Novel function of keratins 5 and 14 in proliferation and differentiation of stratified epithelial cells

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ABSTRACT Keratins are cytoplasmic intermediate filament proteins preferentially expressed by epithelial tissues in a site-specific and differentiation-dependent manner. The complex network of keratin filaments in stratified epithelia is tightly regulated during squamous cell differentiation. Keratin 14 (K14) is expressed in mitotically active basal layer cells, along with its partner keratin 5 (K5), and their expression is down-regulated as cells differentiate. Apart from the cytoprotective functions of K14, very little is known about K14 regulatory functions, since the K14 knockout mice show postnatal lethality. In this study, K14 expression was inhibited using RNA interference in cell lines derived from stratified epithelia to study the K14 functions in epithelial homeostasis. The K14 knockdown clones demonstrated substantial decreases in the levels of the K14 partner K5. These cells showed reduction in cell proliferation and delay in cell cycle progression, along with decreased phosphorylated Akt levels. K14 knockdown cells also exhibited enhanced levels of activated Notch1, involucrin, and K1. In addition, K14 knockdown AW13516 cells showed significant reduction in tumorigenicity. Our results suggest that K5 and K14 may have a role in maintenance of cell proliferation potential in the basal layer of stratified epithelia, modulating phosphatidylinositol 3-kinase/Akt-mediated cell proliferation and/or Notch1-dependent cell differentiation.

INTRODUCTION Keratins (Ks) are the largest subgroup of intermediate filament (IF) proteins preferentially expressed in epithelial tissues (Moll et al., 1982; Coulombe and Omary, 2002). They are subdivided into type I acidic (K9–K28) and type II basic (K1–K8 and K71–K74) keratins (Moll et al., 1982; Schweizer et al., 2006) on the basis of biochemical properties, such as molecular weight and isoelectric point (Hesse et al., 2004). They are obligatory heteropolymers and are assembled in 1:1 molar ratio, consisting of one type I and one type II keratin (Moll et al., 1982; Coulombe and Omary, 2002; Herrmann and Aeberli, 2004). Epithelial tissues express different pairs of keratins depending upon the cell type, that is, all stratified squamous epithelia express K5/K14 (Nelson and Sun, 1983), while K8/K18 are seen in all simple epithelia (Omary et al., 2009).

Keratins have a basic molecular structure common to all cytoplasmic IF proteins. They have a highly conserved central coil-coil α-helical “rod” domain flanked by a non-α-helical, N-terminal “head” and C-terminal “tail” domains of various lengths (Fuchs and Weber, 1994; Herrmann and Aeberli, 2004). The central rod domain plays a critical role in filament assembly. The heptad-repeat structure within the central domain of keratin monomers facilitates filament formation (Albers, 1996). The point mutations in human keratin genes (in rod domain) are associated with different epithelial disorders in multiple tissue types. These inherited human diseases exhibit cytolysis of epithelial cells, resulting in blistering of the corresponding epithelial sheets (Fuchs and Cleveland, 1998; Moll et al., 2009). These observations, along with several studies conducted on...
keratin knockout mice and mice carrying dominant keratin mutations, suggest that keratin filaments provide mechanical support to tissue architecture and are critical for the maintenance of cell viability (Fuchs and Cleveland, 1998; Coulombe et al., 2004; Gu and Coulombe, 2007). In addition to their cytoprotective functions, they also perform some important regulatory functions by modulating certain signaling pathways (Paramio and Jorcano, 2002; Magin et al., 2007). They form complex signaling platforms and interact with various proteins, such as kinases, adaptors, and receptors (Pallari and Eriksson, 2006), and thus regulate/modulate different signaling pathways associated with various cellular processes, such as protein synthesis, cell growth, and cell differentiation (Ku et al., 2002; Oshima, 2002; Koch and Roop, 2004; Kim et al., 2006).

In stratified epithelia, keratins exhibit a complex expression pattern tightly regulated by the differentiation program of the tissue. The keratins in the basal proliferating layer of these epithelia are K5/K14 (Moll et al., 1982). As these cells move upward and differentiate, K5/K14 levels are gradually reduced and expression of a new pair of keratins is induced, depending upon the tissue type (Fuchs and Green, 1980). Differentiating cells express K1/K10 in skin; K4/13 in internal stratified epithelia, such as esophagus; and K3/K12 in corneal cells (Albers, 1996).

Keratin 14 (K14) is a prototypic marker of dividing basal keratinocytes and helps in the maintenance of epidermal cell shape; it also provides resistance to mechanical stress. Interestingly, the K5/K14 pair is expressed in the basal layer of the epidermis, which contains epidermal stem cells and transient amplifying (TA) cells, while the K1/K10 pair is synthesized only in postmitotic keratinocytes (Coulombe et al., 1989; Byrne et al., 1994). K14 mutations have been associated with a hereditary skin blistering disease called epidermolysis bullosa simplex (EBS; Lane, 1994; Omary et al., 2004) and mice harboring a K14 null mutation also exhibit a phenotype similar to EBS (Lloyd et al., 1995). K14 null mice demonstrate extensive blistering and die ~2 d after birth, indicating the functional importance of K14 in maintaining mechanical integrity of the stratified epithelial cells (Chan et al., 1994; Rugg et al., 1994). In addition, K14 is also implicated in regulating certain apoptosis-associated signaling pathways via TRADD (tumor necrosis factor (TNF)–associated death domain) and extracellular signal–regulated protein kinase (ERK) (Yoneda et al., 2004; Lugassy et al., 2008; Russell et al., 2010). Transgenic and in vitro studies indicate that loss of K14 cannot be completely compensated for by the ectopic expression of other keratins (Hutton et al., 1998; Paladini and Coulombe, 1998). These observations imply that the K5/K14 pair is required for normal development and functioning of the basal cells and performs some unique regulatory functions. However, unique tissue-specific functions of this pair in relation to cell proliferation and keratinocyte differentiation are not yet fully understood.

In this study, we have investigated the role of K14 in cell proliferation, differentiation, and neoplastic progression using RNA interference. K14 expression was stably inhibited in both HaCaT and AW13516 cells. K14 knockdown cells showed depletion in its normal partner K5. K14-deficient cells demonstrated substantial reduction in cell proliferation, decrease in phospho-Akt levels, increase in activated Notch1 levels, and increase in levels of keratinocyte differentiation markers. K14 knockdown also led to reduction in tumorigenic potential in AW13516 cells.

RESULTS

Generation of stable K14 knockdown cells
To generate efficient shRNA constructs for knockdown of K14, four different sequences were used to generate pTU6 PURO-based short hairpin RNA (shRNA) constructs. The constructs were validated by testing their efficiency to inhibit the expression of an exogenously expressed green fluorescent protein (GFP)-tagged K14 construct. shRNAK14.2 and shRNAK14.4 showed significant knockdown of exogenous GFP-tagged K14 expression (Supplemental Figure S1). The most effective shRNA construct (shRNAK14.4) or the empty vector control was transfected into the HaCaT and AW13516 cell lines, to obtain stable K14 knockdown and vector control clones. K14 expression (both mRNA and protein) in shRNAK14.4-transfected stable clones (shRK14-D1, -D2, and -D6 derived from HaCaT and shRK14-K7, -K9, and -K16 derived from AW13516) was determined by RT-PCR and Western blot analysis respectively. K14 expression in K14.4-shRNA transfected HaCaT stable clones (shRK14-D1, -D2, and -D6) was significantly reduced compared with the vector control (pTU6-Hac) generated upon transfection of pTU6-PURO alone (Figure 1, A and B). A substantial reduction in K14 level was also observed in shRNAK14.4-transfected AW13516 stable clones (shRK14-K7, -K9, and -K16), as compared with the vector control clone (pTU6-AW1; Figure 1, A and B). Moreover, K14 filaments were absent in K14 knockdown clones, while vector control clones showed K5/K14 filaments, as determined by confocal microscopy (Figure 1C). These clones demonstrated significant reduction in protein level (Figure 1A) and ~60% decrease in filament formation of K14's normal partner K5 (Figure 1C). K5 was still able to form some filaments, possibly because of its pairing with some other type I keratin in these cells. No significant difference in protein expression (Figure S2, A and C) and filament formation of K8 or K18 was observed in these clones (Figure S2, B and D). This observation ensures the specificity of our K14 shRNA, especially since the keratins share a great deal of homology at the sequence level.

K14 knockdown leads to reduction in cell proliferation
To determine the effects of K14 downregulation on cell proliferation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed on the K14 knockdown (shRK14-D1 and -D2; shRK14-K7 and -K9 derived from HaCaT and AW13516, respectively) and the respective vector control clones (pTU6-Hac and pTU6-AW1). K14 knockdown in both cell lines resulted in significant reduction in cell proliferation (Figure 2A and B). A substantial knockdown of K14 expression was also observed in shRNAK14.4-transfected stable clones (shRK14-D1, -D2, and -D6 derived from HaCaT and AW13516, respectively) and the respective vector control clones (pTU6-Hac and pTU6-AW1). K14 knockdown in both cell lines resulted in significant reduction in cell proliferation (Figure 2A and B). In addition, colony-forming ability of K14 knockdown and vector control clones was assessed. K14 knockdown clones demonstrated considerable reduction in colony-forming efficiency compared with their vector control clones. Moreover, the average size of colonies formed by K14 knockdown cells was smaller than for colonies formed by vector control cells (Figure 2, C and D), which correlates with the slower proliferation rate. Our results therefore suggest that K14 knockdown leads to substantial reduction in cell proliferation.

K14 knockdown cells show delayed cell cycle progression
The K14 knockdown clones (shRK14-D1 and -D2) showed decreased cell proliferation in comparison with vector control cells, as determined by MTT assay (Figure 2, A and B). To understand whether the decrease in cell proliferation was due to delay in cell cycle, we analyzed cell cycle progression through different phases at different time points by flow cytometry (Figure S3) and Western blotting. The vector control and K14 knockdown clones showed approximately the same percentage of cells in G1-phase upon serum starvation (Figure S3). However, the percentage of cells in S-phase at 18 h for K14 knockdown clones was significantly reduced compared with vector control (Figure 3A). Using Western blot analysis, we also observed reduction in the levels of cyclin D1 (G1/S phase–specific cyclin) and proliferating cell nuclear antigen (PCNA; S phase–specific...
To understand whether K14 modulates cell proliferation in these cells via the PI3K/Akt pathway, activation of Akt, that is, the level of phosphorylation of Akt, was studied using Western blot analysis. K14 knockdown cells showed significantly decreased levels of phosphorylation at serine 473 residue of Akt compared with the vector control cells, whereas total Akt expression levels remained unchanged in these cells (Figure 3E). The reduced phosphorylation of Akt upon K14 knockdown correlated well with the reduced cell proliferation rate and delay in cell cycle progression. Thus our results suggest that K14 modulates cell proliferation in stratified epithelia, possibly through the PI3K/Akt pathway.

K14 knockdown results in induction of keratinocyte differentiation
K5 and K14 are expressed in the mitotically active basal layer of stratified epithelia. When these cells move upward and differentiate, K5/K14 expression is decreased and K1/K10 expression is induced

Reduced activation of Akt in K14 knockdown cells
The phosphatidylinositol 3-kinase (PI3K) pathway is known to regulate cell proliferation in epithelial cells and phosphorylation of Akt is an important downstream event of PI3K activation (Vivanco and Sawyers, 2002). Recent reports clearly suggest that keratins modulate the Akt pathway during different cellular processes (Lloyd et al., 1995; Kim et al., 2006; Kippenberger et al., 2010; Ku et al., 2011).

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**FIGURE 1:** Generation of K14 knockdown clones of HaCaT and AW13516 cells. (A) Western blot analysis of stable K14 knockdown clones (shRK14-D1, -D2, and -D6 derived from HaCaT; shRK14-K7, -K9, and -K16 derived from AW13516) and vector control (pTU6-HaC and pTU6-AW1 derived from HaCaT and AW13516, respectively) cells with antibodies to K14 and K5. β-Actin was used as a loading control. (B) RT-PCR analysis of K14 in stable K14 knockdown and the respective vector control clones. GAPDH was used as internal control. (C and D) Confocal analysis of K14 and K5 levels and filament networks in the indicated clones. Scale bars: 10 μm. The mean fluorescence intensity (+SD) of K5 and K14 was calculated per cell by measuring fluorescence intensity of 20 cells of each experiment (using LSM10 software; Carl Zeiss MicroImaging GmbH, Jena, Germany) and is shown in the graph on the right-hand side. ***, p < 0.001 for K14; ***, p < 0.001 for K5.
K14 depletion leads to increased activation of Notch1

To investigate the possible mechanism of K14-mediated modulation of differentiation program and its associated molecules, such as involucrin and K1, we studied the Notch1 signaling pathway. Notch1, that is, notch intracellular domain (NICD), levels were determined in K14 knockdown cells by Western blot analysis. An increase in the levels of NICD was observed in K14 knockdown clones (Figure 5, A and B). Cell surface levels of Notch1 in the K14 knockdown clones were elevated, as analyzed by confocal microscopy (Figure 5C). Furthermore, K14 knockdown cells showed increased nuclear levels of Notch1 compared with vector control cells as visualized using confocal microscopy (Figure 5D). Elevated nuclear levels of Notch1 correlated with increased NICD levels. No significant difference in mRNA levels of Notch1 and Notch2 were observed by real-time PCR in the K14 knockdown cells when compared with the vector control cells (Figure S3A). These results suggest that K14 knockdown leads to increase in NICD levels, possibly modulating the levels of differentiation markers, such as involucrin and K1. Activation of Notch1 signaling was also correlated with reduced cell proliferation.

Effect of K14 knockdown cell–extracellular matrix and cell–cell adhesion

Since cell differentiation and Notch 1 activation have been shown to be associated with cell adhesion (Watt et al., 2008), we analyzed cell–extracellular matrix (ECM) and cell–cell adhesion in K14 knockdown and vector control cells (methods described in Supplemental Material). K14 knockdown cells did not show significant increase in cell adhesion to ECM substrates such as matrigel, collagen, and fibronectin in comparison with vector control cells (Figure S4C). Further, cell–cell adhesion was analyzed by hanging drop assay. The K14 knockdown cells formed smaller aggregates compared with vector control cells (Table S3).

Reduction in tumorigenicity of the K14 knockdown AW13516 cells

To determine whether reduced cell proliferation and enhanced activated-Notch1 levels resulting from K14 knockdown have any effect on tumorigenetic potential of cells, in vivo tumorigenesis assays were performed. The tumorigenic potential of K14 knockdown cells was determined by tumor formation upon subcutaneous injection in nude mice. The volume and frequency of tumor formation were significantly decreased in mice injected with K14-knockdown cells (shRK14-K9 and -K16) compared with mice injected with vector control cells (pTU6-AW1; Figure 6, A and B). Immunohistochemistry was used to analyze the levels of K14 in tumors. The tumors derived from the vector control (pTU6-AW1) showed higher levels of K14 as compared with the tumors derived from the K14 knockdown clones (shRK14-K7 and -K9; Figure 6C). In summary, the results suggest that K14 depletion leads to reduction in tumorigenic potential of the cells.

Rescue of K14 knockdown phenotypes by reexpression of shRNA-resistant K14

To ensure that the phenotypic changes observed upon K14 knockdown were not due to off-target effects of the shRNA construct, a rescue experiment was performed. Resistance of mutated GFP-tagged-K14 construct (K14GFP-RR) to shRNA14.4 was confirmed by cotransfecting it in HEK-293 cells and measuring the intensity of GFP and Western blot analysis. K14GFP-RR demonstrated resistance to shRNA14.4 (Figure S5, A and B). To investigate the reversal in phenotypic and molecular changes, K14GFP-RR and empty vector pEGFP were transfected in a shRK14-K1 clone. Stable clones D1-pEGFP and D1-K14GFP (R11, R12) were obtained after transfection with pEGFP and K14GFP-RR, respectively. K14 expression and filament formation were analyzed by Western blotting and confocal microscopy. K14GFP-RR–transfected cells showed increased K14...
and K5 levels as compared with pEGFP-transfected cells (Figure 7, A and B). GFP-tagged exogenous K14 also showed proper filament formation with its normal partner K5 in K14GFP-RR stable clones (Figure 7C). These clones demonstrated increases in both cell proliferation rate (analyzed by MTT assay; Figure 7D) and phosphorylated-Akt levels (Figure 7E). Furthermore, reduced involucrin and activated Notch1 levels were observed in K14GFP-RR–transfected cells (Figure 7E). Taken together, these results confirmed that molecular and phenotypic changes observed after K14 knockdown are due to depletion in K5 and K14 levels.
as involucrin and K1, were also found to be increased, as was activation of Notch1 signaling in these cells. In addition, the tumorigenic potential of the K14 knockdown clones of AW13516 cells was found to be significantly reduced. Reexpression of shRNA-resistant K14 resulted in reversal of the phenotypic and molecular changes, such as increase in cell proliferation, increased phosphorylated Akt, reduction in involucrin, and a decrease in NICD levels.

**DISCUSSION**

The keratin 5 and 14 pair is normally expressed in the basal layer of all stratified epithelia, and its expression decreases as these cells differentiate and lose their proliferation potential. Previous in vitro and transgenic mouse model studies, where the loss of K14 cannot be completely compensated by the ectopic expression of other keratins, suggest that K14 may have some unique tissue-specific functions (Hutton et al., 1998; Paladini and Coulombe, 1998) that are not yet well understood. In this study, we show decreased cell proliferation rate (Figure 2, A and B) in response to K14 knockdown. Furthermore, we also observed delayed cell cycle progression (Figure S3) and reduction in the levels of cyclin D1, PCNA, and Ki67 with concomitant increases in p21 and p27 levels (Figure 3, B and C). Consistent with these findings, K14 knockdown cells also showed delayed M-phase entry (Figure 2, A and B) and reduction in the levels of histone-H3 phosphorylation at serine 10 (Figure 3B). Previous reports have shown that K5 and K14 are specifically expressed in the mitotically active basal layer of the stratified epithelia, which contains epidermal stem cells and TA cells (Fuchs and Green, 1980). In another in vitro study, primary cultures developed from K14-null mice showed reduction in cell proliferation (Troy and Turksen, 1999). These observations suggest that K5 and K14 may be involved in control of cell proliferation. Our results are in agreement with these findings and provide experimental evidence for this hypothesis. To confirm that the reduction in cell proliferation seen in K14 knockdown cells was not the effect of increased apoptosis, we analyzed caspase-3 and poly(ADP-ribose) polymerase-1 (PARP-1) activity in these cells. Our results showed that there was no substantial difference in caspase-3 and PARP activities between these cells (Figure S5C). These observations suggest that K5 and K14 have some role in promoting and/or maintaining cell proliferation in basal cells.

PI3K signaling has been shown to be a key regulator of various cellular processes, including cell growth and cell fate decisions, such as differentiation and cell survival (Vivanco and Sawyers, 2002).
Akt activation in K10 knockout mice. The authors suggest that it is possible that the activation of Akt is not seen in the suprabasal layers of K10-knockout mice because activated Akt expression is restricted to basal layer of the epidermis. Recently K8 has also been shown to interact with Akt1, and regulate its phosphorylation in a glycosylation-dependent manner in simple epithelial cells (Ku et al., 2011). On the basis of these findings, we hypothesized that K14 may directly interact with Akt in basal layer of stratified epithelia, thus regulating its phosphorylation. Moreover, reduction in phosphorylated Akt levels in K14-depleted cells indicates the involvement of the PI3K/Akt signaling pathway in K5/ K14-mediated modulation of cell proliferation.

During epidermal differentiation, as the cells move upward from the basal layer, K5/14 expression is down-regulated and expression of cell differentiation markers such as K1/K10 and involucrin is induced (Fuchs and Green, 1980). In this study, we found increased levels of involucrin and K1 in K14 knockdown cells (Figure 4). In a previous study, Dakir et al. (2008) showed that transgenic mice expressing human K14 in the lung epithelium initiated a squamous differentiation program but failed to promote squamous maturation, suggesting K14 may have a role in cell differentiation of squamous epithelia. Previously, some other keratins, such as K16 and K10, have also been shown to modulate cell proliferation/differentiation in the transgenic mouse model (Paladini and Coulombe, 1998; Wawersik and Coulombe, 2000; Santos et al., 2002; Koch and Roop, 2004). There were no alterations in terminal differentiation during fetal development in K14 and K5 knockout mice (Lloyd et al., 1995; Peters et al., 2001). It is possible that K14 may not be the determinant of terminal differentiation, especially during fetal development, but K5 and K14 may act as modulators of terminal differentiation in the adult stage. Our study indicates that K5 and K14 act as negative regulators of cell differentiation and thus are possible controls for the squamous differentiation program.

The process of squamous differentiation requires precision in coordinating the molecular events that lead to generation of a stratified epithelium. In recent years, increasing evidence suggests that Notch1 plays a key role in controlling epidermal proliferation and differentiation and acts as direct determinant of keratinocyte growth arrest and entry into the program of cell differentiation (Rangarajan et al., 2001). Activation of Notch 1 results in release and then entry of a functionally active form of Notch1 (NICD) into the nucleus. This in turn induces expression of target genes, which leads to cell cycle arrest and initiation of cell differentiation (Bray, 2006; Watt et al., 2008). Our results have documented that K14 knockdown led to an increase in levels of NICD (Figure 5) that correlated with reduced cell proliferation and delay in expression of target genes, like involucrin and K1 (Figure 4). We have also observed increased p21 and p27 levels, which are known to be direct targets of NICD. Activation of p21 and p27 leads to cell cycle arrest (Rangarajan et al., 2001; Sriuranpong et al., 2001). In a recent report, it was proposed that transgenic mice expressing K10 under K5 promoter display altered differentiation and changes in expression of the Notch family of proteins in thymic epithelial cells (Santos et al., 2005). Expression of Notch pathway components is dynamic in epidermis, and most studies find that Notch expression is upregulated in the suprabasal cells. Loss of K5 and K14 expression during epidermal differentiation coincides with increased Notch expression in these cells. Thus our data indicate that the K5- and K14-mediated modulation of keratinocyte differentiation is possibly via Notch1 signaling.

Increase in tumorigenicity in response to increased cell proliferation and PI3K/Akt activation has been well documented (Vivanco et al., 2005).
and Sawyers, 2002). In addition, aberrant Notch signaling has been linked to a wide variety of tumors (Allenspach et al., 2002) and its function as a tumor suppressor in the epidermis has been illustrated by transgenic studies (Proweller et al., 2006; Dotto, 2008). In this study, we have demonstrated that K14 knockdown leads to reduction in tumorigenesis in K14 knockdown clones (*, p < 0.05). HaCaT (derived from normal human skin) and HEK293 (ATCC) cells were cultured in DMEM (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Thermo Scientific, Lafayette, CO), and antibiotics (12.5 U/ml penicillin, 4 μg/ml streptomycin 0.05 μg/ml amphotericin B) at 37°C and 5% CO2. The cell line AW13516 derived from the SCC of human tongue was cultured in Iscove's Modified Dulbecco’s Medium (IMDM; Gibco; Tatakke et al., 1990) supplemented with 10% FBS (Hyclone) and antibiotics at 37°C and 5% CO2.

FIGURE 6: Tumorigenicity assays for K14 knockdown clones. (A) Representative images of nude mice bearing tumor of indicated clones 8 wk after the injection. (B) Tumor growth was plotted against time. Results are mean ± SD for five animals injected for each clone. Note decreased tumorigenicity in K14 knockdown clones (*, p < 0.05). (C) Haematoxylin and eosin along with immunohistochemical staining (with antibody against K14) of paraffin-embedded sections of tumor tissues obtained from nude mice injected with indicated clones.

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Validation of shRNAs and selection of stable clones
To select the most effective shRNA construct, we cotransfected 1 μg of shRNA constructs with GFP-tagged K14 plasmid in HEK293 cells using the calcium phosphate precipitation method described previously (Alam et al., 2011). Efficiency of knockdown was checked by green fluorescence intensity and Western blot analysis of exogenous K14-GFP 50 h posttransfection. To generate stable K14 knockdown clones, 2 μg of shRNA constructs or the empty vector were transfected into HaCaT and AW13516 cells using liposome-based FuGENE HD transfection reagent (Roche, Indianapolis, IN), and stable clones were selected in 0.5 μg/ml puromycin-containing medium (according to the manufacturer’s protocol).

Site-directed mutagenesis and validation of mutant
shRNA-resistant GFP-tagged K14 construct was generated by introducing a silent mutation in the target sequence of shRNAs using the Quick change site-directed mutagenesis kit (Stratagene, Agilent, Santa Clara, CA). Oligonucleotides 5′-CAGCGGACCTGTGGCAATC-TAACGGCCAGAGCGAGATCTCG3′ from Sigma) were designed according to the manufacturer’s protocol. The resulting mutant construct was verified by direct DNA sequencing. To determine the resistance of the resultant mutant, we cotransfected mutated GFP-tagged K14 construct and shRNA K14.4 in HEK293 cells. Expression
Hybond, GE Healthcare, Waukesha, WI) and probed first with the primary antibody and then with the secondary antibody conjugated with horseradish peroxidase (HRP; Amersham Pharmacia Biotech, Uppsala, Sweden). The signals were detected by using ECL Plus detection system (Amersham) according to the manufacturer’s protocol.

**Antibodies**

The following antibodies were used: K14 (mouse monoclonal, clone LL002; AbD Serotec, Oxford, UK; working dilution 1:5000), K5 (mouse monoclonal, clone XM26; working dilution 1:1000; NovoCastra, Newcastle, UK), K8 (mouse monoclonal, clone M20; working dilution 1:5000; Sigma, St. Louis, MO) K18 (mouse monoclonal, clone CY-90; working dilution 1:10,000; Sigma), β-actin (mouse monoclonal, clone AC-74; working dilution 1:8000; Sigma), phosphoAkt (rabbit polyclonal; working dilution 1:1000; Abcam, Cambridge, UK), Akt (rabbit polyclonal; working dilution 1:1000; Abcam), Notch 1 (rabbit polyclonal, working dilution 1:1000; Abcam), Notch1 (sheep polyclonal; working dilution 1:1000; R&D Systems, Minneapolis, MN), Ki67 (mouse monoclonal; NovoCastra; Bond ready to use), p21 (rabbit polyclonal, clone C-19; working dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), p27 (rabbit polyclonal, clone C-19; working dilution 1:100; Santa Cruz Biotechnology), secondary antibody HRP-conjugated anti-mouse, anti-rabbit (working dilution 1:8000; GE Healthcare UK), anti-sheep (working dilution 1:2000; Sigma).

**Immunofluorescence and laser confocal microscopy**

To detect the localization of proteins and filament organization of keratins in cells, immunofluorescence assay was performed as described previously (Raul et al., 2004). Working dilutions for different antibodies were as follows K5, K14, K8, and K18: 1:200; Notch1, p63, involucrin, and K1: 1:100; and Ki67 was ready to use (antibody details described in Antibodies). The secondary antibodies, Alexa Fluor 488–conjugated anti–mouse immunoglobulin G (IgG) and Alexa Fluor 568–conjugated anti–rabbit IgG (Molecular Probes, Eugene, OR) and fluoroscein isothiocyanate (FITC)-conjugated anti–sheep IgG (BD Biosciences), were used at a dilution of 1:200. Confocal images were obtained using a LSM 510 Meta Carl Zeiss confocal system (Carl Zeiss MicroImaging GmbH, Jena, Germany).

**RNA isolation and reverse transcriptase PCR**

RNA was isolated by TRI reagent (Sigma), and RT-PCR was conducted using the RevertAid First Strand cDNA Synthesis Kit.

of exogenous mutated GFP-tagged K14 was examined by fluorescence and Western blot analysis 50 h posttransfection.

**Western blot analysis**

Whole-cell lysates were prepared in SDS lysis buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 0.1% BME, and 10% glycerol) with protease inhibitor cocktail (Calbiochem, San Diego, CA). An equal amount of protein (20 μg per well) was loaded and run on SDS–PAGE. The gels were transferred on polyvinylidene difluoride membrane (Amersham (Molecular Probes, Eugene, OR) and fluoroscein isothiocyanate (FITC)-conjugated anti–sheep IgG (BD Biosciences), were used at a dilution of 1:200. Confocal images were obtained using a LSM 510 Meta Carl Zeiss confocal system (Carl Zeiss MicroImaging GmbH, Jena, Germany).
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(Fermentas, Thermo Scientific, Waltham, MA) according to the manufacturer’s protocol. The primers used to amplify K14 and GAPDH (as internal control) and product size are shown in Supplemental Table S2. RT-PCR conditions were as follows: denaturation at 94°C for 30 s; annealing at 56°C and 58°C for K14 and GAPDH, respectively, for 30 s; and extension at 72°C for 60 s. RT-PCR products were run on agarose gel electrophoresis to compare RNA levels.

Real-time quantitative PCR

cDNA was prepared as described in the preceding section and used as templates for PCR. SYBR Green Master Mix (Applied Biosystems, Bedford, MA) was used with 200 nM of forward and reverse primers (Supplemental Table S3). Real-time quantitative PCR was performed with the ABI PRISM7700 Sequence Detection System. A standard curve was generated for each primer pair, and genes of interest were assigned a relative expression value interpolated from this standard curve using the cycle threshold, as per standard protocol. All expression values were normalized against GAPDH and β-actin. All amplifications were done three times in triplicate.

Cell proliferation assay (MTT assay)

Cells were seeded (n = 1500 cells per well), in triplicate in a 96-well microtiter plate in 100 μl complete medium. Proliferation was studied every 24 h up to a period of 5 d using MTT assay. At the desired time points, 100 μl of the medium was replenished from the designated wells, and 20 μl MTT solution was added to each well. The plate was incubated at 37°C in a CO2 incubator for 4 h, then 100 μl of acidified SDS (10% SDS in 0.01 N HCl) was added to each well, and the plate was incubated overnight at 37°C. The next day, the optical density (OD) was measured on an enzyme-linked immunosorbent assay plate reader at 540 nm against a reference wavelength of 690 nm. A growth curve was prepared from three independent experiments by plotting OD at 540 nm (on y-axis) against time (on x-axis).

Colony-forming assay

Cells (n = 400) were plated in 60-mm tissue culture plates in triplicate. Cells were grown in complete DMEM/IMDM medium for 14 d, with medium changes every 2–3 d. Cells were fixed with 4% paraformaldehyde for 1 h, which was followed by staining with 0.5% crystal violet (Himedia, in 70% ethanol) for 1 h at room temperature. Stained plates were then washed with 1X PBS, and images were captured using a high-resolution Nikon D70 camera (Nikon, Tokyo, Japan).

Cell cycle analysis

For cell cycle analysis, cells were plated in 60-mm dishes at 20% confluency. The next day, the cells were serum-starved for 72 h to synchronize the cells in G1 phase. The cells were then washed twice with PBS and fed with fresh complete medium with 10% fetal bovine serum (FBS). One set of cells was kept unsynchronized (with 10% FBS) as control. Cells were harvested at the indicated time points for the next 42 h and fixed with propidium iodide as described earlier (Hosing et al., 2008). Cell cycle profile was determined by flow cytometry. Ten thousand events were acquired per plate. The data were analyzed using ModFit software (Verity, Topsham, ME).

Tumor formation in nude mice

To test the tumorigenicity of the cells, NMRI nude mice (6–8 wk old) were used. AW135167 clones (both K14-knockdown and vector control cells) were suspended in plain IMDM without serum, 3 x 106 cells were injected subcutaneously in the dorsal flank of 6- to 8-wk-old NMRI nude mice. Six mice were injected for each clone and were observed for 2 mo for tumor formation. The ellipsoid volume formula ½ × l × w × h was used to calculate the tumor volume (Tomayko and Reynolds, 1989). All protocols for animal studies were reviewed and approved by the Institutional Animal Ethics committee constituted under the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

Histology and immunohistochemistry

The mice were killed 8 wk after subcutaneous injection, and the tumor tissues were fixed in 10% Formalin buffer and processed for histology. Five-micron sections of Formalin-fixed and paraffin-embedded tissues were stained with hematoxylin/eosin for histology. Immunohistochemical staining was performed as described previously (Ranganathan et al., 2006). Signals were detected by an avidin-biotin–based immunoperoxidase technique (Elite ABC Kit; Vector Laboratories, Burlingame, CA).

Statistical analysis

Two groups of data were statistically analyzed by t test using GraphPad Prism 5 software (La Jolla, CA). A p value less than 0.05 was considered statistically significant.

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