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Tumor Suppressor Gene ARHI and its Role in Carcinogenesis

A Major Qualifying Project Report

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ABSTRACT

ARHI is a tumor suppressor gene inactive in up to 60% of breast, pancreatic and ovarian cancers. To test its role in sebaceous tumors, immunofluorescence, Western blots, and PCR were used to assay the presence of ARHI mRNA and protein in normal human sebaceous and telogen cells, and its absence in human sebaceous tumor cells. Sebaceous tumors have been found to contain Lef/TCF defects, and using a microarray analysis the Lyle Lab found ARHI to be down regulated in sebaceous tumor cells. To test the role of Lef/TCF transcription factors on ARHI expression, the levels of ARHI mRNA in normal sebaceous, telogen stem cells, and tumor cells were compared with ARHI levels in a LEF/TCF knockdown cell line. The data indicated significantly lower ARHI expression in the LEF/TCF knockdown cells, evidence of a possible relationship between the Lef/TCF transcription factors and regulation of ARHI. The data support the role of ARHI in sebaceous tumor formation in tissues, but indicate no significant difference in ARHI mRNA or protein levels in cultured cells.

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BACKGROUND

Carcinogenesis

Cancer is the result of the uncontrolled growth of cells whose regulatory genes have been damaged. These tumorigenic cells are said to originate from normal precursor cells which have endured extensive genetic damage. This damage is the result of mutations either passed on from an individual's parents, DNA mutations caused by an agent of the environment, or random errors in the DNA undetected by the cell's proofreading system (Weinberg, 2007, pp.46-49). Spontaneous mutations include random replication errors and random molecular events that go unrepaired, while induced mutations result from a cell being exposed to an agent that causes cancer. Induced mutations can result from exposure to UV rays, x-rays, mutagens, chronic inflammation, or oxygen radicals (McKinnell et al., 1998, pp. 125). UV rays cause point mutations in the genetic code, while x-rays cause breaks in the DNA helix, and mutagens bind to the DNA, interfering with replication and transcription (*CancerQuest,* 2008). In chronic inflammation, the cells of the immune system make their own mutagens, which can lead to genetic damage in nearby healthy cells. Also, oxidative phosphorylation produces free radicals which are very energetic and reactive and may cause genetic damage. Throughout carcinogenesis, gene regulation is a key factor, since a tumor originates from a cell whose cell cycle has been altered, and either programmed cell death does not occur, or the normal checks and balances have been damaged.

Regulation of the Cell Cycle

The cell cycle is divided into two parts: mitosis and interphase. Interphase begins after cytokinesis, and consists of three phases: G1 (gap 1) where the cell grows and is metabolically active, S (synthesis) where DNA is replicated to pass onto the daughter cell, and G2 (gap 2) where the cell grows and prepares for mitosis. The cell cycle is mediated by checkpoints, protein complexes that ensure the previous phase is complete before continuing, and the DNA is not damaged (Cooper, 2004, pp. 596). Upon detection of damaged or unreplicated DNA, these checkpoints will stall the cell cycle to fix the problem. These checkpoint complexes consist of protein kinases called cyclin dependent kinases (cdk).

Cyclins are proteins that control the entry and exit from mitosis by accumulating throughout interphase, and rapidly degrading toward the end of mitosis. Cyclins are cofactors for protein kinases, which phosphorylate the target proteins to enter the next phase of the cell cycle (Weinberg, 2007, pp. 263). The cdk:cyclin interaction is self limiting in that cdk activity causes cyclin breakdown though ubiquitin mediated proteolysis, which in turn inactivates cdk causing the transitions from mitosis, into cytokinesis and interphase (Cooper, 2004, pp. 603).

There are multiple cyclins and cdks, each regulating a different phase of the cell cycle. Progression from G1 to S phase is regulated by Cdk2, 4, and 6 in complex with cyclins D and E (Cooper, 2004, pp. 605). The cyclins at the G1 restriction point respond to growth factors, which control the cell cycle through intracellular pathways. The Ras/Raf/ERK pathway (Figure-1) induces responses in the D cyclins, causing this cyclin group to be considered crucial in growth regulation. Defects in the regulation of cyclin D

could contribute the loss of regulation and lead to cancerous cell growth. This is significant because ARHI, as a member of the Ras super family, downregulates cyclin D1 expression (Wang et al. 2003). Cyclin D1 is also a target of β catenin regulation through the Wnt/Wg pathway, utilizing the transcription factor Lef-1/TCF-1.

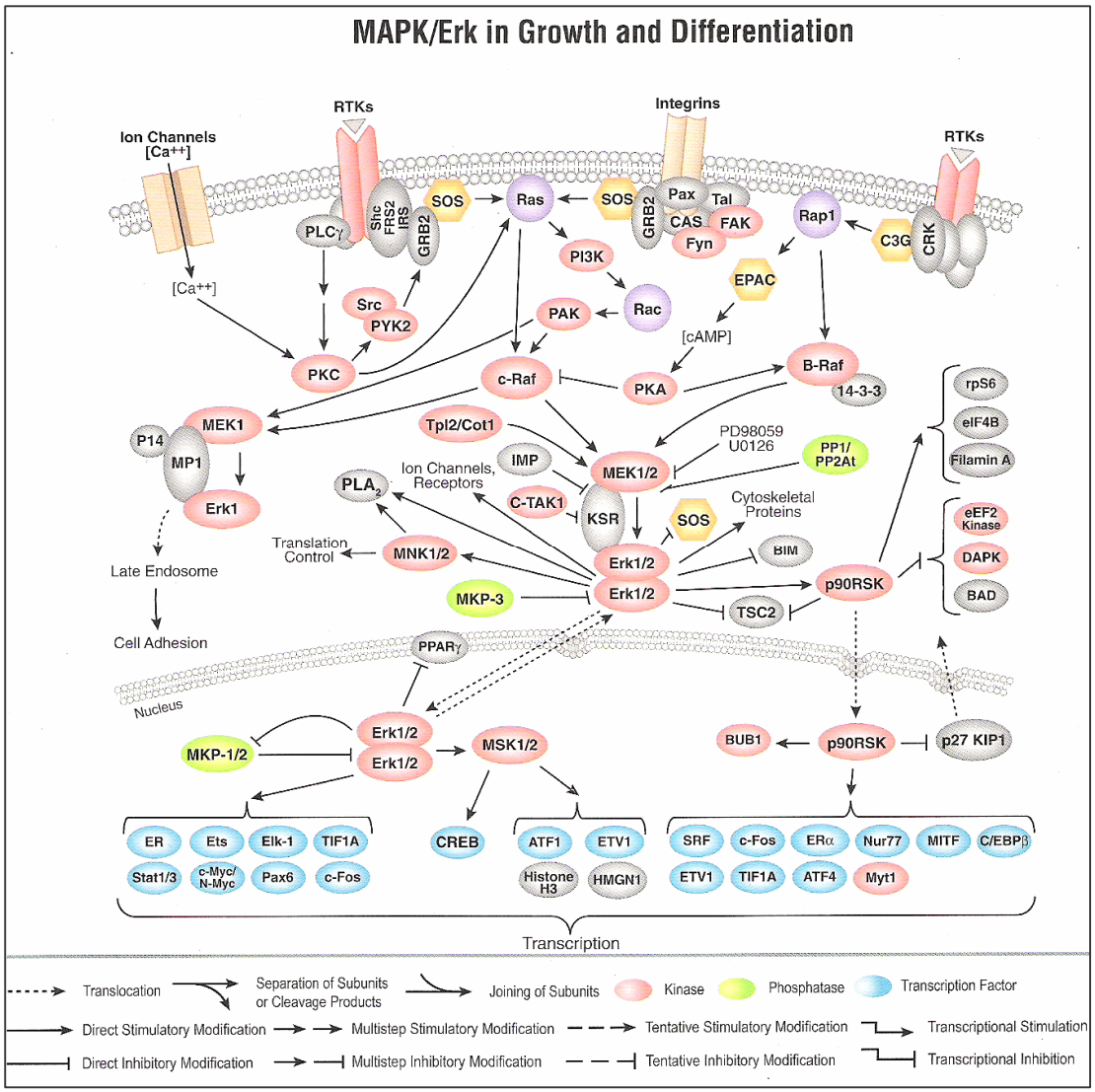


Figure 1: Diagram of Mitogen-Activated Protein Kinase (MAPK) Signaling. Taken from Cell Signaling Technologies, 2008.

Lef-1/TCF-1

Lef/TCF is a transcription factor in the Wnt pathway. Members of the Wnt family are paracrine factors that interact with the transmembrane receptor family Frizzled (Gilbert, 2003, pp. 161). The pathway can be summarized (Figure-2) as Wnt protein binding its receptor, Frizzled, which activates Disheveled- an inhibitor of glycogen synthase kinase 3, GSK3. GSK3 normally inhibits β catenin, preventing its release from the APC protein, but upon Wnt activation, once GSK3 is inhibited, β catenin is free to associate with Lef/TCF and initiate transcription of the target genes (Gilbert, 2003, pp. 62).

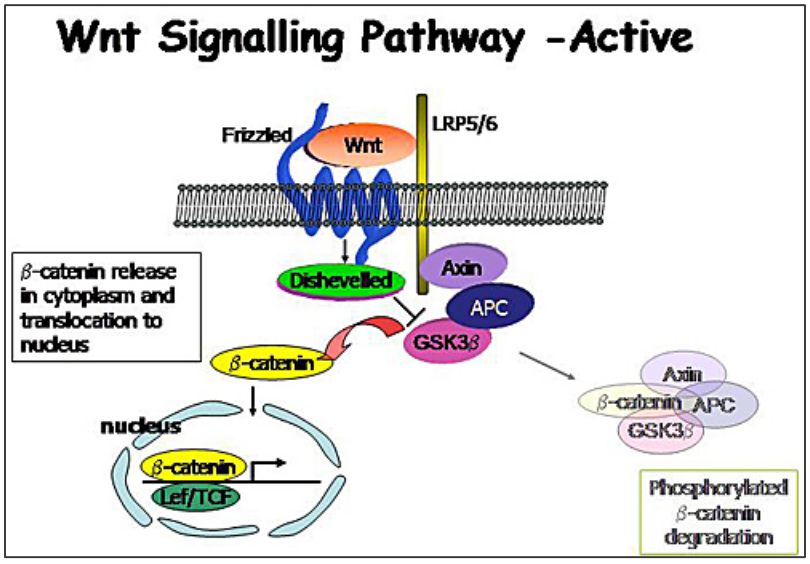


Figure 2: Diagram of the Wnt Pathway. Activation of the pathway leads to the movement of β-catenin into the nucleus. Binding of β-catenin to Lef/TCF forms a complex that binds DNA promoters to induce the activation of a variety of genes related to cell growth and cancer. (University of Melbourne, 2008)

T-cell specific transcription factor (TCF-1) and lymphoid enhancer factor (LEF-1) regulate T-cell specific genes, and bind DNA thru interaction with the minor groove of

DNA (Zhou et al., 1995). This interaction provides a DNA binding moiety which helps activate transcription. Mutations in the Wnt pathway have been identified with colorectal cancer. For example, a mutation in the APC gene causes β catenin levels to accumulate, which allows binding to Lef/TCF, and the subsequent transcription of various genes lead to colorectal cancerous growth. The target genes for this pathway include c-myc and cyclin D1. Over-expression of β catenin in mouse keratinocytes leads to cell cycle arrest in G2 phase and eventual apoptosis (Zhang et al., 2005), clearly linking β catenin to the cell cycle regulator cyclin D. Inhibitors of β catenin also inhibit cyclin D1 promoter activity (Shtutman et al., 1999), solidifying the link between β catenin over-expression and cyclin D1. The expression of cyclin D1 anti-sense cDNA stopped the growth of colon cancer cells in nude mice (Shtutman et al., 1999). Cyclin D1 also contains Lef/TCF binding sequences that are activated in human colorectal cancers (Shtutman et al., 1999) and can be found in a variety of gene promoters (Figure 3.)

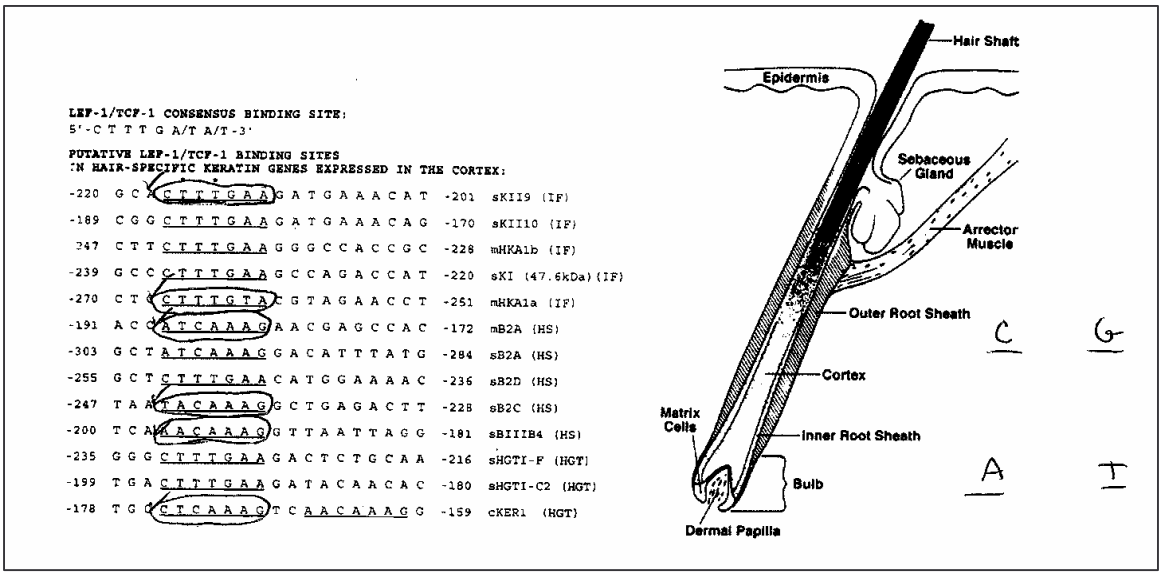


Figure 3: Lef-1/TCF-1 Binding Sites in a Variety of Gene Promoters. Diagram shows a variety of genes related to keratin expressed in the cortex of a hair shaft. (Zhou et al., 1995)

Oncogenes

Proto-oncogenes often control cell division or cell death. When they incur damage and are unable to perform their function and cause a cell to divide uncontrollably, they are termed oncogenes. Often the transcribed protein retains some of its function, but is unable to control normal growth patterns (*CancerQuest,* 2008). Only one copy of the proto-oncogene need be damaged for irregular growth to ensue, as opposed to tumor suppressor genes, in which both copies must be damaged for uncontrolled proliferation. Oncogenes include ras, myc and src.

As described by Campbell et al. (1998) humans contain three ras genes: H-ras, N-ras and K-ras which respond to GTP/GDP levels to act as a switch for signal transduction in cell growth, differentiation, and apoptosis (Figure 4). Ras is connected to many pathways and responds to factors such as cytokines, growth factors, hormones and

neurotransmitters (Campbell et al., 1998), including receptor tyrosine kinases (RTK). Once activated, the RTK (upper center of diagram) creates recognition sites for GRB2 to bind SHC, translocating SOS to the plasma membrane and raising GTP levels to bind and activate RAS, inducing the cascade shown in Figure 5 to control protein expression.

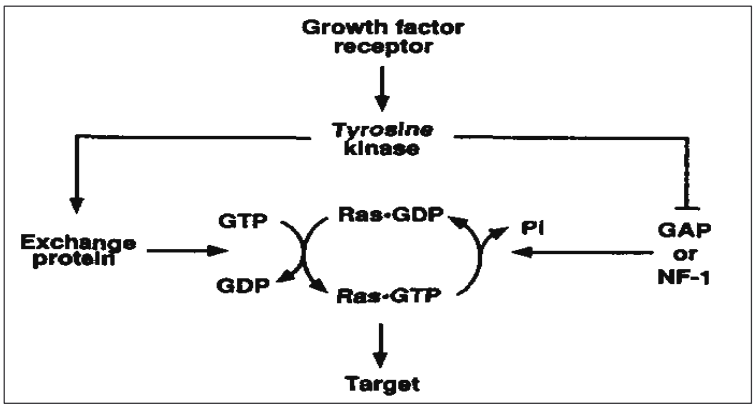


Figure 4: Possible Mechanism of RAS Activation. Note the key role of receptor tyrosine kinases (RTKs). (Satoh et al. 1992)

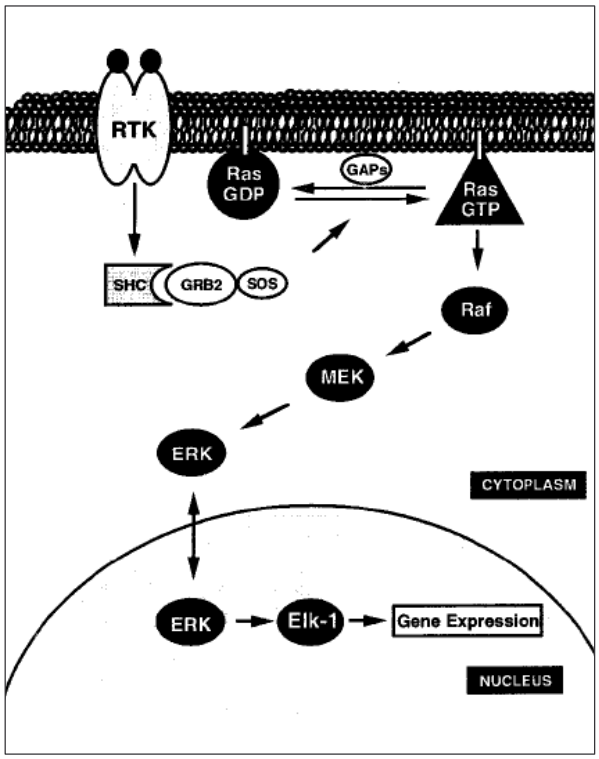


Figure 5: Diagram of the Ras Signaling Pathway (Campbell et al., 1998).

The Ras protein (diagram upper center) is activated by binding GTP, and inactivated when GTP is broken down to GDP. Point mutations often occur that prevent Ras from releasing GTP, leaving it always on and initiating cell proliferation despite the presence of inhibitory signals. This over-expression can lead to cancer. ARHI is a known ras-related protein which acts as a tumor suppressor whose protein is inactivated or down regulated in many cancer cases.

Tumor Suppressors

Tumor suppressors act to regulate cell proliferation by producing products which inhibit cell growth when conditions are not met, as opposed to oncogenes which promote cell growth. Tumor suppressors respond to factors such as DNA damage, lack of factors or damaged division machinery (CancerQuest, 2008), and stop cell growth to either correct the problem or trigger apoptosis. Cancer sometimes occurs when a mutation renders a tumor suppressor inactive, and there is a loss of function or decreased expression via various methods such as methylation, deacetylation or hypermethylation. This allows mutations in the cell to accumulate and cell division to proceed unchecked.

ARHI

Discovery

ARHI, also known as NOEY2 and DIRAS3, is a maternally imprinted tumor suppressor gene with high homology to RAS (Figure-6) that encodes a 26 kDa GTP binding protein (Luo et al., 2003). The gene was first discovered by Yinhua Yu et al.

(1999) using differential display PCR, and it was found to have high homology to ras and rap genes. Yu also found that it was expressed in breast and ovarian epithelial cells, but not found in breast and ovarian cancers (Yu et al., 1999). Re-expression of ARHI led to suppressed growth in breast and ovarian cancer cells, along with down regulation of cyclin D1 promoter and induction of $p21^{WAF1/CIP1}$ (Yu et al., 1999).

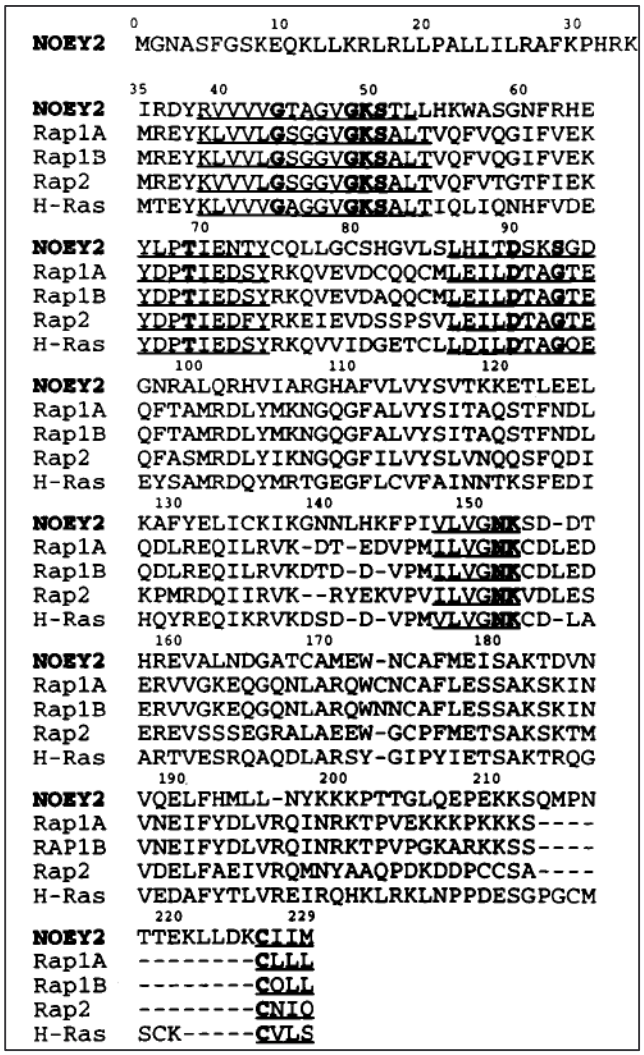


Figure 6: Diagram Demonstrating ARHI's Homology to RAS and RAP. In this diagram, ARHI is designated NOEY2. Yu et al., 1999.

ARHI Location

Using metaphase mapping and *in situ* hybridization, the Yu team located the ARHI gene on human chromosome 1p31 (Yu et al., 1999) (Figure-7).

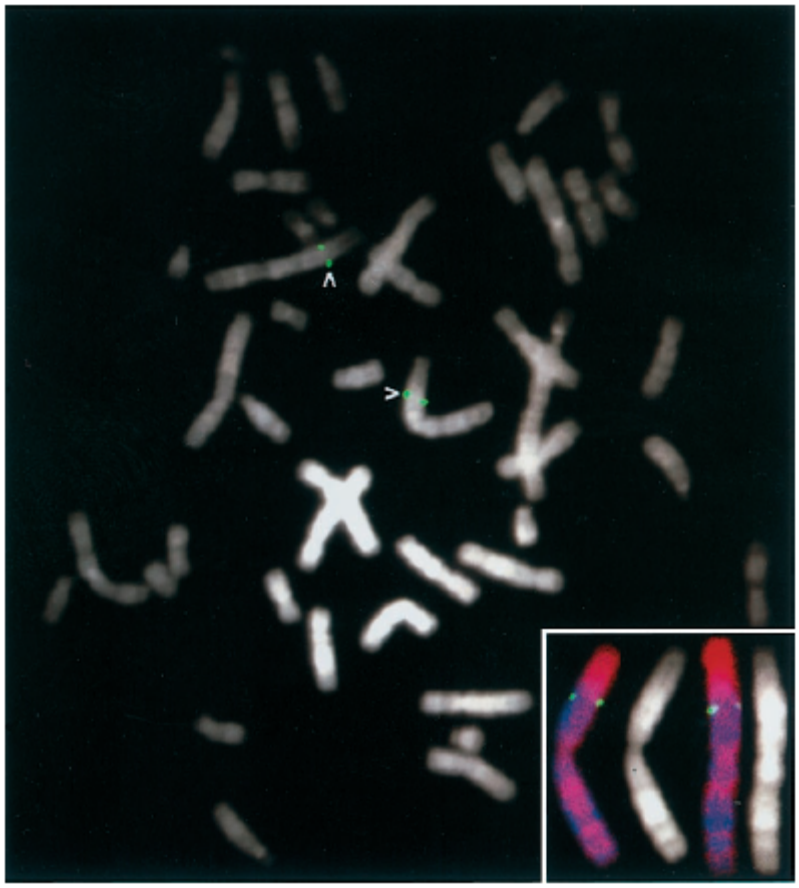


Figure 7: Location of ARHI on Chromosome 1p31. In this diagram ARHI fluoresces green. (Yu et al. 1999).

ARHI Function

ARHI functions as a tumor suppressor gene which encodes a small GTP binding protein, and due to sequence differences with its ras homologs it is most likely in an active constitutive GTP-bound form and has several binding partners. ARHI has been

shown to interact with signal transducers and activators of transcription such as STAT3 (Nishimoto et al., 2005). STAT3 transduces signals from the cell surface to the nucleus to induce transcription. It is most commonly activated by cytokines or growth factors, but proteins from many pathways also interact with it. ARHI "truncates signaling through Ras/mitogen-activated protein kinase (MAPK), activates c-Jun NH2-terminal kinase (JNK), induces p21WAF1/CIP1, down-regulates cyclin D1, and triggers apoptosis" (Nishimoto et al., 2005) in defective cells.

ARHI Significance in Cancers

ARHI is significant due to its loss or down regulation in so many breast, ovarian, and thyroid cancers. Loss of heterozygosity, CpG methylation, or allelic deletion, resulting in complete loss of expression or down regulation of ARHI, was found in up to 80% of breast and ovarian cancer cases (Yu et al., 2003), and 69% of follicular thyroid carcinomas (Weber et al., 2005). Loss of heterozygosity was found in up to 40% of breast and ovarian cases.

Prior to 1985, breast cancer claimed more women than any other cancer (McKinnell et al., 1998, pp. 126), although presently lung cancer has surpassed it. As of 2008, breast cancer is the most common cancer afflicting women after skin cancer, and is the second most common cancer cause of death after lung cancer. The chance of getting breast cancer is 1 in 8, while the chances of dying from it are 1 in 35, and getting lower each year due to earlier detection methods. "About 182,460 women in the United States will be found to have invasive breast cancer in 2008. About 40,480 women will die from the disease this year" (*American Cancer Society, 2008*).

The prevalence of ARHI down regulation in so many breast cancer patients, and the frequency of this type of cancer make understanding the mechanisms behind ARHI loss of function crucial to further research.

ARHI Loss and Restoration of Function

Since ARHI is maternally imprinted, the maternal allele is silenced early in development, with only the paternal allele expressed in normal cells (Yu et al., 2003). The fact that ARHI is maternally imprinted is significant due to the two hit theory of Knudson, a mutation resulting in the deletion of the remaining functional allele will result in silencing ARHI. ARHIs location on chromosome 1p31 is significant in its role of breast and ovarian cases, as this region is deleted in 28-50% of breast cancer cases (Peng et al. 2000). Silencing of ARHI also occurs through DNA hypermethylation, histone 3 deacetylation, and K9 methylation. Of the three CpG islands on the ARHI gene, I and II in the promoter region, III in the coding, hypermethylation of islands I and III result in downregulation of ARHI, while CpG II hypermethylation completely silenced ARHI expression in cancer cells (Yuan et al. 2003). ARHI expression can be restored through demethylation, histone deacetylation inhibition, and adenoviral infection (Yu et al., 2003). Re-expression of ARHI induces $p21^{WAF1/CAP1}$, the cyclin dependent kinase inhibitor, and down regulates cyclin D1 (Rosen et al. 2004) and can induce apoptosis in ovarian and breast cancer cells via a capase-dependent, calpain-independent pathway (Bao et al., 2002). This has strong implications for use of ARHI re-expression in gene therapy for breast and ovarian cancer patients.

PROJECT PURPOSE

The specific aim of this project was to test the hypothesis that ARHI under expression plays a role in sebaceous skin cell tumor formation. If so, this data would extend the previous findings of ARHI's role in ovarian, pancreatic, and breast cancer cases. This hypothesis was tested by analyzing ARHI protein levels in immortal normal sebaceous cells, telogenic sebaceous cells, and sebaceous tumor cells using immunofluorescence, fluorescence activated cell sorting and immunoblots. The relationship between Lef-1/TCF-1 expression and ARHI levels was also explored to determine this transcription factor's influence on the tumor suppressor using semi-quantitative RT-PCR.

METHODS

Cells

The cells cultured and sampled for this project included: human immortalized (but non-cancerous) sebaceous E6E7, human sebaceous tumor 1-C, and human immortalized telogen E6E7 cells for assay of ARHI protein and mRNA. The human sebaceous and telogen cells were previously immortalized using the E6E7 gene of the Human Papilloma Virus (HPV). J2 3T3 fibroblasts were used as feeder cells for the previously mentioned cell lines. *E. coli* cells were used to cultivate the plasmid used in later Bing-293 transformations. Finally, sebaceous human 92-93, which had been transformed retrovirally for lef-1 knockdown, were used for ARHI mRNA comparison in the final RT-PCR.

Cell Culture

Immortalized human sebaceous E6E7, sebaceous tumor 1-C, and telogen E6E7 progenitor cells were cultured in 10 cm^2 dishes with J2 3T3 fibroblast feeder cells. The cells were suspended in KCM without EGF, plated, and incubated overnight to allow cell adhesion. The next day, media was replaced with KCM containing EGF, and media renewed every 48 hrs until confluence was achieved. Dishes were split upon confluence, with re-plating of about one million cells to a new dish, and harvest of remaining cells for later analysis, either in pellet form stored in -80°C, or stored in RNAlater solution at 4°C.

Immunoflourescence

Immunoflourescent microscopy was used to detect ARHI protein using a mouse anti-ARHI antibody on sebaceous E6E7 cells, sebaceous tumor 1-C, and human telogen E6E7 progenitor cells initially provided by the Lyle lab.

Fixing Coverslips

All three cell lines were cultured in two wells each of a six well dish, with three coverslips arranged on the bottom of the well. Pre-prepared cultures were split, and 2 ml aliquots of each were applied to the corresponding well in KCM without EGF. After overnight incubation, the media was changed to KCM with EGF. Cells were grown in this manner for one week, with media changes on average every 48 hours. After this growth period, the cells were washed in PBS, and the coverslips fixed. The coverslips in the top row were fixed with formalin by adding 1 ml formalin to the three wells, and incubating for 5 minutes. The lower row was fixed using 2 ml of acetone and 10 minute incubation. The formalin coverslips were washed twice with PBS and allowed to air dry. The acetone slips were air dried after removal of acetone, no wash. These fixed slips were stored at 4°C until use.

FACS

Fluorescence activated cell sorting was used to probe for ARHI expression within immortalized normal sebaceous E6E7, telogen E6E7, and sebaceous tumor 1-C cells. About 10^6 of each cell type were cultured and collected in 96 U bottomed tubes, to provide a cell only control, an IgG control, and an ARHI:FITC sample. All tubes were

centrifuged at 1000 rpm for 5 minutes to pellet cells, and the supernatant was discarded. Cells were washed in FACS buffer (sodium azide 1 mM, BSA 0.05 g/ml, PBS 1X) twice before resuspension in 100 µl Fix/Perm buffer (4% paraformaldehyde, PBS 1X), and incubated on ice for 20 minutes in the dark. After incubation, cells were washed twice in Perm/Wash buffer 1X (0.5% Saponin, sodium azide 1 mM, BSA 0.05 g/ml, PBS 1X) and incubated on ice for 30 minutes with 100 µl of the following: cell only control FACS buffer, IgG control 1:500 rabbit IgG, and ARHI 1:50 primary antibody. After incubation, cells were again washed twice in Perm/Wash 1X, resuspended in 100 µl secondary antibody (or FACS buffer for cell only control) anti-mouse FITC 1:300 and incubated on ice, in the dark, for 30 minutes. Cells were then washed twice with Perm/Wash, resuspended in 400 µl FACS buffer, and sent to the FACS lab for analysis.

Transformation with FuGene and Effectene

To over-express ARHI protein, a plasmid containing the human ARHI gene was grown in *E. coli*, then transfected into competent Bing fibroblast cells using Effectene (Qiagen) and FuGene 6 (Roche) transfection reagents. The plasmid pcDNA3-1+ ARHI was supplied by Dr. Alex Lazar (MD Anderson, Houston) containing the human ARHI gene of interest, and which responded to G418 glutamycin and ampicillin selection.

Transforming Competent Cells

The plasmid and two *E. coli* cell tubes were placed on ice to thaw. 1 µl of plasmid was added to one tube, vortexed gently, and incubated on ice for 30 minutes. Cells were heat shocked for 20 seconds at 37°C, and placed on ice for 2 minutes. 900 µl

of LB broth was added to the tubes, and 100 µl from each (half transformed, half control) were plated on an LB agar dish, and incubated at 37°C overnight.

Inoculation

175 ml LB media and 200 µl ampicillin were added to a new flask. Using the tip of a pipette, 1 colony was swabbed and mixed into the media. The flask was placed in a 37°C shaking incubator overnight.

Plasmid Prep

For higher plasmid yields, transformed cells cultured in a flask were centrifuged at 6000X g for 45 minutes at 4°C. The supernatant was discarded and the pellet frozen at -20°C overnight.

Plasmid Extraction

Utilizing a Plasmid Isolation Kit (Sigma) the pelleted cells were resuspended in 12 ml resuspension solution, then 12 ml of lysis solution was added, and the flask was inverted 4-6 times and incubated at room temperature for 5 minutes. Samples were neutralized by adding 12 ml chilled neutralization buffer, inverting 4-6 times to form a white aggregate. 9 ml of binding solution G was added, poured into a filter syringe and allowed to sit for 5 minutes. Lysate was expelled through the syringe into the binding column and centrifuged at 3000X g for 2 minutes. The column was washed twice, once with Wash O, and once with Wash Solution, and the flow through was discarded. The elution solution was added, DNA was collected and quantified using a

spectrophotometer. The DNA concentration was found to be 23.18 µg/ml and as it had been diluted 20 times for the spectrophotometer DNA program, the original concentration was calculated and recorded to be 0.4636 µg/µl. Tubes labeled and stored at -20°C until later use.

Transfection

Bing-293 fibroblast cells were cultured in 6 well plates in preparation of transfection with Effectene and FuGene 6 transfection reagents. 1 µg of DNA was diluted with DNA condensation buffer EC to a total volume of 150 µl, 8 µl of enhancer was added, vortexed, then incubated at room temperature for 5 minutes. 25 µl of Effectene reagent was added to the 2nd and 3rd wells of the top row, and FuGene 6 was added at varying dilutions (Figure 8) to the bottom wells with fresh media.

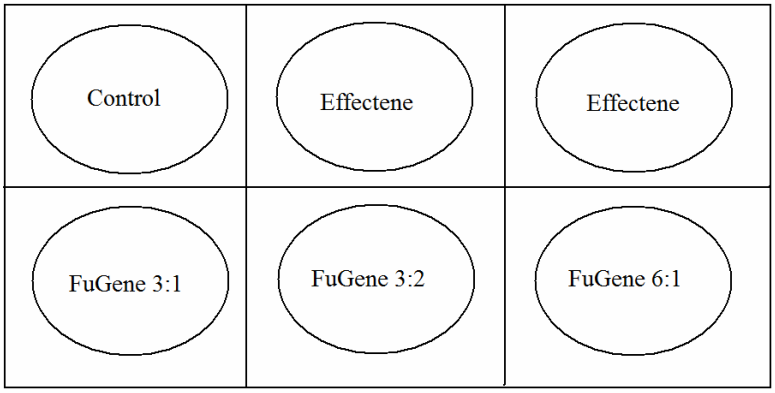


Figure 8: Transfection Reagent Distribution

Selection

Once fibroblast cells were confluent, selection began with G418 glutamycin at a 1:100 dilution. After two weeks of continuing selection, cells were harvested for Western Blots.

Western Blots

Immunoblot analysis was performed several times on the series of cultured cells**,** transformed and untransformed, to detect the 26 kDa ARHI protein.

Protein Quantification and Sample Prep

Samples were first quantified using the Bradford program of the spectrophotometer. 5 µl of sample and 995 µl of Bradford protein assay solution were added to a cuvette, and the concentration was determined by spectrophotometry at 495 nm. All samples were calculated for loading amount of 20 µg per well, one third of which was added in loading dye. Samples were heated at 100°C for 5 minutes, immediately placed on ice for 5 minutes afterward, spun down and stored in -20° C until use.

SDS PAGE Gel

A 12% SDS PAGE gel was prepared. Samples were added to their assigned wells and run in an upright unit at 80 V through the stacking gel until they entered the resolving gel, and then voltage was increased to 200 V for 1 hour.

Transfer

A sandwich was assembled to transfer the separated samples to a PDBF membrane, assembled in an upright unit, and run in transfer buffer, in the cold room, at 27 V overnight or 100 V for 1-2 hours.

Blocking

Upon complete transfer to membrane and labeling, blocking ensued with blocking buffer (2.5 g of nonfat dried milk, 50 ml 1X PBST) in a plastic case and placed on a rocking shaker at room temperature for 1 hour.

Primary Antibody Incubation

Primary antibodies were prepared at the following dilutions:

Antibody	Dilution
Mouse anti-ARHI (Abcam)	1:500
ID8 (MD Anderson)	1:5000

Blocking buffer was drained, and the membrane was washed three times in PBS-T, for 10 minutes each. The membrane was cut in half along the ladder, placed in separate bags and probed with primary anti-ARHI and ID8 overnight in the cold room.

Secondary Antibody Incubation

Primary antibody was recovered and stored in 4°C for later use. Membranes were washed three times in PBS-T for 10 minutes each and placed in separate bags. Secondary anti-mouse HRP antibody at 1:5000 dilution was added to each membrane, and incubated on a rocking shaker at room temperature for 1 hour, or in the cold room overnight.

Development

Membranes were washed three times in PBS-T for 10 minutes each. 1 ml of horse radish peroxidase (HRP) substrate (GE) was added to each blot, and incubated for 1 minute at room temperature. Samples were developed in a cassette using ECL film at varying exposure times until optimal intervals were found. Blots were saved at 4°C, in the dark, for reference.

Primer Design with MacVector and ARHI mRNA Analysis

The human genomic sequence for ARHI was obtained from NCBI's database, and opened on MacVector. Many primer pairs were provided for ARHI mRNA constructs and were selected on product size basis (less than 600 nt). Primers were then ordered through XX IDT Integrated DNA Technologies, and stored at -20°C until use.

RNA Extraction

Total cellular RNA extraction was performed using the RNeasy Kit from Qiagen. To loosen the cell pellet 200 µl of PBS 1X was added to tubes, spun at 0.3 rcf for 5 minutes and flicked gently to loosen. 600 µl Buffer RLT (10 µl β-mercaptoethanol per 1 ml buffer) was added, mixed via pipet, and homogenized 1-2 minutes via vortexing. 600 µl 70 % ethanol was added, and the 700 µl sample was added to 2 columns (for large pellet) and centrifuged at 10,000 rpm for 15 seconds. Columns were washed using 700 µl Buffer RW1 and again spun down. 500 µl Buffer RPE was added to the columns, spun for 15 seconds at 10,000 rpm, and repeated for 2 minutes to dry the column. The columns

were then transferred to a new 1.5 ml Eppendorf and 50 µl RNase-free water added, spun 1 minute at 10,000 rpm, and repeated for high yield samples (>30 µg). Samples were labeled and stored -20°C until later use.

RNA Quantification

Using the RNA program on the spectrophotometer, the RNA concentration was found by UV absorbance by adding 2 µl of sample to 98 µl of water (50X dilution) in a quartz cuvette. This was repeated for all samples and total RNA concentration was calculated and recorded.

Semi-Quantification RT PCR for ARHI mRNA (Normal Sebaceous vs. Lef-TCF Knockdown)

Primers for ARHI were diluted to a stock solution 50 µM, then to a working solution concentration 5 µM, and added to master mix for a final concentration of 0.6 µM. The master mix was prepared via Table 1 seen below and added to tubes.

Table 1: RT-PCR Master Mix Components

Tube	RNase-free water (µl)	RT PCR Buffer (µl)	dNTP Mix (µl)	Primer A (1F) (µl)	Primer B (1R) (µl)	Enzyme Mix (µl)	Template RNA (µl)
1	21.4	10.0	2.0	6.0	6.0	2.0	2.6
2	18.87	10.0	2.0	6.0	6.0	2.0	5.13
3	22.14	10.0	2.0	6.0	6.0	2.0	1.86
4	23.065	10.0	2.0	6.0	6.0	2.0	0.935

The PCR thermocycler was programmed as described in Table 2:

Table 2: RT-PCR Program LEF

Program- LEF	**Time (min)**	**Temp (°C)**	**Notes**
Reverse Transcription	30	50	
PCR Activation	15	95	
3 step cycle: Denaturation	0.5-1	94	
Annealing	0.5-1	50	5°C below Tm of primers
Extension	1	70	
# cycles	42		
Final extension	10	72	
Hold	99.99	4	

Template RNA was added, and the tubes were placed in the PCR machine. 5 µl aliquots were taken from each sample at cycles 26, 29, 32, 35 and 40 and all were stored at -20°C.

Agarose Gel Electrophoresis

A 1.0% agarose gel with ethidium bromide was prepared. The 5X loading buffer (Biorad) was diluted with running buffer, and 5 µl droplets were added to each sample. Samples were loaded with a 1 Kb ladder according to Table 3 below. The gel was run at 100 V for about an hour, and a picture taken for records.

Table 3: Agarose Gel Sample Loading Assignments

Lane	Sample	Lane	Sample	Lane	Sample
1	1 kb ladder	12	T1C Cyc 40	22	Seb92-93 Cyc 29
2	Seb Cyc 26	13	T1C Cyc 42	23	Seb92-93 Cyc 32
3	Seb Cyc 29	14	1 kb ladder	24	Seb92-93 Cyc 35
4	Seb Cyc 32	15	TEL Cyc 26	25	Seb92-93 Cyc 40
5	Seb Cyc 35	16	TEL Cyc 29	26	Seb92-93 Cyc 42
6	Seb Cyc 40	17	TEL Cyc 32	27	-empty-
7	Seb Cyc 42	18	TEL Cyc 35		
8	T1C Cyc 26	19	TEL Cyc 40		
9	T1C Cyc 39	20	TEL Cyc 42		
10	T1C Cyc 32	21	Seb92-93 Cyc 26		
11	T1C Cyc 35				

RESULTS

The specific aim of this project was to test the hypothesis that ARHI expression is lower in sebaceous tumor cells than in normal cells and whether altered LEF/TCF signaling affects ARHI expression. This project used a combination of immunoflourescence, FACS, Western Blots, and semi-quantitative RT-PCR to detect ARHI protein and mRNA levels in immortalized but non-tumorigenic sebaceous and telogen cells versus sebaceous tumor cells.

With respect to the microscopy results, immunoflourescence of cultured cells fixed on coverslips and probed with ARHI antibodies yielded insignificant results, with little to no fluorescence above background, so this approach was discontinued.

Fluorescence-activated cell sorting (FACS) using flourescein isothiocynate (FITC)-labeled ARHI antibody was performed on immortalized sebaceous, telogenic, and tumor 1-C cultures. Negative controls included no antibody control and an immunoglobulin G control. The results were contrary to the proposed hypothesis in that fluorescent levels were essentially equivalent throughout all samples, as can be seen below in Figure-9.

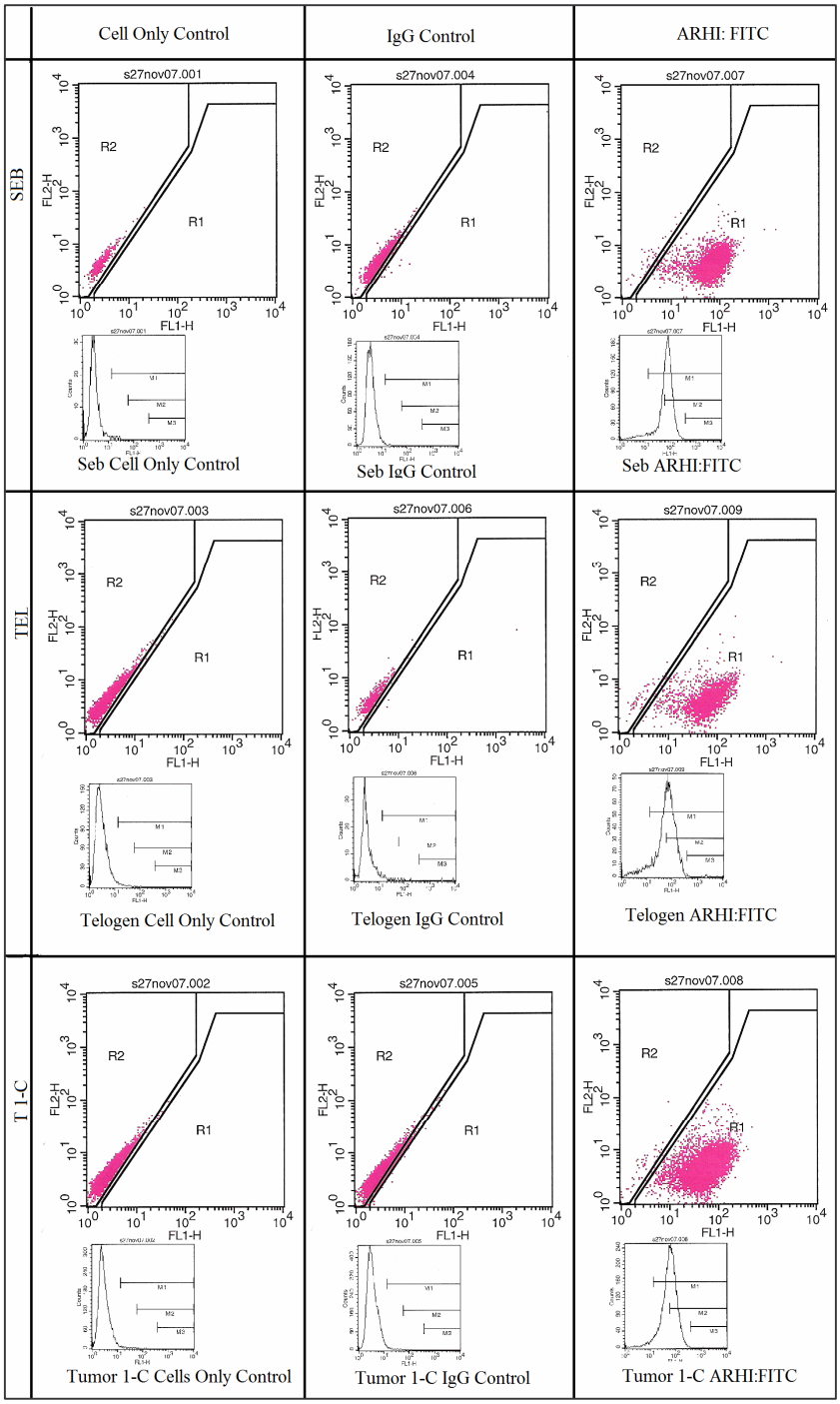
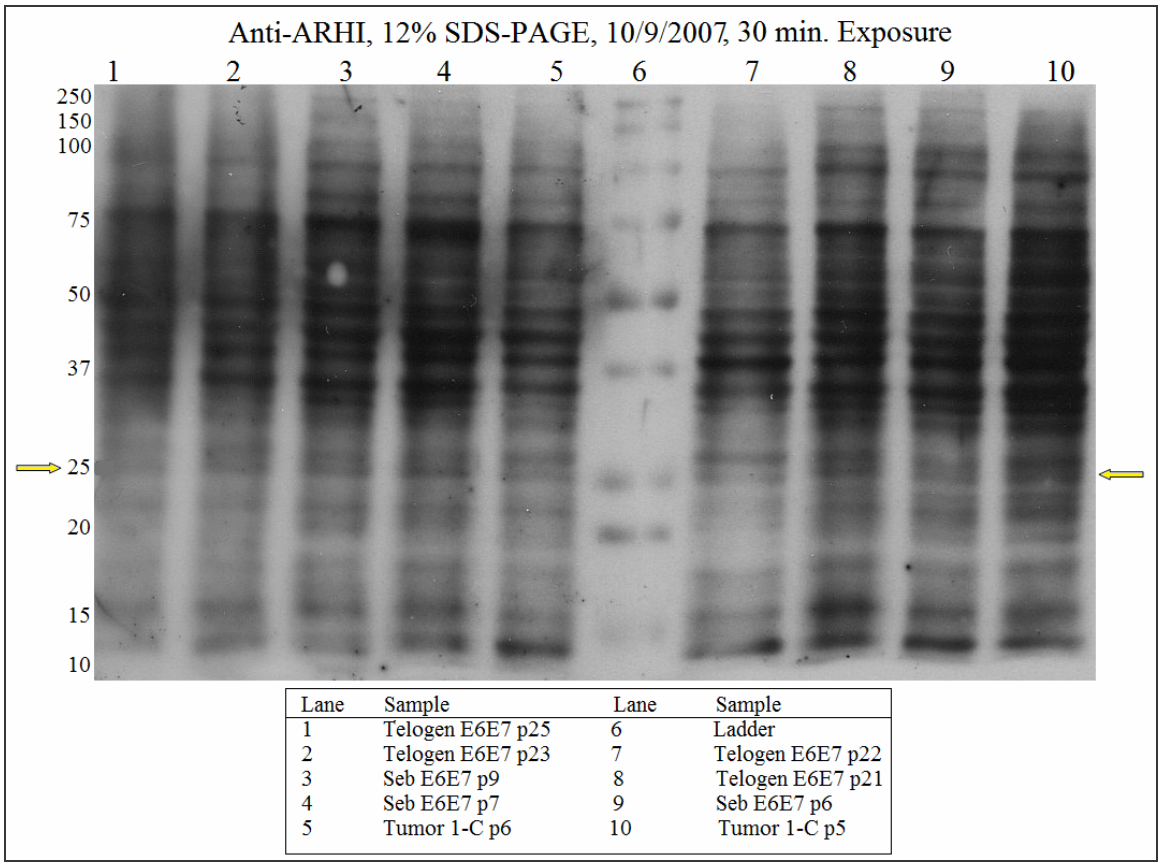


Figure 9: FACS Results Comparing ARHI Tumor Suppressor Levels in Various Sebaceous Cells. The FACS plots show cell counts versus fluorescence (lower panels) and R1 versus R2 fractions (upper panels). Left column shows negative controls with no antibody added. Middle column shows IgG controls. Right column shows ARHI antibody treated samples.

Immunoblot results of untransformed Sebaceous E6E7, Telogen E6E7, and Tumor 1-C cells are shown below in Figure 10. The yellow arrows identify the 25 kDa band location, near which the ARHI band should be seen. Lane 6 is the protein marker ladder. Lanes 1-4, and 7-9 contain the non-tumorigenic cells, while lanes 5 and 10 contain the sebaceous tumor 1-C cells. A comparison of these lanes shows no clear change in signal between the normal sebaceous and tumor sebaceous cells, which did not support the hypothesis.



Lane	Sample	Lane	Sample
1	Telogen E6E7 p25	6	Ladder
2	Telogen E6E7 p23	7	Telogen E6E7 p22
3	Seb E6E7 p9	8	Telogen E6E7 p21
4	Seb E6E7 p7	9	Seb E6E7 p6
5	Tumor 1-C p6	10	Tumor 1-C p5

Figure 10: ARHI Western Blot Comparison of Untransformed Sebaceous and Telogen Cells to Sebaceous Tumor Cells.

Bing-293 fibroblasts were transformed with a plasmid encoding human ARHI to amplify ARHI expression to produce a clearer marker band of interest. Immunoblots of

transformed Bing-293 fibroblasts (Figure 11) showed the plasmid DNA transformation to be successful. In Figure 11 below, Lanes 1-4, and 6 were the transformed Bing cells, Lane 5 the protein ladder, and Lanes 7 and 8 were untransformed sebaceous and telogen, Lanes 9 and 10 untransformed sebaceous tumor 1-C. The 25 kDa band representing endogenous ARHI encoded from the plasmid, marked again by the yellow arrows, is still faint, but is clearly stronger in the transformed lanes and is indicative of a successful transformation. However, the difference between normal and tumor cells was again insignificant.

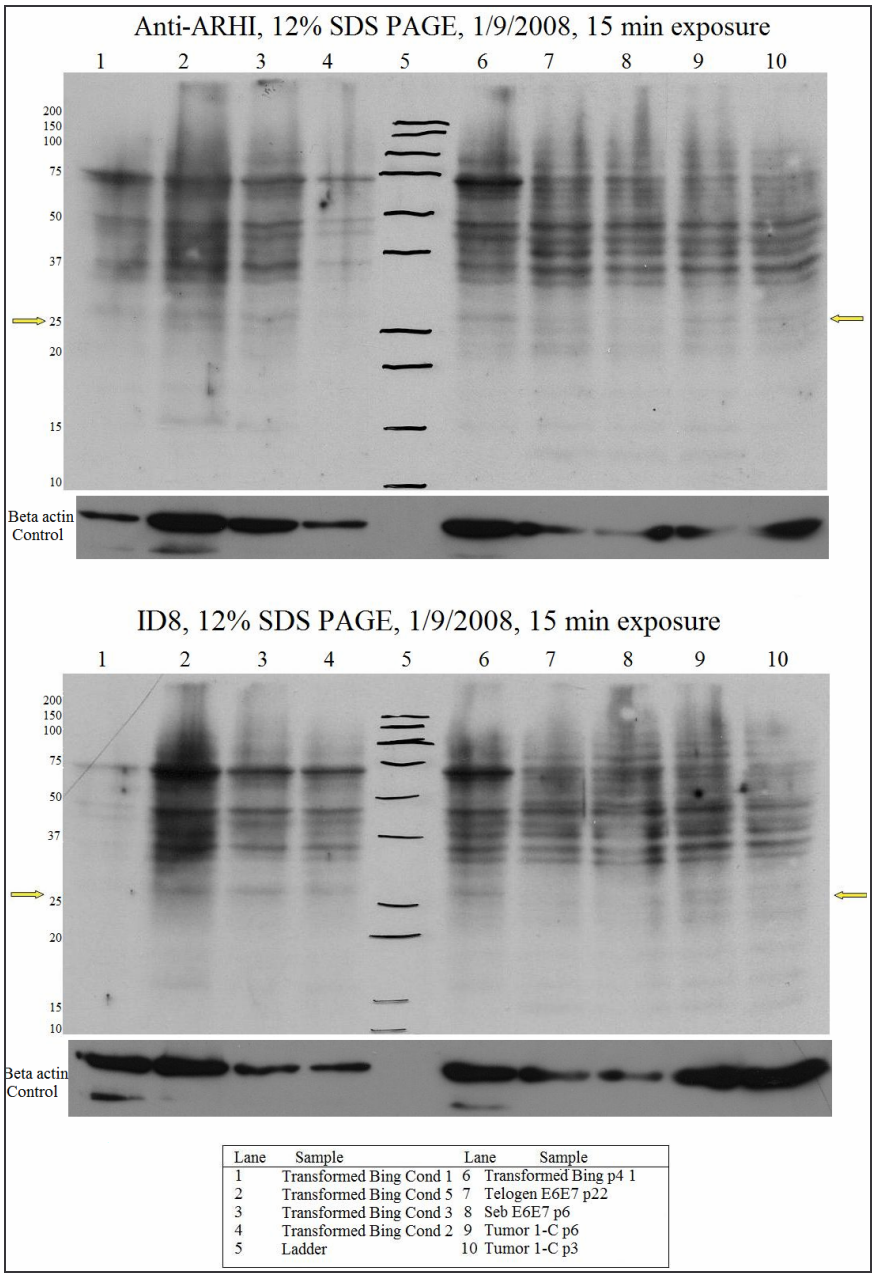


Figure 11: ARHI Immunoblot Comparisons of Plasmid-Transformed and Untransformed Bing Fibroblast Cells. The lanes containing cells transformed with plasmid encoding ARHI (lanes 1-4 and 6) show darker signals at both 25 kDa and 75 kDa than non-plasmid transformed cells (lanes 7-10).

As discussed in the Background, Lef/TCF defects had been found in sebaceous tumors, and re-expression analysis showed tumors to have lower levels of ARHI. A possible relationship between Lef/TCF defects and ARHI expression was hypothesized.

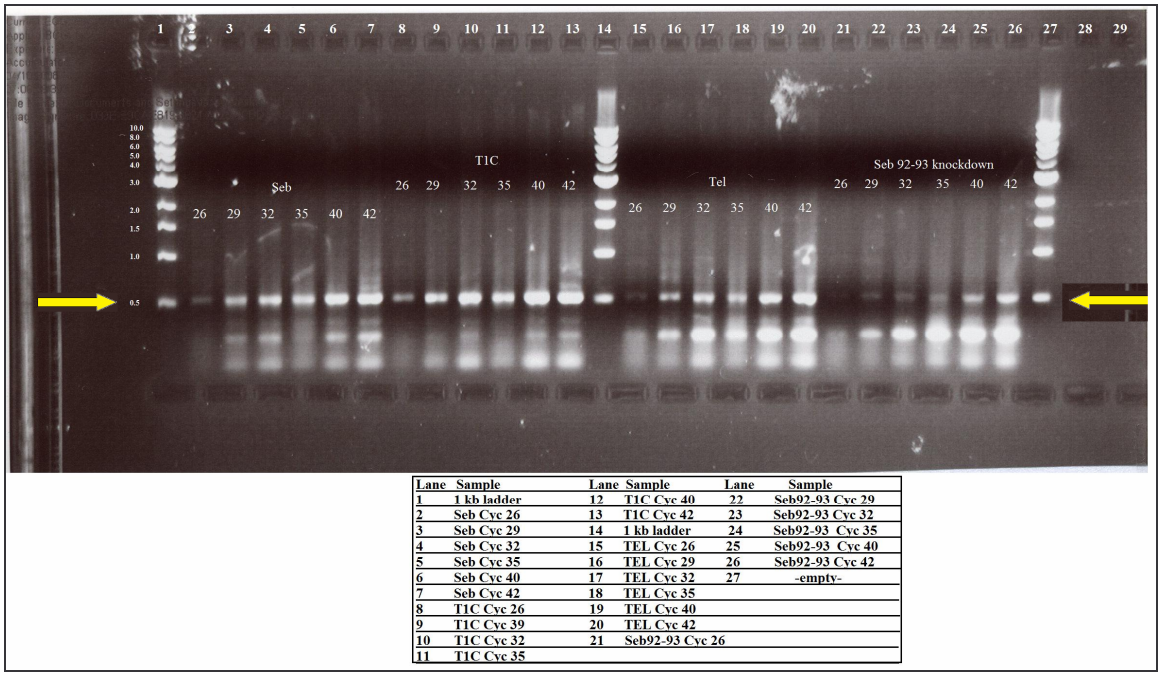
To test this relationship MacVector was used to locate Lef/TCF binding sites in the ARHI promoter. All eight Lef-1/TCF-1 binding sites, shown below in Table 4, were found in the ARHI promoter and form clusters within 100 bp of each other. Cluster 1: sites 3,5, and 6, cluster 2 sites 2 and 4, and the 3rd sites 1,7 and 8. In addition RT PCR was performed for ARHI mRNA levels in normal sebaceous, telogen and sebaceous tumor versus the sebaceous 92-93 Lef/TCF knockdown.

Table 4: Identification of Lef-1/TCF-1 Binding Sites in the ARHI Promoter

Lef/TCF Site #	Sequence cDNA	ARHI Promoter Location
1	CTTGAA	14504-14511
2	CTTTGTT	3554-3561
3	CTTTGTA	979-986
4	CTTTGAT	3472-3479
5	ATCAAAG	1573-1580
6	TACAAAG	1091-1098
7	AACAAAG	14283-14290
8	CTCAAAG	12853-12860

The semi-quantitative RT PCR results for ARHI mRNA levels in immortalized normal sebaceous, telogen and sebaceous tumor cells, versus ARHI mRNA levels in the sebaceous 92-93 Lef/TCF knockdown line are shown in Figure 12. The 1st, 14th and 27th rows contain a 1 kb ladder. The intervening lanes compare Sebaceous E6E7, Tumor 1-C, Telogen E6E7, and Sebaceous 92-93 Lef-1 Knockdown at cycles 26, 29, 32, 35, 40 and 42. The primers designed produced a 527 nucleotide product, the band located near the 0.5 kb ladder mark and denoted by the yellow arrows (Figure 12). As can be clearly seen, there is a steady increase in signal from cycle 26 to 42 for all samples. The signal strength is approximately equal between Seb, Tumor, and Telogen cells, but the Lef-1 knockdown (cells 92-93) show a significant reduction in ARHI signal. By comparing

cycle 32 for each cell line the variation in signal is clear. Cycle 32 for sebaceous and telogen lines are nearly identical, while in the sebaceous tumor line the signal is stronger and in the sebaceous 92-93 Lef/TCF Knockdown line it is very faint. These results indicate a possible relationship between ARHI expression and Lef-1/TCF-1 regulation, as the sebaceous Lef/TCF knockdown line was the only sample to show weaker ARHI mRNA expression.



Lane	Sample	Lane	Sample	Lane	Sample
1	1 kb ladder	12	T1C Cyc 40	22	Seb92-93 Cyc 29
2	Seb Cyc 26	13	T1C Cyc 42	23	Seb92-93 Cyc 32
3	Seb Cyc 29	14	1 kb ladder	24	Seb92-93 Cyc 35
4	Seb Cyc 32	15	TEL Cyc 26	25	Seb92-93 Cyc 40
5	Seb Cyc 35	16	TEL Cyc 29	26	Seb92-93 Cyc 42
6	Seb Cyc 40	17	TEL Cyc 32	27	-empty-
7	Seb Cyc 42	18	TEL Cyc 35		
8	T1C Cyc 26	19	TEL Cyc 40		
9	T1C Cyc 39	20	TEL Cyc 42		
10	T1C Cyc 32	21	Seb92-93 Cyc 26		
11	T1C Cyc 35				

Figure 12: Semi Quantitative RT-PCR Results for ARHI mRNA Levels. The band at approximately 0.5 kb represents the expected 527 bp amplicon size for ARHI. The numbers above each lane (26, 29, 32, 35, 40, and 42) denote the number of RT-PCR cycles at which the sample was taken for analysis. Seb (lanes 2-7) denote non-transformed primary sebaceous cells. T1C (lanes 8-13) denote sebaceous tumor cells. TEL (lanes 15-20) denotes sebaceous telogen progenitor cells. Seb92-93 (lanes 21-26) denotes cells in which Lef/TCF expression has been knocked down via retroviral treatment with siRNA.

DISCUSSION

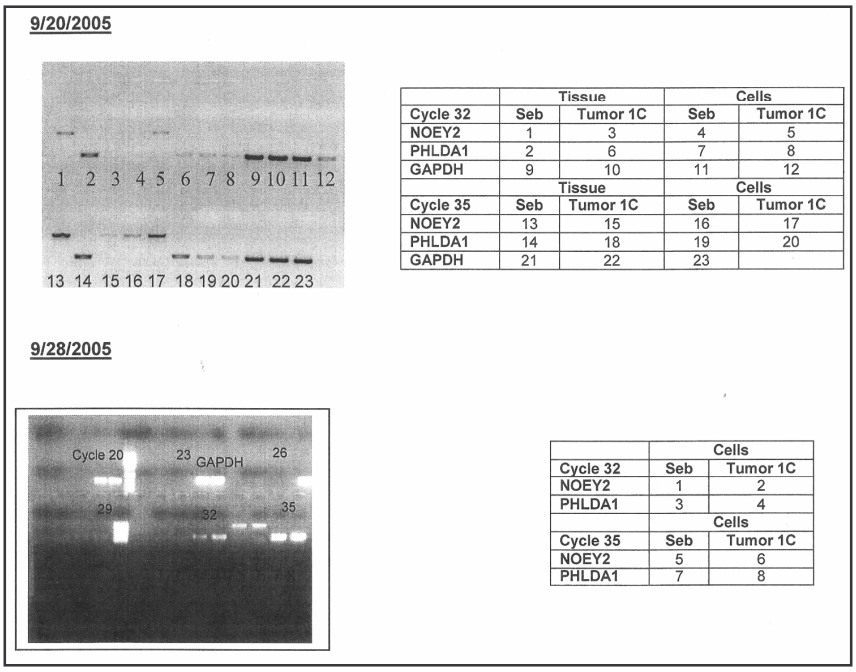
The results from this project appear to refute the hypothesis that ARHI expression is lower in sebaceous tumor cells than in normal sebaceous cells. Thus the earlier work of Yu et al. 2003 and others, which found lower levels of ARHI in other types of cancer tissue, apparently cannot be extended to cultured sebaceous cells. This project utilized cultured immortalized cells, a factor that greatly influenced the results, while Yu et. al did not.

The fluorescence activated cell sorting showed relative equal amounts of fluorescence for ARHI between normal sebaceous, telogen, and sebaceous tumor cells. Similarly, the faint 26 kDa band found in Western Blot analysis showed no decrease in signal between normal sebaceous cells and sebaceous tumor cells.

The data of this project correlate with a previous analysis of ARHI levels in sebaceous cells by Sanya Sanduja, an associate of the Lyle Lab. In an analysis of human sebaceous tumors, Lef1 was found to be down-regulated, and in re-expression analysis ARHI was also found to be down-regulated and confirmed in tissue (Figure 13). The question remained whether this was true in cell lines, whether ARHI would be down regulated in cultured cells. Semi Quantitative RT-PCR was used to compare ARHI expression in normal vs. tumor cells, in both primary tissue and cultured cells (Figure-13). In the top gel, compare lanes 1 and 3 for ARHI in sebaceous vs. tumor tissues. The ARHI band is not visible in lane 3, indicating loss of ARHI expression in the sebaceous tumor tissue. Lanes 4 and 5 compare sebaceous and tumor in cultured cells. Expression is not visible in lane 4, the normal cells, but a faint band is seen in the tumor lane 5, the opposite of what is seen in the primary tissue. The lower gel in the figure shows how

expression of ARHI stays constant in cultured normal and tumor cells, see lanes 1 and 2, 5 and 6 and table for reference. Therefore the cell lines showed no significant difference in ARHI expression between normal sebaceous cells and sebaceous tumor cells.

From the re-expression analysis the question remained whether Lef/TCF had any impact on ARHI expression. The identification of Lef/TCF binding sites in the ARHI promoter shows that Lef/TCF may have some control. Furthermore, when Lef/TCF was downregulated using siRNA, a significant effect was seen on ARHI expression. The results of semi-quantitative RT PCR showed a much weaker signal for ARHI mRNA in the Lef/TCF knockdown cell line than in the normal sebaceous, telogen and sebaceous tumor cells. The knockdown line did show low ARHI expression, and in Lef/TCF complete knockouts the tumor cells have been found to be more aggressive than those with low ARHI expression (Dr. Stephen Lyle, PhD, MD, Personal Communication). The cultured knockdowns can still proliferate with ARHI expression, but if this expression is reduced further or removed completely the cells grow even more aggressively, indicating ARHI possibly still holds some control over cell proliferation in cultured immortalized cells.



	Tissue		Cells	
Cycle 32	Seb	Tumor 1C	Seb	Tumor 1C
NOEY2	1	3	4	5
PHLDA1	2	6	7	8
GAPDH	9	10	11	12
	Tissue		Cells	
Cycle 35	Seb	Tumor 1C	Seb	Tumor 1C
NOEY2	13	15	16	17
PHLDA1	14	18	19	20
GAPDH	21	22	23	

	Cells	
Cycle 32	Seb	Tumor 1C
NOEY2	1	2
PHLDA1	3	4
	Cells	
Cycle 35	Seb	Tumor 1C
NOEY2	5	6
PHLDA1	7	8

Figure 13: Reduction of ARHI mRNA Levels in Primary Sebaceous Tumor Cells vs. Cultured Cells. Figure shows the research results of Sanya Sanduja (personal communication through Dr. Stephen Lyle, MD, PhD).

The implications of this data are significant because the impact of Lef/TCF transcription factors on the tumor suppressor ARHI is unknown. Lef/TCF transcription factors are known players in a number of cancer cases, and both ARHI and Lef/TCF regulate the cell cycle through cyclin D1, so a possible relationship between the two fits the current model. In sebaceous tumors Lef/TCF factors have been known to contain defects, and ARHI has been found to be inactive or down regulated in these tumors as well. The model that these Lef/TCF defects may cause ARHI expression down regulation, and that Lef/TCF may exhibit control over ARHI, is possible.

The data support previous findings in sebaceous tumors. It is surprising that there is no change in ARHI expression between normal sebaceous and sebaceous tumor cells in culture. Tissue cells grow in their natural environment, and their cell cycle is normal, therefore ARHI expression is on in normal cells and off or down regulated in tumor cells. Cultured cells are grown in an artificial environment and the cell lines used were immortalized using the Human Papilloma Virus gene E6E7, causing the cell cycle to be

disrupted, up-regulation of cell cycle promoters, overriding the normal cell cycling. The re-expression of ARHI associated with this immortalization had no deleterious effects on the cell, E6E7 is a more powerful oncogene than ARHI is a tumor suppressor, so ARHI expression did not hinder cell proliferation.

Most interesting were the presence of Lef-1/TCF-1 binding sites in the ARHI promoter and the impact of Lef/TCF knockdown on ARHI mRNA expression. The Lyle Lab will continue its investigation of this relationship by testing whether Lef/TCF actually binds ARHI through chromatin immunoprecipitation, the next step in showing Lef/TCF's control over ARHI expression. Work with ARHI is recommended to proceed using either un-immortalized cells or tissue samples, with either a new, improved primary antibody or the diluted antibodies which were recovered from previous procedures to improve the clarity of results.

BIBLIOGRAPHY

American Cancer Society. Rosen, Leo and Gloria. American Cancer Society, Inc. 9 February 2008. < http://www.cancer.org/docroot/home/index.asp?level=0>.

Bao, J., Le, X., Wang, R., Yuan, J., Wang, L., Atkinson, E., LaPushin, R., Andreeff, M., Fang, B., Yu, Y., and Bast, R. 2002. Reexpression of the Tumor Suppressor Gene *ARHI* Induces Apoptosis in Ovarian and Breast Cancer Cells through a Caspase-independent Calpain-dependent Pathway. *Cancer Research.* 62: 7264-7272.

Campbell, S., Khosravi-Far, R., Rossman, K., Clark, G., and Der, C. 1998. Increasing complexity of Ras signaling. *Oncogene.* 17: 1395-1413.

Cancer Quest. Emory Winship Cancer Institute. Emory University. 22 Jan. 2008. < http://www.cancerquest.org/index.cfm?page=261>.

Cooper, Geoffrey, and Hausman, Robert. *The Cell: A Molecular Approach 3rd Edition.* Washington, DC: ASM Press, 2004.

Gilbert, Scott. *Developmental Biology, 7th Edition.* Sunderland, MA: Sinauer Associates, 2003.

Lu, Z., Luo, R., Peng, H., Huang, M., Nishimoto, A., Hunt, K., Helin, K., Liao, W., and Yu, Y. 2006. E2F–HDAC complexes negatively regulate the tumor suppressor gene ARHI in breast cancer. *Oncogene.* 25: 230-239.

Luo, R., Peng, H., Xu, F., Bao, J., Pang, Y., Pershad, R., Issa, J., Liao, W., Bast, R., Yu, Y. 2001. Genomic structure and promoter characterization of an imprinted tumor suppressor gene ARHI. *Biochimica et Biophysica Acta*. 1519: 216-222.

Luo, R., Fang, X., Marquez, R., Liu, S., Mills, G., Liao, W., Yu, Y., and Bast, R. 2003. ARHI is a Ras-related small G-protein with a novel N-terminal extension that inhibits growth of ovarian and breast cancers. *Oncogene.* 22: 2897-2909.

McKinnell, Robert, Parchment, Ralph, Perantoni, Alan, and Pierce, G. *The Biological Basis of Cancer.* New York: Cambridge University Press, 1998.

NCBI. Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, Ke Z, Krylov D, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Thanki N, Yamashita RA, Yin JJ, Zhang D, Bryant SH. (2007) *CDD: a conserved domain database for interactive domain family analysis.* Nucleic Acids Res. 35: D237-40. 22 Jan. 2008. < http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=58023>.

Nishimoto, A., Yu, Y., Lu Z., Mao, X., Ren, Z., Watowich, S., Mills, G., Liao, W., Chen, X., Bast, R., and Luo, R. 2005. A Ras Homologue Member I Directly Inhibits Signal Transducers and Activators of Transcription 3 Translocation and Activity in Human Breast and Ovarian Cancer Cells. *Cancer Research.* 65: 6701-6710.

Peng, H., Xu, F., Pershad, R., Hunt, K, Frazier, M., Berchuck, A., Gray, J., Hogg, D., Bast, R., Yu, Y. 2000. ARHI is the center of allelic deletion on chromosome 1p31 in ovarian and breast cancers. *Int. J. Cancer.* 86: 690-694.

Rosen, D., Wang, L., Jain, A., Lu, K., Luo, R., Yu, Y., Liu, J., and Bast, R. 2004. Expression of the Tumor Suppressor Gene ARHI in Epithelial Ovarian Cancer Is Associated with Increased Expression of p21WAF1/CIP1 and Prolonged Progression-Free Survival. *Clinical Cancer Research.* 10: 6559-6566.

Satoh, T., Nakafuku, M., and Kaziro, Y. 1992. Function of Ras as a Molecular Switch in Signal Transduction. *The Journal of Biological Chemistry.* 267: 24149-24152.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., Amico, M., Pestell, R., and Ben-Ze'ev, A. 1999. The Cyclin D1 Gene is a Target of the β-catenin/LEF-1 Pathway. *Proc. Natl. Acd. Sci. USA.* 96: 5522-5527.

University of Melbourne (2008) *Department of Anatomy and Cell Biology.* Goodwin, Tony. The University of Melbourne ABN. 20 March 2008. <http://www.anatomy.unimelb.edu.au/ researchlabs/deIongh/projects.html>.

Wang, L., Hoque, A., Luo, R., Yuan, J., Lu, Z., Nishimoto, A., Liu, J., Sahin, A., Lippman, S., Bast, R., and Yu, Y. 2003. Loss of the Expression of the Tumor Suppressor Gene *ARHI* Is Associated with Progression of Breast Cancer. *Clinical Cancer Research*. 9: 3660-3666.

Weber, F., Aldred, M., Morrison, C., Plass, C, Frilling, A., Broelsch, C., Waite, K., and Eng, C. 2005. Silencing of the Maternally Imprinted Tumor Suppressor ARHI Contributes to Follicular Thyroid Carcinogenesis. *The Journal of Clinical Endocrniology and Metabolism.* 90: 1149-1155.

Weinberg, Robert. *The Biology of Cancer.* New York: Garland Science, Taylor and Francis Group, LLC. 2007.

Yuan, J., Luo, R., Fujii, S., Wang, L., Hu, W., Andreef, M., Pan, Y., Kadota, M., Oshimura, M., Sahin, A., Issa, J., Bast, R., and Yu, Y. 2003. Aberrant Methylation and Silencing of *ARHI*, an Imprinted Tumor Suppressor Gene in which the Function Is Lost in Breast Cancers. *Cancer Research.* 63: 4174-4180.

Yu, Y., Xu, F., Peng, H., Fang, X., Zhao, S., Li, Y., Cuevas, B., Kuo, W., Gray. J., Siciliano, M., Mills, G., and Bast, R. 1999. *NOEY2* (ARHI), an imprinted

putative tumor suppressor gene in ovarian and breast carcinomas. *Proc. Natl. Acad. Sci. USA.* 96: 214-219.

Yu, Y., Fujii, S., Juan, J., Luo, R., Wang, L., Bao, J., Kadota, M., Oshimura, M., Dent, S., Issa, J., and Bast, R. 2003. Epigenetic regulation of ARHI in Breast and Ovarian Cancer Cells. *Ann. N.Y. Acad. Sci.* 983: 268-277.

Zhang, W., Jullig, M., Connolly, A., and Stott, N. 2005. Early Gene Response in Lithium Chloride Induced Apoptosis. *Apoptosis.* 10: 75-90.

Zhou, P., Byrne, C., Jacobs, J., and Fuchs, E. 1995. Lymphoid Enhancer Factor 1 Directs Hair Follicle Patterning and Epithelial Cell Fate. *Genes and Development.* 9: 700-713.