KLF4 AND RETINOID RECEPTOR SIGNALING IN CANCER

by

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A DISSERTATION

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PREVENTION OF KLF4-MEDIATED TUMOR INITIATION AND MALIGNANT TRANSFORMATION BY UAB30 REXINOID

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ABSTRACT

The fight against cancer has generated wide interest in understanding the genetic mechanisms behind the disease. One group of oncogenes – transcription factors – offers many opportunities for treatment or prevention. Among these transcription factors is KLF4, which functions in post-mitotic epithelial cells to promote differentiation. KLF4 has tumorigenic activity in both in vitro and in vivo models. In analyzing transcripts that were induced by KLF4, we noted the induction of multiple nuclear receptors, including retinoid receptors. Retinoids are well-established as chemopreventive agents, but the mechanisms by which they act to prevent cancer remain unclear.

Previous studies have shown that KLF4 is co-expressed with the nuclear receptors RAR\(\gamma\) and RXR\(\alpha\) in the skin, and the formation of the skin permeability barrier is a common function of these three proteins. To examine functional interactions between KLF4 and retinoic acid receptors, we utilized a tetracycline-inducible KLF4-transgenic mouse model of cutaneous squamous cell carcinoma (SCC) together with cultured epithelial cells.

Employing an RK3E epithelial cell model and multiple oncogenes (KLF4, ErbB2, Notch1, Gli1), we found that retinoids, including an RAR-selective agonist (all-trans RA), an RXR-selective agonist (9-cis UAB30, rexinoid), and a pan agonist (9-cis RA), specifically inhibit KLF4-mediated epithelial cell transformation, implicating distinct nuclear receptor heterodimers as modulators of KLF4 transforming activity. When
RXRα expression was suppressed by RNAi in cultured cells, transformation was promoted and the inhibitory effect of 9cUAB30 was attenuated. Meanwhile, we also found that KLF4 rapidly induces transcripts encoding RXRα and RARγ using a conditional approach.

In KLF4-transgenic mice, we found that rexinoid successfully prevented the initiation of cutaneous SCC. We further analyzed rexinoid effects in KLF4-induced epithelial cell cycling and differentiation in vivo. These studies indicate that 9cUAB30 permits KLF4 expression and cell cycling, consistent with the results of in vitro FACS analysis and cell growth assays; however, it may restrict the cell fate changes induced by KLF4, as indicated by attenuation in the misexpression of cytokeratin 1 in basal cells. Taken together, these results identify antagonism of KLF4-induced tumor initiation as a novel effect of retinoids that may contribute to their activities in cancer chemoprevention and therapy.
DEDICATION

To my dearest parents, Mom and Dad and my beloved husband Xiaojia!
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I would like to acknowledge many people who helped me in the past five years of my graduate studies at UAB.

First, I would like to thank my parents for their constant support through my entire life. They have always encouraged me, even though they live far away in China. Their selfless love for me is my source of courage, creativity, persistence and confidence.

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<td>9cUAB30</td>
<td>9-cis UAB30</td>
</tr>
<tr>
<td>9cRA</td>
<td>9-cis Retinoic Acid</td>
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<tr>
<td>AK</td>
<td>actinic keratosis</td>
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<td>ALL</td>
<td>acute lymphocytic leukemia</td>
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<td>AML</td>
<td>acute myelogenous leukemia</td>
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<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
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<td>APL</td>
<td>acute promyelocytic leukemia</td>
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<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>ATRA</td>
<td>all-trans Retinoic Acid</td>
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<tr>
<td>BCC</td>
<td>basal cell carcinoma</td>
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<td>BMP</td>
<td>bone morphogenic proteins</td>
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<tr>
<td>BTE</td>
<td>basic transcription element</td>
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<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<td>CREB</td>
<td>cAMP response element binding</td>
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<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>dox</td>
<td>doxycycline</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EPU</td>
<td>epidermal proliferating unit</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<td>ES</td>
<td>embryonic stem</td>
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<td>EZF</td>
<td>epithelial zinc finger</td>
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<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>HAT</td>
<td>histone acetyltransferase</td>
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<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor-2</td>
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<td>HRE</td>
<td>hormone response element</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>iPSC</td>
<td>inducible pluripotent stem</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>KLF</td>
<td>Krüppel-Like Factors</td>
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<td>LBD</td>
<td>ligand binding domain</td>
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<td>LXR</td>
<td>liver-X-receptor</td>
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<tr>
<td>MMR</td>
<td>mismatch repair</td>
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<tr>
<td>MMS</td>
<td>methyl methanesulfonate</td>
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<tr>
<td>MNU</td>
<td>1-methyl-1-nitrosourea</td>
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<tr>
<td>NCoR</td>
<td>nuclear receptor co-repressor</td>
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<tr>
<td>NER</td>
<td>nucleotide-excision repair</td>
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<td>NLS</td>
<td>nuclear localization signals</td>
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<td>NPM</td>
<td>nucleophosim</td>
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<tr>
<td>NR</td>
<td>nuclear receptor</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>NUMA</td>
<td>nuclear mitotic apparatus protein</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PEST</td>
<td>proline (P), glutamic acid (E), serine (S), and threonine (T)</td>
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<tr>
<td>PLZF</td>
<td>PML zinc finger</td>
</tr>
<tr>
<td>PML</td>
<td>promyelocytic leukaemia</td>
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<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
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<td>PR</td>
<td>progesterone receptor</td>
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<td>RAR</td>
<td>retinoic acid receptor</td>
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<td>RNAi</td>
<td>ribonucleic acid interference</td>
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<td>RXR</td>
<td>retinoid X receptor</td>
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<td>SCC</td>
<td>squamous cell carcinoma</td>
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<td>SERM</td>
<td>selective estrogen receptor modulator</td>
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<tr>
<td>shRNA</td>
<td>short hairpin Ribonucleic Acid</td>
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<tr>
<td>SMRT</td>
<td>silencing mediator for retinoid and thyroid hormone receptor</td>
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<tr>
<td>SPR</td>
<td>small proline-rich</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>STAT5B</td>
<td>signal transducer and activator of transcription 5B</td>
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<td>TGF β1</td>
<td>transforming growth factor β1</td>
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<td>TR</td>
<td>thyroid hormone receptor</td>
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<td>VDR</td>
<td>vitamin D receptor</td>
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<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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<td>WT</td>
<td>wild type</td>
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INTRODUCTION

CANCER

The second leading cause of death in the United States, cancer is not just one disease, but a large, complex group of almost one hundred diseases. Each year, about 1.2 million Americans are diagnosed with some form of cancer, and more than 500,000 die of it. Medically defined as a malignant neoplasm, cancer owes its deadly effect to three properties -- uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and in the most serious cases, metastasis (spread to distant sites in the body via lymph or blood). The most common types attack the skin, lungs, colon, breast (in women), and prostate (in men). The risk of cancer increases with age and the presence of other known risk factors, such as smoking, overexposure to sunlight, radiation or carcinogenic chemicals, hormonal imbalance, viruses, or a family history of the disease. While progress has been made in the fight against certain types of cancer, the disease remains poorly understood.

Carcinogenesis: A Multistage Process

Cancer in human is thought to take many years to develop (Vogelstein and Kinzler 789-99; Vogelstein and Kinzler 138-41), emerging in as many as four distinct stages: initiation; promotion; progression; and malignant conversion. The first stage, initiation, involves genetic or epigenetic changes in the somatic cells of normal tissue. These early
changes may not cause cancer right away, but may alter and predispose a tissue to further alterations (Beachy, Karhadkar, and Berman 324-31; Trosko et al. 192-201). Promotion is a stage in which cell division may be prominent, due to an external growth stimulus. Eventually these cells produce a benign neoplasm. In the skin, for example, such a neoplasm could be a papilloma or else a more diffuse lesion called actinic keratosis. These lesions may be reversible after termination of the stimulus (such as sun exposure) (Trosko et al. 192-201). During progression, it is thought that additional mutation and epigenetic alteration render the tumor independent of external signals (growth factors), which creates the environment for uncontrolled growth (Trosko et al. 192-201; Hanahan and Weinberg 57-70). The resulting imbalance of cell growth and cell death leads to the final stage, malignant conversion, in which tumor cells acquire the ability to invade locally and/or to metastasize to distant sites. Invasion and successful metastasis require the acquisition of multiple new tumor cell properties, including cell motility, remodeling of the extracellular matrix, entry into the circulation, exit from the vasculature, growth at a distant site, and neo-angiogenesis (Chambers, Groom, and MacDonald 563-72; Hood and Cheresh 91-100; Sasisekharan et al. 521-28; Thiery 740-46). Each of the multiple stages of tumor progression, as well as the properties required for metastasis, provides opportunities for prevention of or intervention in the progress of cancer.

In a more detailed view of this process, Hanahan and Weinberg reviewed the essential features of a cancer cell: self-sufficiency in growth signals; insensitivity to anti-growth signals; inhibition of apoptosis; unrestricted replicative potential; constant angiogenesis; and tissue invasion (Hanahan and Weinberg 57-70). Each of these
alterations may have genetic or epigenetic origins, consistent with studies of age and incidence showing that human tumorigenesis may proceed through 6-10 rate-limiting steps (Finkel, Serrano, and Blasco 767-74).

Cancer as a Genetic Disease

Molecular evidence in support of a genetic basis for cancer was first documented in the 1970s with the discovery of the oncogene Src, and it has been confirmed by subsequent studies in the period since then (Nobel Prize in Physiology or Medicine for 1989 jointly to J. Michael Bishop and Harold E. Varmus for their discovery of "the cellular origin of retroviral oncogenes", Robert A. Weinberg). In fact, cancer is now thought to be essentially a genetic disease in which key biological players -- oncogenes, tumor suppressor genes, or DNA repair genes -- are damaged or compromised (Vogelstein and Kinzler 789-99). This perspective opens the door to many more potential prevention and treatment strategies than were not thought possible only a few decades ago, when cancer was considered capricious and incurable.

1. Oncogenes

Among the key players in tumor progression are oncogenes, wild type or mutated genes that are activated in cancer cells by point mutation, gene amplification, gene rearrangement, or by loss of function of upstream regulators (e.g., tumor suppressors). For example, the common skin cancer, basal cell carcinoma (BCC), almost always
upregulates expression of wild-type Gli1, usually as a consequence of loss-of-function mutation of its upstream regulators, Patched (Ptc) or Smoothened (Smo).

The mutated version of the Src oncogene was first characterized by isolating an avian retrovirus that could rapidly induce sarcomas when injected into animals (Nobel Prize in Physiology or Medicine for 1989 jointly to J. Michael Bishop and Harold E. Varmus for their discovery of "the cellular origin of retroviral oncogenes"). Src is the prototype of a family of tyrosine kinases. The discovery of Src suggested that oncogenes were mutated versions of proto-oncogenes that normally regulate critical processes, including embryonic development, cell division, and cellular differentiation.

The development of recombinant DNA technology in the 1980s led to the isolation of over 30 retroviral oncogenes that could transform mammalian cells in tissue culture. In addition, oncogenes were identified by a variety of other approaches, including functional expression cloning, by analysis of amplified regions in tumors, and by characterization of translocations (Robert A. Weinberg). Of these genes, multiple oncogenes are now well-established to be important in human tumors, including RAS, GLI, c-MYC and others (Foster et al. 423-34; Foster et al. 1491-500; Louro et al. 503-16; Louro et al. 5867-73; Shih and Weinberg 161-69). Recently, microRNA genes have been identified as potential oncogenes through the regulation of protein-coding genes such as c-Myc or E2F (Esquela-Kerscher and Slack 259-69).

Today, researchers recognize at least six functional categories of oncogenes: 1. growth factors (e.g. platelet-derived growth factor [PDGF], c-Sis); 2. receptor tyrosine kinases (e.g. epidermal growth factor receptor [EGFR], platelet-derived growth factor
receptor [PDGFR], and vascular endothelial growth factor receptor [VEGFR]; 3. signal transducers (including cytoplasmic tyrosine kinases [e.g. Src-family, BTK family], cytoplasmic serine/threonine kinases [e.g. Raf, AKT, cyclin-dependent kinases], and the Ras protein); 4. chromatin remodelers (e.g. the fusion of ALL1 with 1 of more than 50 proteins leads to a multi-protein complex, which remodels chromatin in acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML)); 5. apoptosis regulators (e.g. BCL2); 6. and transcription factors (e.g. c-MYC, Gli, KLF4) (Croce 502-11).

While all oncogene categories warrant further study, transcription factors have attracted much interest. One such factor, KLF4, was recently isolated as a novel transforming oncogene in the E1A-immortalized rat kidney epithelial cell line (RK3E cells), which marked the first reported use of these cells for oncogene expression cloning (Adams M123-M133;Bagchi, Weinmann, and Raychaudhuri 1063-72;Huang et al. 1563-66;Kinzler and Vogelstein 761). The significance of the use of epithelial cells is that most adult cancers are derived from epithelia, and commonly used fibroblast models often fail to show transformation in response to carcinoma oncogenes (e.g., KLF4, β-catenin, Gli1).

2. Tumor Suppressor Genes

Tumor suppressor genes are usually inactivated in cancer cells, typically by point mutation, deletion, chromosomal allelic loss, or promoter methylation, which is termed epigenetic silencing. Unlike oncogenes, tumor suppressor genes generally follow A.G. Knudson’s "two-hit" model for carcinogenesis, which stems from the analysis of the age-
incidence of retinoblastoma (Knudson, Jr. 820-23). Corresponding to the two hits, mutations in both the maternal and paternal alleles of a tumor-suppressor gene are generally observed in cancer cells. Mutant tumor suppressor alleles are usually “recessive”, permitting normal development and surfacing in postnatal or adult mammals following loss-of-function of the second copy. The high frequency of these second hits (often through allelic loss) accounts for the autosomal dominant inheritance pattern observed in cancer families for diseases such as Adenomatous Polyposis Coli (APC gene) or Gorlin’s Basal Cell Carcinoma (Patched gene). In contrast, mutant oncogenes are more often dominant over the wild type allele, interfering with normal development. Therefore inherited gain-of-function mutations are consequently less common.

The first tumor suppressor, the Retinoblastoma protein (p105Rb or pRb or Rb), was discovered in 1984, and many studies since then have documented its crucial role (Murphree and Benedict 1028-33). Primarily, a tumor suppressor acts at the “restriction point” to prevent cell cycle. Rb function is now thought to be inactivated by direct or indirect mechanisms in all or nearly all human cancers. In quiescent or early G1-phase cells, pRB is hypophosphorylated and associated with members of the E2F transcription factor family, forming a co-repressor complex (Bagchi, Weinmann, and Raychaudhuri 1063-72). When cyclin/CDK4/CDK6 complexes phosphorylate pRB, E2Fs are released from pRB and function as transcriptional activators, the subsequent loss of pRB leads to deregulated cell proliferation and apoptosis (Adams M123-M133). Thus, dysfunctional pRb is one of the critical contributing factors in a number of types of cancer.

Another important example of a tumor suppressor is the transcription factor p53, encoded by the TP53 gene and located on chromosome 17. Central to many of the cell's
anti-cancer mechanisms, p53 is able to not only inhibit cell growth through cell cycle arrest (allowing the time to repair damaged DNA), but also stimulate apoptosis or senescence when induced by cellular stress (Oren 431-42; Prives and Hall 112-26; Vogelstein, Lane, and Levine 307-10). Mutation of the p53 gene has been found in most tumor types. Hence, the normal and mutant functions of pRb and p53 reflect their significant roles in the pathogenesis of human cancers, making them potential targets for cancer therapy and prevention.

3. Stability or Caretaker Genes

A third class of cancer genes, stability or caretaker genes, encode products that can minimize genetic alterations. Fundamentally, mutations in caretaker genes result in genomic instability and promote tumorigenesis in a completely different way. This class includes genes responsible for specific DNA maintenance operations, such as the mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes. Other genes that regulate chromosome stability are also included in this class, such as BRCA1, BLM and ATM (Vogelstein and Kinzler 789-99).

An inherited predisposition to cancer can lead to early onset, a feature that leads Knudson to propose two hits in retinoblastoma. In contrast, when the mutations must accumulate in somatic cells, the resulting cancers may occur at a later age.
Cutaneous Squamous Cell Carcinoma (SCC)

It is known that 90 percent of human cancers arise in epithelial cells. The cutaneous epithelium is a readily accessible and experimentally tractable setting to model the general processes of epithelial neoplasia. It is also the site of the most common cancer in the United States -- non-melanoma skin cancer -- including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), with over 1.3 million cases per year (Alam and Ratner 975-83). Twenty percent of these are SCC, the second most common cancer among Caucasians, with a yearly incidence estimated at more than 250,000. Its lifetime risk is 9-14% (men) or 4-9% (women). SCC is an aggressive tumor that has a substantial risk of recurrence (8% at 5 years) or metastasis (5% at 5 years). These rates are increased by 2-5 fold by high risk features, such as large tumor size or depth, poor differentiation, perineural invasion, a history of previous SCC, or location on the lip or ear (Alam and Ratner 975-83). Avoidance of sunburn can help prevent this disease, but no practical chemoprevention exists. Thus, SCC represents a considerable burden in terms of morbidity and mortality, accounting for much of the current interest in genetic analysis of this disease and potential interventions.

Chemoprevention of Cancer

Among the newest therapies emerging over the last decade, chemoprevention of cancer relies on pharmacological agents to preempt the process of carcinogenesis (Sporn 6215-18). Because they are administered proactively, before tumors emerge, these agents offer the potential to interact with all stages of carcinogenesis -- initiation, promotion,
progression, or malignant conversion – and dramatically improve survival rates and quality of life.

Significantly, chemoprevention, which is recommended for high-risk populations long before cancer develops, differs from more traditional chemotherapy that is used to kill existing cancer cells. Therefore, success with chemoprevention depends on recognition of a patient’s risk for a cancer at the early stage. At the same time, any preventive agent that is given long term to at-risk but otherwise “healthy” people must meet safety standards and demonstrate proven benefits.

Chemoprevention has been successfully administered in many animal experiments for over 20 years, and certain types of these agents are currently undergoing human clinical trials (Prevention of cancer in the next millennium: Report of the Chemoprevention Working Group to the American Association for Cancer Research 4743-58). Many agents target molecular and cellular processes known to be implicated in carcinogenesis. Among these, agonists of the nuclear receptor superfamily regulate cell differentiation and proliferation (Mangelsdorf et al. 835-39). For example, tamoxifen, which is a selective estrogen receptor modulator (SERM), can inhibit the growth effect estrogen has on estrogen receptor positive breast cancers (Fisher et al. 1371-88). Clinical trials have also shown that it can prevent breast cancer in women at high risk. As a result, tamoxifen has been approved by the U.S. Food and Drug Administration (FDA) as a preventive as well as treatment agent to reduce the risk of breast cancer.

Another group of highly promising chemopreventive agents in skin and breast cancer are retinoids and rexinoids, agonists of the retinoic acid receptors (RARs) and
retinoid X receptors (RXRs), respectively. RARs and RXRs function as heterodimeric transcription factors. As a result, the successful development of chemopreventive agents like these may identify and target critical signaling pathways in genetically unstable and genetically heterogeneous populations of tumors cells capable of adapting to and surviving diverse clinical regimens.

**KLF4**

**Family of Krüppel-Like Factors (KLFs)**

Krüppel-like factors (KLFs) are a family of transcription factors that play important roles in many fundamental biologic processes, including development, proliferation, differentiation and apoptosis. The KLF family is distinct in that these proteins contain three highly conserved C-terminal C2H2-type zinc fingers that bind DNA. Therefore, KLFs recognize and bind to very similar “GT-box” or “CACCC element” consensus sequences (Dang, Pevsner, and Yang 1103-21). They were named “Krüppel-like” due to strong regional homology with the *Drosophila* gene product Krüppel, which is important in segmentation of the developing embryo. Not surprisingly, genetic deletion of Krüppel results in a “crippled” phenotype (Preiss et al. 27-32). In human, there are at least 25 members of the KLF family. For example, KLF4 exhibits a high degree of homology in the Krüppel region of the protein with KLF2 (Lung Kruppel Like Factor (Anderson et al. 5957-65)) and KLF1 (Erythroid Kruppel Like Factor (Miller and Bieker 2776-86)). However, the specificity of their activities is determined by different amino termini and/or by tissue-specific expression. Additionally, they have
different transcriptional properties depending on the co-regulators with which they interact. KLF family members can be both oncogenic and tumor suppressive, and function in tumorigenesis, apoptosis and angiogenesis (Black, Black, and Azizkhan-Clifford 143-60). Therefore, a better understanding of the mechanism between these transcription factors and cancer development will lead to more effective diagnostic strategies and to drug design of more promising therapeutic targets.

**KLF4 Discovery and its Structure**

KLF4 (Krüppel-like factor 4) was identified independently by two groups in 1996. At that time, it was given two different names -- gut-enriched Krüppel-like factor (GKLF) due to its high expression in the intestine (Shields, Christy, and Yang 2009-17) and epithelial zinc finger (EZF) due to its high expression in the skin epithelium (Garrett-Sinha et al. 31384-90). To avoid confusion, it was later renamed KLF4, as the protein expression is also detectable in many other organs and tissues, including lung (Shields, Christy, and Yang 2009-17), testis (Shields, Christy, and Yang 2009-17), thymus (Panigada et al. 103-13), cornea (Chiambaretta et al. 901-09), cardiac myocytes (Cullingford et al. 1229-36), and lymphocytes (Fruman et al. 359-64). KLF4 is dispensable for embryonic development but is required in some tissues for normal epithelial differentiation. KLF4 deficient animals show defects in goblet cell formation in the gut and fail to form a permeability barrier in the skin (Ton-That et al. 239-43; Segre, Bauer, and Fuchs 356-60).
Structural organization of the *KLF4* gene and the corresponding protein functional domains are shown in Fig.1 (Wei et al. 23-31). The human KLF4 gene locus is located at chromosome 9q31. Human and mouse KLF4 are 470 and 483 amino acids in length, respectively, and produce a protein of approximately 55 kDa. Several functional domains have been characterized in the KLF4 protein, including a transcriptional activation domain at the N-terminus, the C-terminal DNA-binding domain, two nuclear localization signals and transcriptional repression domains adjacent to the zinc fingers. In addition, there are two potential PEST sequences located between the transcriptional activation and transcriptional inhibitory domains, suggesting that KLF4 may be degraded through the ubiquitin-proteosome pathway (Chen et al. 10394-400). The DNA-binding domain consists of three successive zinc fingers, each containing an anti-parallel β-sheet, followed by a short loop and an α-helix. The single zinc ion stabilizes the figure through working together with two cysteines within the β-sheet and two histidines within the α-helix (Yang 2045-51). As shown by highly purified recombinant GKLF in a target detection assay, KLF4 interacts with the double-stranded DNA containing the sequence 5’ RRGGYGY 3’ (R=purine, Y=pyrimidine) (Shields and Yang 796-802).

**KLF4-Mediated Activation and Repression: Gene-Dependent**

Transcription factors activate or repress downstream target gene expression by binding to promoter regions and recruiting co-activators or co-repressors to the promoters. KLF4 has both activation and repression domains that could mediate either activity in transcription. To activate transcription, KLF4 can interact with p300/CBP (Geiman et al.
CBP (CREB-binding protein) and its homologue p300 contain the catalytic histone acetyltransferase (HAT) domain (Ogryzko et al. 953-59) and act as transcriptional co-activator for many sequence-specific transcriptional factors (Eckner et al. 869-84). They can acetylate the histone to activate transcription (Allfrey, Faulkner, and Mirsky 786-94) and they can directly acetylate transcription factors as well (Chan and La Thangue 2363-73). p300/CBP play a key role in the regulation of cell proliferation and differentiation (Giordano and Avantaggiati 218-30; Kouzarides 40-48), and have also been shown to interact with the tumor suppressor p53, thereby modulating its cell cycle regulator activity (Gu, Shi, and Roeder 819-23; Lill et al. 823-27). Additionally, it has been shown that there is physical interaction between KLF4 and CBP both in vivo and in vitro to activate transcription by targeting histones for acetylation (Geiman et al. 1106-13). Site-directed mutagenesis of two clusters of acidic amino acid residues within N-terminus of KLF4 also provides substantial association between two primary biological activities of KLF4, i.e. transcription activation and cell growth suppression (Geiman et al. 1106-13). Furthermore, direct acetylation of KLF4 by p300 is also critical for KLF4-mediated transactivation (Evans et al. 33994-4002).

In other contexts, however, KLF4 can be a potent repressor of transcription. KLF4 has been shown to suppress the activity of the CYP1A1 promoter in a basic transcription element (BTE)—dependent fashion. It does this by competing with the binding of Sp1 to BTE and physically interacting with Sp1, which is a potent activator of CYP1A1 (Zhang et al. 17917-25). KLF4 can also repress cyclin B, in part by promoting co-repressor proteins including (histone deacetylase) HDAC3 (Evans et al. 33994-4002). KLF4 has also been shown to interact with Tip60 and HDAC7. The repressive complex
formed by these three proteins can inhibit histidine decarboxylase (HDC) promoter activity (Ai et al. 6137-49). Finally, KLF4 represses transcriptional targets of Wnt signaling by directly interacting with β-catenin/TCF-4 (Zhang et al. 2055-64). These observations suggest that KLF4-mediated activation and/or repression is dependent upon the context.

**Role of KLF4 in Inhibiting Cellular Proliferation**

In many cell lines, KLF4 inhibits cell proliferation. Consistently, the highest expression of KLF4 is detected in terminally differentiated, post-mitotic epithelial cells, such as skin and gut, rather than in proliferating cells. In NIH3T3 cells, KLF4 expression peaks as the cells become growth-arrested by serum starvation or contact inhibition, and drops to the lowest level in dividing cells (Shields, Christy, and Yang 20009-17). The mechanism by which KLF4 suppresses cell proliferation may derive from the function of KLF4 in DNA damage induced cell-cycle arrest. DNA damage induced by methyl methanesulfonate (MMS) (Zhang et al. 18391-98) or γ irradiation (Yoon, Chen, and Yang 2101-05) gives rise to the induction of KLF4 expression in a p53-dependent pattern. The *KLF4* mRNA level increases simultaneously with *p21WAF1/Cip1* mRNA, of which protein product is a major cyclin-dependent kinase inhibitor (Zhang et al. 18391-98). KLF4 activates p21 via a specific Sp1-like *cis*-element in the *p21* proximal promoter, through which p53 activates *p21* promoter as well. As a matter of fact, p53 and KLF4 do interact with each other physically, allowing p53 to gain access to the *p21* promoter and activate *p21* transcription (Zhang et al. 18391-98). Thus, by transcriptionally activating
KLF4 can act as an important mediator of p53 checkpoint function at both the G1/S and G2/M transition points after DNA damage occurs. KLF4 has also been shown to directly inhibit cyclin D1 (Shie et al. 2969-76) and cyclin B1 expression (Yoon and Yang 5035-41), which are fundamental elements in the G1/S and G2/M transitions, respectively. Furthermore, after DNA damage caused by γ irradiation, KLF4 is both necessary and sufficient to prevent chromosomal replication by transcriptionally suppressing the cyclin E gene (Yoon et al. 4017-25).

Transcriptional profiling of KLF4 using cDNA microarray analysis confirms that KLF4 activates or suppresses transcription of a wide range of genes encoding regulators of the cell cycle, including p21, p57kip2, IGFBP6, p27Kip1, CyclinD1, CDC2/CDK1, KIA, MCM2, CENPE, MAD2L1, BUB1B and RAD21, etc (Chen et al. 665-77).

In addition to the essential roles of KLF4 in cell-cycle regulation, KLF4 is also one of down-stream targets of interferon-γ (IFN-γ) (Chen, Shie, and Tseng 67-72). IFN-γ can mediate its growth inhibitory and apoptotic properties by inducing the expression of a number of effector genes (Chin et al. 719-22; Chin et al. 5328-37). In colon cancer cells, IFN-γ upregulates KLF4 expression in a dose and time dependent manner, a process which is p53-independent. It has also been suggested that upregulation of KLF4 expression can be mediated through the intracellular signaling pathway triggered by the activation of IFNs and its downstream signaling such as Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway (Leaman et al. 1578-88; Shuai 253-59). Indeed, this effect is mediated by phosphorylation of STAT1, partly through interaction of STAT1 with the GAS element on the KLF4 promoter (Chen, Shie, and
Taken together, these studies point to the important roles of KLF4 in regulating cellular proliferation.

**Role of KLF4 in Promoting Differentiation**

KLF4 is an important factor involved in the differentiation of many tissues and organs, such as skin, gut, cornea and vascular smooth muscle cell. Under physiological conditions, KLF4 is highly expressed in the suprabasal, differentiating layers of the epidermis. In fact, it is necessary for survival to establish the mouse skin permeability barrier; defects in this barrier can lead to death shortly after birth due to dehydration, as seen in Klf4−/− mice (Segre, Bauer, and Fuchs 356-60). On the contrary, accelerated formation of the skin permeability barrier can be achieved when KLF4 is overexpressed in the epidermis (Jaubert, Cheng, and Segre 2767-77).

The role of KLF4 in forming the skin barrier may be attributed to its regulation of Sprr, as expression of this protein is reduced in KLF4-deficient skin (Patel, Kartasova, and Segre 140-48). Another potential mechanism is that KLF4 and corticosteroids can co-regulate many of the same cellular genes (Patel et al. 18668-73). Steroids are known inducers of epithelial maturation and barrier formation. Consistent with these findings, krüppel-like transcription factors and glucocorticoid receptors can act synergistically at the promoters to superactivate transcription (Turner and Crossley 5129-40).

KLF4 is also expressed in the colonic and corneal epithelium, where it is crucial for goblet cell terminal differentiation. For example, as high as 90% reduction in the total number of colonic goblet cells, as well as abnormal expression of goblet cell-specific
markers, is observed in the colonic epithelium of Klf4−/− mice (Katz et al. 2619-28). The fact that KLF4 is able to interact with β-catenin and suppress Wnt signaling pathway, which is essential in intestinal epithelium proliferation (Batlle et al. 251-63; Korinek et al. 379-83; van de Wetering et al. 241-50), suggests KLF4 may also be crucial in regulating the transition process from transit-amplifying cells to the diversified differentiated cells in the colonic crypts. In the eye, targeted deletion of KLF4 leads to corneal fragility, edema and a shortage of goblet cells in the conjunctiva (Swamynathan et al. 182-94).

In the regulation of vascular smooth muscle cell (VSMC) differentiation, KLF4 is a target of bone morphogenic proteins (BMP) and transforming growth factor β1 (TGF β1) (King et al. 11661-69). Both TGFβ1 and BMP belong to transforming growth factor β superfamily, which plays fundamental roles in the control of growth, development and differentiation. They can interact with membrane complex formed by type I and type II serine/threonine kinase receptors, and transduce their signals via SMAD proteins (Miyazono 1101-09; Miyazono, Kusanagi, and Inoue 265-76; Moustakas, Souchelnytskyi, and Heldin 4359-69). To modulate the VSMC phenotype, TGF β1 and BMP-2, -4, -6 induce KLF4 to bind to the promoters of smooth muscle cell marker genes (King et al. 11661-69).

**KLF4 Can Serve Dual Roles: Tumor Suppressor or Oncogene**

Because of KLF4’s critical roles in inhibiting proliferation and promoting differentiation, it is logical to consider the transcription factor a tumor suppressor. In fact, KLF4 can function as a tumor suppressor in the gastrointestinal tract (Rowland and
Consistently, conditional knockout of KLF4 in the gastric epithelium leads to increased proliferation, altered differentiation and precancerous changes in the adult stomach (Katz et al. 935-45). Conversely, overexpression of KLF4 in the human colon cancer cell line RKO results in decreased colony formation, cell migration and invasion, and in vivo tumorigenicity (Dang et al. 3424-30). Examination of KLF4 expression in mouse models of colorectal cancer has yielded similar results. The multiple intestinal neoplasia (APC\textsuperscript{min/+}) mouse develops multiple intestinal adenomas early in life and is a widely used model of intestinal tumorigenesis (Moser, Pitot, and Dove 322-24; Su et al. 668-70). In adenomas from these mice, expression of KLF4 is reduced and is inversely related to the size of the tumor (Dang et al. 203-07; Ton-That et al. 239-43). In addition, crossing APC\textsuperscript{min/+} mice with KLF4\textsuperscript{+/−} heterozygotes resulted in significantly more adenomas than in APC\textsuperscript{min/+} mice alone (Ghaleb et al. 7147-54).

In addition to its established role as a tumor suppressor, KLF4 can also act as an oncogene in certain contexts, as identified by three independent labs through unbiased genetic screens. First, KLF4 was identified as an oncogene by the Ruppert laboratory in the late 1990s, using E1A-immortalized rat kidney epithelial cells to screen for oncogenes that could induce transformation (Foster et al. 423-34). In 2002, KLF4 was identified as a retroviral insertion site in murine leukemias and lymphomas (Suzuki et al. 166-74). In 2005, KLF4 was identified in a screen for genes of which products can prevent induction of senescence by an activated form of Ras (RasV12) (Rowland, Bernards, and Peeper 1074-82). Indeed, KLF4 is up-regulated early during progression of specific human tumor types, including cutaneous and oral SCCs (Foster et al. 423-
Importantly, up to 70% of primary human breast cancers show KLF4 overexpression (Foster et al. 423-34; Foster et al. 6488-95). KLF4 overexpression becomes evident at the stage of ductal carcinoma in situ, indicating that, similar to SCC, this represents an early event in breast cancer progression (Foster et al. 6488-95). Furthermore, in early stage breast cancer, increased nuclear expression of KLF4 was correlated with a poor outcome (Pandya et al. 2709-19).

When expressed in basal layers of the skin, KLF4 results in hyperplasia and dysplasia, which rapidly lead to SCC (Foster et al. 1491-500; Huang et al. 1401-08). We use the term “maturation-independent” to describe SCC lesions in which KLF4 protein is not restricted to the post-mitotic, differentiating compartment. This failed regulation represents a potential early event in human SCC tumor progression (Huang et al. 1401-08). Using a distinct KLF4 antibody for immune-staining assays and complementary studies, such as western blot and RNA analysis, Chen and colleagues have confirmed the maturation-independent expression of KLF4 (Chen et al. 777-82). Expression of KLF4, especially nuclear expression, is associated with an aggressive skin cancer phenotype and even metastasis.

As KLF4 transcripts are upregulated in HNSCC, particularly within the basal cell layer of adjacent dysplastic epithelium, our lab generated an inducible transgenic mouse model that ectopically expresses KLF4 in basal epithelium of skin. These animals develop SCC-like lesions rapidly (within 4 weeks) in which there is prominent nuclear
staining of KLF4 (Foster et al. 1491-500). The biggest advantage of this mouse model is that it can develop lesions rapidly—within days of induction. The resulting tumors shared molecular and genetic similarities to human SCC, including reliance upon p53 inactivation. In contrast to this simple model, traditional in vivo skin carcinogenesis models, which relied on the use of chemical carcinogenesis in mice, develop over a prolonged time and are likely to contain additional genetic and epigenetic changes. To study KLF4-proximal signaling mechanisms, we generated a KLF4-estrogen receptor fusion protein (KLF4-ER), which is 4-hydroxytamoxifen (4-OHT)-dependent for both transformation and nuclear localization (Foster et al. 1491-500). Thus, when KLF4 is active in transformation, it is localized to the nucleus.

**Critical Determinants of KLF4 Function as an Oncogene (p53 and p21)**

Whether KLF4 acts as a tumor suppressor or an oncogene depends on different cell contexts influenced by many factors, such as the cell type and the expression of other genes.

To study the KLF4 oncogenic mechanisms in transformation, two major contexts offer useful examples: adenovirus E1A (Foster et al. 423-34; Foster et al. 1491-500) and RasV12 (Rowland, Bernard, and Peeper 1074-82), either of which renders cells susceptible to KLF4-mediated transformation. In these studies, known transcriptional targets of KLF4 include the tumor suppressor p53 (repressed by KLF4) and p21Waf1/Cip1 (upregulated by KLF4). Knockout studies suggest that each of these
activities are critical determinants of KLF4 function as an oncogene (Foster et al. 1491-500; Rowland, Bernards, and Peeper 1074-82; Zhang et al. 18391-98).

In both context of E1A and RasV12 mediated transformation, KLF4 suppresses the expression of p53, possibly providing resistance to DNA-damage-induced apoptosis (Rowland, Bernards, and Peeper 1074-82). p53 plays crucial role in determining KLF4 function as an oncogene. Induction of KLF4 in p53 hemizygous knockout animals (p53+/−) stimulates distinct gross and microscopic changes of both the dorsal and ventral skin. Meanwhile, in p53 wild-type male mice (p53+/+), the phenotype is mainly limited to ventral skin (Foster et al. 1491-500). When patchy (rather than homogeneous) expression of KLF4 was engineered in the skin (using X-linked mosaicism), loss of p53 was absolutely essential for a skin phenotype. These observations support that the suppression of p53 expression may represent a critical oncogenic function of KLF4.

As a principle regulator of cell cycle, p21Cip1/Waf1 seems to be another effective determinant for KLF4 oncogenic capacity. Indeed, Cdkn1a−/− mouse embryonic fibroblasts are transformed by KLF4 in vitro (Rowland, Bernards, and Peeper 1074-82). In the context of RasV12-cyclin D pathway, overexpression of cyclin D may bypass growth-inhibitory effects of p21Cip1/Waf1 in KLF4-induced cell cycle arrest (Rowland, Bernards, and Peeper 1074-82). These may include the inactivation of p21 by sequestration, in complexes with CDK4 or CDK6 (Sherr and Roberts 1501-12). In the transformation screen that was performed in RK3E cells (Foster et al. 1491-500), the functional neutralization of p21 was perhaps mediated by the adenoviral E1A oncoprotein through its ability to inhibit p105Rb and promote cell cycle progression (Whyte et al. 124-29).
Roles of KLF4 in Stem Cell Renewal and Reprogramming

Recently, it was found that overexpression of KLF4, in combination with three other transcription factors Oct3/4, Sox2, and c-Myc, could generate inducible pluripotent stem cells (iPS) from adult mouse or human fibroblasts (Takahashi and Yamanaka 663-76; Takahashi et al. 861-72).

The characteristics of Human iPS cells are similar to human embryonic stem (ES) cells in many ways, including morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. What is more, these cells could also differentiate into the cell types of the three germ layers \textit{in vitro} and in teratomas.

The role of KLF4 in reprogramming is transcriptional control, probably through downregulation of p53 transcription (Rowland, Bernards, and Peeper 1074-82), which is known to regulate expression of Nanog, a gene required for ESC self-renewal (Lin et al. 165-71). Repression of p53 also inhibits c-Myc-induced apoptotic pathways (Zindy et al. 2424-33), suggesting that there may be a finely tuned balance between c-Myc and KLF4 for successful reprogramming. Obviously, the exact role of KLF4 in ES cell self-renewal and iPS cell reprogramming needs further study.

RETINOID RECEPTORS

Retinoids and Nuclear Receptors (NRs)

Retinoids are a class of chemical compounds related chemically to vitamin A, consisting of the natural vitamin A-like compounds (retinol, retinal, retinoic acid) and
their synthetic derivatives. They help control normal growth, differentiation of epithelial tissues, and also play critical roles in the prevention and treatment of a variety of cancers and other diseases (Altucci and Gronemeyer 181-93; Fisher and Voorhees 1002-13). These pleiotropic activities are primarily mediated by hormone nuclear receptors (NRs) to regulate gene transcription (Bastien and Rochette-Egly 1-16; Niles 81-96).

Nuclear Receptors (NRs) are a class of transcription factors that can directly bind to DNA in response to some specific hormones or molecules. These receptors work together with other proteins to regulate the expression of certain genes, thereby controlling the development, homeostasis, and metabolism of the organism.

NRs contain a structure of 5-6 conserved regions, designated A-F (Fig.2) (Gronemeyer, et al. 950-64). The variable N-terminal A/B domain contains the activation function 1 (AF-1) motif responsible for ligand-independent transcription. The highly conserved C region is the DNA binding domain (DBD), including two zinc-fingers which bind to specific sequences of DNA. Region E is the ligand binding domain (LBD). It has complex functions, including mediating dimerization, ligand binding and ligand-dependent transactivation (through the activation function 2, AF-2 motif). Region D serves as a hinge between the DBD and the LBD, allowing rotation of the DBD. It also harbors nuclear localization signals (NLS). Region F in the C-terminus is variable in sequence between different NRs.

The NR superfamily contains several liganded receptors but also “orphan receptors”, for which no ligand has yet been discovered. These NRs need to be homodimerized or heterodimerized with each other to mediate different effects (Bastien
and Rochette-Egly 1-16; Glass 391-407). There are two types of NRs: Type 1 and Type 2. Type 1 NRs are located in the cytosol in the absence of ligands; in the presence of ligands, they translocate into nucleus where the ligand-receptor complex bind to a specific sequence of DNA known as a hormone response element (HRE). This type of NRs includes the estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR). In contrast, Type 2 NRs are retained in the nucleus bound to DNA regardless of the ligand binding and are usually heterodimerized with RXR to bind to DNA. Unliganded as well as DNA-bound retinoid receptors repress transcription through the recruitment of co-repressors, such as NCoR1(nuclear receptor co-repressor)and NCoR2/SMRT (silencing mediator for retinoid and thyroid hormone receptor) (Bastien and Rochette-Egly 1-16;Glass and Rosenfeld 121-41;Perissi and Rosenfeld 542-54). Target genes are initially silent and packed in a dense chromatin structure. To activate gene expression, ligand-induced conformational changes cause the dissociation of co-repressors and recruitment of a variety of co-activators (such as the p160 family and TRAP/DRIP/SMCC complex), chromatin remodelers, and modifiers. This, in turn, results in the binding of RNA Polymerase II machinery and transcription activation (Fig.3) (Bastien and Rochette-Egly 1-16; Glass and Rosenfeld 121-41;Perissi and Rosenfeld 542-54). Type 2 NRs consist of the retinoid X receptor (RXR), retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), vitamin D receptor (VDR), and thyroid hormone receptor (TR).
RXR and its Functions in the Skin

RXR heterodimers exhibit three modes of activation (Chambon 1418-28; Germain et al. 760-72). The permissive heterodimers (e.g. RXR/PPAR and RXR/LXR) demonstrate dual ligand permissive pattern, in which both receptors are independently responsive but are synergistically activated in the presence of both ligands. The RXR/RAR heterodimer is conditionally permissive, in which transcription activities for RXR can be achieved only in the presence of an RAR agonist. The requirement for the presence of RAR agonist for RXR/RAR heterodimer to activate transcription is called “RXR subordination” (Chambon 940-54; Gronemeyer, Gustafsson, and Laudet 950-64). In the third mode, RXR can act as a silent partner in the non-permissive RXR/VDR or RXR/TR heterodimer, meaning that transcription cannot be activated by an RXR agonist. RXRs can also heterodimerize with the orphan nuclear receptor family comprising NUR77, Nor1 and Nurr1. RXR, as the common partner for type 2 NRs, can modulate diverse signaling pathways through its ability to form homodimers or heterodimers (Germain et al. 760-72; Glass 391-407).

Both RXRs and RARs possess three isotypes (α, β, γ) encoded by separate genes. RXRα is the most abundant RXR isotype expressed in epidermis and hair follicle ORS (outer root sheath) keratinocytes (Fisher and Voorhees 1002-13), and it plays crucial role in their development and maintenance. Knockout of RXRα is lethal in utero before skin formation (Kastner et al. 987-1003; Sucov et al. 1007-18). Conditional RXRα knockout (Li et al. 633-36) suggests biological roles of RXR in adult tissues, further highlighting the relevance of RXR signaling pathways to cancer. For example, selective ablation of RXRα in adult mice keratiocytes results in epidermal interfollicular
hyperplasia, keratinocyte hyperproliferation, aberrant terminal differentiation in skin (Li et al. 675-88). Consistently, conditional knockout mice that lack RXRα in their epidermis are hypersensitive to DMBA/TPA-induced skin tumorigenesis (Indra et al. 1250-60). In the prostate, RXRα deficiency results in multifocal hyperplasia in the prostatic epithelium (Huang et al. 4812-19), and reduction of RXRα expression in basal cells occurs in the early stage of prostate carcinogenesis (Mao et al. 383-90). These results identify RXRα as a tumor suppressor in cutaneous SCC and prostate cancer.

Whether the effects of RXR result from the autonomous RXR signaling (Benoit et al. 7011-18) or from the RXR signaling as a heterodimer with other NRs remains to be established. Among them, RARs, especially RARα and RARγ, also express in the normal epidermis and cultured keratinocytes. In the skin, heterodimers of RARγ-RXRα represent the major signal transducer (Altucci and Gronemeyer 181-93; Chapellier et al. 3402-13; Darwiche et al. 2774-82). Like KLF4, which is most abundant in suprabasal keratinocytes, the repressor form of RARγ/RXRα heterodimers (i.e., unliganded RARs) are required in suprabasal keratinocytes for the generation of lamellar granules (LG), the small keratinocyte organelles instrumental to the formation of the skin permeability barrier (Calleja et al. 1525-38). Besides that, PPARs and VDR are also highly potential dimerization partners for RXR in regulation of homeostasis of epidermal proliferation and differentiation (Altucci and Gronemeyer 181-93; Calleja et al. 1525-38; Chapellier et al. 3402-13). However, it remains unclear whether the signaling pathways intersect or else operate in a parallel, independent fashion.
Retinoids in Cancer Therapy/Prevention and Potential Mechanisms

Retinoids display distinct anticarcinogenic activities. They are currently used and being tested in clinical trials as therapeutic and/or preventive agents in many types of cancer, including leukemia, breast cancer and skin cancer (Altucci and Gronemeyer 181-93). For example, retinoids are used for treatment of skin disorders including psoriasis and acne (Livrea M.A.), and also used as chemopreventive agents for epidermal cancer (Altucci and Gronemeyer 181-93; Sun and Lotan 41-55). When delivered to animals or cell lines, exogenous retinoids can regulate gene expression through activating specific NRs to induce growth arrest, differentiation, or apoptosis (Donato, Suh, and Noy 609-15; Rochette-Egly and Chambon 909-22; Schug et al. 723-33); Still, the molecular mechanisms behind retinoids are poorly understood.

The cell-cycle inhibitor p21WAF1/CIP1 (ref to Part1) could be one of the crucial mediator molecules; this is suggested by the role of p21 as a common transcriptional target of retinoid receptors, through either RAR/RXR heterodimer or RXR/RXR homodimer (Liu, Iavarone, and Freedman 31723-28; Tanaka et al. 29987-97). Indeed, the effects of p21 induction on retinoid induced cell differentiation, growth inhibition or apoptosis remain to be established.

The successful treatment of acute promyelocytic leukemia (APL) with all-trans RA (ATRA) provides an example of differentiation therapy, which tries to reactivate transduction pathways that are bypassed, inactivated or suppressed during tumorigenesis. In this way, it can force a tumor cell to adopt a more differentiated phenotype, thereby optimally induces terminal differentiation and/or apoptosis. (Lengfelder et al. 261-74).
The RAR agonist, ATRA, when administered with anthracycline, cures 70–80% of APL patients. The most common cause of APL is a somatic translocation between chromosomes 15 and 17 that fuses the promyelocytic leukaemia gene (PML) with the RARα gene such that a fusion protein (PML-RARα) is produced that includes the RARα ligand-binding domain (LBD). This fusion protein PML-RARα causes enhanced recruitment of co-repressor–histone deacetylase (HDAC) complexes that epigenetically silence gene programs, resulting in a block in differentiation of promyelocytes (Piazza, Gurrieri, and Pandolfi 7216-22; Slack and Gallagher 75-124). Rarely, it has been identified in APL patients for other RAR-fusion proteins, in which PML is replaced with PLZF (PML zinc finger), NUMA (nuclear mitotic apparatus protein), NPM (nucleophosim) or STAT5B (signal transducer and activator of transcription 5B). Recent work has revealed that the formation of higher-order Stat5b-RARα fusion protein complexes with RXR can induce RXR desubordination, therefore, besides RAR, RXR can also be a drug target in APL or even more types of cancer (Zeisig et al. 36-51; Zhu et al. 23-35).

**Rexinoids and their Anti-carcinogenic Effects**

As the common partner for Type 2 NRs, RXRs are master regulators in mediating the action of RA on cell function, directly reflected by the growth inhibiting effects of RXR ligand in multiple cancer cell lines (Balasubramanian, Chandraratna, and Eckert 1377-85; Davis et al. 7105-10; Naka et al. 313-20). RXRα-null F9 embryonic carcinoma cells are resistant to retinoid-mediated cellular differentiation, anti-proliferation and apoptosis (Clifford et al. 4142-55). Similarly, mice that lack epidermal RXRs do not
respond to chemoprevention by RA, indicating that RXRs are involved in the chemopreventive activity of RA in experimental skin carcinogenesis model (Altucci and Gronemeyer 181-93).

Indeed, RXR-selective ligands such as Targretin represent a class of drugs now collectively termed rexinoids. These have shown promising chemopreventive and chemotherapeutic activities in several cancer types with fewer side effects than that are normally associated with retinoid therapy; what is more, RXR expression is rarely lost in human tumors (Altucci and Gronemeyer 181-93; Cheng and Kupper 649-52; Lawrence et al. 29-35). Rexinoids can effectively suppress tumor development in carcinogen-induced and transgenic mouse breast cancer models (Gottardis et al. 5566-70; Wu et al. 6376-80). In cutaneous T cell lymphoma clinical trials and non-small cell lung cancer phase I and II clinical trials, rexinoids show anti-tumor activity with moderate toxicity (Cheng and Kupper 649-52; Khuri et al. 2626-37).

Rexinoids are increasingly valued because that they can synergize with retinoids as well as provide RAR-independent activity (Chen et al. 819-22). These effects are consistent with the molecular basis that RXRs can act not simply as silent heterodimerization partners of RARs, but also as active transducers of tumor-suppressive signals.

Rexinoid: 9cUAB30

Among the most promising rexinoids, one selective agonist for RXRs, 9-cis UAB30 (9cUAB30) (Muccio et al. 1679-87), shows chemopreventive efficacy in
mammary cancers without known toxicity (Atigadda et al. 3766-69; Grubbs et al. 17-24).

In contrast to the aforementioned Targretin, 9cUAB30 does not induce hyperlipidemia. The absence of such side effects is an important prerequisite for administration of a drug to at-risk but otherwise healthy people.

9cUAB30 offers several benefits. First, 9cUAB30, RA [(2E,4E,6Z,8E)-8-(3′,4′-dihydro-1′(2′H)-napthalen-1′-ylidene)-3,7-dimethyl-2,4,6-octatrienoic acid)], is rapidly taken up in the serum of mice, and it has a much longer half-life than 9cRA (13.6 h vs 15 min). Moreover, this rexinoid does not elevate triglycerides (TGs), alter estrus cycles or uterine or ovarian weights, or increase liver retinyl palmitate levels (Grubbs et al. 17-24). Of the most importance, it does not accumulate in the serum with either increasing doses or increasing the length of dosing. Such disposition of 9cUAB30 may partially account for the lack of toxicity that was observed.

QUESTIONS ADDRESSED IN THIS DISSERTATION

Despite the promising anti-carcinogenic actions of RXR ligands, the molecular mechanisms underlying their efficacy remain to be understood. This dissertation attempts to document what is known to date from the publication of two recent articles on the subject. The first paper contains characterization of the rexinoid 9cUAB30 as a small molecule antagonist in KLF4-mediated tumor initiation and malignant transformation, identifying the KLF4 pathway as a potential mediator of retinoid effects in chemoprevention or therapy. The second paper covers the relationship between the oncogene KLF4 and cutaneous SCC initiation and progression, especially in the basal
cell layer of skin, providing basis for pharmaceutical interventions that impact KLF4 oncogenic activity. Summary part offers a discussion of these findings, implications, and options for future research.
Fig 1. Structural organization of the klf4 gene and the corresponding protein. (A) The Klf4 gene is located on chromosome 9q31, which covers a 6.3 kb region. (B) The dark red bars mark the locations of the five identified exons of the klf4 gene. (C) Below the genomic map is the klf4 RNA transcript. The five boxes represent corresponding exons, whereas the solid red boxes show the klf4 open reading frame within the 2639 bp cDNA. (D) The Klf4 open reading frame encodes a protein of 470 amino acids with several functional domains, including the transcriptional activation domain (AD), transcriptional inhibitory domain (ID), zinc finger DNA-binding domain, nuclear localization signal (NLS) and potential PEST sequence. Several point mutations have been identified in tumor cells, which are shown in blue.

Fig 1.
Fig 2. Schematic illustration of the structural and functional organization of nuclear receptors. The evolutionarily conserved regions C and E are indicated as boxes (green and orange, respectively), and a black bar represents the divergent regions A/B, D and F. Domain functions are depicted above and below the scheme. AD, activation domain; AF1, activation function 1; AF2: activation function 2; NLS, nuclear localization signal.

Fig 2.
**Fig 3. Mechanism of nuclear receptor action.** This figure depicts the mechanism of a class II nuclear receptor (NR) which, regardless of ligand binding status is located in the nucleus bound to DNA. For the purpose of illustration, the nuclear receptor shown here is the thyroid hormone receptor (TR) heterodimerized to the RXR. In the absence of ligand, the TR is bound to corepressor protein. Ligand binding to TR causes a dissociation of corepressor and recruitment of coactivator protein which in turn recruit additional proteins such as RNA polymerase that are responsible for transcription of downstream DNA into RNA and eventually protein which results in a change in cell function.
Fig 3.
PREVENTION OF KLF4-MEDIATED TUMOR INITIATION AND MALIGNANT TRANSFORMATION BY UAB30 REXINOID

by


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Abstract

The transcription factor KLF4 functions in post-mitotic epithelial cells to promote differentiation, and acts in a context-dependent fashion as an oncogene. In the skin, KLF4 is co-expressed with the nuclear receptors RARγ and RXRα, and formation of the skin permeability barrier is a common function of these three proteins. We utilized a KLF4-transgenic mouse model of skin cancer in combination with cultured epithelial cells to examine functional interactions between KLF4 and retinoic acid receptors. In cultured cells, activation of a conditional, KLF4-estrogen receptor fusion protein by 4-hydroxytamoxifen resulted in rapid upregulation of transcripts for nuclear receptors including RARγ and RXRα. We tested retinoids in epithelial cell transformation assays, including an RAR-selective agonist (all-trans RA), an RXR-selective agonist (9-cis UAB30, rexinoid), and a pan agonist (9-cis RA). Unlike for several other genes, transformation by KLF4 was inhibited by each retinoid, implicating distinct nuclear receptor heterodimers as modulators of KLF4 transforming activity. When RXRα expression was suppressed by RNAi in cultured cells, transformation was promoted and the inhibitory effect of 9-cis UAB30 was attenuated. Similarly as shown for other mouse models of skin cancer, rexinoid prevented skin tumor initiation resulting from induction of KLF4 in basal keratinocytes. Rexinoid permitted KLF4 expression and KLF4-induced cell cycling, but attenuated the KLF4-induced misexpression of cytokeratin 1 in basal cells. Neoplastic lesions including hyperplasia, dysplasia and squamous cell carcinoma-like lesions were prevented for up to 30 days. Taken together, the results identify retinoid receptors including RXRα as ligand-dependent inhibitors of KLF4-mediated transformation or tumorigenesis.
Introduction

The zinc finger protein KLF4/GKLF functions as a transcription factor to regulate cell fate and differentiation (1-4). KLF4 is prominently expressed in the post-mitotic, differentiating cells of epithelia including the skin and gut (5-7). KLF4 knockout mice die from dehydration shortly after birth, a consequence of a defective cutaneous water permeability barrier (3). These mice also fail to form goblet cells, a specialized epithelial cell type in the gut (4).

Unbiased, functional expression-cloning assays identified KLF4 as a prominent transforming activity in libraries derived from human tumor cell lines (7,8). These studies used adenovirus E1A or RasV12 to render cells susceptible to KLF4-mediated transformation. Known transcriptional targets of KLF4 include p21Waf1/Cip1 (upregulated by KLF4) and the tumor suppressor p53 (repressed by KLF4), and knockout studies demonstrate that each of these activities is a critical determinant of KLF4 function as an oncogene (1,8,9). Unlike wild type cells, primary rodent cells that are deficient in p21Waf1/Cip1 are susceptible to transformation by KLF4 in vitro (8). In cells that express E1A or RasV12, induction of p21Waf1/Cip1 does not prevent transformation by KLF4. p21Waf1/Cip1 may contribute to the slower growth rate observed for KLF4-transduced cells, but these cells efficiently generate transformed foci in vitro and are tumorigenic in athymic mice (7).

KLF4 transcripts and protein are increased in human tumors including squamous cell carcinoma (SCC) of the oropharynx, breast cancer, and cutaneous SCC (1,7,10,11). Compared with normal epithelium, KLF4 upregulation is also observed in premalignant lesions such as dysplasia or carcinoma in situ. In these lesions, the normal pattern of
maturation-dependent expression is profoundly altered, with mitotically-active basal cells and post-mitotic suprabasal cells expressing KLF4 (7,11). Modeling this aberrant pattern in the skin of transgenic mice reproduced several aspects of tumor progression, rapidly inducing hyperplasia, dysplasia, SCC in situ, and SCC-like invasive lesions (1,11). These results identified deregulation of KLF4 as a potentially early step in skin tumor progression.

Like KLF4, the retinoic acid receptors (RARs) regulate cell fate, cell growth, and differentiation in normal or malignant cell types (12-14). RARs (including RARα, β, and γ) function in concert with RXRs (including RXRα, β and γ) as ligand-dependent, heterodimeric transcription factors. All-trans retinoic acid (ATRA), the active vitamin A derivative, functions as an RAR agonist. 9-cis retinoic acid (9cRA) is an agonist for RARs and RXRs. RXR selective ‘rexinoids’ such as LGD1069 (Targretin) or 9-cis-UAB30 (9cUAB30) are effective in cancer prevention or therapy, and have fewer side effects compared to ATRA or 9cRA (15-21). 9cUAB30 does not display lipid toxicities associated with retinoids and most rexinoids (e.g., Targretin), and is entering Phase I clinical trials as a preventive agent for cancer (22,23).

Because RXRs interact with various partners including RARs, PPARs, and other NRs such as Nur77, rexinoids affect diverse signaling pathways (15,16,24). The RAR/RXR heterodimer is typically conditional in its response to rexinoid (i.e., activation by rexinoid requires the presence of an RAR agonist) (24). In contrast, the RXR/PPAR heterodimer is permissive (i.e., activated by ligands for either subunit). Retinoids are effective in the prevention of human carcinomas, including tumors such as oral SCC, cutaneous SCC and breast cancer (12,25). Mouse models of these tumor types have been
used to show efficacy of retinoids and rexinoids in both prevention and treatment (12,16,17,21). Keratinocyte-selective knockout of Rxra (encoding RXRα) or Pparg (encoding PPARγ) promotes SCC progression in response to topical DMBA/TPA, identifying these receptors as tumor suppressors in mouse skin (26). Similarly, PPARγ deficiency promotes carcinogen-induced tumor progression in the breast (27). Consequently, nuclear receptors (NR) represent ligand-inducible tumor suppressors for tumor types that show upregulation of KLF4.

In the skin, KLF4 is co-expressed in post-mitotic cells with RXRα and several RXRα dimerization partners, including RARγ, PPAR β/δ, and PPARγ (28-30). Gene knockout studies indicate that KLF4, RXRα, RARγ, or PPARβ/δ each contributes to formation of the skin permeability barrier (3,30). However, it remains unclear whether the signaling pathways intersect or else act in a parallel, independent fashion. In the current study we utilized a conditional allele of KLF4 to demonstrate regulation in vitro of multiple NRs, including RXRα and RARγ. Of several oncogenes that independently induce malignant transformation of RK3E epithelial cells, only KLF4 showed substantial sensitivity to retinoids. The rexinoid 9cUAB30 inhibited KLF4-induced transformation in vitro in an RXRα-dependent fashion. In vivo, rexinoid prevented the induction of skin tumorigenesis in the rapid-onset, KLF4 transgenic mouse model of SCC, arresting tumor progression at an early stage of rapid cell cycling and cell turnover. These results identify antagonism of KLF4-induced tumor initiation as a novel effect of retinoids that may contribute to their activity in cancer chemoprevention.
Results

Retinoids selectively inhibit KLF4-mediated epithelial cell transformation. To test retinoids as gene-selective modulators of transformation in vitro, we utilized RK3E epithelial cells. These were previously derived from primary rat kidney cells by immortalization with adenovirus E1a, which induces epithelial features (36,37). In this model, oncogene transformed cells form dense foci surrounded by adjacent, contact inhibited parental cells that constitute an epithelial sheet, as shown by electron microscopy (7). Rather than indicating mere increases in cell growth rate, the RK3E focus assay detects other characteristics of the malignant phenotype, such as insensitivity to growth inhibitory signals. Unlike several other assays, it identifies oncogenes such as KLF4 and Gli1 that, at subconfluence, induce a slower growth phenotype compared with parental cells [see Table 3 in reference (7)]. This genetically tractable model has been productive for in vitro functional analysis of carcinoma-derived oncogenes including Gli1 (36,38), KLF4 (7), β-catenin (39), Notch1 (40-42), and others (43), and functional observations made in these cells have been consistently supported by analysis of epithelial tissues (7,10,31,38,44).

To test a series of oncogenes for retinoid sensitivity, we transduced RK3E cells with retroviruses encoding a conditional, 4OHT-inducible KLF4 allele (1), or with Gli1, ErbB2, or Notch1. In the initial experiments we used KLF4-ER because it induces foci at 3 weeks, similar to the other oncogenes. In contrast, the wild type KLF4 assay requires 4-5 weeks (7). Following incubation in non-selective growth media, cells were fixed and stained and transformed foci were counted (Fig. 1 A-B). Transformation by KLF4-ER was markedly reduced by each of the retinoids tested (≥85% inhibition), including ATRA,
9cRA, and 9cUAB30. In an independent series of experiments, we transduced cells with wild-type KLF4 or sensitive/resistant controls (KLF4-ER, Gli1). Compared with DMSO, 9cUAB30 inhibited transformation by wild type KLF4 (mean ± SD, 83% ± 20) to a similar degree as observed for KLF4-ER (83% ± 3.1). Gli1 was inhibited by only 19% ± 12.5 (N=4 for each condition).

In contrast to its effect on KLF4-ER or KLF4, 9cUAB30 showed little or no inhibitory effect on transformation by Gli1, Notch1, or ErbB2 in this model (Fig. 1A; Fig. 1B, upper panel). Because 4OHT might synergize with 9cUAB30 to inhibit transformation, assays for Gli1, Notch1, and ErbB2 were repeated to determine the effect of 9cUAB30 in the presence/absence of 4OHT (4 conditions for each oncogene, N=2 for each condition). Compared with the DMSO control, neither 4OHT, 9cUAB30, nor the combination altered the activity of these oncogenes (data not shown).

Titration studies (0.01 μM-1.0 μM) indicated an EC50 of ≥100 nM for 9cUAB30 in KLF4-ER transformation assays (Fig. 1B, upper panel). This concentration is in agreement with our previous analysis of 9cUAB30 by transcriptional activation of an RXR-responsive promoter reporter construct (EC50=118 nM) and by receptor binding assays in competition with radiolabelled 9cRA (IC50=284 nM) (19).

ATRA and 9cRA, more potent agonists than 9cUAB30 (19), showed selective, marked inhibition of KLF4-ER (similar to 80%) at the lowest concentration tested (0.01μM; Fig. S1 B,C). At this concentration, ErbB2 was inhibited by 30% or less. When used at 1.0 μM, a concentration well above the EC50 in transcriptional reporter assays, inhibition of KLF4 was >95% (Fig. 1B, lower panel). In contrast, ErbB2 inhibition was only 55% for ATRA and 46% for 9cRA (Fig. 1B, lower panel). These studies indicate
that KLF4 transforming activity is selectively abrogated by agonists of either RAR or RXR.

9cUAB30 is an RXR-selective retinoid. 9cUAB30 was previously demonstrated to be RXR selective in direct binding assays and in transcriptional activation of a promoter-reporter by wild type retinoid receptors (19). To confirm this, we tested 9cUAB30 for its ability to promote the transcriptional activity of fusion proteins composed of the ligand binding domains of RARα or RXRα and the Gal4 DNA-binding domain. HEK293 cells were transfected with a vector encoding the indicated fusion protein, a luciferase reporter plasmid containing Gal4 binding sites in the promoter, and an internal control (Fig. 1C). 9cUAB30 showed 14-fold transcriptional activation of Gal4-RXRα, but had little effect on RARα activity. As expected, the RAR-agonist TTNPB (or ATRA, not shown) selectively activated RARα, and 9cRA activated each fusion protein to a similar degree.

Maintenance of KLF4-ER expression and transcriptional activity in cells treated with 9cUAB30. To determine whether 9cUAB30 affects the intrinsic activity of the KLF4-ER fusion protein, we examined the expression of KLF4-ER and its ability to regulate its transcriptional targets, p53 (repressed by KLF4) and p21Waf1/Cip1 (induced by KLF4) (8,9). RK3E cells stably transduced with KLF4-ER were treated with 4-OHT in the presence of 9cUAB30 or vehicle (Fig. 1D). KLF4-ER levels were unchanged by any treatment. In the presence or absence of 9cUAB30, KLF4 repressed p53 protein levels beginning 6-24 hours post-induction. 9cUAB30 did not alter the stability or the phosphorylation of p53 (at Ser15; not shown), a potent inhibitor of KLF4-induced tumor initiation (1). p21 levels were upregulated within 3h of addition of 4OHT and were
independent of 9cUAB30 treatment. These results show that the KLF4-ER fusion exhibits the transcriptional regulatory properties previously reported for the wild type protein, and that neither the expression nor the intrinsic activity of the KLF4-ER fusion is altered by 9cUAB30.

Although retinoids can inhibit cell growth and promote differentiation or apoptosis in certain settings, the effect of 9cUAB30 on KLF4-ER cell growth was only minor, similar to the effect on vector control cells (5 day growth assays, \( \leq 10\% \) growth inhibition, data not shown). In addition, 9cUAB30 did not alter the cell cycle profile, as shown by propidium iodide staining. These results indicate that retinoids enforce contact inhibition in KLF4 transformation assays without direct cytotoxicity to KLF4-expressing cells.

**Rexinoid prevents initiation of SCC-like lesions by KLF4.** To determine the effect of 9cUAB30 *in vivo*, we utilized the dox-inducible, KLF4 transgenic mouse model of SCC, in which KLF4 is induced in basal keratinocytes (1). This well characterized, single-gene model has 4 distinct phases that are readily identified by morphology and/or immunostaining (1,11). Phase 1, evident at 48 hours, shows increased cell proliferation and turnover, indicated by BrdU incorporation and prominent TUNEL staining (1). Phase 2, hyperplasia, shows additional epithelial cell layers, with KLF4 staining largely restricted to the more superficial, PCNA-negative cells (11). Phase 3, prominent at 7-14 days, shows dysplastic lesions with loss of differentiation, increased nuclear:cytoplasmic ratios, and clear co-expression of KLF4 and PCNA in basal cells. Phase 4, SCC-like, shows high level, nuclear KLF4 staining in PCNA positive cells that appear to detach from the interfollicular skin and infiltrate the superficial dermis.
Post-transcriptional controls normally suppress KLF4 mRNA and protein in dividing cells (5,11,45). Thus, in this model the KLF4-induced increase of cell cycling limits the expression of the exogenous protein in the early phases (1,11). The apparent loss of these controls leads to the progressive increase of KLF4 expression in successive phases.

Administration of 9cUAB30 to mice for 30 days yielded no apparent side effects such as weight loss or gross or microscopic changes of the hair or skin, and did not alter cell proliferation in the skin as indicated by BrdU incorporation (not shown). Following 30 days of treatment with dox + 9cUAB30 or dox + placebo, the skin phenotype was examined. By both gross and microscopic exam, the placebo-treated mice all developed SCC-like skin lesions, as reported for this highly penetrant model (1). In contrast, induction of the skin phenotype was completely blocked in 8 of the 9 animals treated with 9cUAB30 (Fig. 2A; p=0.0004, Fisher’s exact test). For the single animal that showed a phenotype, there was no abnormality by gross examination, and only an attenuated, hyperplastic skin phenotype by microscopic analysis (not shown). Therefore, as in other mouse models of skin cancer, SCC is prevented by administration of retinoid (16).

9cUAB30 enforces the epithelial differentiation of KLF4-induced skin lesions in vivo. The diffuse, rapid nature of this single-gene model, and the reproducible progression of the lesions enable the identification of the phase(s) at which preventive agents exert their effect. To analyze the effects of 9cUAB30 on the KLF4 skin phenotype at earlier timepoints, we induced KLF4 transgenic animals with dox + 9cUAB30 or placebo, and skin samples were isolated at days 0, 2 or 7 (i.e., 2 treatments x 3 timepoints = 6
treatment groups; 2 animals per treatment group). Following fixation and embedding, we performed microscopic exam of 8 skin samples per animal. Four representative samples per treatment group were sectioned and analyzed by immunostaining with antibody to KLF4, K1, BrdU, or normal IgG.

Control sections (day 0) showed only low-level immunostaining of endogenous KLF4 at the antibody concentration used (Fig. 2B, left column). Animals treated for 7 days with dox + placebo exhibited diffuse hyperplasia, dysplasia, and focal, SCC-like lesions that involved the dermis (Fig. 2B, middle column). These SCC-like lesions showed prominent staining of KLF4 in cell nuclei and greatly reduced staining of K1, indicating a loss of differentiation (Fig. 2B). Whereas control sections showed BrdU staining in ≤5% of basal cells, in dox-treated animals BrdU staining was estimated as >20% (Fig. 2B). These features of the model were all as previously reported (1,11).

In contrast to mice treated with dox + placebo, mice treated with dox + 9cUAB30 for 7 days rarely showed any abnormality by examination of H&E-stained sections. Focally there was early hyperplasia of the interfollicular epithelium, with moderate levels of nuclear KLF4 in suprabasal cells as reported previously (11). SCC-like lesions, which express high levels of KLF4, were not identified. The most advanced lesion revealed cystic structures in the dermis (Fig 2B, right column). In this lesion suprabasal K1 was similar to that of uninduced skin (Fig. 2B, right column), indicating that 9cUAB30 enforces normal differentiation in these KLF4- induced skin lesions. The moderate level of KLF4 expression in this lesion is similar to that observed in early hyperplastic lesions of animals untreated with 9cUAB30 [(11) and data not shown]. Therefore, consistent with its effect in vitro (Fig. 1D), 9cUAB30 did not inhibit KLF4 expression per se, but
rather appeared to arrest tumor progression at an early phase in which KLF4 expression is restricted by cellular controls (5,11,45).

**9cUAB30 permits the induction of cell proliferation by KLF4 in vivo.** Either with or without retinoid treatment, BrdU incorporation was similarly elevated in the 7 day skin samples (Fig. 2B, αBrdU; mean ± SD: dox+placebo, 119 ± 9.2 positive cells/field; dox+retinoid, 117 ± 44 positive cells/field; no dox, 2.5 ± 0.87 positive cells/field). To further examine the ability of KLF4 to trigger cell cycle progression in mice treated with 9cUAB30, we analyzed sections of mice treated for only 48h. As reported previously (11), at this early timepoint KLF4 expression appeared patchy, with some areas below the sensitivity of the staining assay (Fig. 3A, column 2, KLF4 detected; column 3, KLF4 not detected). However, BrdU incorporation was markedly increased in all the sections examined (Fig. 3A, columns 2-4; dox+placebo, 109 ± 20 positive cells/field; dox+retinoid, 97 ± 31 positive cells/field; no dox, 2.5 ± 0.87 positive cells/field), indicating that cell proliferation in response to KLF4 occurs independently of drug treatment or KLF4 expression levels. In summary, induction of cell cycle progression by KLF4 is not perturbed by 9cUAB30, and persists through at least day 7. As hyperplastic skin lesions were only rarely observed (e.g., Fig 2B), rexinoid blocked tumor progression at Phase 1, a stage of increased cell cycling and cell turnover (11).

**9cUAB30 suppresses the early induction of K1 by KLF4 in basal keratinocytes in vivo.** In normal skin, K1 is present in KLF4-positive, suprabasal cells that are committed to terminal differentiation, and is low or absent in basal cells. When KLF4 is misexpressed in basal keratinocytes in vivo, basal induction of K1 induction is one of the
earliest known responses (1). After 48h of dox, both KLF4 and K1 were prominent in the follicle outer root sheath cells that comprise the basal cell layer (Fig. 3B), where cell proliferation was not yet apparent (Fig. 3B, BrdU). At 7 days post-induction the misexpression of K1 in basal cells of the interfollicular epithelium was a consistent feature of the sections that we examined (Fig. 3C, compare left and middle panels). However, in mice treated with 9cUAB30, K1 misexpression was partially blocked, and patches of K1-negative basal cells were consistently found next to areas with K1-positive cells (Fig. 3C, right panel). These results indicate that 9cUAB30 attenuates an early response to KLF4 in vivo.

**KLF4 induces NR expression.** While KLF4 is coexpressed with several NRs in epithelial cells of the skin and mammary gland, and gene knockouts have similar effects on formation of the permeability barrier, it is unknown whether KLF4 signaling affects NR levels. We utilized RK3E-KLF4-ER cells and performed microarray (Fig. 4A) and immunoblot analyses (Fig. 4B). For microarray studies we treated cells with 4OHT.(45) When KLF4-ER cells were treated for 2 h with 4OHT, RARγ (mean fold induction, 4.0) and Nur77 (mean fold induction, 14) transcripts were rapidly upregulated in each of two independent experiments (Fig. 4A). In these studies p21 served as a positive control (mean fold induction, 6.5).

Because RXRα transcripts were not successfully detected by the microarray, we used immunoblot analysis to examine its response when KLF4-ER cells were treated with 4OHT (Fig. 4B). Compared to the β-actin control, RXRα was induced by 12 h, the earliest timepoint examined, and remained elevated for at least 48 h. Vector cells served as a control and showed no response.
To better determine the temporal relationship between KLF4 activation and NR mRNA levels, we treated KLF4-ER cells with 4OHT and examined the response of RXRα and other NRs by sqRT-PCR (Fig. 4C). In comparison to the Gapd control, three NRs, including RXRα, RARγ and Nur77, were induced by KLF4 at 3 h (lane 2). In contrast, there was little or no induction of RARα or RARβ in KLF4-ER cells (lanes 1–6). Vector cells served as a control (lanes 7–12).

**9cUAB30 acts through RXRα to inhibit KLF4-mediated transformation in vitro.** The above studies indicate that the RXR-selective agonist 9cUAB30 is an antagonist of KLF4-mediated transformation, and that KLF4 induces expression of a 9cUAB30 receptor, RXRα. To determine whether inhibitory effects of 9cUAB30 are mediated by RXRα, we suppressed this receptor in RK3E cells using lentiviral shRNAs (Fig. 5A, Table 1). Compared with Vector-transduced cells, RK3E cells stably transduced with shRNA constructs RXRαsh1 (contains 1 mismatch, outside of the seed sequence) or RXRαsh2 (no mismatches) showed reduced RXRα by immunoblot. RXRαsh3 (2 mismatches in the seed sequence) failed to suppress expression and served as a control in the studies below.

We next determined the efficiency of transformation of each cell line in the presence and absence of 9cUAB30 (Fig. 5B and C). RXRα knockdown promoted transformation *in vitro* by KLF4 (Fig. 5C, 4OHT + DMSO, compare Vector control cells to RXRαsh1 and RXRαsh2 cells). This enhanced susceptibility to transformation is similar to results obtained in the skin of RXRα-deficient mice, which show hyperplasia and an increased susceptibility to tumor initiation or promotion (26,46).
Compared to control lines (Vector, RXRαsh3), in RXRα-knockdown cells (RXRαsh1, RXRαsh2) the suppressive effect of 9cUAB30 was attenuated (Fig. 5D). Residual suppression in the knockdown lines may be due to incomplete or transient knockdown of RXRα (Fig. 5A). These results support a role for RXRα as a rexinoid-dependent antagonist of KLF4-mediated transformation.

Discussion

Retinoids have a well-established role in cancer prevention and therapy(12,17,25). Remaining challenges include identifying the relevant cellular mechanisms, and identifying preclinical or clinical settings in which retinoids have efficacy. The current study identifies the KLF4 pathway as a potential effector of retinoid signaling in tumor prevention. We utilized a genetically tractable epithelial model to link retinoid sensitivity to the function of KLF4. In RK3E cells there was little or no effect of retinoids on transformation induced by the Hedgehog pathway transcription factor Gli1, the receptor tyrosine kinase ErbB2, or the cell fate determinant Notch1. In contrast, transformation by KLF4 was markedly suppressed.

Suppression was observed for agonists with diverse receptor selectivity, including ATRA, 9cRA and 9cUAB30. While certain conditions can promote dissociation of co-repressors from RAR/RXR heterodimers, leading to a rexinoid response in the absence of RAR ligand (i.e., desubordination16), we found that enforced expression of KLF4 did not do so (transcriptional reporter studies not shown). Therefore, the ability of functionally distinct retinoids to prevent KLF4-mediated transformation implicates distinct heterodimers as KLF4 antagonists. These may include RXRα bound to RARγ, to the
PPAR family members, or to other NRs such as Nur77. The most selective KLF4 inhibitor, the rexinoid, was further analyzed \textit{in vivo}.

During initiation of SCC tumor progression by KLF4 in mouse skin, 9cUAB30 enforced normal patterns of K1 expression and arrested lesions at a stage in which the majority of the skin appeared morphologically normal by light microscopy, but showed prominent mitotic activity as indicated by BrdU incorporation. Consistent with this rapid proliferation \textit{in vivo}, 9cUAB30 was non-cytotoxic to KLF4-expressing cell lines in culture. However, similar to other rexinoids such as Targretin\cite{17,21}, 9cUAB30 is effective in treating established, MNU-induced breast cancers that express KLF4 (unpublished observations). The distinct effects observed in the different models may indicate context-specific signaling, and support roles for this rexinoid in both prevention and treatment.

Using conditional KLF4-ER cells we found that KLF4 rapidly induces transcripts encoding RXR\textalpha and RAR\gamma. This result identified NR induction as an early transcriptional response to KLF4 \textit{in vitro}, a result consistent with their co-expression in the skin\cite{3,11,28} and breast\cite{10,47}. shRNA-mediated receptor knockdown indicated that the KLF4-inhibitory effects of rexinoid are mediated, at least in part, by RXR\textalpha. RXR\textalpha knockout in the skin promotes proliferation, and enhanced tumor growth was seen in animals treated with topical DMBA/TPA, identifying roles for RXR\textalpha in skin homeostasis and tumor suppression\cite{26,46}. Taken together, these observations indicate that RXR\textalpha represents a KLF4-induced tumor suppressor that can be further activated by use of exogenous ligand.

p21Waf1/Cip1 and p53 are transcriptionally regulated by KLF4, and in turn they regulate its function as an oncogene\cite{1,8,9}. 9cUAB30 did not alter KLF4-ER expression,
its ability to induce p21Waf1/Cip1, or its ability to repress p53, suggesting that downstream, convergent signaling by retinoid receptors and KLF4 may impact common targets. The current study implicates a single pathway in retinoid mediated tumor prevention, and provides genetically tractable models for further analysis of mechanisms. We are currently using shRNA and gene knockout approaches to evaluate the role of p21, p53, PPARs or Nur77 as potential mediators of retinoid effects on KLF4 activity.

As this work was in progress, Wang and colleagues reported an essential role for KLF4 in retinoid-induced differentiation of vascular smooth muscle cells (VSMCs).48 Whereas KLF4 suppresses p53 in other cell types(8), in ATRA-treated VSMCs KLF4 instead upregulated the expression of p53 and promoted differentiation. Although specific mechanisms remain to be identified, the study by Wang and colleagues supports the idea that KLF4 and the NRs have convergent roles in regulation of cellular differentiation and tumorigenesis.

Materials and Methods

Cell culture and retroviral transduction. Culture of RK3E cells, retroviral transduction, and in vitro transformation assays were performed as described(7). Expression vectors encoding KLF4-ER fusion protein, wild type human KLF4, human ErbB2, mouse Notch1 or human Gli1 were previously described(1,7,31). pBpuro vector served as a control(32). 9cUAB30 was synthesized as described(20). 4-Hydroxytamoxifen (4OHT; 0.3 μM; Calbiochem), 9cUAB30, 9cRA, ATRA, the RAR agonist TTNPB (Sigma), and/or vehicle (DMSO) was added to the culture medium every other day.
Knockdown of RXRα in RK3E cells utilized pLKO.1 lentiviral expression vector encoding shRNAs against mouse RXRα (Mission® TRC shRNA, Sigma) (33). Constructs were co-transfected with helper plasmids (pCMV-Gag-Pol, pCMV-VSVg) into HEK293T cells. Lentiviral supernatants were applied to RK3E cells, and transduced cells were selected in puromycin (1.0 μg/ml) for three days prior to western blot analysis and transformation assays. shRNA sequences are provided in Table 1.

**Animal studies.** For assessment of skin tumor chemoprevention by 9cUAB30, a doxycycline (dox)-inducible, transgenic KLF4 mouse model of SCC was used as described (1). One day prior to administration of dox in drinking water, 9cUAB30 (1,000 mg/kg diet) was added to the feed as described(18).

**Transient transfection and luciferase reporter assays.** At 24 h prior to transfection, human embryonic kidney (HEK) 293 cells were plated at 2 x 105 cells per well in six-well plates. Transfection mixtures included 0.2 μg of the Gal4 reporter plasmid pGL4.31[luc2P/Gal4UAS/Hygro] (Promega), 0.5 μg of pCMXGal4-hRARα or pCMX-Gal4-hRXRα expression vector (gifts from Dr. Thomas Perlmann), and 0.01 μg of Renilla luciferase reporter plasmid, pRL-TK. TransIT-LT1 Transfection Reagent (Mirus) was used according to the manufacturer’s instructions. At 24 h posttransfection, retinoid was added to the culture medium. At 48 h post-transfection, reporter activity was determined using the Dual- Luciferase Reporter Assay (Promega).

**Immunoblot analysis.** Cultured cells at 70% confluence were washed twice in PBS and then incubated 30 min in cold lysis buffer [50 mM HEPES-KOH (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 0.1% (v/v) NP40, 0.4 mM sodium orthovanadate, 0.25 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM
pepstatin and 1 mM DTT]. Extracts were centrifuged at 13,000 xg for 15 min at 4°C. Protein was quantitated using the Bradford assay (Bio-Rad).

Extracts of frozen tissue were prepared from 50 mg fragments by addition of 1.0 mm glass beads and 2X Laemmli buffer, followed by disruption using a MiniBead-Beater-1 (BioSpec Products, Bartsville, OK). Extracts in 1X Laemmli buffer were quantitated as described (34).

Following electrophoresis, proteins were transferred to nitrocellulose. p53 antibody (CM5, Novocastra) was used at 1:1000. KLF4 antibody (H180, Santa Cruz) and p21 antibody (C-19, Santa Cruz) were used at 0.2 μg/ml. β-actin antibody (AC74, Sigma) was used at 0.26 μg/ml. RXRα antibody (D20, Santa Cruz) was used at 0.4 μg/ml. Bound antibodies were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific).

mRNA expression studies. Analysis of Affymetrix RAE U230A arrays was performed in the Gene Expression Shared Facility (UAB Comprehensive Cancer Center). Two independent experiments were performed without technical replicates. For confirmation of microarray and western blot analyses, semi-quantitative RT-PCR analysis of cultured cells was performed as described(31). For selected samples, variation of input cDNA confirmed the semi-quantitative nature of the reactions. Primer sequences are shown in Table 2.

Immunostaining and imaging. Skin samples (~1.0 cm) were fixed in neutral buffered formalin and embedded in paraffin. Immunodetection of KLF4, the differentiation marker cytokeratin 1, or the S-phase marker BrdU was performed as described(1,11). Normal rabbit immunoglobulin (Dako) was used as a control and showed no background of
nonspecific staining. For scoring of BrdU positive cells, a field diameter of 0.7 mm was used to assess 4 randomly selected areas per section. Digital images were captured as described(11).

Acknowledgements

We thank Thomas Perlmann for providing reagents for transcriptional reporter assays, and members of the Ruppert Laboratory for helpful comments on the manuscript. We thank Reddy Atigadda for the preparation of 9-cis-UAB30 used in this study. Funded by NIH grants R01 CA127405, RO1 CA094030, P50 CA89019, AR050948, and by The American Cancer Society RSG-05-207-01-TBE.

References


18. Grubbs CJ, Hill DL, Bland KI, Beenken SW, Lin TH, Eto I, Atigadda VR, Vines KK,


**Figure 1.** The RXR-selective retinoid 9cUAB30 inhibits KLF4-mediated *in vitro* transformation. (A) Retroviral supernatants were generated using the expression vectors indicated on the left and applied to RK3E cells. 9cUAB30 or vehicle (DMSO) was added to the culture media every two days. For dishes transduced with KLF4-ER, 4OHT was added to the culture media. Transformed foci were quantified by staining and counting of foci. *Columns*, mean (n = 4); *bars*, SE; *p value*, unpaired t test (two-tailed). (B) Dose response analysis (upper) and analysis of functionally distinct retinoids (lower and see Suppl. Fig. 1). KLF4-transformed foci were generated and quantified as described in (A), with addition of retinoid or DMSO to the culture media. *Columns*, mean (n = 2); *bars*, range. (C) Receptor selectivity of retinoid agonists. HEK293 cells were transfected with expression vectors encoding the indicated Gal4 fusion proteins, a luciferase reporter plasmid containing Gal4 binding sites, and an internal control. Following treatment with the indicated agonist for 24 h, normalized firefly luciferase activity was determined. *Columns*, mean (n = 4); *bars*, SE. (D) Immunoblot analysis of KLF4-ER and KLF4-regulated genes. RK3E cells were stably transduced with KLF4-ER retrovirus, encoding a 4OHT-dependent allele. Cells were treated with 4OHT + DMSO or with 4OHT + 9cUAB30 for the indicated interval. β-actin served as a control for loading.
Fig. 1
Figure 2. (A) Skin tumor initiation by wild type human KLF4 is inhibited by 9cUAB30. Dox-inducible KLF4 transgenic mice were fed chow admixed with 9cUAB30 or placebo. Following dox administration for 30 days, the skin phenotype was determined by gross examination and by histologic analysis of fixed tissues. Penetrance of the skin phenotype is defined as any observed abnormality (gross or microscopic). (B) Analysis of 9cUAB30 effects on skin tumor initiation/progression. Mice (N = 2 per treatment group) were treated for 7 days as indicated and skin samples were analyzed by microscopic examination and immunostaining. Asterisks indicate SCC-like cells within the dermis. Arrowheads indicate the DEJ. Insets show a portion of the section at a higher magnification. Scale bars, 100 μ.
A

Penetration of skin lesions (percentage of animals)

<table>
<thead>
<tr>
<th>Chow:</th>
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<th>Placebo</th>
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</table>

p=0.0004

B

Dox (7d):
- - + +
9cUAB30:
- - + +

KLF4

K1

BrdU

Fig. 2
Figure 3. Reprogramming of basal keratinocytes in TO-KLF4 transgenic mice is inhibited by 9cUAB30. (A) Immunostaining analysis of KLF4 transgenic mouse skin. Mice were treated with Dox for 48 h. Insets show a portion of the section at a higher magnification. (B) K1 antibody stained the outer root sheath cells following induction of KLF4, but did not stain these cells in samples from control mice (not shown). (C) Mice were treated as indicated for 7 days, and expression of K1 in basal keratinocytes of the interfollicular skin was examined by immunostaining. Arrows indicate the DEJ. Scale bars, 50 μ.
**Figure 4.** Regulation of NRs gene expression by KLF4. (A) RK3E epithelial cells stably transduced with a KLF4-ER expression vector were treated with 4OHT for two hours. RNA was isolated and assayed by microarray. Fold changes were calculated using DMSO-treated KLF4-ER cells as the control. (B) KLF4-ER cells or Vector-transduced RK3E cells were treated with 4OHT as indicated, and RXRα was analyzed by immunoblot. (C) Cultures were treated as indicated, and mRNA expression was analyzed by sqRT-PCR. Gapd served as a loading control.
Fig. 4
Figure 5. RXRα knockdown attenuates the rexinoid inhibitory effect on KLF4. (A) Lentiviral vectors encoding shRNAs to RXRα were transduced into RK3E cells, and stable populations were selected using puromycin. Cell extracts were isolated at 96 h post-transduction and RXRα was analyzed by immunoblot. β-actin served as a loading control. (B) KLF4 transforming activity was assessed in knockdown cells and control cells in the presence or absence of 9cUAB30 using similar methods as in Figure 1A. (C) Quantitation of KLF4 transforming activity in the presence or absence of 9cUAB30. Each lentiviral construct was introduced into RK3E cells in two independent transduction experiments. Columns, mean (n = 2); bars, range. (D) The data shown in (C) was used to calculate the relative KLF4 transformation efficiency (i.e., the ratio of transformed foci observed in dishes treated with either 9cUAB30 or DMSO). Columns, mean (n = 4); bars, SE; p value, one way ANOVA with Tukey’s Multiple Comparison Test, comparing each shRNA-expressing cell line to Vector control cells).
**Fig. 5**

A. Western blot analysis showing expression levels of RXRα and β-actin in different cell lines: Mock, Vector, RXRα1, RXRα2, RXRα3. The molecular weight (MW) is indicated in KD (kilodaltons): 55 for RXRα and 40 for β-actin.

B. Images of colony formation assay using KLF4-ER retroviruses. Columns represent 4OHT+DMSO and 4OHT+9cUAB30 conditions, showing the effect of RXRα shRNA transduction on colony formation.

C. Bar graphs showing transformed foci counts for Lentiviral shRNA Transduction #1. Cell lines: Vector, RXRα1, RXRα2, RXRα3. Conditions: 4OHT+DMSO, 4OHT+9cUAB30.

D. Bar graphs showing transformed foci counts for Lentiviral shRNA Transduction #2. Conditions: 4OHT+DMSO, 4OHT+9cUAB30. Efficiency of transformation (9cUAB30/DMSO control) is compared across cell lines: Vector, RXRα1, RXRα2, RXRα3. Statistical significance indicated: *P<0.01, **P<0.001.
Table 1: Structure of RXRα shRNAs

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</table>

\(^*\) Numbers refers to rat RXRα mRNA (Genbank accession NM_012805)

** The strand shown corresponds to the final RNA Polymerase III transcript. Constructs were designed to target mouse RXRα (Mission® TRC shRNA, Sigma). Mismatches to rat are shown in lower case. RNAi seed sequences are indicated in italics.
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<th>Transcript</th>
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<th>Antisense oligonucleotide (5'-3')</th>
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Supplemental Fig 1. Functionally distinct retinoids inhibit transformation by KLF4. RK3E cells were transduced with retroviruses encoding either KLF4-ER, ErbB2, or a Vector control (not shown). Dishes were treated with the indicated retinoid every other day until foci were apparent. For each treatment group the relative transformation efficiency was determined as the ratio of transformed foci observed in dishes treated with either retinoid or DMSO. Columns, mean (n=2); bars, range.
Supplement Fig. 1
BAD THINGS HAPPEN IN THE BASAL LAYER--KLF4 AND SQUAMOUS CELL CARCINOMA

by

Wen Jiang, Susan M. Lobo-Ruppert and J. Michael Ruppert

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Format adapted for dissertation
Squamous epithelium of the skin is generated by a highly regulated series of events that occur within ‘epidermal proliferating units’ (EPUs) thought to be associated with a single stem cell. Upon commitment to the keratinocyte pathway, cells enter into the ‘transit amplifying’ compartment, located in the basal layer, and undergo several rounds of mitosis. These cells then undergo a proliferation differentiation switch and gradually approach the skin surface as they differentiate. Even subtle alterations in the rates of cell division, cell death or transit time could lead to pathologic conditions. The regulatory pathways that control these cell fate decisions would seem among the likeliest to contribute to malignancy.

However, Nature is full of surprises, and an article by Chen and colleagues in the current issue highlights yet another one. A molecule that normally lives in the upper, post-mitotic cell layers of the skin, and which has no major function in these developmental processes, is aberrantly expressed in the basal layer of neoplastic skin lesions and has other properties consistent with an oncogene important in skin tumor progression. Kruppel-like factor 4 (KLF4, also called GKLF) is a zinc finger transcription factor that is dispensable for normal embryogenesis. It is expressed in the post-mitotic, differentiating cells of a variety of epithelia including the epidermis. Although mechanisms remain poorly understood, KLF4 can function as a transforming oncogene in vitro, and can likewise cooperate with just three other genes (c-MYC, Sox2 and Oct3/4) to convert adult fibroblasts into pluripotent embryonic stem cells.

In spite of its negligible role in skin morphogenesis, KLF4 mRNA and protein are upregulated early and often in squamous cell carcinoma (SCC) progression. In fact, KLF4 is expressed in the early, potentially reversible lesion termed actinic keratosis (AK).
In these premalignant lesions, KLF4 is expressed in all cell layers, including the basal layer where it is normally not detected. Previously, we used the term ‘maturation-independent’ to describe SCC and AK lesions in which KLF4 protein is not restricted to the post-mitotic, differentiating compartment.9 This failed regulation represents a potential early event in SCC tumor progression. Chen and colleagues now confirm these studies using a distinct KLF4 antibody for immunostaining assays, and report complementary studies such as western blot and RNA analysis. They show that maturation-independent expression of KLF4, especially nuclear expression, is associated with an aggressive skin cancer phenotype, and even metastasis. Taken together, the published data support a model in which SCC results when a cancer precursor cell acquires properties normally segregated among three distinct epithelial compartments: the self-renewal property of stem cells, the proliferative capacity of transit amplifying cells, and the transforming activity of oncogenes such as KLF4 that are normally restricted to the post-mitotic, differentiating compartment.

KLF4 appears sufficient to recapitulate several aspects of SCC tumor progression. Previously, we engineered transgenic mice to conditionally express KLF4 in the proliferation-competent basal cell layer.10 These mice develop lesions over a period of days to weeks that include hyperplasia, dysplasia and SCC-like tumors. Unlike the latent, focal tumors that are observed in most models of cancer, these lesions evolved rapidly—within days of induction. Lesions involved the majority of the skin surface in a relatively uniform, diffuse fashion, suggesting that other genetic changes may not be required for this rapid-onset phenotype. The resulting tumors shared molecular and genetic similarities to human SCC, including reliance upon p53 inactivation. Compared to this
simple model, human SCCs develop over a prolonged interval and are likely to contain additional genetic and epigenetic changes.

Similar to mouse models of SCC, in human SCCs the role of p53 appears prominent. Morphologically normal human skin, especially from sun-exposed areas, contains frequent ‘p53 clones’. These small clusters of 60–3000 keratinocytes are identified by their increased staining of p53 protein, and ~50% of the clones contain a mutated p53 gene. p53 clones are often shaped like an inverted cone, as might be expected if they derive from a single stem cell, and show evidence of spreading beyond the architectural boundaries of a normal EPU. Otherwise, p53 clones are morphologically similar to normal cells. Separately, genetic studies in mice indicate strong links between p53 deficiency and progression to cutaneous SCC, with p53 functioning somewhat like a cutaneous SCC ‘gatekeeper’. These results point to p53 alteration as an early step, or perhaps the first step, in the genesis of cutaneous SCC. The subsequent steps that may convert a p53 clone into a morphologically-distinctive, neoplastic lesion such as AK remain poorly understood. While a role for an oncogene in this conversion seems likely, frequent mutation of Ras or other known oncogenes has not been reported.

The ability of KLF4 to induce diffuse SCC-like skin lesions within a short timeframe, and the strict dependence upon p53 status, point to KLF4 as a likely step that cooperates with p53 alteration in human SCC tumorigenesis. Normally, KLF4 and p53 appear to be mutually antagonistic. KLF4 can directly suppress p53 expression. Conversely, p53 suppresses KLF4-induced dysplasia if the size of the skin patch containing deregulated KLF4 is too small. For example, uniform expression of KLF4 across the skin is sufficient to initiate dysplasia even in p53 WT animals, but ‘patchy’ or
mosaic KLF4 expression (utilizing X-linked inactivation) yielded no skin phenotype unless p53 was altered by loss of either one or both copies. This straightforward mouse genetic experiment suggests a model in which p53 alteration (e.g., mutation and/or allelic loss) would render an EPU competent to expand in response to KLF4 expression in basal cells. Thus, the p53 clones that exist in human skin represent an ideal setting in which deregulation of KLF4 could promote tumor formation.

As shown for p53 in other tumor types,16,17 p53 is haplo-insufficient for suppression of KLF4-induced skin tumors.10 There is increased cancer susceptibility even though one wild type p53 gene remains. For p53, this haplo-insufficiency could be related to its function as a multimer in equilibrium with monomer, as small fold changes in monomer concentration would result in larger-fold changes in the active homotetramer.18 Rowland and colleagues showed that KLF4 can directly lower p53 protein concentration by interacting with the p53 promoter and suppressing p53 transcription.7 Since many p53 clones in the skin appear to contain copies of wild type p53,1,12 suppression by KLF4 may be functionally important and could contribute to p53 haploinsufficiency.

How does p53 block the transforming activity of KLF4 in vivo? Although KLF4 can directly suppress p53 expression, it is unknown whether p53 signaling may likewise affect activity of the KLF4 protein. Mosaic expression of basal KLF4 shows that the required patch size is smaller in p53-deficient skin.10 This permissive effect may indicate competition between adjacent EPUs, with cell arrest/death signals predominating when the patch size is small and p53 is wild type. The timing of onset of maturation-independent KLF4 expression can now be mapped by analysis of earlier lesions. It will
be interesting to see whether reversible actinic keratosis lesions or the p53 clones of 60–3000 cells that are frequent in sun-exposed skin show KLF4 deregulation.

It is currently unclear how KLF4 expression is suppressed in normal, proliferating squamous epithelial cells. Not only KLF4 protein, but also the mRNA is low or absent in the normal basal cells of the skin and oral mucosa. While a role for transcriptional control seems likely, results that we obtained in KLF4 transgenic mice suggest that posttranscriptional mechanisms suppress the mRNA in dividing cells (see Fig. 1). In addition, Chen and colleagues showed that the KLF4 protein is actively targeted for degradation in dividing cells. Given the potential for KLF4 to induce malignant transformation and to promote pluripotency when co-expressed with genes such as c-MYC, it is perhaps not surprising that multiple mechanisms operate to prevent high-level KLF4 expression in dividing cells. Each of these mechanisms somehow gets bypassed during tumor progression, leading to maturation-independent expression.

One attractive possibility is that KLF4 induces the cancer stem cell phenotype in the skin, just as it induces the stem cell phenotype in fibroblasts. Basal expression of KLF4 could promote expansion of stem cells within an EPU, leading to a clonal outgrowth of a tumor. If so, then molecularly targeted therapies aimed at KLF4 could effectively target cancer stem cells. These same therapies may reverse lesions that show maturation independent expression and nuclear localization of KLF4.

KLF4 deficient mice develop normally in utero but subsequently die in the early postpartum period due to dehydration, a consequence of failure to form an adequate skin permeability barrier. The specific stage of skin development at which KLF4 executes this function has not been identified experimentally. KLF4 can have dramatic effects at
even low levels of expression, and might exert its barrier effect prior to onset of the high level expression seen in post-mitotic cells. Also unknown at the present time is whether KLF4 is normally required strictly for formation of the skin permeability barrier during development, or whether it has a more general role in maintenance of the barrier in postpartum animals or in adults. This question is ultimately important for understanding potential toxicities in patients undergoing KLF4-directed therapies.

References


**Figure 1.** A KLF4 transgenic mouse model of SCC shows progression to maturation-independent, constitutive nuclear staining of KLF4, as seen in human skin tumors (Chen, Y.J. et al., this issue). KLF4 was induced in the skin of transgenic mice by administration of doxycycline for 14 days, using a keratin 14 (K14) promoter-dependent expression strategy. Skin sections were analyzed by immunostaining, showing that all the epidermal cell layers had become K14-positive. Even though KLF4 transcription is driven by doxycycline in all K14-positive cell layers, only low level KLF4 is detected in the basal, PCNA-positive, epidermal cells of hyperplastic skin (upper panels, see open arrowheads). This result suggests that restriction of KLF4 expression to post-mitotic cells is, at least in part, a post-transcriptional effect. This restriction is lost during tumor progression, as dysplastic skin shows frequent basal expression of nuclear KLF4 (lower panels). Insets show the cell-type specific staining at increased magnification. Arrows indicate the demo-epidermal junction, and open arrowheads indicate examples of KLF4-positive, basal keratinocytes. Adapted from reference 10. Scale bars 100µ (upper panels) or 50µ (lower panels).
Figure 1.
Dissertation Summary

This dissertation describes the identification and characterization of KLF4 as a determinant of retinoid receptor expression and function in cancer prevention. There are four key findings: first, by employing a genetically tractable epithelial model to link retinoid sensitivity to the function of KLF4, we discovered that KLF4 transforming activity was specifically suppressed by rexinoid 9cUAB30 and by other retinoids. Second, using conditional KLF4-ER cells, we found that KLF4 rapidly induces transcripts encoding RXRα, RARγ, and Nur77. Third, shRNA-mediated receptor knockdown indicated that the KLF4-inhibitory effects of rexinoid are mediated, at least in part, by RXRα. Finally, in the dox-inducible, KLF4 transgenic mouse model of SCC, 9cUAB30 prevented KLF4-mediated tumor initiation, arresting cells at a stage of increased cell cycling and cell turnover.

These results demonstrate for the first time that retinoid anti-tumor effects intersect with KLF4 oncogenic signaling. We used RK3E epithelial cells immortalized with adenovirus E1a, a model previously used for in vitro functional analysis of carcinoma-derived oncogenes including Gli1(Li et al. 4489-98;Nilsson et al. 3438-43;Ruppert, Vogelstein, and Kinzler 1724-28), KLF4(Foster et al. 423-34), Notch1 (Ascano, Beverly, and Capobianco 8771-79;Capobianco et al. 6265-73;Tonon et al. 208-13), and ErbB2(Menard et al. 2965-78). The transcription factor Gli1 is a major effector of Hedgehog (Hh) signaling and its overexpression is sufficient for tumor development.
The cell membrane surface-bound receptor tyrosine kinase ErbB2 (also known as HER2, human epidermal growth factor receptor-2) is normally involved in the signal pathways leading to cell growth and differentiation. The cell fate determinant Notch1 is a receptor for membrane bound ligands in the Notch signaling pathway, an intercellular signaling involved in a wide variety of human neoplasms. Strikingly, in this model there was no inhibitory effect of rexinoid on the ability of these other oncogenes (Notch, Gli, ErbB2) to induce transformation. In contrast, transformation of KLF4 was greatly suppressed (Fig 1A). This result, albeit in vitro and utilizing an artificial model, provides genetic data that links retinoid chemopreventive activity and the KLF4 pathway. A similar previous study showed that retinoic acid greatly inhibits transformed focus formation transfected with another oncogene H-ras (Cox et al. 102-08). However, in this study, there was neither genetic evidence linking retinoid effects selectively to the H-ras pathway, nor association with changes in the expression of the exogenous H-ras or endogenous c-myc or c-fos oncogenes. KLF4 expression was not assessed in this study as well.

In the KLF4 pathway, p21Waf1/Cip1 and p53 are known transcriptional targets, and, in turn, they are critical determinants of KLF4 function as an oncogene. In our study, we have found out that 9cUAB30 did not alter KLF4-ER expression, its ability to induce p21Waf1/Cip1, or its ability to repress p53 (Fig 1D). This suggests that downstream, convergent signaling by retinoid receptors and KLF4 may impact common targets. Indeed, a recent study of vascular smooth muscle cells (VSMCs) shows that KLF4 is one of the key mediators of retinoid activity in these cells, which inhibit proliferation and induce differentiation (Wang et al. 313-21). They found that treatment of VSMCs with ATRA leads to up-regulation of KLF4, and its target gene p53, as well as the VSMC
differentiation marker genes SM22α and SM α-Actin (α-SMA). These results suggest us to understand the mechanisms of retinoid effects on KLF4 activity in distinct context and to find common targets for both retinoid receptors and KLF4. Recently, we have been using shRNA and gene knockout approaches to evaluate the role of p21, p53, RAR, PPARs, or Nur77 as potential mediators of retinoid effects on KLF4 activity. In the future, our laboratory will utilize this simple genetic model in combination with microarray analysis to identify overlapping sets of genes that are regulated by both retinoid and KLF4.

Not only in cellular differentiation and tumorigenesis, but also in skin water permeability barrier, KLF4 and the NRs may have convergent roles. Previous studies have also shown that KLF4 represents the first transcription factor that is essential for establishing the skin’s water permeability barrier function (Segre, Bauer, and Fuchs 356-60). In normal skin, KLF4 is expressed in spinous cell layers, which also express repressor forms of RARγ2/RXRα heterodimers that are required in suprabasal keratinocytes for the generation of barrier formation (Calleja et al. 1525-38). In conditional RK3E-KLF4-ER cells, we have also found out that KLF4 rapidly induces transcripts encoding RXRα and RARγ by microarray and immunoblot analyses. (Fig 4)

These observations raise new questions for future research. First, does KLF4 induce RAR/RXR expression in adult mouse skin? Using conditional KLF4 knockout (KLF4<sup>−/−</sup>) in mouse skin, one could employ Cre-mediated excision to analyze the effect of KLF4 on RAR/RXR expression and signaling (Calleja et al. 1525-38;Katz et al. 2619-28;Katz et al. 935-45). An interesting aspect of this approach is that it allows one to determine the role of KLF4 in maintenance of the skin permeability barrier in adult mice.
To date, it is clear that KLF4 is required to form the barrier in utero, but its role in maintenance of the existing barrier in adults has not been assessed. This question is important since therapies that inhibit KLF4 activity in tumors might lead to skin effects in adults. For these studies, the skin from KLF4 knockout and control mice will be analyzed for retinoid receptor expression (RXRα, RARγ) by mRNA in situ hybridization (ISH) and real-time RT-PCR. Second, does KLF4 induce the permeability barrier through regulation of RARγ/RXRα heterodimers? It will be important to compare the barrier phenotype of KLF4 knockout skin to that in barrier-defective skin induced by modulation of retinoid signaling. The barrier-defective skin in KLF4 knockout mouse will be treated with NRs agonists and/or antagonists, followed by an analysis of phenotype changes. To discover whether KLF4 modulates skin permeability barrier function by acting through the NRs, one could test the ability of KLF4-/- keratinocytes to form a barrier using in vitro raft cultures (Cheng et al. 2335-49; Parker et al. 751-62). If retinoid receptor expression is deficient, then RAR/RXR vectors could be used to test for reconstitution of barrier assembly. This same model could be used to conditionally delete KLF4 or the RARs to test for loss of the barrier in vitro. These studies would demonstrate whether KLF4 barrier formation acts through the retinoid receptors. Thus, in these two settings (KLF4-mediated transformation or tumor progression, and KLF4-mediated barrier formation), KLF4 may be linked specifically to the retinoid pathways.

Among multiple type 2 NRs, RXRα is the most abundant RXR isotype expressed in epidermal keratinocytes (Fisher and Voorhees 1002-13). RXRs impact diverse signaling pathways by heterodimerizing with PPARs, RARs, Nur77, VDR, LXRα etc. To identify a functional mediator of rexinoid effects on KLF4-mediated transformation, we
used lentiviral shRNA-mediated receptor knockdown. This approach suggested that transformation by KLF4 in vitro is inhibited by RXRα (i.e., even in the absence of exogenous rexinoid) (Fig 5). Consistent with our in vitro studies, previous studies in vivo have shown that conditional RXRα knockout in mouse skin leads to hyperplasia and to an increased susceptibility to tumor initiation or promotion (Indra et al. 1250-60; Li et al. 633-36). This enhanced susceptibility to transformation confirms the tumor suppressor role of RXRα. Consistent with our knockdown studies, complementary studies in which exogenous RXRα was introduced into RK3E cells showed that overexpression of RXRα in RK3E cells greatly inhibits KLF4 transforming activity (data not shown).

These studies, performed in the absence of exogenous ligand, demonstrate that RXRα has a pronounced effect on its own, perhaps by associating with endogenous ligands. In their often-cited paper that examined the role of RXRs and RARs on the permeability barrier formation in the skin, Chambon and colleagues concluded that RXR functioned as a transcriptional activator in PPAR β(δ)/RXRα heterodimers to promote barrier formation, while RARγ/RXRα heterodimers functioned as transcriptional repressors to promote same process (Calleja et al. 1525-38). Thus, within a single tissue, RXR participates in distinct complexes that mediate barrier formation. In the repressor form (RARγ/RXRα), ligand binding to RXRα would still permit transcriptional repression because of subordination. However, in the active form (RXRα/PPARβ(δ) heterodimer), there may be endogenous ligands that can bind and activate both PPAR β(δ) and RXRα since this heterodimer complex is ligand-permissive. Since RXR clearly has effects both on barrier formation and on tumor suppression, it will
be important to understand more about the complexes that mediate these processes and the endogenous ligands that may activate RXR signaling.

Whereas rexinoid normally inhibits transformation by KLF4, we found out that RXRα knockdown attenuated this effect (Fig 5). Thus, RXRα is a ligand-dependent inhibitor of KLF4-mediated transformation. We also compared the oncogene-transformation inhibiting effects of 9cUAB30 with other retinoids, such as ATRA, 9cRA, which are agonists with diverse nuclear receptor selectivity (Fig 1B). Suppression of KLF4-mediated transformation was observed for all of these agonists, indicating distinct NRs heterodimers as KLF4 antagonists. So the next question is which could be the potential heterodimerized partner for RXR. In the skin, RARγ-RXRα represents a major signal transducer. Like KLF4, which is most abundant in suprabasal keratinocytes, the repressor form of RARγ/RXRα heterodimers (i.e., unliganded RARs) contributes to the formation of the skin permeability barrier. While under some conditions rexinoid can activate RAR/RXR heterodimers in the absence of RAR ligand (called desubordination; (Altucci et al. 793-810)), enforced expression of KLF4 is not sufficient to accomplish this (transcriptional reporter studies not shown). Therefore, the exact role of RARγ/RXRα in skin cancer and in the prevention/treatment effects of retinoid/rexinoid on KLF4-induced tumor transformation poses an interesting and promising question.

Nur77, another potential dimerization partner for RXR, was identified in our studies as being induced by KLF4 in vitro, and has been shown to mediate apoptosis by transcriptional induction of apoptotic genes and by translocation of RXRα-Nur77 to mitochondria (Cao et al. 9705-25;Lee et al. 16942-48;Rajpal et al. 6526-36). The binding of Nur77 to Bcl2 converts the latter from a blocker of apoptosis to a promoter of cell
death (Lin et al. 527-40). Whether rexinoid 9cUAB30 acts through RXR/Nur77 heterodimer to activate apoptosis, and therefore, to mediate anti-tumor effect is unknown; it could be another potential mechanism. In addition to RAR and Nur77, RXRs are also known to heterodimerize with some other members of NR superfamily expressed in the epidermis, such as PPARs, VDR, LXRs (liver-X-receptors) (Fowler et al. 246-55; Li et al. 633-36; Mao-Qiang et al. 305-12; Schmuth et al. 971-83; Sheu et al. 94-101). Previous observations have shown that ligands of PPARs and VDR can inhibit the growth of SCC cell lines in vitro and multistage skin tumorigenesis in vivo (Kensler et al. 1341-45; Kopelovich et al. 357-63; Nikitakis et al. 817-23). LXR ligands have anti-inflammatory effect against TPA-induced cutaneous inflammation (Fowler et al. 246-55). Thus, whether and precisely how rexinoid acts through these signaling pathways to control skin carcinogenesis remains to be investigated.

Our group has previously established a dox-inducible mouse model of SCC, in which KLF4 was induced in the proliferation-competent basal keratinocytes (Foster et al. 1491-500). This well characterized, transgenic mice model virtually reproduced the skin cancer progression process, rapidly inducing hyperplasia, dysplasia, SCC in situ, and SCC-like invasive lesions. It supports a role for KLF4 as a substantially critical factor in the early stage of cutaneous SCC development (Foster et al. 1491-500; Huang et al. 1401-08; Jiang, Lobo-Ruppert, and Ruppert 783-85). The fact that all four distinct stages in skin tumor progression can be easily identified by means of morphological examination and/or immunostaining makes it a favorable single-gene model, to characterize the chemopreventive effects of retinoids on KLF4 oncogenic activity. During the initiation of SCC tumor progression by KLF4 in our transgenic mouse skin, 9cUAB30, the most
selective KLF4 inhibitor, greatly diminished KLF4’s ability to alter cell fate, as seen from the normal K1 expression pattern and lesions arrested at the stage when the skin appeared morphologically normal by light microscopy (Fig 2, 3). Furthermore, substantial mitotic activity was seen from these lesions as shown by BrdU incorporation. Taken together, rexinoid shows the ability to block tumor progression at Phase 1 from our SCC model, a stage of enhanced cell cycling and cell turnover. These \textit{in vivo} observations suggest that 9cUAB30 prevents KLF4 induced-SCC initiation probably through enforcing of normal differentiation but not through direct suppression of proliferation. This is consistent with \textit{in vitro} results that 9cUAB30 had almost little or no effects on cell growth assay or cell cycle progression in KLF4-expressing cells (data not shown). To confirm the enforcing differentiation effects in the lesions in the presence of 9cUAB30, one could examine the expression of terminal differentiation markers by more specific immunostaining in the skin (i.e., transglutaminase type 1 (TGase 1), cornifin, involucrin, loricrin, small proline-rich protein (SPR)1A, SPR2H). Moreover, expression levels of potential NRs (such as RARs, RXRs) in these lesions in the presence and absence of 9cUAB30 could also be determined. This test would reveal whether KLF4 can induce specific NRs \textit{in vivo} and whether 9cUAB30 mediates its chemopreventive effects through certain NRs heterodimers. Not only in prevention but also in treatment, similar to other rexinoids such as Targretin (Li and Brown 203-15; Liby, Yore, and Sporn 357-69), 9cUAB30 was effective in inhibiting growth of the established, MNU-induced breast cancers that express KLF4 (data not shown). Thus, the distinct effects observed in the different models may indicate context-specific signaling.
Concluding Remarks

While questions remain about the relevant cellular mechanisms for retinoids, these natural and synthetic agents clearly play promising anti-tumor roles in multiple cancer types, including skin and breast cancers. Critical components in distinct signaling pathways that mediate early steps in tumor progression provide especially attractive targets for therapy or prevention of cancer. Deregulation of KLF4 seems to be one of the early events in SCC (Foster et al. 1491-500; Huang et al. 1401-08; Jiang, Lobo-Ruppert, and Ruppert 783-85). The study in this dissertation implicates a single pathway, KLF4, in retinoid-mediated tumor prevention. The rapid, single-gene, genetically tractable models that we describe provide in vitro and in vivo systems that will allow others to better understand the chemopreventive activity of retinoids. In addition, by analysis of KLF4 signaling in tumor cells, these studies may prompt improved patient selection for more effective treatment, leading to superior therapeutic responses and/or to fewer recurrences.

GENERAL LIST OF REFERENCES


Nobel Prize in Physiology or Medicine for 1989 jointly to J. Michael Bishop and Harold E. Varmus for their discovery of "the cellular origin of retroviral oncogenes". 2009. PressRelease.


NOTICE OF APPROVAL

DATE: January 13, 2009

TO: J. Michael Ruppert, M.D., Ph.D.
WTI-570 3300
FAX: 975-5650

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: SPORE: Spore in Breast Cancer (Bland); Project # 3: Biomarkers in the KLF4 Signaling Pathway
Sponsor: NIH
Animal Project Number: 071008307

On January 17, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>B</td>
<td>200</td>
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Animal use is scheduled for review one year from October 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 071008307 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.