, PTHrP, and Fibroblasts

A screen of proteins secreted by Snail-expressing keratinocytes showed that one of the proteins whose secretion is increased compared to that of wild type keratinocytes is parathyroid hormone related protein (PTHrP). PTHrP is a protein that shares structural similarities with parathyroid hormone, and in addition to having the ability to activate parathyroid hormone receptor, it also plays a physiological role in many different tissues, including epithelia, mesenchyme, endocrine glands and the central nervous system, exemplifying the wide variety of roles PTHrP has in the body (Strewler 2000).

PTHrP was first discovered in the 1980's as a tumor-secreted factor that causes hypercalcemia in cases of bone metastasis (Kremer 2011). Since then many studies have shown that high PTHrP expression is common among many carcinomas, including prostatic, lung, and colorectal carcinomas, independent of hypercalcemia (Kremer 2011). However, although many studies have shown that there is a positive correlation between PTHrP expression and cancer progression, almost as many studies have shown a negative correlation between the two as well (Nishihara 2007). As such, the role of PTHrP in cancer development has yet to be elucidated.

Putting these ideas together, though, could lead to a mechanism by which carcinoma cells activate fibroblasts. If Snail-expressing keratinocytes can activate fibroblasts through a secreted factor, and one of the factors that shows increased secretion in such keratinocytes is PTHrP, then it is possible that Snail-expressing carcinoma cells secrete PTHrP in levels that activate fibroblasts and subsequently creates a microenvironment that is conducive to tumor growth.

To show this, both cancerous and non-cancerous keratinocytes will first be assayed for Snail expression. Snail expression can be assayed by RT-PCR, Western blot, and immunofluorescence. Once Snail expression is assayed among the various cell lines, conditioned media will be taken from cell cultures and added to fibroblasts, which will then be assayed for activation. The three activation assays will be a collagen contraction assay, a proliferation assay, and a gene expression assay for α -SMA.

Materials & Methods

Cell line selection and cultivation

The squamous cell carcinoma lines A3886 and SCL-1, along with the immortal human keratinocyte line HaCaT, were cultured in DMEM containing pyruvate with the addition of 10% fetal bovine serum and penicillin and streptomycin. The squamous cell carcinoma line COLO-16 was cultured in RPMI 1640 with the addition of 10% fetal bovine serum and penicillin and streptomycin. 3T3J2 feeder cells were cultured with pyruvate-free DMEM with the addition of 10% bovine calf serum and penicillin and streptomycin. Primary human keratinocytes were obtained from Columbia University Skin Research Center and were cultivated on 3T3J2 feeder cells with EpiLife media (M-EPI-500-CA, Gibco, Grand Island, NY) with the addition of human keratinocyte growth supplement (S0015, Gibco, Grand Island, NY) and penicillin and streptomycin. All cells were cultured in 5% CO₂ at 37°C.

RNA extraction and synthesis of cDNA

Cell cultures were grown to ~80% confluency and collected in TRIzol reagent (15596-026, Life Technologies, Grand Island, NY). 1mL TRIzol was used for a 10cm dish. 500µl TRIzol was used for each well of a 24-well plate. RNA was extracted from each sample and quantified using a spectrophotometer. Synthesis of cDNA was subsequently performed using iScript cDNA synthesis kit according to manufacturer's protocol (170-8890, Bio-Rad, Hercules, CA).

Western blot

Cell cultures were grown to ~80% confluency and collected in sample buffer with RIPA buffer (150mM NaCl, 1M Tris pH 7.2, 10% SDS, Triton X-100,

Deoxycholate, 0.5M EDTA) and 2-mercaptoethanol. Whole cell lysates were run on a 15% polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane at 200mA for two hours. Membranes were blocked with 3% non-fat milk for 30 minutes at room temperature and probed with primary antibodies overnight at 4°C. Primary anti-SNAI1 antibodies screened included #AP2054a, Abgent, San Diego, CA; G-7 #sc-271977, Santa Cruz Biotechnology, Santa Cruz, CA; H-130 sc-28199, Santa Cruz Biotechnology, Santa Cruz, CA; UC71 and UC66, lab-derived. Membranes were probed with secondary antibodies for 20 minutes at room temperature. Secondary antibodies include Chemiluminescence was elicited by ECL solution (90mM p-coumaric acid, 250mM Luminol, 1M Tris pH 8.5) with the addition of a small amount of hydrogen peroxide.

Immunofluorescence

Glass coverslips were placed in 10cm dishes, which were then used to grow cell cultures of SCL-1, COLO-16, A3886, HaCaT, and primary human keratinocytes. Upon ~80% confluency, these coverslips were fixed with 40% formaldehyde, and blocked with IF block for 30 minutes at room temperature. Samples were incubated in primary antibody for 45 minutes at room temperature. Primary antibodies include the same Snail antibodies as mentioned for Western blot and anti- α -SMA (a2547, Sigma-Aldrich, St. Louis, MO). Samples were incubated with secondary antibodies for 20 minutes at room temperature. Secondary antibodies include rhodamine phalloiden (R415, Invitrogen, Grand Island, NY), Alexa Fluor 488 goat anti-rabbit (A11008, Invitrogen, Grand Island, NY), and Alexa Fluor 488 donkey anti-mouse (A21202, Invitrogen, Grand Island, NY). 3T3J2 cells that were grown on PDMS-coated 24-well

plates were fixed, blocked, and stained on the PDMS surface in the well under the same conditions.

Conditioned media experiments

Conditioned media was collected after 16 hours of culture with COLO-16, SCL-1, A3886, HaCaT, and primary human keratinocytes. Conditioned media was centrifuged at 1500 rpm for 5 minutes and added directly to fibroblasts for their respective assays. If serum-free media was to be collected, cells were grown in serum with 10% FBS until 16 hours before media was to be collected at which point media was replaced with serum-free media. Serum-free media was prepared exactly the same as normal growth media but without the addition of serum. All conditioned media experiments were with media containing serum unless otherwise noted.

Collagen contraction assay

To create collagen plugs, collagen I was diluted with 0.1% acetic acid to 3mg/ml and 3T3J2 fibroblasts were diluted to 1.5×10^5 cells/ml. Collagen I, 3T3J2 fibroblasts, and NaOH were combined in proportions of 200 μ l, 400 μ l, and 4 μ l, respectively. 500 μ l aliquots were added to each well of a 24-well plate. The gels were allowed to solidify for 20 minutes. Conditioned media from COLO-16, A3886, SCL-1, HaCaT, and human epidermal keratinocytes was added to gels, which were incubated in 5% CO₂ at 37°C for 5 to 7 days.

Proliferation assay

3T3J2 cells were grown and 100 μ l aliquots containing 3,000 cells were added to each well of a 96-well plate. Cells were allowed to incubate for 24 hours before being treated with conditioned media. Proliferation was assayed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (G3582, Promega, Madison, WI)

according to manufacturer's protocol. Cells were plated every day for 4 days, and conditioned media was added 24 hours after plating. On the fifth day, all media was replaced with serum-free growth media for one hour, cells were incubated in CellTiter reagent for one hour, and absorbance readings were subsequently taken.

Generation of PDMS base

The Sylgard 184 silicone elastomer base (3097366-1004, Dow Corning, Midland, MI) and curing agent (3097358-1004, Dow Corning, Midland, MI) were combined in a ratio of 50:1, respectively, for a stiffness of 150kPa. After thorough agitation, 100 μ l was added to each well of a 24-well plate and the plate was degassed. The plate was then cured at 80°C overnight. For cell seeding, collagen I was attached to the PDMS surface using sulfo-SANPAH as a linker. Each well had 500 μ l of a 0.2mg/ml solution of sulfo-SANPAH in milli-Q water added to it. The plate was then irradiated for 15 minutes using 365nm UV light. The solution was removed and sulfo-SANPAH was added and irradiated once more for 30 minutes. The wells were then washed with 50mM HEPES in PBS twice followed by coating with 400 μ l of 50 μ g/mL collagen I in PBS overnight. The fibroblast cell line 3T3J2 was cultured on these specially coated wells to minimize tensile stresses.

cAMP Assay

3T3J2 cells were grown and 100 μ l aliquots containing 3,000 cells were added to each well of a 96-well plate. Cells were allowed to incubate for 24 hours before being treated with serum-free conditioned media from all cell cultures. cAMP levels were measured using the cAMP-Glo (V1501, Promega, Madison, WI) kit according to manufacturer's protocol.

Results

Identification of Snail-Expressing Cell Types

Our lab has shown that Snail transgenic skin in mouse can activate α -smooth muscle actin expression in fibroblasts through the secretion of some factor that makes its way to the dermis. In addition, numerous human carcinomas also express Snail (Becker 2007). To see if Snail-expressing carcinomas also secrete a factor that can activate α -smooth muscle actin, Snail expression was first assayed by RT-PCR.

RNA was extracted from COLO-16, SCL-1, A3886, HaCaT, and primary human keratinocyte cells for reverse transcription into cDNA that could be amplified using PCR. The absorbance readings at 260nm and 280nm were taken of all RNA samples to quantitate RNA concentration and purity. Since all A260/A280 ratios were between 1.8 and 2.1, all RNA samples could be considered pure enough for cDNA synthesis (Table 1).

Table 1: RNA Concentration and Purity

Sample	ng/ μ l	A260/A280
HaCaT (+ serum)	678.5	1.97
HaCaT (- serum)	428.4	2.03
COLO-16 (+ serum)	112.6	1.93
COLO-16 (- serum)	144.3	1.86
SCL-1 (+ serum)	398.3	2.04
SCL-1 (- serum)	274.5	2.03
A3886 (+ serum)	1,361	2.02
A3886 (- serum)	582.8	2.03

Various primers were screened for identification of Snail transcription (Table 2). The first set of primers yielded many nonspecific bands (Fig. 1A) under initial PCR conditions. PCR was performed with only the SCL culture grown in serum-free conditions under a range of annealing temperatures to optimize the PCR product.

None of the temperatures used were able to give a specific band (Fig. 1B). A screen of three more primers was done, with expected band sizes of 134, 106, and 129, respectively (Table 2). The 134-base pair primer did not show the strong bands that the other two primers showed, so it was ruled out (Fig. 1C). Performing PCR with the 129-base pair primer yielded additional nonspecific bands as well, most prominently seen in lane 3 with COLO-16 grown with serum (Fig. 1D). Performing PCR with the 106-base pair primer, however, yielded single, discrete bands.

Table 2: Snail Primers Tested

Primer Set	Direction	Sequence (5'→ 3')	Expected Band Size
1	Forward	TTCTTCTGCGCTACTGCTGCG	883
	Reverse	GGGCAGGTATGGAGAGGAAGA	
2	Forward	CATCTGAGTGGGTCTGGAGG	134
	Reverse	CTTCTCTAGGCCCTGGCTG	
3	Forward	AGGTTGGAGCGGTCAGC	106
	Reverse	CCTTCTCTAGGCCCTGGCT	
4	Forward	TCTGAGTGGGTCTGGAGGTG	129
	Reverse	CTCTAGGCCCTGGCTGCTAC	

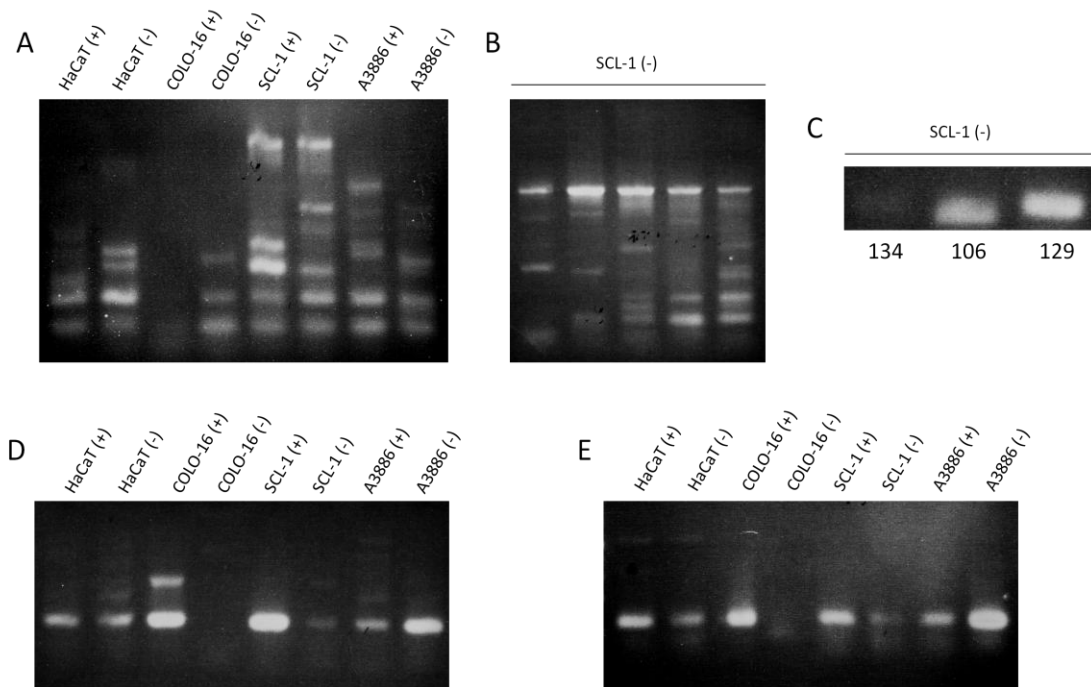


Figure 1. Initial primer screen for Snail. (A) PCR was conducted on HaCaT, COLO-16, SCL-1, and A3886 cells incubated with (+) and without (-) serum for 16 hours with primer set 1, yielding multiple nonspecific bands. (B) PCR was conducted on SCL-1 cells grown without serum for 16 hours under a range of annealing temperatures for optimization. Nonspecific banding still occurred. (C) PCR was performed with three different primers on SCL-1 cells cultured in serum free media. The primer that gave a 134-base pair product did not show a band. (D) Using the 129-base pair primer, PCR was performed on HaCaT, COLO-16, SCL-1, and A3886 cells incubated with serum (+) and without (-) for 16 hours, but nonspecific banding occurred in lanes 2, 3, 6, and 7. (E) PCR was performed on HaCaT, COLO-16, SCL-1, and A3886 cells incubated with (+) and without (-) serum for 16 hours, and single discrete bands appeared in all lanes.

After optimizing the PCR conditions, the primer that gave a 106-base pair product showed low transcriptional levels of Snail in HaCaT and A3886 cells and high transcriptional levels of Snail in COLO-16 and SCL-1 cells (Fig. 2). Additionally, primary human keratinocytes showed an intermediate level of Snail transcription. Of note is the increased Snail expression in A3886 and SCL-1 cells cultured without serum for 16 hours. This could be a stress response to the serum-free conditions.

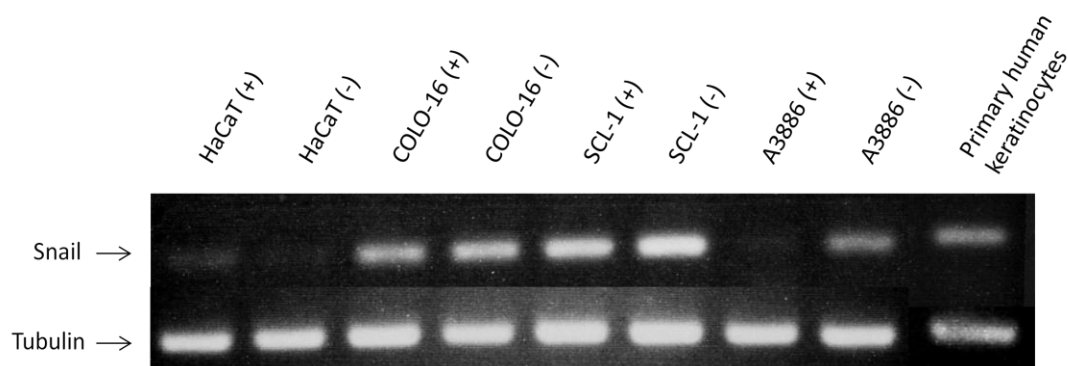


Figure 2. PCR of Snail using 106-base pair primer. PCR was performed on HaCaT, COLO-16, SCL-1, and A3886 cells cultured both with (+) and without (-) serum for 16 hours, as well as primary human keratinocytes grown in EpiLife. Of note is an increased expression level of Snail in A3886 and SCL-1 cells grown in serum-free conditions.

The Snail protein is a highly unstable protein (Wu 2010). In the cytoplasm it is quickly phosphorylated by glycogen synthase kinase-3 beta (GSK3 β), which marks it for proteosome degradation, giving it a half-life of only about 25 minutes (Zhou 2004). As such, the presence of the Snail protein was verified by Western blot. A screen of various antibodies was used to prove samples for Snail expression.

Probing for Snail with most antibodies resulted in either nonspecific binding of antibodies or no binding of antibodies. The Abgent anti-Snail antibody gave preliminary results to confirm Snail expression in HaCaT, COLO-16, SCL-1, and A3886 cell lines, although bands seemed to bleed over into other lanes, leaving the data inconclusive (Fig. 3). Of note is the size at which human Snail protein ran compared to the size of the positive control, which was purified Snail protein derived from mouse. The positive control ran at the known size of Snail, 29kDa. The human Snail protein samples, however, ran at 45kDa.

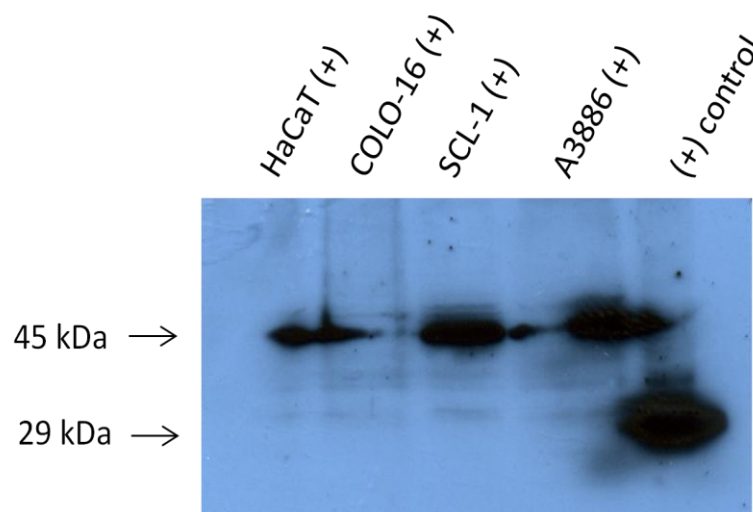


Figure 3. Western blot of Snail using UC71 antibody. Western blot was performed on HaCaT, COLO-16, SCL-1, and A3886 cells grown 10% FBS media and probed with anti-Snail antibody from Abgent. A band for the positive control appeared at the expected size of 29kDa. Bands from the samples, however, appeared at 45kDa. This could be due to post-translational modifications that occur in human cells but not in mouse cells.

The only antibody that gave discrete bands upon probing was the H-130 anti-Snail antibody (Fig. 4). This antibody showed expression levels of Snail protein that followed a similar expression pattern of Snail transcription, with HaCaT and A3886 expressing low amounts of Snail and COLO-16 and SCL-1 expressing the highest amount of Snail protein.

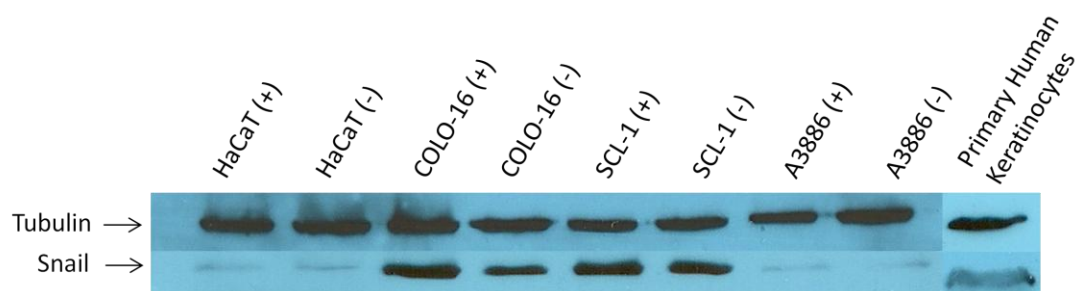


Figure 4. Western blot of Snail expression using H-130 antibody. Western blot was performed on HaCaT, COLO-16, SCL-1, A3886 and primary human keratinocyte cells cultured

both with (+) and without (-) serum for 16 hours. β -tubulin was used as the 55kDa loading control.

The activity of Snail is also regulated by its intracellular location. Being a transcription factor, Snail would only be playing its role as a regulator of gene expression when it is localized in the nucleus (Yamashita 2004), and its subcellular localization is tightly controlled: phosphorylation of Snail by p21-activated kinase 1 (PAK1) targets Snail to the nucleus (Yang 2005), while phosphorylation of Snail by GSK3 β targets Snail to the cytoplasm (Zhou 2004). To confirm nuclear localization of Snail in these cell lines, immunofluorescent staining was performed using the UC71, Abgent-provided, and H-130 antibodies that were used in Western blot (Table 4). The UC71 antibody showed nuclear staining in SCL-1 and COLO-16 cells. Staining in A3886 cells occurred in both the nucleus and the cytoplasm. There was no nuclear staining of Snail in HaCaT (Fig. 5A-D). The Abgent antibody showed nuclear staining in COLO-16, SCL-1, A3886 and HaCaT cells (Fig. 5A'-D').

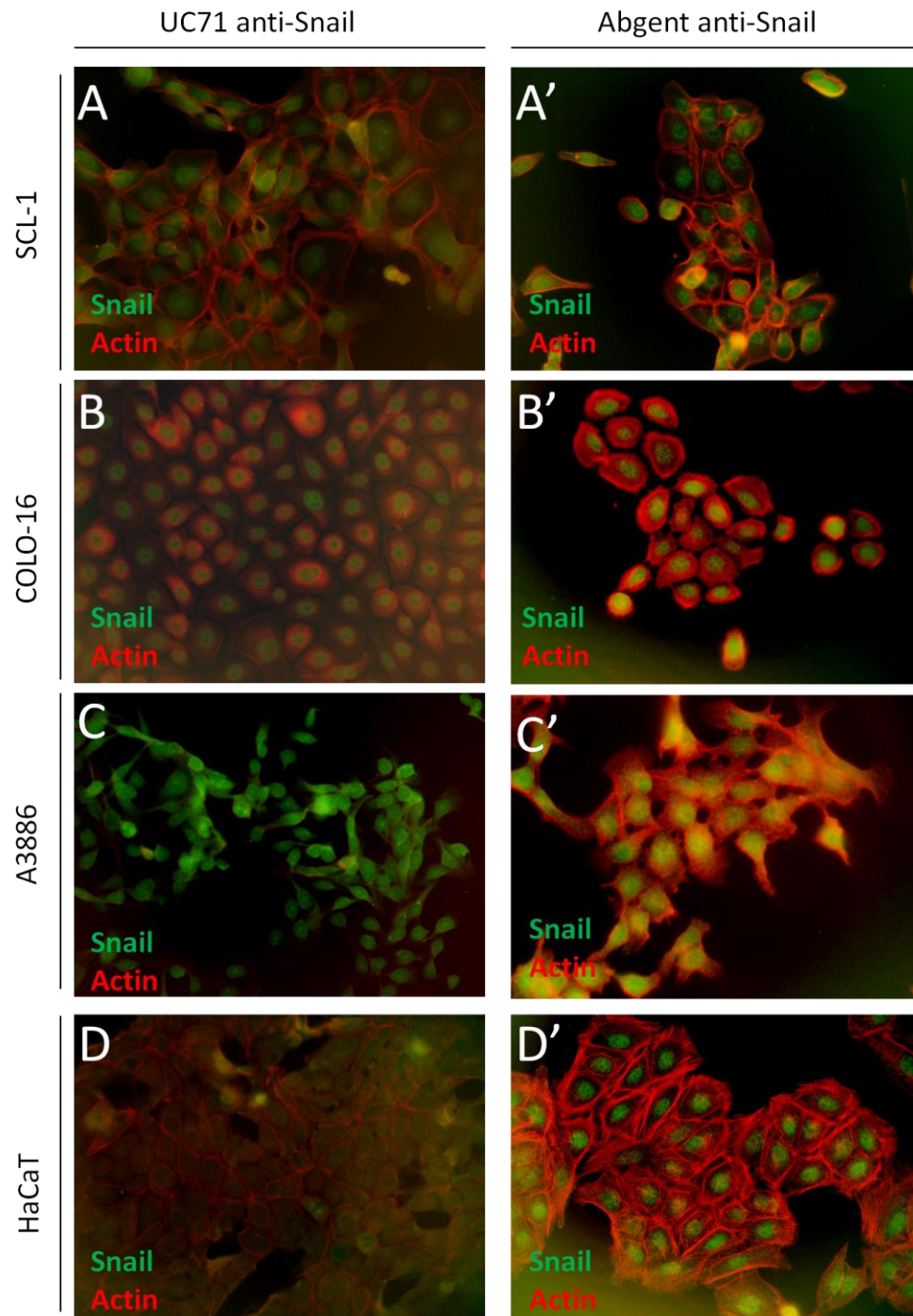


Figure 5. Immunofluorescence staining of Snail. Rhodamine phalloiden was used to stain actin in all cells. **(A-D)** UC71 anti-Snail antibody was used to stain snail SCL-1, COLO-16, A3886, and HaCaT cells. Nuclear staining occurred in SCL-1, COLO-16, and A3886 cells, although some cytoplasmic staining occurred in A3886 cells as well. HaCaT cells had no nuclear staining of Snail. **(A'-D')** The anti-Snail antibody from Abgent was used to stain for Snail as well. SCL-1, COLO-16, A3886, and HaCaT cells all showed nuclear staining of Snail.

The H-130 anti-Snail antibody showed nuclear staining in SCL-1, COLO-16, A3886, and HaCaT cells, confirming the results seen with the Abgent anti-Snail antibody. In addition, the H-130 antibody showed cytoplasmic expression of Snail in primary human keratinocytes (Fig. 6).

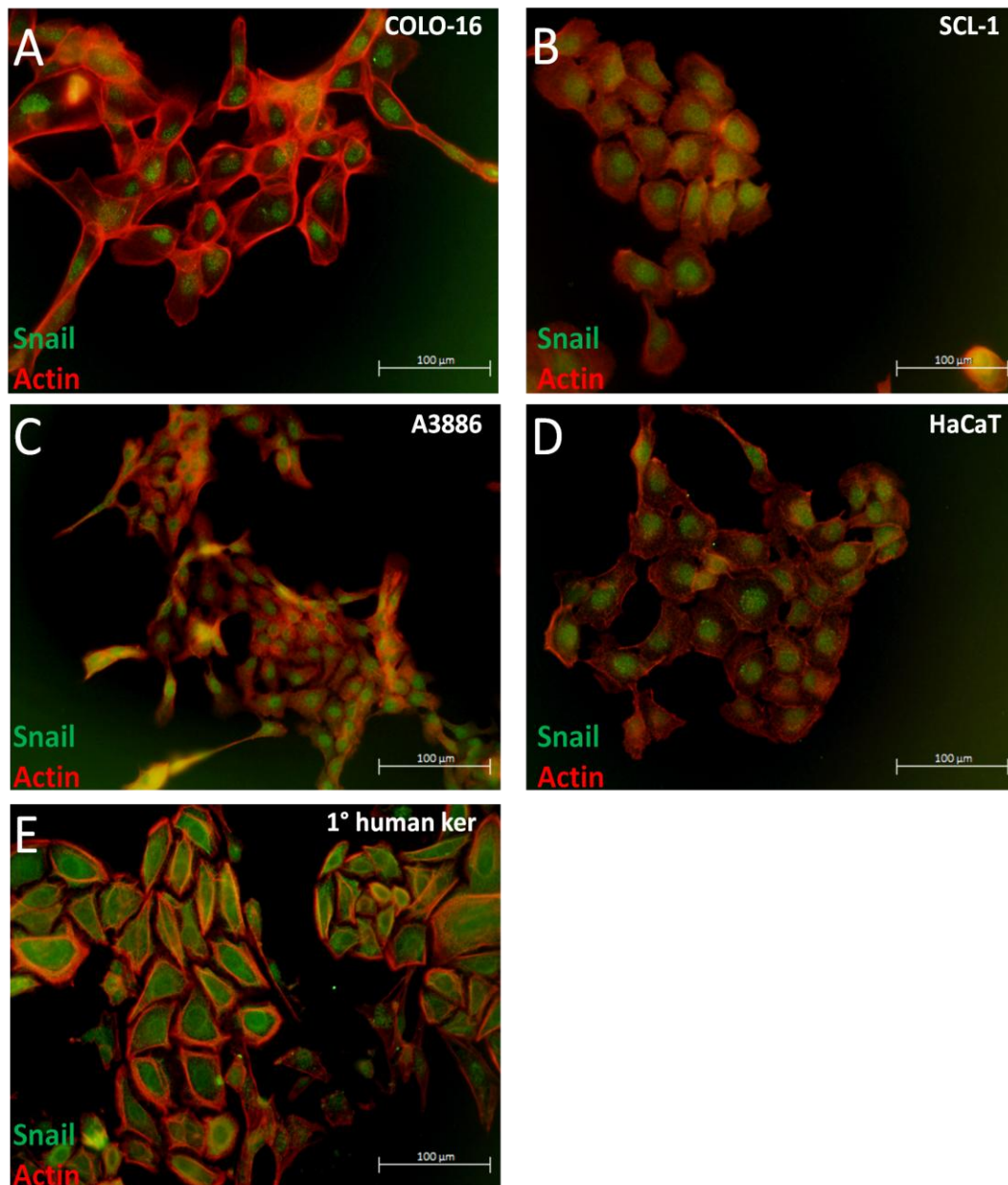


Figure 6. Immunofluorescence staining of Snail with H-130 antibody. Rhodamine phalloiden was used to stain actin in all cells. UC71 anti-Snail antibody was used to stain snail SCL-1, COLO-16, A3886, HaCaT, and primary human keratinocyte cells. **(A-D)** Nuclear staining occurred in SCL-1, COLO-16, A3886 and HaCaT cells. **(E)** Snail staining occurred in the cytoplasm in primary human keratinocytes. As such, primary human keratinocytes may be used as a Snail-negative control in the following experiments.

Collagen Contraction Assay

One of the functional characteristics of activated fibroblasts is their ability to contract collagen, which is how they perform their in contracting wounds during the wound-healing process (Vaughan 2000). To test whether Snail-expressing cancer cells can cause fibroblasts to contract collagen, I added conditioned media from SCL-1, COLO-16, A3886, HaCaT, and primary human keratinocyte cell cultures to collagen plugs with fibroblasts embedded in them. Since TGF β -2 is a known activator of fibroblasts (Vaughan 2000), it was used as a positive control.

In the first collagen contraction assay, I added conditioned media to the collagen plugs in both 1:1 and 1:5 ratios with normal fibroblast growth media. After of 5-7 days, only plugs treated with TGF β -2 showed a significant decrease in surface area. None of the collagen plugs treated with conditioned media showed significant contraction (Fig. 7).

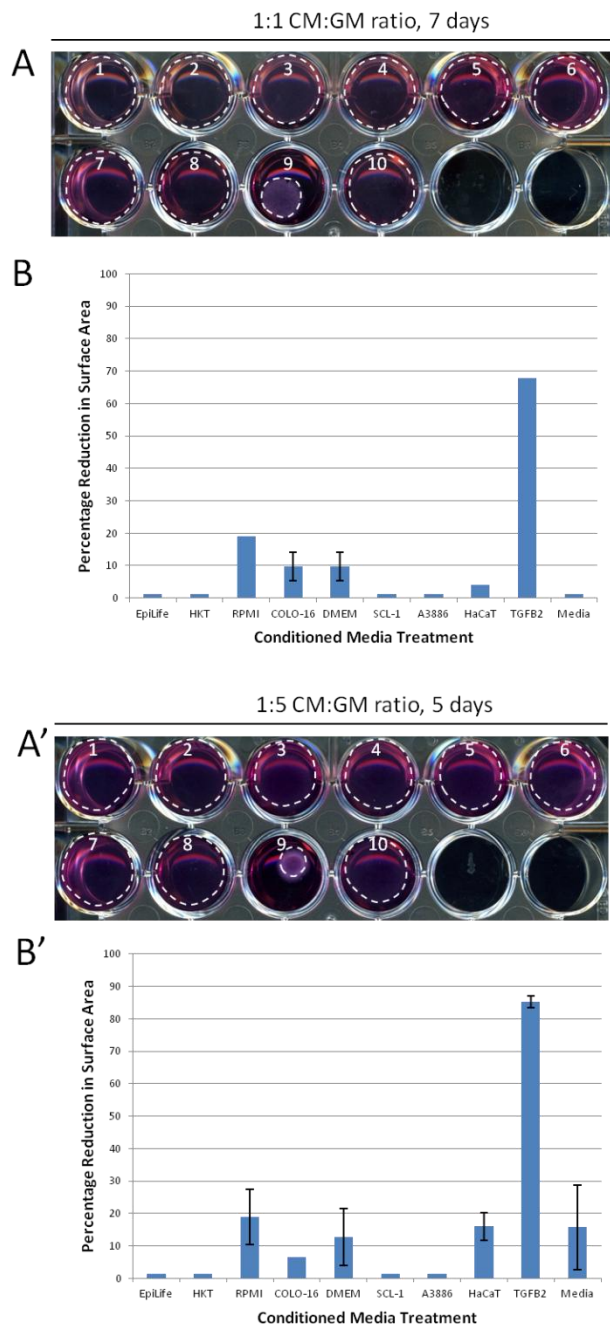


Figure 7. Collagen Contraction Assay. Fibroblast-embedded collagen plugs were treated with various media treatments. Well 1: HKT GM, Well 2: HKT CM, Well 3: COLO-16 GM, Well 4: COLO-16 CM, Well 5: SCL-1/A3886/HaCaT GM, Well 6: SCL-1 CM, Well 7: A3886 CM, Well 8: HaCaT CM, Well 9: TGFβ-2, Well 10: Fibroblast GM. **(A-B)** Collagen plugs were treated with media in a 1:1 ratio with fibroblast GM over a period of 7 days. Plugs will naturally contract slightly over time. **(A'-B')** Collagen plugs were treated with media in a 1:5 ratio with fibroblast GM over a period of 5 days. GM = growth media, CM = conditioned media.

α -Smooth Muscle Actin Activation Assay

One of the most reliable markers for identifying fully differentiated myofibroblasts is the presence of α -smooth muscle actin (Darby 1990). Fibroblasts were plated on glass coverslips and incubated in either control media or conditioned media for 16 hours. Immunofluorescence staining was then performed using an anti- α -SMA antibody.

No increase in α -SMA was seen in any of the treatments, including the TGF β -2 treatment (Fig. 9). Notably, there was already a significant basal expression of α -SMA in all fibroblast cultures, regardless of treatment. One explanation for this is the tension conferred to the fibroblasts by the glass coverslips. It is known that mechanical tension can induce α -SMA expression in fibroblasts. Indeed, this mechanical tension manifests in wounds when the ECM around the fibroblast is damaged and no longer insulates fibroblasts from external tensile forces (Hinz 2001).

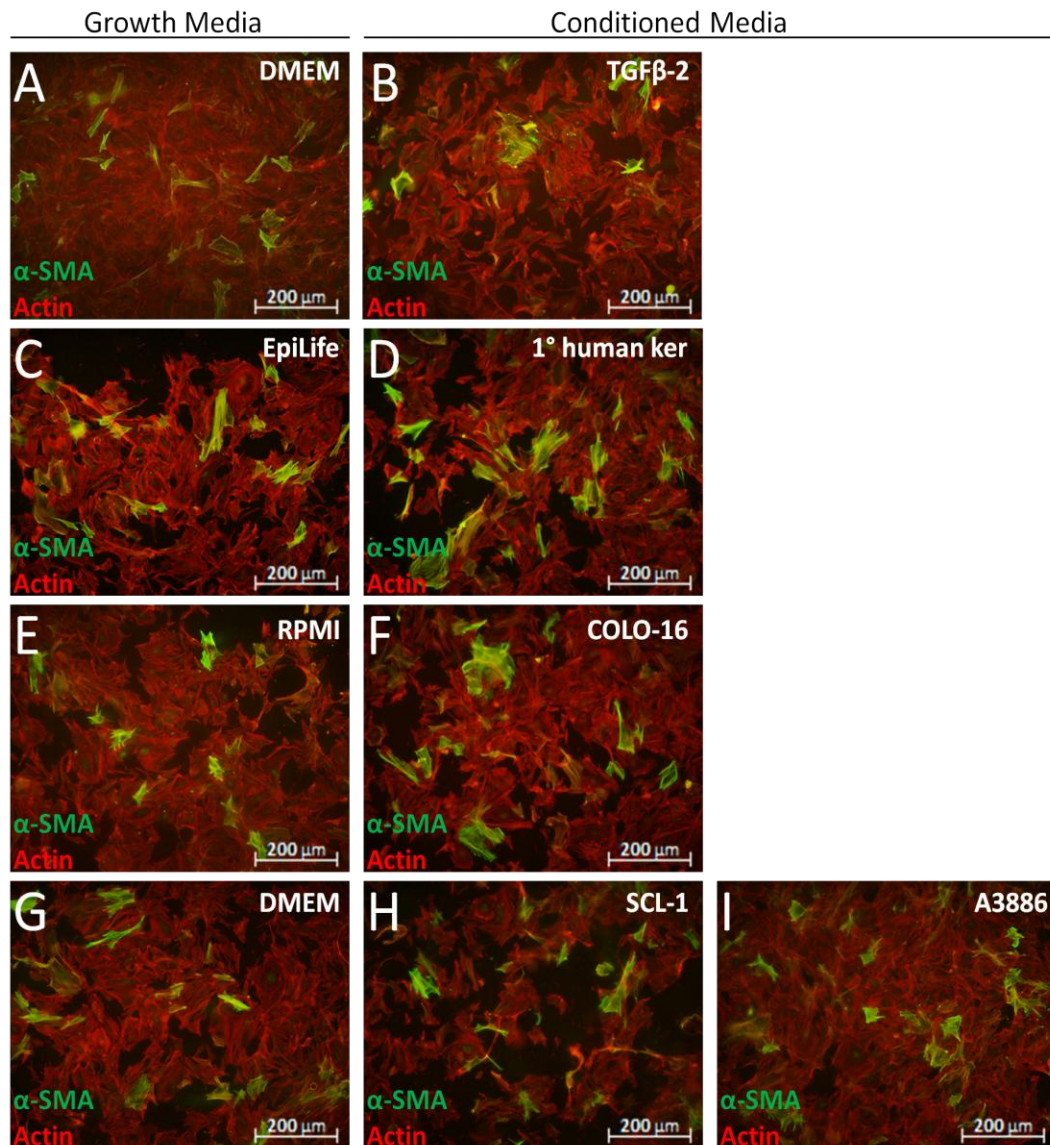


Figure 8. Immunofluorescence staining of α -SMA on glass (16h). Fibroblasts were seeded on glass coverslips. After 24 hours, fibroblasts were treated with either TGF β 2, conditioned media, and controlled media. Smooth muscle actin was stained after a 16-hour incubation in treatment media.

The rigidity of the adherent surface was addressed by creating a polydimethylsiloxane (PDMS) base that could be used to coat 24-well plates. The advantage of creating such a material is the rigidity of the material can be controlled. Whereas the rigidity of glass can be measured in units of gigapascals, the PDMS

base was created to have a rigidity of 150 kilopascals, which is about the same rigidity as that of human skin.

Conditioned media-treated fibroblasts grown on PDMS showed a decreased basal level α -SMA expression overall compared to fibroblasts grown on glass coverslips (Fig. 10). However, there was no increase in α -SMA in conditioned media treatments compared to media control treatments. Additionally, there was no α -SMA expression in TGF β -2-treated fibroblasts.

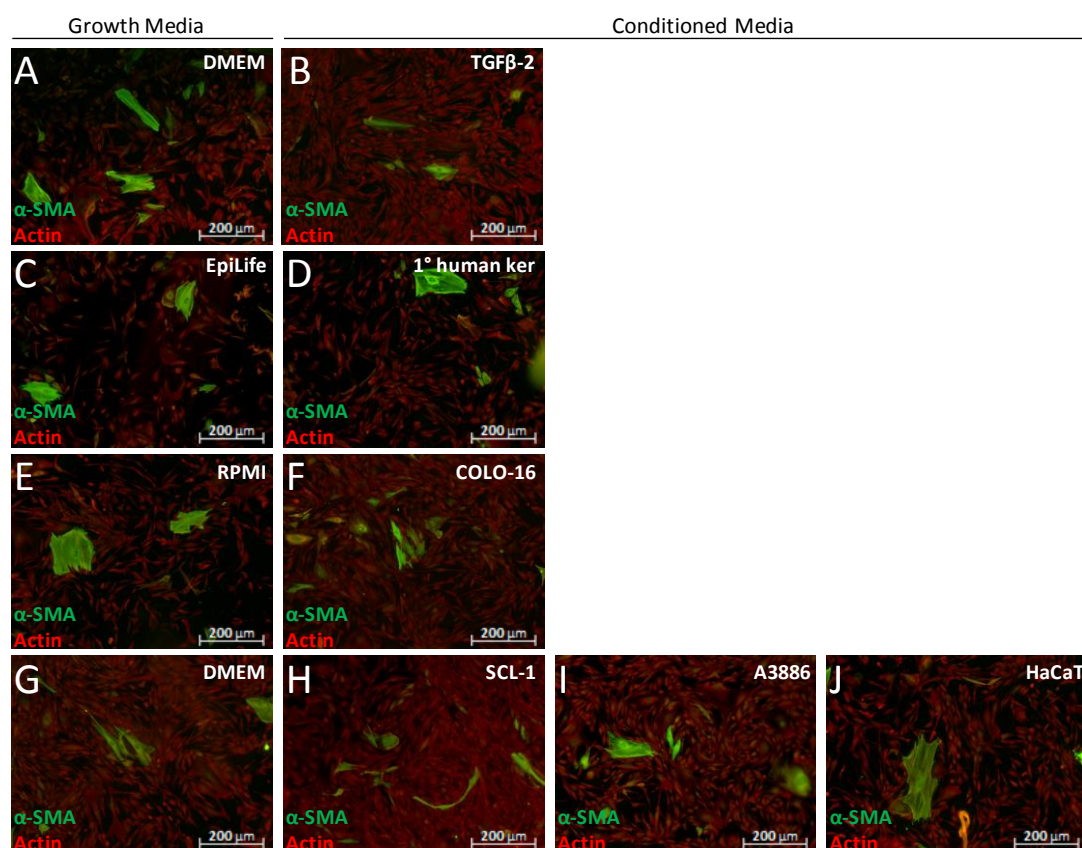


Figure 9. Immunofluorescence staining of α -SMA on PDMS (16h). Fibroblasts were seeded in the wells of a 24-well plate coated with PDMS. After 24 hours, fibroblasts were treated with either TGF β 2, conditioned media, and controlled media. Smooth muscle actin was stained after a 16-hour incubation in treatment media.

One explanation for the lack of an increase in α -SMA expression even with TGF β -2-treated fibroblasts is the short 16-hour incubation time. Although it has

previously been shown that α -SMA mRNA levels increase after only 16 hours of treatment with conditioned media from Snail transgenic skin, it may take longer for the protein levels to increase. Indeed, it takes at least 3 days before TGF β -2 elicits collagen contraction. Similar conditioned media experiments with fibroblasts have been done with an incubation time of 72 hours before α -SMA activation was assayed (Chen 2009). To address this issue, fibroblasts were treated for 72 hours with conditioned media before being stained with an anti- α -SMA antibody. Fibroblasts treated with TGF β -2 showed increased expression of α -SMA, while fibroblasts treated with conditioned media from primary human keratinocytes and COLO-16 cells did not show increased levels of α -SMA expression (Fig. 11).

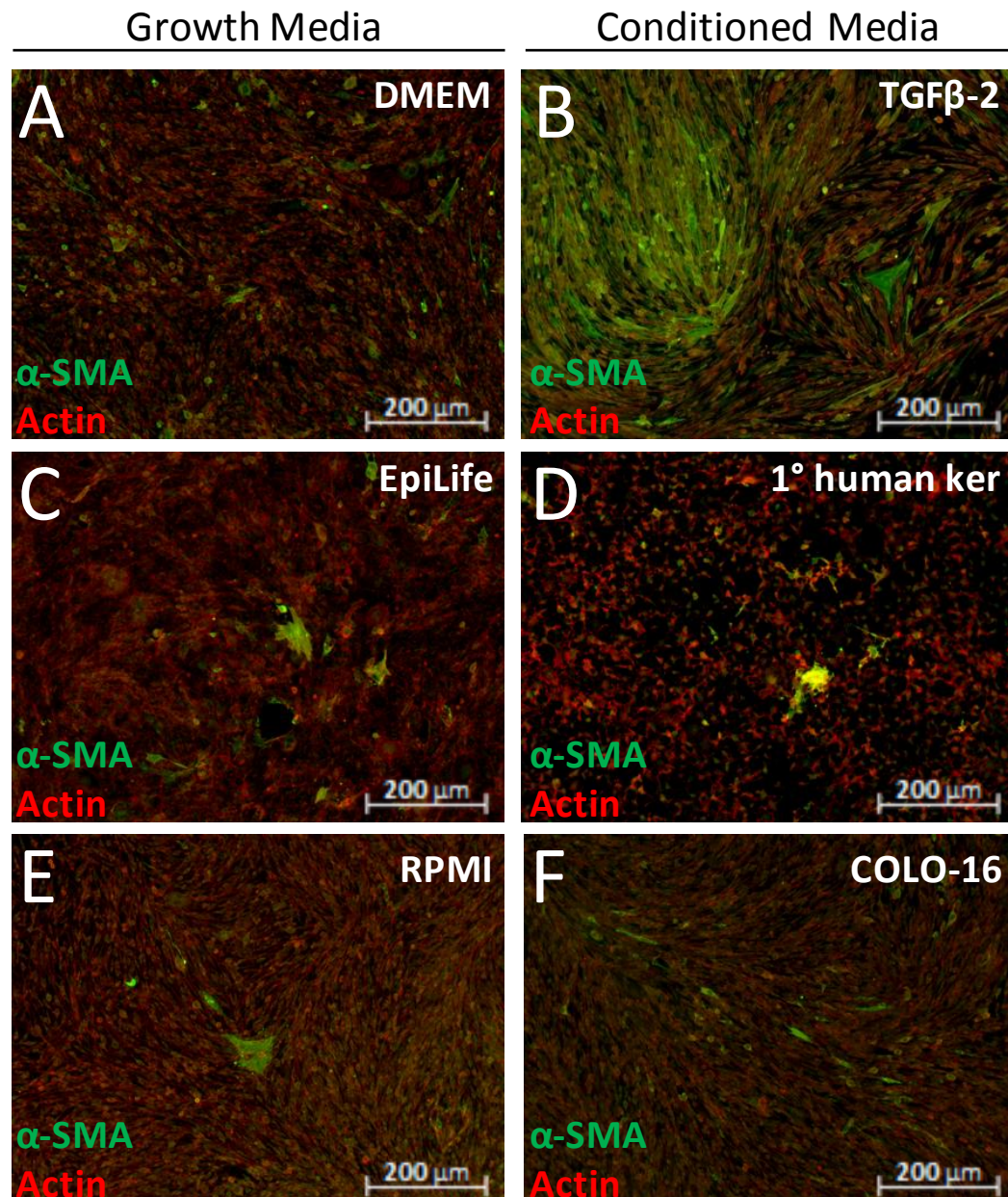


Figure 10. Immunofluorescence staining of α-SMA on PDMS (72h). Fibroblasts were seeded in the wells of a 24-well plate coated with PDMS. After 24 hours, fibroblasts were treated with either TGFβ2, conditioned media, and controlled media. All treatments were replaced after 36 hours. Smooth muscle actin was stained after an additional 36 hours for a total 72-hour incubation time.

Proliferation Assay

It has been shown that fibroblasts that are present in the stroma of cancer cells proliferate at a higher rate than fibroblasts located in otherwise normal dermis (Kuperwasser 2004). Despite the lack of any other activating markers, some molecules, such as PDGF, have been shown to increase fibroblast proliferation without inducing other characteristics of an activated phenotype (Shao 2000). To test whether Snail-expressing cells secrete a factor that induces increased proliferation of fibroblasts, conditioned media was taken from SCL-1, COLO-16, A3886, HaCaT, and primary human keratinocyte cell cultures and frozen in liquid nitrogen to be added to fibroblasts at a later time for subsequent proliferation measurements. Unfortunately, the glycerol used to maintain the conformation of proteins in the conditioned media during the freezing process caused the fibroblasts to round up and die. Additionally, in an attempt to slow the overall growth rate, fibroblasts were cultured in less than 10% serum. Fibroblasts did not proliferate in the initial 2% serum conditions, but they did proliferate in 5% serum conditions. As such, the proliferation assay was done under 5% serum conditions.

Conditioned media was taken straight from plates of COLO-16 and primary human keratinocyte cultures and added to cultures of fibroblasts. TGF β -2 was also added to serve as a positive control. The proliferation rates of the treated fibroblasts were then measured over a 4-day period. Fibroblasts treated with conditioned media from primary human keratinocytes did not proliferate faster than their media control (Fig. 8A), and fibroblasts treated with TGF β -2 had a significantly higher proliferation rate (Fig. 8B). Fibroblasts that were treated with conditioned media from COLO-16, however, did not proliferate at a higher rate (Fig. 8C).

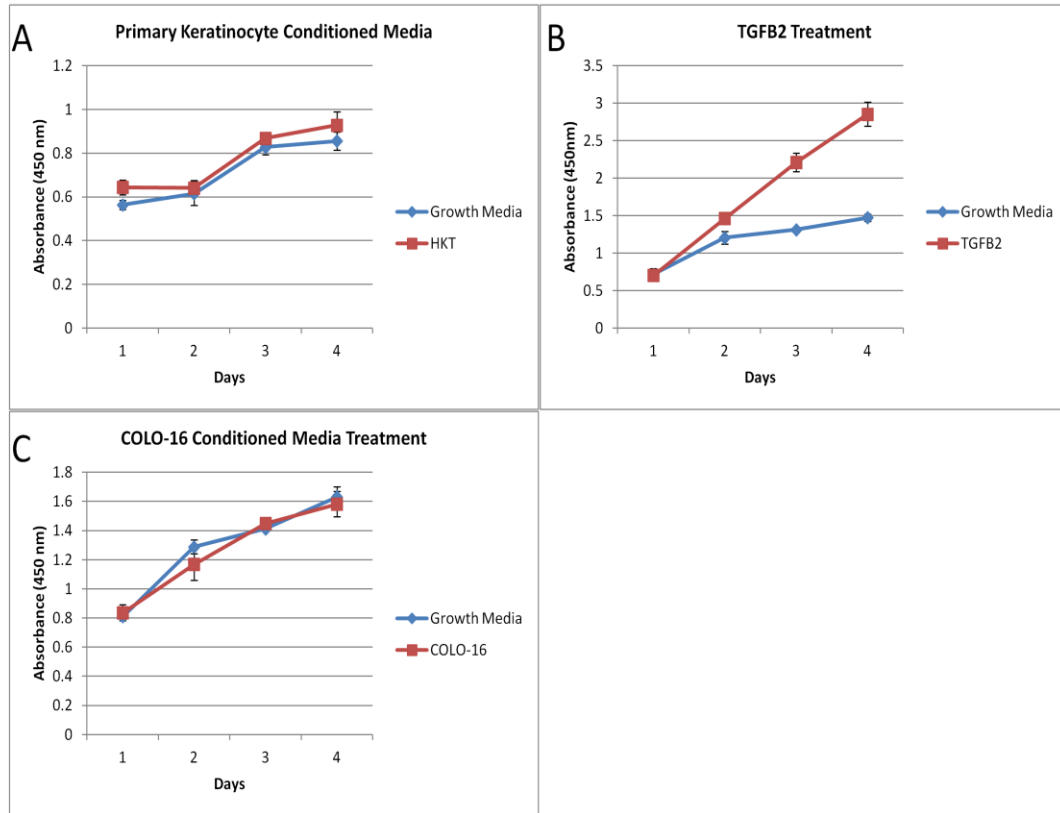


Figure 11. Fibroblast proliferation assay. Fibroblasts were seeded in each well of a 96-well plate and treated with conditioned media or control media each day. Cells were harvested and proliferation rates were measured every 24 hours for a period of 4 days.

Parathyroid hormone-related protein

A screen done by my lab of proteins secreted by Snail-transgenic skin showed secretion of PTHrP. As such, we hypothesized that PTHrP could be a factor that is released by Snail-expressing cells that causes a response in fibroblasts. RT-PCR results showed that all cell lines expressed PTHrP, although the level of PTHrP expression did not correlate with the level of Snail expression (Fig. 9).

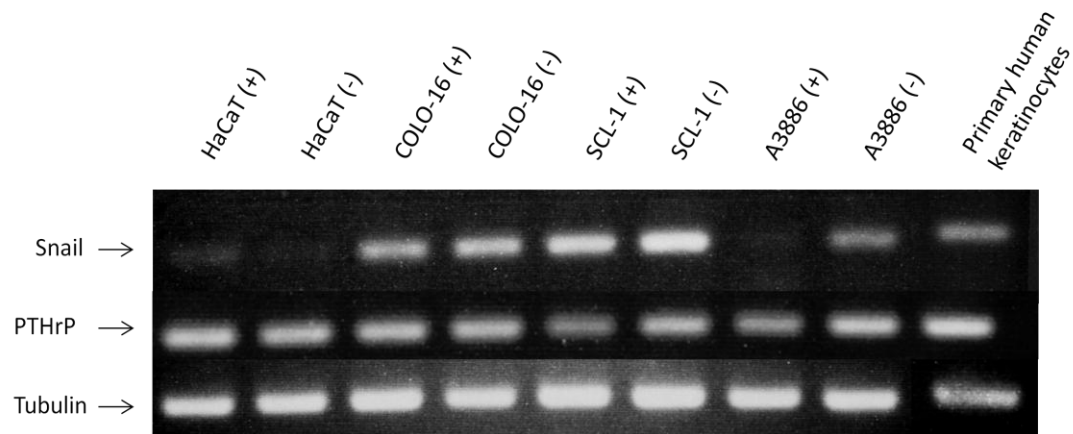


Figure 12. RT-PCR of PTHrP. PCR was performed on cells to determine PTHrP transcription.

It has previously been shown that PTHrP causes cAMP levels in fibroblasts to rise.

Conditioned media from cell cultures was added to fibroblasts, which were assayed for cAMP production. cAMP levels did not increase upon treatment with any conditioned media (Fig. 13).

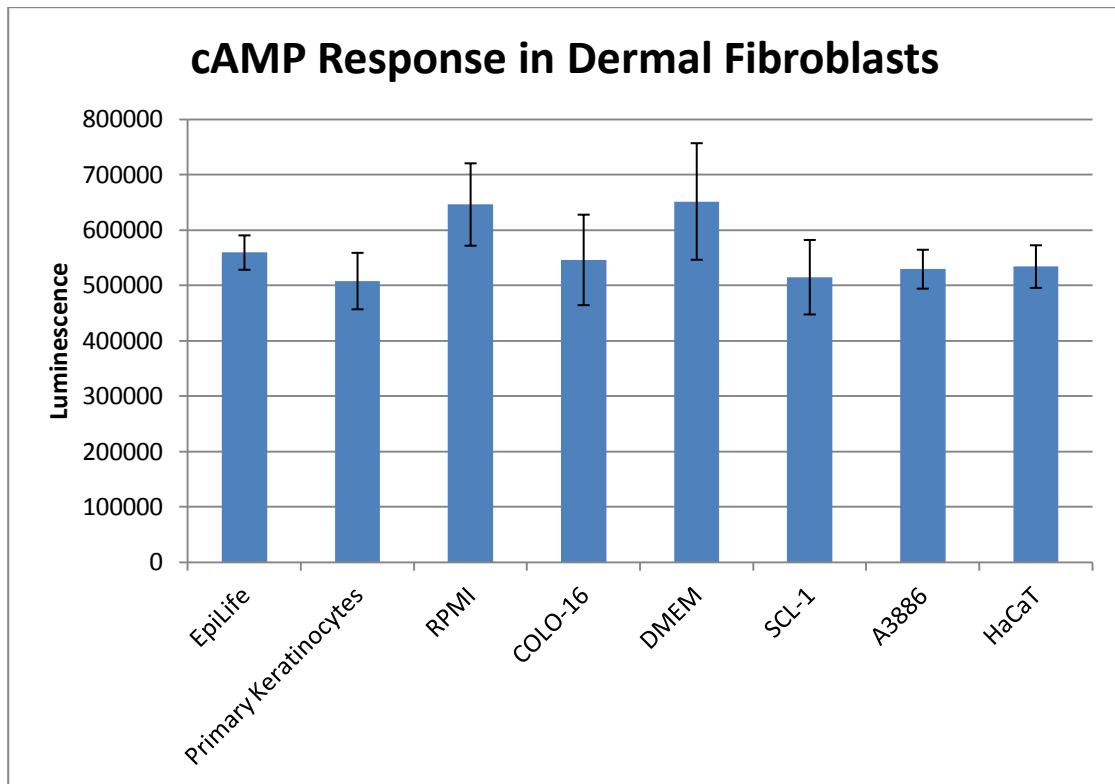


Figure 13. cAMP Assay Upon Treatment of Fibroblasts with Conditioned Media. cAMP levels were determined using a luminescence assay. The luminescence level is inversely proportional to the cAMP level.

Discussion

With multiple studies showing over 2,000 cases of Snail being upregulated in 9 different tumor types, it is clear that Snail plays an important role in tumor development (Becker 2007). Since Snail is well-known for its role in inducing an EMT during embryogenesis, it has been widely extrapolated that Snail's role in tumor progression can be attributed to its ability to repress epithelial markers in carcinoma cells and induce expression of mesenchymal markers. The outcome of this can be either increased invasiveness of carcinoma cells or the transformation of Snail-expressing keratinocytes into activated fibroblasts. However, such transformation does not necessarily have to be the only source of activated fibroblasts in cases of Snail-expressing carcinomas.

As studies in my lab have shown, conditioned media from both Snail-transgenic epidermis and Snail-transfected keratinocytes have the ability to increase α -SMA expression in fibroblasts. This suggests that Snail-expressing cells communicate with surrounding fibroblasts in a paracrine-like fashion. If Snail-expressing keratinocytes secrete a factor that activates fibroblasts, then it is possible that Snail-expressing cancer cells also secrete a factor that causes nearby fibroblasts to gain an activated phenotype. To test this hypothesis, I first tested normal and cancerous keratinocytes for Snail expression to determine which cells expressed Snail in the nucleus and which did not. I then performed conditioned media experiments with three different Snail-positive carcinoma lines and a fourth Snail-positive keratinocyte line. Although activation of fibroblasts was not observed using a collagen contraction assay, proliferation assay, or analysis of α -SMA expression,

fundamental limitations of this study should be kept in mind when deciding whether or not Snail induces the secretion of a fibroblast-activating factor.

The first main limitation of this experiment was the use of cell lines as a source of Snail-expressing carcinomas. Being rapidly dividing cells that have been passaged many times over many years, it is possible that an accumulation of mutations has changed the gene expression profile of the cells such that any fibroblast-activating factor is no longer expressed. The second major limitation was the environment in which the cell lines were grown. It is important to remember that signaling is not a one-way road. Tumor cells have been shown to respond to factors secreted by activated fibroblasts (Chen 2009), and it is possible that by growing these cells in isolation from chemical signals they would otherwise be in contact with, these cells are not behaving as they normally would in vivo. A third limitation was that the conditioned media was not conditioned long enough. Although 16 hours was sufficient to condition media that was used to grow Snail transgenic epidermis, it may not have been enough to condition media that used to grow cells that naturally expressed Snail. The importance of time was shown when it took 72 hours for smooth muscle actin to be expressed at the protein level although it only took 16 hours to be expressed at the mRNA level in the study using Snail-transgenic conditioned media. Other studies have used conditioned media using 24-hour incubation times instead (Chen 2009), so future studies may benefit from increased incubation times.

To address these issues it would be more appropriate to look at Snail-expressing carcinomas in a setting that is more reminiscent of an in vivo environment. Cell lines could be co-cultured with fibroblasts to induce a response in cancer cells,

similar to what was seen by Chen, *et al.* Conditioned media from these cells may contain additional factors that could cause fibroblast activation.

It would also be useful to look at Snail *in vivo*. It has previously been shown that Snail transgenic mice are more susceptible to inflammation-driven skin cancer than their wild-type counterparts when subjected to a 2-stage chemical carcinogenesis protocol (Du 2010). In addition preliminary findings have shown that Snail and other E-box binding proteins are expressed when mouse skin is subjected to the same chemical carcinogenesis protocol. It would thus be interesting to look at a Snail knockout mouse that is then treated to induce cancer to determine what the effect is in cancer induction and whether fibroblast activation still occurs.

Despite the lack of evidence of fibroblast activation in this study, it is important to note the importance of tensile stress on fibroblasts when assaying activation, especially by α -SMA expression. It has previously shown that tension increases fibroblast activation markers, including α -SMA (Hinz 2001). Indeed, this study showed that plating fibroblasts on a substrate as rigid as glass induced a level of α -SMA expression that made it difficult to determine different amounts of expression and the use of the softer PDMS material was able to reduce basal α -SMA expression. As such the use of polymers, such as PDMS, whose rigidity can be controlled, proves to be a highly beneficial tool in studying fibroblasts.

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