Ski protein levels increase during in vitro progression of HPV16-immortalized human keratinocytes and in cervical cancer

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ABSTRACT

We compared the levels of the Ski oncoprotein, an inhibitor of transforming growth factor-beta (TGF-β) signaling, in normal human keratinocytes (HKc), HPV16 immortalized HKc (HKc/HPV16), and differentiation resistant HKc/HPV16 (HKc/DR) in the absence and presence of TGF-β. Steady-state Ski protein levels increased in HKc/HPV16 and even further in HKc/DR, compared to HKc. TGF-β treatment of HKc, HKc/HPV16, and HKc/DR dramatically decreased Ski. TGF-β-induced Ski degradation was delayed in HKc/DR. Ski and phospho-Ski protein levels are cell cycle dependent with maximal Ski expression and localization to centrosomes and mitotic spindles during G2/M. ShRNA knock down of Ski in HKc/DR inhibited cell proliferation. More intense nuclear and cytoplasmic Ski staining and altered Ski localization were found in cervical cancer samples compared to adjacent normal tissue in a cervical cancer tissue array. Overall, these studies demonstrate altered Ski protein levels, degradation and localization in HPV16-transformed human keratinocytes and in cervical cancer.

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Introduction

C-Ski plays an important role in diverse processes including; proliferation, differentiation, transformation and tumor progression (Li et al., 1986; Reed et al., 2005; Colmenares and Stavnezer, 1989; He et al., 2003; Larsen et al., 1992, 1993). Both c-ski and v-ski, a truncated version of c-ski, can induce transformation when expressed at high levels (Colmenares et al., 1991). Increased Ski protein has been observed in several human cancer cell lines and cancer including melanoma (Reed et al., 2005; Fumagalli et al., 1993; Hussein 2005; Medrano, 2003; Reed et al., 2001), colon cancer (Buess et al., 2004) and esophageal carcinoma (Fukuchi et al., 2004). One important mechanism by which Ski promotes transformation is by inhibiting the transforming growth factor-beta (TGF-β) pathway (Luo et al., 1999).

Cervical cancer is the second most common cancer in women worldwide and infection with high-risk human papillomavirus (HPV) drives cervical carcinogenesis (zur Hausen, 2000). HPV type 16 (HPV16) is responsible for about 50% of cervical cancers worldwide and we have used a model system of HPV16-mediated transformation of human epithelial cells to study the cellular and molecular events that mark in vitro progression. In this model system normal human keratinocytes (HKc) are immortalized by transfection with a plasmid containing a dimer of HPV16 DNA (HKc/HPV16) (Pirisi et al., 1987, 1988). HKc/HPV16 progress in vitro towards malignancy through growth factor independent (HKc/GFI) and differentiation resistant (HKc/DR) stages (Pirisi et al., 1988; Zyzak et al., 1994). HKc/HPV16 are as sensitive to the growth inhibitory effects of TGF-β as normal HKc, but become increasingly resistant to TGF-β during in vitro progression towards the HKc/DR stage (Creek et al., 1995). This loss of sensitivity to growth inhibition by TGF-β is due to a partial loss of the TGF-β receptor type 1 (TβRI) that decreases but does not entirely abolish Smad signaling (Mi et al., 2000) [and unpublished results].

The expression of the HPV E6 and E7 oncoproteins is controlled by the upstream regulatory region (URR) of the virus, which contains binding sites for a myriad of transcription factors, including nuclear factor I (NFI) (Apt et al., 1993). In previous studies using HKc/HPV16, we reported that Ski can interact with and increase the transcriptional activity of NFI, which increases E6 and E7 expression (Baldwin et al., 2004). On the other hand the cytokine TGF-β promotes Ski degradation, which reduces NFI transcriptional activity, and E6 and E7 expression (Baldwin et al., 2004). In these studies we also found that Ski protein levels increased several fold in TGF-β resistant HKc/DR compared to TGF-β sensitive HKc/HPV16 (Baldwin et al., 2004).

The focus of the present study was to explore in detail Ski protein levels, turnover and cellular localization in HKc/HPV16 and HKc/DR, both in the absence and the presence of TGF-β. These
studies determined that Ski protein and phosphoprotein levels are cell cycle regulated in both HKc/HPV16 and HKc/DR and that Ski is localized to centrosomes and mitotic spindles during mitosis. Although basal Ski levels are much greater in HKc/DR compared to HKc/HPV16, and while treatment with TGF-β resulted in degradation of Ski in both HKc/HPV16 and HKc/DR, Ski degradation in response to TGF-β was delayed in HKc/DR. Finally, using tissue arrays, we show that Ski protein levels are also increased in cervical cancer.

Results

Ski protein levels increase during in vitro progression of HPV16-immortalized HKc and decrease following TGF-β treatment

In previous studies exploring the mechanism of TGF-β modulation of HPV16 early gene expression we reported that Ski protein levels increase during in vitro progression of HKc/HPV16 to HKc/DR (Baldwin et al., 2004). Activation of TGF-β signaling promotes Ski degradation through the ubiquitin-mediated pathway (Le Scolan et al., 2008). HKc/DR are resistant to the antiproliferative effects of TGF-β due to a partial loss of TpRI (Mi et al., 2000), however Ski degradation in response to TGF-β still occurs in HKc/DR (Baldwin et al., 2004). This observation, together with additional studies in our laboratory using a Smad reporter construct suggest that some TGF-β signaling remains intact in HKc/DR, despite the partial loss of TpRI. To explore in more detail alterations in Ski protein levels during HPV16-mediated transformation and the modulation of Ski protein levels by TGF-β in HPV16-transformed cells, we conducted both Western blot analysis (Fig. 1a) and immunofluorescence confocal microscopy studies (Fig. 1b) in normal HKc, HKc/HPV16 and HKc/DR treated for 24 h with or without TGF-β (40 pM). Ski protein levels increased in HKc/HPV16 relative to normal HKc, and increased further in HKc/DR (Fig. 1a). Immunofluorescence staining and confocal microscopy also showed that Ski levels increase markedly from normal HKc to HKc/HPV16 to HKc/DR (Fig. 1b). Ski was found in both nuclei and cytoplasm and no notable difference in the subcellular localization of Ski was found between normal HKc and HKc/HPV16 or between HKc/HPV16 and HKc/DR (Fig. 1b).

TGF-β treatment of normal HKc, HKc/HPV16, and HKc/DR resulted in a dramatic decrease in Ski protein as shown by Western blot analysis (Fig. 1a) and immunofluorescence (Fig. 1b). However, it should be noted that the basal levels of Ski in HKc/DR are initially very high, so that the levels of Ski remaining in HKc/DR following TGF-β treatment are roughly equivalent to those found in normal HKc prior to TGF-β exposure (Fig. 1).

To explore in more detail the subcellular trafficking of Ski following TGF-β treatment, we conducted immunofluorescence staining and confocal microscopy for Ski in normal HKc and HKc/DR treated for various times (5 to 60 min) with TGF-β (40 pM). In normal HKc nuclear Ski relocated to the cytoplasm by 30 min of TGF-β treatment and was mostly degraded by 60 min (Fig. 2A). However, HKc/DR exhibited a delay in both the loss of nuclear Ski and Ski degradation in response to TGF-β treatment (Fig. 2B). Ski was still clearly detected in both the nucleus and the cytoplasm in HKc/DR after 60 min of TGF-β treatment (Fig. 2B).

Ski protein and Ski phosphorylation levels increase dramatically between the G0/G1 and G2/M phases of the cell cycle without changes in Ski mRNA levels

The confocal microscopy studies of Ski described above used unsynchronized cells. During the course of these studies we noticed that cellular Ski protein levels could vary quite dramatically among cells on the same dish, even cells in the same confocal field. This observation suggested that Ski protein levels vary in a cell cycle-dependent manner. To explore whether Ski protein levels are cell cycle-dependent, HKc/DR were synchronized by maintaining them in growth factor-depleted medium (serum, insulin, EGF, and BPE free). As determined by flow cytometry, about 90% of HKc/DR were in G0/G1 following 48 h of growth factor deprivation (data not shown). Following refeeding with complete medium, S phase reached a maximum at 20 h, while G2/M was maximal at 24 h (data not shown). Total Ski levels in synchronized HKc/DR were determined by Western blot in cell extracts obtained at various times after refeeding (0 to 27 h). As shown in Fig. 3a. Ski protein levels were lowest in HKc/DR following growth factor depletion (time 0), increased dramatically by 6 h after refeeding, reached maximal levels between 18 and 24 h, and then decreased. We also followed Ski protein levels by Western blot in synchronized HKc/HPV16, with similar results (data not shown). In addition, we followed Ski levels by immunofluorescence staining and confocal microscopy in synchronized normal HKc, HKc/HPV16 and HKc/DR. Similar to our Western immunoblot results, Ski levels were lowest following growth factor depletion and both cytoplasmic and nuclear Ski increased dramatically over a 24 h period after refeeding (data not shown).

We always detected Ski as two closely migrating bands on Western immunoblots, consistent with Ski phosphorylation. The slower migrating band of Ski showed the greatest increase during cell cycle progression, suggesting that not only total protein levels of Ski, but also phospho-Ski levels increase with cell cycle progression (Fig. 3a). To verify Ski phosphorylation we treated nuclear extracts collected from HKc/DR with λ-phosphatase (400 IU, 30 C for 1 h) and then conducted Western blots for Ski. As shown in Fig. 3b, after λ-phosphatase treatment (+PP), the upper slower migrating band of Ski was mostly lost, consistent with that band being a phosphorylated form of Ski. Importantly, the slower migrating band of Ski was not affected by incubation with λ-phosphatase and the phosphatase inhibitors NaF (50 mM) and Na3VO4 (10 mM) (Fig. 3b, +PP+PPI).

We next explored whether the increased levels in Ski protein that we observed during cell cycle progression could be attributed to increased levels of Ski mRNA. Total RNA was extracted from synchronized HKc/DR at 6 to 24 h after refeeding with complete medium. Ski mRNA levels were determined by Real Time PCR. Ski mRNA levels fluctuated, but increased by less than 2-fold at any point during the 24 h refeeding period (data not shown). Thus, it is likely that the observed increase in cellular levels of Ski and phosphorylated Ski protein as cells progress through the cell cycle is the result of changes in the rate of Ski degradation, rather than increased steady-state levels of Ski mRNA.

Ski protein is located in centrosomes and mitotic spindles of HKc/DR during the G2/M phase of the cell cycle

Since we found that Ski protein levels peak at the G2/M phase of the cell cycle, we next studied cellular localization of Ski during G2/M. HKc/DR were synchronized in G2/M by treatment with the microtubule inhibitor nocodazole (10 ng/ml) for 16 h. Ski protein was then visualized by immunofluorescence staining and confocal microscopy. We found that virtually all of Ski is localized in centrosomes and mitotic spindles in HKc/DR (Fig. 4). Similarly, we found that Ski is localized in the centrosomes and mitotic spindle in nocodazole-treated normal HKc (data not shown).

Ski shRNA inhibits cell proliferation in HKc/DR

To further explore the possible functions of Ski, a Ski shRNA was used to knock down Ski levels in HKc/DR. The pSUPER.retro
SkiRNAi vector or the control pSUPER.retro vector was stably transfected into HKc/DR and Ski protein levels were determined by Western blot analysis. The Ski shRNA reduced the levels of Ski protein by about 70% (Fig. 5a). These results were confirmed by immunofluorescence and confocal microscopy for Ski in HKc/DR stably transfected with Ski shRNA vector, in comparison with those transfected with the control pSUPER.retro (data not shown).

To assess whether knocking down Ski protein affects cell proliferation, we compared the growth of HKc/DR transfected with the pSUPER.retro SkiRNAi vector to those containing the control pSUPER.retro vector by determining cell numbers every 24 h for eight days. As shown in Fig. 5b, control HKc/DR doubled approximately every 24 h, while the proliferation of cells expressing Ski shRNAi was markedly reduced.

**Ski protein is overexpressed and mislocalized in cervical cancer**

We used a tissue array to explore Ski protein levels in cervical squamous cell carcinoma tissue specimens and corresponding adjacent normal tissue specimens by immunofluorescence staining and confocal microscopy. In normal cervical tissue Ski was expressed at the highest level in the basal layer of the epithelium and Ski protein gradually decreased in the more differentiated layers (Fig. 6, adjacent normal cervical tissue). Also, most of the Ski was localized to the nucleus in the normal epithelium, with some cytoplasmic staining (Fig. 6, adjacent normal cervical tissue). In cervical squamous cell carcinoma Ski was present throughout the entire tissue and most of the Ski was localized to the cytoplasm rather than the nucleus (Fig. 6, cervical squamous cell carcinoma). The tissue microarray contained 80 tissue samples, including 42 cervical squamous cell carcinoma samples, and 38 adjacent normal cervical tissue samples. Ski staining was generally more intense in cervical squamous cell carcinoma samples than in adjacent normal cervical tissue. Multiple images from each sample were taken at the same settings using a confocal microscope and images were scored from + to ++++ based upon Ski signal intensity as described in Materials and methods. The results of this study are presented in Table 1. Most of the normal tissue (about 74%) had staining intensities of ++ or less, while almost 79% of cancers stained ++++. None of the cancers were found to have staining intensity of +.

**Discussion**

-c-Ski is involved in diverse cellular processes ranging from proliferation, differentiation, transformation and ultimately tumor progression [see (Bonnon and Atanasoski, 2012) for a recent
Overexpression of Ski has been found in premalignant conditions such as Barrett's esophagus (Villanacci et al., 2008), as well as numerous cancers such as pancreatic (Heider et al., 2007), melanoma (Reed et al., 2005, 2001), esophageal (Fukuchi et al., 2004), colorectal (Buess et al., 2004), and acute myeloid leukemia (Ritter et al., 2006). Increased levels of Ski protein correlate to tumor progression in esophageal cancer (Fukuchi et al., 2004) and amplification of the Ski gene is a negative prognostic marker in early-stage colorectal cancer (Buess et al., 2004).

The most important function described to date for Ski is to negatively regulate TGF-β signaling [reviewed in (Liu et al., 2001; Deheuninck and Luo, 2009)]. Specifically Ski interacts with Smad2, Smad3, and Smad4, forming a complex that binds to Smad binding elements (SBEs) on DNA, and represses the transcription of TGF-β responsive genes through the recruitment of the nuclear repressor N-CoR and mSin3a; the latter associates with histone deacetylase (HDAC) (Luo et al., 1999; Xu et al., 2000; Sun et al., 1999; Akiyoshi et al., 1999; Nomura et al., 1999; Suzuki et al., 2004). This is in contrast to the interaction of Ski with nuclear factor I (NFI), which potentiates NFI-stimulated transcriptional activation (Baldwin et al., 2004; Tarapore et al., 1997).

A role for Ski in HPV-mediated transformation and cervical cancer has not been investigated. In previous studies, exploring TGF-β modulation of HPV16 early genes expression in HPV16-immortalized...
human keratinocytes (HKc/HPV16), we found that Ski protein levels increased dramatically during in vitro progression to a differentiation resistant phenotype (HKc/DR) (Baldwin et al., 2004). Furthermore, HKc/DR are resistant to the antiproliferative effects of TGF-β (Creek et al., 1995; Mi et al., 2000). In the present study we sought to explore in detail Ski protein levels, turnover and cellular localization in HKc/HPV16 and HKc/DR, both in the absence and the presence of TGF-β.

Both Western blot and immunofluorescence confocal microscopy studies showed that Ski protein levels are higher in HKc/HPV16 than normal HKc and further increased in HKc/DR. Interestingly, although most functions described for Ski are nuclear, Ski was present in both the cytoplasm and nucleus, with no large differences in subcellular distribution as HKc/HPV16 progress to HKc/DR. Several studies, in a variety of tumors and tumor-derived cells, have reported Ski in the cytoplasm (Reed et al., 2001; Fukuchi et al., 2004; Villanacci et al., 2008; Heider et al., 2007). What function cytoplasmic Ski plays remains to be determined. However, nuclear and cytoplasmic Ski likely modulate cellular function differently (Nagata et al., 2006). Cytoplasmic Ski may promote growth-promoting properties by sequestering the tumor suppressor Rb, which is normally localized to the nucleus, in the cytoplasm (Jacob et al., 2008). In addition, cytoplasmic Ski may interfere with TGF-β signaling by: (1) inhibiting the translocation of Smad2 to the nucleus (Kokura et al., 2003), (2) interfering with the phosphorylation of Smad 2 and Smad3 by the activated TGF-β type I receptor (TβRI) (Prunier et al., 2003), and (3) interacting directly with TβRI, forming a stable association between TβRI and the R-Smad/Smad4 complex, and preventing the nuclear accumulation of R-Smad/Smad4 (Ferrand et al., 2010).

Ski is rapidly degraded in response to TGF-β signaling through the ubiquitin-dependent proteasome pathway via the E3 ligase Arkadia in a Smad-dependent manner (Le Scolan et al., 2008; Sun et al., 1999; Nagano et al., 2010). Ski is efficiently degraded in

![Fig. 3](image-url)  
Fig. 3. Ski protein levels in HKc/DR increase throughout the cell cycle and Ski is a phosphoprotein. (A) HKc/DR were synchronized by feeding with growth factor-free medium (no serum, EGF, insulin, or BPE) for 48 h. Cells were then refed with complete medium and total cell protein collected at different times (0 to 27 h) following refedding for detection of Ski by Western Blot. TFII-D was used as a loading control. (B) Nuclear extracts from HKc/DR were incubated in the absence (-PP) or the presence (+PP) of λ-phosphatase for 1 h at 30°C or in the presence of λ-phosphatase and the phosphatase inhibitors NaF and Na3VO4 (+PP+PPI). Ski protein was detected by Western blot and TFII-D was used as a loading control.

![Fig. 4](image-url)  
Fig. 4. Ski protein is localized to the centrosomes and spindles in HKc/DR. HKc/DR were treated with nocodazole for 16 h, to block the cells at prophase. Ski was then detected by indirect immunofluorescence (green) and nuclei were stained with DAPI (blue). (a) Ski (green) staining only; (b) merged Ski (green) and nuclei (blue) staining.

![Fig. 5](image-url)  
Fig. 5. Ski protein levels and cell proliferation are decreased by a Ski shRNA in HKc/DR. (a) HKc/DR were stably transfected with either the pSUPER.retro vector (Control) or a pSUPER.retro SkirNAI plasmid, encoding a Ski shRNA (Ski shRNA). Ski protein levels in cell extracts were determined by Western blot. TFII-D was used as a loading control. (b) HKc/DR were plated in 6-well plates in equal numbers, transfected with either the control pSUPER.retro vector (closed circles) or the pSUPER.retro SkirNAI plasmid (squares), and selected with puromycin for three days. Cell numbers were determined by manually counting the cells beginning 3 days after the end of puromycin selection.
response to TGF-β in normal HKc, HKc/HPV16 and HKc/DR (Baldwin et al., 2004). However in HKc/DR, which are completely resistant to the anti-proliferative effects of TGF-β, we found that translocation of Ski from the nucleus to the cytoplasm and Ski degradation in response to TGF-β treatment was delayed compared to normal HKc.

The steady-state levels of Ski fluctuate during the cell cycle, being low during G1 and S phases and accumulating during G2/M (Marcelain and Hayman, 2005; Macdonald et al., 2004). The cell cycle-dependent degradation of Ski is mediated by the ubiquitin-conjugating enzyme Cdc34 (Macdonald et al., 2004). In our studies, we followed endogenous Ski protein levels in partially synchronized HKc/HPV16 and HKc/DR, and found that Ski levels reached a maximum between 18–24 h following refeeding after growth factor depletion. Thus, Ski levels were greatest during the G2/M phase of the cell cycle in HPV16-immortalized HKc. RNA levels of Ski did not significantly change in HKc/DR during a 24 h period after refeeding synchronized cells. These results further support the concept that alterations in Ski levels during the cell cycle are the result of changes in Ski degradation, rather than changes in the rates of Ski transcription. However, we cannot rule out changes in the rate of translation of Ski mRNA during the cell cycle.

Ski is a phosphoprotein (Marcelain and Hayman, 2005) and two sites of phosphorylation have been identified: serine 515 (Nagata et al., 2010) and threonine 458 (Band et al., 2009). Phosphorylation of Ski at serine 515 did not affect its stability or its ability to suppress TGF-β signaling (Nagata et al., 2010). However, phosphorylation of Ski at threonine 458, by Akt kinase, caused its destabilization (Band et al., 2009). Ski is also a target of the serine/threonine kinase Aurora A (Mosquera et al., 2011). In the present studies we demonstrated that endogenous Ski was phosphorylated in HPV16-immortalized cells and consistent with Ski protein levels, phosphorylation of Ski was greatest during mitosis. It should be noted, however, that phosphatase treatment of cell extracts did not result in a single Ski band on Western blots (Fig. 3b), indicating that either some sites of phosphorylation are resistant to phosphatase treatment, or that other post-translational modifications of Ski are present. Further studies are necessary to define the physiological significance of Ski phosphorylation in HPV-transformed cells.

These studies, which found that nuclear Ski localized to the centrosomes and the mitotic spindle during mitosis of HKc/DR, are in agreement with those of Marcelain and Hayman (Marcelain and Hayman, 2005). The increased levels of Ski protein and the increased phosphorylation of Ski found during mitosis, along with its localization to centrosomes and the mitotic spindle, suggest that Ski might play an important role in the mitotic process, including chromosomal segregation. This is further supported by the co-localization of Aurora A kinase and Ski at the centrosomal level and Ski being a target of Aurora A phosphorylation (Mosquera et al., 2011). Interestingly, a recent report found

Table 1

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Fig. 6. Ski protein levels increase in cervical squamous cell carcinoma. Ski protein (green) in adjacent normal cervical tissue and cervical squamous cell carcinoma tissue was detected by indirect immunofluorescence and confocal microscopy. Nuclei were stained with DAPI 405 (blue). (a) and (b) Ski staining only (green); (a′) and (b′) nuclei staining only (blue); (a″) and (b″) merged Ski and nuclei staining.
that mouse embryonic fibroblasts null for Ski exhibit high levels of aneuploidy, the result of defects in chromosome segregation and a weakened spindle assembly checkpoint (Marcelain et al., 2012). These results suggest that Ski not only acts as an oncogene but also has tumor suppressor function in certain situations. This is consistent with the finding that ski-deficient heterozygous mice have an increased susceptibility to chemical carcinogens (Shinagawa et al., 2001) and that reducing Ski expression in breast and lung cancer cells does not affect tumor growth but enhances tumor metastasis in vivo (Le Scolan et al., 2008). In the present study we found that knocking down Ski in HKc/DR, with a shRNA, markedly reduced cell proliferation, likely by sensitizing the cells to growth control by endogenous TGF-β.

One may ask what is the contribution of Ski overexpression to the observed resistance to growth inhibition by TGF-β in HKc/DR. In this regard, we have previously shown that loss of TβRI plays a pivotal role, as re-expression of the receptor restores growth responses to TGF-β in HKc/DR (Mi et al., 2000). It is likely that Ski levels increase in HKc/DR because TGF-β signaling is reduced in these cells, and TGF-β is known to promote Ski degradation (Baldwin et al., 2004; Le Scolan et al., 2008; Sun et al., 1999; Nagano et al., 2010). At the same time, however, Ski can contribute to further decreasing TGF-β signaling in HKc/DR through its many interactions with components of the TGF-β signaling pathway (Nagata et al., 2006; Jacob et al., 2008; Kokura et al., 2003; Prunier et al., 2003; Ferrand et al., 2010). In addition, increased levels of Ski are likely to promote E6/E7 expression: We have previously demonstrated that Ski cooperates with NF1 to increase HPV16 URR activity in HKc/HPV16 and HKc/DR, leading to increased expression of E6 and E7 (Baldwin et al., 2004). Higher levels of E7 lead to inactivation of Rb and, as we have previously shown, decreased activity of the TβRI promoter (Hypes et al., 2009). Taken together, these observations point to a role of HPV16 E6/E7 in repressing TGF-β signaling at multiple levels, which will ultimately lead to increased Ski levels and activity.

To our knowledge these are the first studies that have explored Ski protein in cervical squamous cell carcinoma tissue. Ski staining was greater than normal tissue: about 79% of the cervical tissue samples analyzed scored +++ for ski signal intensity, while only 26% of normal tissue samples scored ++++. Furthermore, similar to what has been found in other cancers (Reed et al., 2001; Fukushima et al., 2004; Villanacci et al., 2008; Heider et al., 2007), over-expressed Ski protein in cervical cancer was localized to the cytoplasm rather than the nucleus.

In conclusion, this is the first study to explore Ski during HPV-mediated transformation. We demonstrate that Ski protein levels increase during in vitro progression of HPV16-immortalized HKc and that Ski protein and phosphoprotein levels are regulated both by TGF-β and the cell cycle, with Ski localized to centrosomes and the mitotic spindle during mitosis. Finally, we show that Ski is overexpressed and primarily localized to the cytoplasm in cervical cancer tissue. Clearly Ski and the TGF-β pathway are in a delicate balance promoting either cellular proliferation and tumor growth, or invasion and metastasis. Further studies are warranted to define more clearly the role each plays during cervical dysplasia and progression to cervical cancer. Whether or not Ski may be useful as a biomarker or prognostic marker in cervical disease also warrants additional study.

**Materials and methods**

**Cell culture and cell lines**

Normal HKc were isolated from neonatal foreskins and immortalized by transfection with a plasmid containing two head-to-tail copies of HPV16 DNA (HKc/HPV16). Establishment and characteristics of the HKc/HPV16 cell lines used in this study; including the TGF-β sensitive HKc/HPV16 and the TGF-β resistant HKc/DR have been described in detail in our previous publications (Piris et al., 1987, 1988; Zyzak et al., 1994) Normal HKc and HKc/HPV16 were cultured in serum-free MCDB153-LB basal medium supplemented with 5 ng/ml of epidermal growth factor (EGF), 35–50 μg protein/ml of bovine pituitary extract (BPE), 0.2 μM hydrocortisone, 0.1 mM calcium chloride, 10 nM triiodothyronine, 10 μg/ml of transferrin, 5 μg/ml of insulin, and 50 μg/ml gentamycin. This medium will be referred to as complete medium. HKc/DR were grown in complete medium containing 1 mM calcium chloride and 5% fetal bovine serum (FBS). Cells were split 1:10 when confluent, and medium was changed every 48 h. The serum free medium used to synchronize cells is complete medium without the insulin, BPE and EGF.

**TGF-β treatment and nuclear protein extractions**

Normal HKc, HKc/HPV16 and HKc/DR were grown on 100-mm tissue culture dishes to 40–50% confluence and treated with or without recombinant human TGF-β1(40 PM, R&D Systems, Minneapolis, MN) for 48 h. After washing with PBS, cells were scraped from the dish and nuclear extracts prepared as described by Baldwin et al. (2004). Nuclear extracts were aliquoted and stored at –80 °C until use. Protein concentration was determined by the DC protein assay (Bio-Rad, Hercules, CA).

**Immunofluorescence and confocal microscopy**

Glass coverslips were pre-coated with 50 μg/ml collagen type I (from calf skin, Sigma, St. Louis, MO) and placed into the wells of a 24-well tissue culture plate. The following day, 5000–10,000 normal HKc, HKc/HPV16, or HKc/DR were plated per coverslip in 1 ml of transferrin, 5 μg/ml gentamycin. This medium used to synchronize cells is complete medium without recombinant human TGF-β1 (40 PM) for 24 h. Cells were then washed with ice-cold phosphate-buffered saline (PBS) and fixed with 4% neutral paraformaldehyde in PBS for 30 min on ice. After fixation, cells were permeabilized and blocked with a solution of 5% normal rabbit serum, 0.5% bovine serum albumin, and 0.1% Triton X-100 in PBS (blocking solution) for 45 min at room temperature with gentle rocking. The cells were incubated with a monoclonal anti-human Ski antibody (diluted 1:500, Cascade Bioscience, Winchester, MA) at 4 °C overnight and then with an Alexa Fluor 488 rabbit anti-mouse IgG (diluted 1:500, Molecular Probes, Eugene, OR) at room temperature for 1 h. DNA was stained with DAPI (Molecular Probes). The cells were viewed on a Zeiss LSM Meta 510 confocal microscope.

**Total protein extraction and western blot analysis**

Lysates were prepared from cells at 70–90% confluency in 100-mm dishes. After washing three times with ice-cold PBS, cells were incubated with RIPA (50 mM Tris–Cl pH7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor (0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) for 30 min on ice. Whole cell lysates were obtained by centrifugation at 14,000xg at 4 °C for 20 min. Supernatants were stored at –80 °C until use. Western blots for Ski (50 μg total protein) were conducted using a monoclonal anti-human Ski antibody (2 μg/ml, Cascade Bioscience). Membranes were incubated with the primary antibody overnight at 4 °C with gentle rocking, rinsed, and then incubated for 1 h at room temperature with a 1:2500 dilution of a HRP-conjugated horse anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Ski was visualized using the Super Signal West Pico chemiluminescence detection kit.
reduction in Ski protein levels was conducted for 48 h. Nuclear protein was extracted from the surviving cells and a transfection, replated, and selected with puromycin (1.0 μg/ml) for protein extraction or flow cytometry on a Beckman Coulter Epics XL Flow Cytometer using EXPO 32 software.

**λ-Phosphatase treatment**

Nuclear extract (35 μg protein) was incubated with λ-phosphatase (800 IU, New England Biolabs, Ipswich, MA) for 1 h at 30 °C in the presence or the absence of the phosphatase inhibitors NaF (50 mM) and Na3VO4 (10 mM).

**Realtime PCR analysis of ski mRNA**

HKc/DR were synchronized as described above, released from growth factor deprivation and total RNA harvested at various times using an RNA Isolation Kit (Agilent Technologies, Santa Clara, CA). RNA quality and quantity was determined on a 2100 Bioanalyzer (Agilent Technologies). Reverse transcription was conducted using an iScript cDNA Synthesis Kit (Bio-Rad) and real-time PCR was performed using an iCycler IQ detection system (Bio-Rad). The Ski primers used were: forward, 5′-GGG TCT GCC GAA AAC ACC AA 3′; reverse, 5′-GCT CTT TCT CAC TGG CTC ACA CT 3′. Control genes were used HPRT1 and GUSB3 which were run in parallel with Ski for each sample, and used for normalization. The samples were run in triplicate, and the experiment was repeated three times.

**Nocodazole treatment**

Normal HKc and HKc/DR were plated on 100-mm dishes and allowed to grow to 50–80% confluency. Cells were then treated with nocodazole (10 ng/ml, Calbiochem, San Diego, CA) for 16 h at 37 °C.

**Ski knock down using shRNA**

The Ski shRNA used in these studies was constructed to target Ski mRNA at nucleotide 1084. The following nucleotides were repeated three times.

The samples were run in triplicate, and the experiment was repeated three times.

**Acknowledgments**

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**References**


(Pierce, Rockford, IL). Bands were quantified using the Image J program.

**Cell cycle synchronization and flow cytometry analysis**

Cells were plated on 100-mm dishes, cultured to 50–70% confluency and then washed with PBS. The cells were synchronized by feeding them with growth factor-free medium (no insulin, PBE or EGF) for 48 h at which time they were released from growth factor deprivation by refeeding with complete medium. At various times after refeeding the cells were harvested for protein extraction or flow cytometry on a Beckman Coulter Epics XL Flow Cytometer using EXPO 32 software.

Cervical cancer tissue arrays were obtained from US Biomax, Inc. (Rockville, MD). Overall, 80 tissue samples are represented on this array which includes 40 cervical squamous carcinoma specimens and 40 adjacent normal cervical epithelial specimens, two of which, however, showed infiltration of the stroma underlying a relatively normal cervical epithelium by frankly cancerous cells. Before immunostaining, antigen retrieval was conducted using 10 mM citrate acid pH 6.0 for 20 min. Monoclonal mouse anti-Ski antibody (Cascade Bioscience) diluted 1:100 was incubated on the slide at room temperature for 1 h. The slide was then incubated with a 1:500 dilution of an Alexa Fluor 488 conjugated rabbit anti-mouse secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 h. After air drying, the slide was viewed under a confocal microscope. Images were collected and scored by three independent reviewers. Results were compared and discrepancy among reviewers resolved by re-examining the images together and reaching consensus. A scoring system of +, ++ and +++ was utilized. In addition, nuclear versus cytoplasmic localization of staining was noted for each sample.