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RESEARCH ARTICLE

Expression of Ski Can Act as a Negative Feedback Mechanism on Retinoic Acid Signaling

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Background: Retinoic acid signaling is essential for many aspects of early development in vertebrates. To control the levels of signaling, several retinoic acid target genes have been identified that act to suppress retinoic acid signaling in a negative feedback loop. The nuclear protein Ski has been extensively studied for its ability to suppress transforming growth factor-beta (TGF-β) signaling but has also been implicated in the repression of retinoic acid signaling. Results: We demonstrate that *ski* expression is up-regulated in response to retinoic acid in both early *Xenopus* embryos and in human cell lines. Blocking retinoic acid signaling using a retinoic acid antagonist results in a corresponding decrease in the levels of *ski* mRNA. Finally, overexpression of *SKI* in human cells results in reduced levels of *CYP26A1* mRNA, a known target of retinoic acid signaling. Conclusions: Our results, coupled with the known ability of Ski to repress retinoic acid signaling, demonstrate that *Ski* expression is a novel negative feedback mechanism acting on retinoic acid signaling. *Developmental Dynamics 242:604–613, 2013*. © 2013 Wiley Periodicals, Inc.

Key words: retinoic acid; ski; *Xenopus*; transcription; negative feedback

Key fingings:

- Addition of retinoic acid increases *Ski* transcript levels in *Xenopus* embryos and mammalian cells.
- Addition of a retinoic acid antagonist results in reduced levels of *Ski* transcripts in *Xenopus* embryos and mammalian cells.
- Addition of retinoic acid appears to directly activate Ski expression.
- Over expression of *Ski* results in reduced transcript levels of the retinoic acid target gene, *Cyp26a1*.

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INTRODUCTION

Retinoic acid (RA) is a key signaling molecule for a wide range of developmental processes (Duester, 2008; Niederreither and Dolle, 2008). The proper development of the embryo requires a tight control over both the spatial localization and levels of RA signaling. During early embryogenesis, it appears that a primary mechanism for controlling both the levels of RA and its spatial distribution is the presence of Aldh1a2 (Raldh2), the enzyme primarily responsible for the conversion of all-*trans* retinaldehyde into RA (Niederreither et al., 1999), and Cyp26a1, the enzyme primarily responsible for metabolizing RA to inactive forms (Sakai et al., 2001). Loss of either of *Aldh1a2* (Niederreither et al., 1999) or *Cyp26a1* (Sakai et al., 2001) results in embryonic lethality closely resembling, but not

Additional Supporting Information may be found in the online version of this article.

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identical to the effects of loss of RA or excess RA, respectively (Mark et al., 2006). Later in development, closely related enzymes will also regulate RA levels (Niederreither and Dolle, 2008) in addition to potential alternate routes of generating RA (Chambers et al., 2007).

The expression of *Aldh1a2* and *Cyp26a1* can act as part of a negative feedback loop that responds to changes in RA levels. Exogenous retinoic acid will suppress expression of *Aldh1a2* (Niederreither et al., 1997) and blocking RA signaling will result in increased expression. Although Cyp26 expression does not require RA, addition of RA greatly increases expression of *Cyp26a1*, whereas reduced levels of RA results in a marked reduction in expression (Hollemann et al., 1998; de Roos et al., 1999; Sirbu et al., 2005; Ross and Zolfaghari, 2011). Negative feedback loops also extend to the enzymes that convert retinol to all-*trans* retinaldehyde (Strate et al., 2009; Feng et al., 2010).

In addition to negative feedback at the level of RA synthesis and degradation, regulation at the level of signaling has been demonstrated. Retinoic acid acts by binding to dimers of specific nuclear receptors, the retinoid receptors (RARs) and retinoid X receptors (RXRs) that are bound to target retinoid acid response elements (RAREs). Binding of the ligand to the receptor results in a recruitment of chromatin remodeling factors that promote activation of transcription whereas the unbound receptor complex is bound to repressors of transcription (Xu et al., 1999). RIP140 is a co-repressor associated with retinoic acid receptors and is directly induced by RA (White et al., 2003; Heim et al., 2007). Expression of *COUP-TF*, encoding another nuclear receptor is directly induced by RA and can compete with RAR-RXR heterodimers on the *Cdx1* promoter thereby acting as a negative feedback on RA induction of *Cdx1* (Beland and Lohnes, 2005).

Ski has also been associated with suppression of the RA signaling pathway through interference with transcription by RARs (Dahl et al., 1998). Ski is a nuclear protein that has been associated with several transcription factors, including Smads and Rb where it acts as a repressor (Tokitou et al., 1999; Luo, 2004; Takeda et al., 2004), and myogenin, where it acts as an activator (Zhang and Stavnezer, 2009). Both gain of function (Amaravadi et al., 1997; Kaufman et al., 2000) and loss of function (Berk et al., 1997) experiments suggest multiple roles for Ski in normal development.

Analysis of acute myeloid leukemia patients also found that the inability to respond to RA therapies is correlated with high levels of *SKI* gene expression (Teichler et al., 2008) and suppression of RA signaling by Ski in COS-1 cells is inhibited when its NCoR binding domain is deleted (Ritter et al., 2006). Ski also colocalizes with the repressor protein, HDAC3, a major component of the co-repressor complex associated with RARs that are not bound by ligand. Degradation of HDAC3 was inhibited by Ski, stabilizing the co-repressor complex associated with the RAR/RXR dimer bound to the RARE (Zhao et al., 2009). The inhibition is attributed to an interaction with Ski and the Seven in Absentia Homologue 2 protein, Siah2, a component of the E3 ubiquitin ligase pathway. Functional Siah2 promotes the degradation of specific proteins, including HDAC3 (Zhao et al., 2009). Therefore, it is proposed that Ski inhibits the degradation of the RA signaling repressor HDAC3 by the E3 ubiquitin ligase pathway, thereby inhibiting RA signaling. The Ski-interacting protein (Skip) has also been implicated in the RA pathway. Skip is a co-regulator of vitamin D receptor/RXR signaling, and more recently has been shown to activate RAR activity by interacting with and stabilizing nuclear co-activators and steroid receptor coactivator (SRC) complexes (Barry et al., 2003; Kang et al., 2010). Taken together, these results suggested to us the hypothesis that expression of *ski* is a link in a negative feedback loop on RA signaling.

In this study, we have found that RA up-regulates *ski* expression in both *Xenopus* embryos and in mammalian cell lines. This regulation appears to be direct and the increased levels of ski appear to reduce levels of RA signaling. Coupled with results from other labs that have demonstrated the ability of Ski to repress transcription through RARs, we provide evidence that Ski can act in a negative feedback loop to modulate RA signaling.

RESULTS

Expression of *ski* in *Xenopus* Embryos

Although *ski* has been studied in *Xenopus*, particularly for its role in suppression of bone morphogenetic protein (BMP) signaling (Sleeman and Laskey, 1993; Amaravadi et al., 1997; Wang et al., 2000; Luo, 2003), the spatial expression of *ski* has not been extensively described. Using whole-mount in situ hybridization, we were able to detect *ski* mRNA in the mesodermal mantle at the beginning of gastrulation and we could also find strong expression in the forming anterior spinal cord and discrete regions of the head. Later in development, significant expression can be found in a variety of sites, including the neural crest, spinal cord, somites, and otic vesicle (Fig. 1). The in situ images suggest that expression may be quite widespread at a low level, although the regions of high expression were very distinct.

Retinoic Acid Causes an Increase in *ski* mRNA Levels in Both *Xenopus* Embryos and in Human Cells

If *ski* expression was part of a negative feedback loop that can regulate RA signaling, then exposure to exogenous RA should increase expression of *ski*. *Xenopus* embryos exposed to 1μM RA showed a marked increase in staining when analyzed by whole-mount in situ hybridization. This increase was easily observed after 6–10 hr of exposure and was observed at all embryonic stages tested (Fig. 2). Importantly, treatment with a pan-RAR antagonist (Teng et al., 1997) resulted in a corresponding decrease in *ski* expression as seen both by in situ hybridization and real-time polymerase chain reaction (PCR) (Fig. 2), suggesting that RA plays a role in the regulation of ski expression in vivo. The veracity of the retinoic acid treatment was confirmed by the marked induction of *cyp26a1* expression by

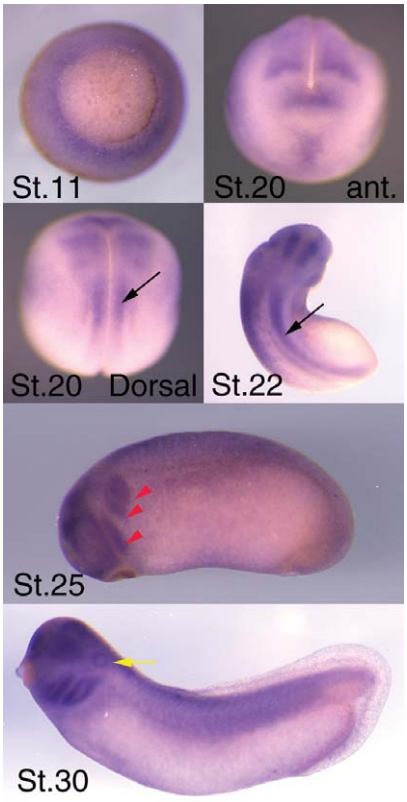

Fig. 1. Endogenous expression of *ski* in *Xenopus*. Ski expression was first detectable in a ring around the blastopore at stage 10/11. At stage 20, strong expression was observed in bands in the forming head in the anterior (ant.) view There is also staining on the ventral midline immediately posterior to the cement gland at that stage. In a dorsal view, strong staining can be seen in the anterior spinal cord (black arrow) and the staining can be seen even further posterior at stage 22. Strong staining was also observed in the migrating anterior neural crest (red arrowheads). At tail bud stages (St. 30) low levels of expression are seen over much of the embryo including somites and higher expression was still viewed in discrete regions including the otic vesicle (yellow arrow).

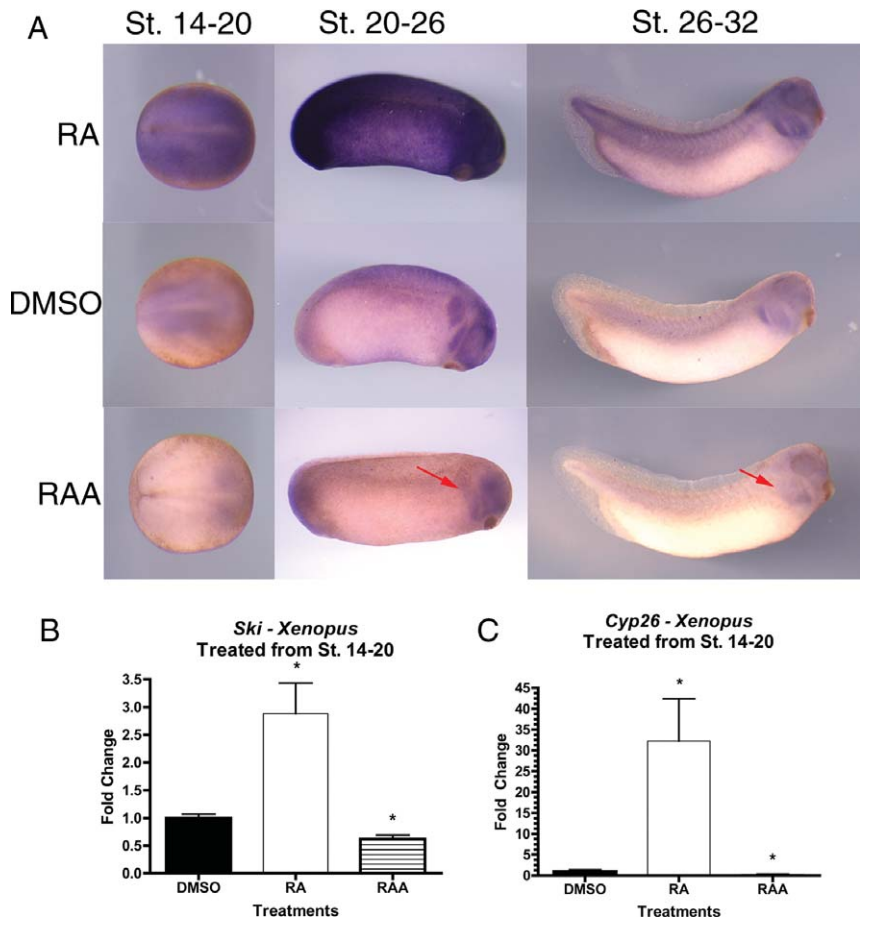

Fig. 2. Exposure to retinoic acid (RA) causes an increase in *ski* expression. **A:** When *Xenopus* embryos were exposed to 1μM retinoic acid from stage 14 to 20, stage 20 to 26, or from stage 26 to 32, there was an increase in *ski* expression as assayed by whole-mount in situ hybridization. If embryos were exposed to 1 μM retinoic acid antagonist expression of *ski* was reduced. This was particularly evident in the pharyngeal region (red arrows). **B:** Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for expression of *ski* in *Xenopus* embryos treated as mentioned previously between stages 14–20. *Ski* is significantly up-regulated in response to RA and down-regulated in embryos treated with RA antagonist. **C:** Positive control quantifying expression of downstream RA signaling target *cyp26a1* in response to RA or RA antagonist treatment showing the expected increase and decrease (respectively) in expression. qRT-PCR is normalized to *h4* mRNA levels. mRNA levels were normalized to the *h4* housekeeping gene. Error bars represent standard error of the mean. n = 5. $^{*}P < 0.05$ $^{**}P < 0.01$.

RA and loss of expression in the presence of the RA antagonist (Fig. 2C). The increase in *ski* expression appeared to be in all tissues as the embryos showed increased expression throughout the embryo. However, regions of novel high expression were also observed. In particular, in control embryos, there was a consistent gap in the expression of *ski* in the spinal cord and expression of *ski* in the forming brain. When embryos were treated with RA, the gap was no longer present and strong *ski* expression was observed along the entire anterior–posterior axis of the nervous system (Fig. 3). As might be predicted, the strong expression in the developing spinal cord was much reduced when embryos treated with the RA antagonist (Fig. 3).

Inhibition of aldh1a2 in embryos using either citral or diethylaminobenzaldehyde (DEAB), resulted in reduced levels of *cyp26a1* expression as expected, although there were regions that were expression was not altered such as the tail bud region. This treatment also resulted in decreased expression of *ski* (Supp. Fig. 1, which is available online). Inhibition of cyp26a1 by ketoconazole treatment caused an increase in *cyp26a1* expression but we were not able to conclude that *ski* expression was increased based on the in situ hybridization results. Taken together, these results support the conclusion that *ski* expression responds to changes in RA levels under physiologically relevant conditions.

To test if the increase in *ski* expression in response to RA treatment was a general phenomenon, we examined ski expression in an immortalized human keratinocyte (HaCaT) cell line in response to altered RA signaling. HaCaT cells were chosen because they have previously been shown to respond to both RA (Torma et al., 1999; Boudjelal et al., 2002) and BMP (Botchkarev, 2003; Gosselet et al., 2007) signaling pathways. HaCaT

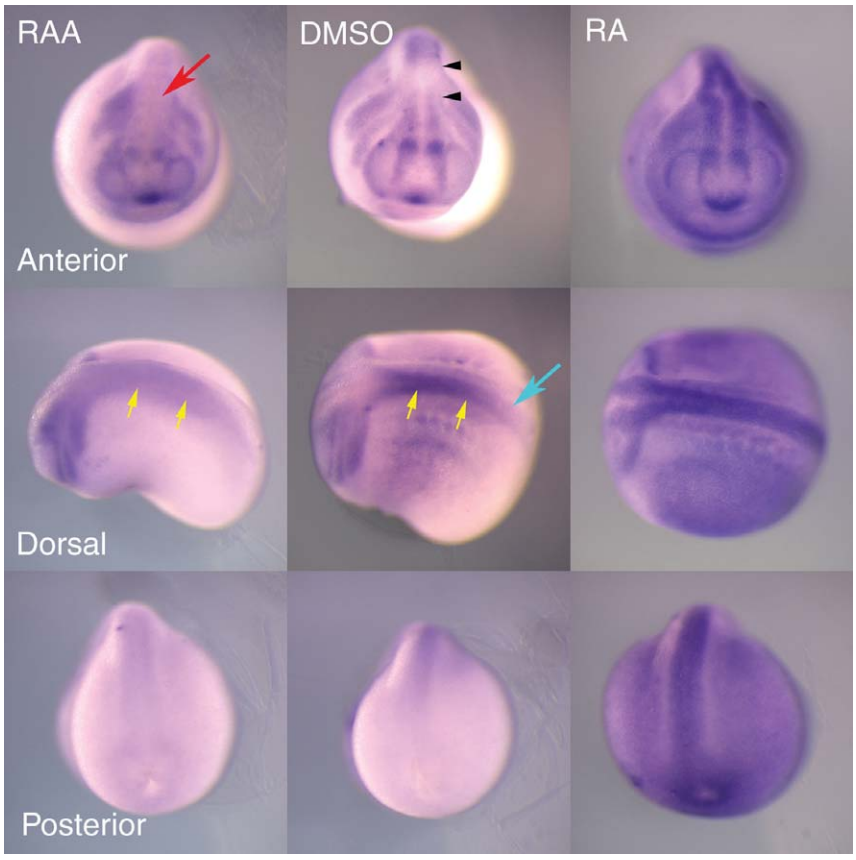

Fig. 3. Different regions of the embryo responded differently to changes in retinoic acid (RA) signaling. Addition of RA to embryos resulted in a general increase in staining by in situ hybridization, suggesting that the most cells are responding to the increased signaling. There was a much greater response at specific sites. In particular, there is a gap in staining in the neural tube (black arrowheads) roughly corresponding to the hindbrain. Addition of RA resulted in that region showing very strong expression of *ski* as assayed by in situ hybridization. The posterior spinal cord (blue arrow) does not normally have strong expression of ski but does so when RA is added. When embryos are treated with an RA antagonist, the strong staining in the brain (red arrow) and spinal cord (yellow arrows) is much reduced. Note that staining in the somites is also markedly reduced in the antagonist treated embryos. DMSO, dimethyl sulfoxide.

cells express *SKI* endogenously and have previously been used to manipulate SKI activity (Denissova and Liu, 2004; Suzuki et al., 2004; Levy et al., 2007). Using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), a significant increase (> two-fold) in *SKI* mRNA levels was observed after 1 hr and that was maintained for at least 8 hr after RA treatment. Treatment with the pan-RAR antagonist caused a decrease in endogenous *SKI* mRNA levels after 4 hr (Fig. 4A). The time course of changes in *SKI* mRNA levels was similar to changes in the levels of *CYP26A1* mRNA, although the magnitude of change was much greater with *CYP26A1* expression and the changes were sustained throughout the 24-hr treatment (Fig. 4B). We were also able to observe an increase in levels of SKI protein after treatment with 1 μM RA. The increase was observed after 8 hr and was sustained to at least 24 hr after exposure to RA (Fig. 4C).

RA Directly Regulates Expression of Ski

Given that the changes in *ski* expression roughly followed the time course of changes in *cyp26a1* expression, we investigated whether RA might directly activate *ski* expression as it does *cyp26a1*. If embryos were cultured in the presence of 1 μM cycloheximide before and during the RA treatment to block protein synthesis, we still observed a significant increase in the expression of *ski*, as assayed both by in situ hybridization and qRT-PCR (Fig. 5). The magnitude of the change in mRNA levels was somewhat reduced as compared to previous experiments because embryos could only be cultured for 2–3 hr in cycloheximide due to toxicity. Again the effectiveness of the RA and cycloheximide treatment was demonstrated using expression of *cyp26a1*, a known direct target of RA signaling (Hollemann et al., 1998), as an assay. The ability of RA to directly activate *ski* expression extends to mammalian cells, as HaCaT cells also up-regulated *SKI* expression in the presence of cycloheximide (Fig. 6), although the magnitude of the change was much smaller than that observed for *CYP26A1* expression.

If ski acts in a negative feedback loop that regulates RA signaling, we would predict that overexpression of *Ski* should inhibit the expression of RA target genes. HaCaT cells were transiently transfected with 2 μg of either *pCS2-FLAG-cSki* or *pCS2-FLAG-Ski-ARPG*, encoding a mutant that is able to inhibit transforming growth factor β (Tgfβ) but not BMP signaling (Takeda et al., 2004). As expected, the transfection with the *Ski-FLAG* construct resulted in reduced expression of *ID3*, a direct target of BMP signaling expression (Hollnagel et al., 1999) whereas the *Ski-APRG-FLAG* mutant did not. Using qRT-PCR to quantify *CYP26A1* mRNA levels, we found a significant decrease in *CYP26A1* expression in cells transfected with either the *Ski-FLAG* or *Ski-APRG-FLAG* constructs when compared with controls (Fig. 7B).

DISCUSSION

RA signaling is essential for multiple developmental events and the level of signaling is critical to many of these events (Duester, 2008; Niederreither and Dolle, 2008). Given the necessity for controlling the level of signaling, it is not surprising that negative feedback loops have been identified that limit the RA signaling once it has been activated. The majority of the identified feedback loops act at the level of ligand availability as exemplified by the expression of *aldh1a2* and *cyp26a1* (Niederreither et al., 1997;

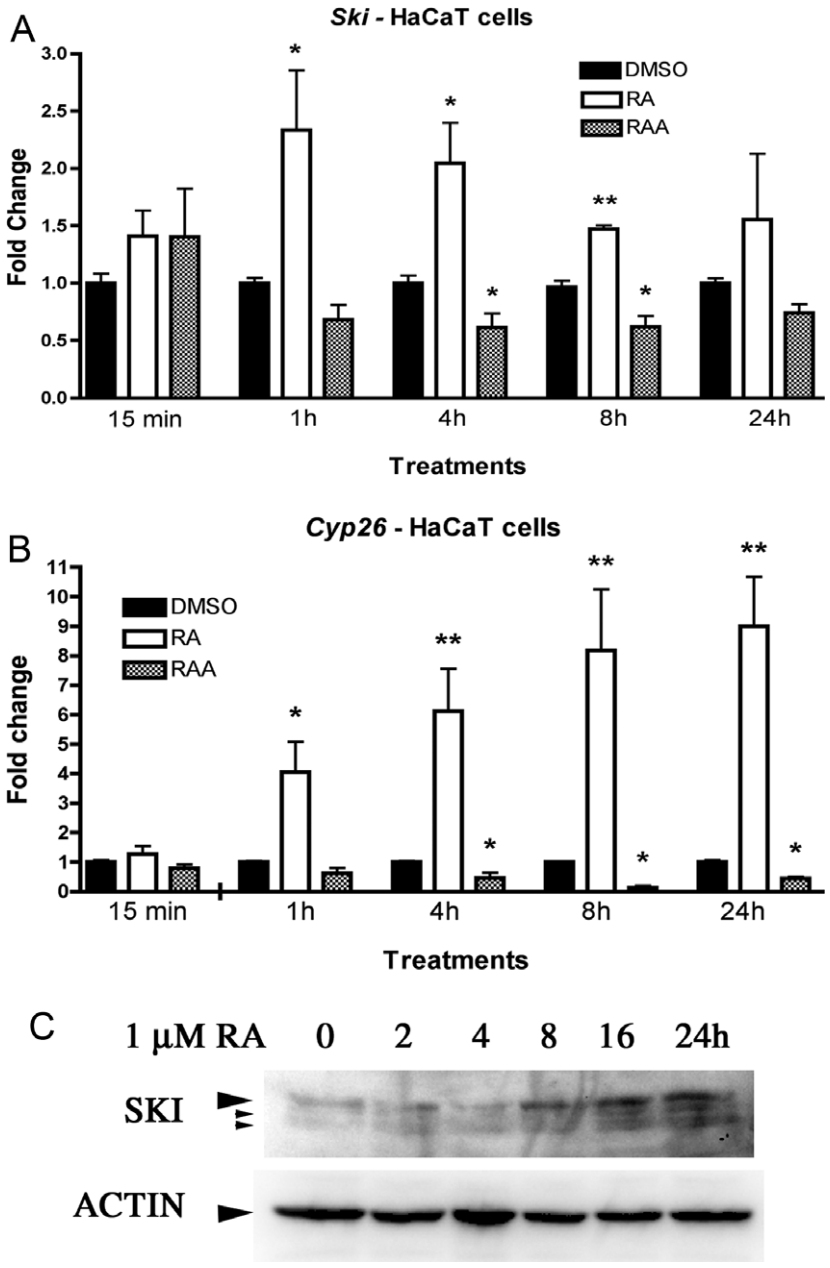

Fig. 4. Retinoic acid (RA) signaling affects *SKI* expression in vitro. **A:** Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) of HaCaT cells treated for 15 min, 1 hr, 4 hr, 8 hr, or 24 hr with dimethyl sulfoxide (DMSO) control, or 1 μM RA or RA antagonist (RAA) quantifying changes in levels of *SKI* mRNA. **B:** Positive control of similarly treated cells quantifying known downstream direct RA signaling target *CYP26A1*. mRNA levels were normalized to *GAPDH* expression. **C:** Western blot showing increase in SKI protein levels in response to RA treatment expression. Increases were noted after 8 hours of treatment compared with the control ACTIN levels. Error bars represent standard error of the mean. n = 5. $^{*}P < 0.05$, $^{**}P < 0.01$.

Hollemann et al., 1998; Strate et al., 2009; Feng et al., 2010; Ross and Zolfaghari, 2011). Here we identify the transcriptional repressor ski as a novel component of feedback loops on RA signaling (Fig. 8). *Ski* expression is directly up-regulated in response to addition of RA in both *Xenopus* embryos (Figs. 2, 3) and in mammalian cells (Fig. 4). Importantly, a normal role for RA in *ski* expression is suggested by the reduction in expression when embryos are exposed to an RA antagonist (Fig. 2) or by changes in RA levels caused by inhibition of aldh1a2, although it is clear that RA is not the only factor regulating its expression.

Our results using cycloheximide suggest that retinoic acid can directly activate the *ski* promoter in both *Xenopus* and mammalian cell lines. A recent analysis of mouse embryonic stem cells exposed to RA identified *Ski* as one of the significantly up-regulated genes. In addition, that study used chromatin immunoprecipitation (ChIP) to identify several enriched binding sites for RARs that were associated with the transcriptional start site of the two protein encoding transcripts of *Ski* (ENMUST00000030917, ENMUST 00000084103), supporting our observations that *Ski* is directly regulated by RA (Moutier et al., 2012). Given that more than 10,000 potential binding sites were identified (Moutier et al., 2012), confirmation of our suggestion that RA directly regulates *Ski* will require ChIP analysis on *Xenopus* embryos and HaCaT cells followed by direct functional analysis of identified binding sites.

We are also able to show that an increase in *Ski* expression is able to reduce the expression of the RA target gene *cyp26a1* (Fig. 7). To complete the feedback loop, a mechanism for the ability of ski to interact with the RA receptor to reduce RA signaling is needed and at least one mechanism, the association with and stabilization of HDAC3, has been elucidated (Zhao et al., 2009, 2010). This represents a similar strategy to transforming growth factor-beta (TGF-β)/BMP signaling where negative feedback loops exist that reduce the ability of ligand by activating expression of *noggin*, while expression of *Ski* can be part of negative feedback loop at the level of the nucleus (Massague and Chen, 2000).

Our observations of a general low level of *ski* expression in most tissues with a high levels in the neural tube and neural crest is in agreement with that observed in mouse (Lyons et al., 1994). It is likely that much of the increase in expression in response to RA exposure is due to the general increase in expression throughout the embryo. The marked increase in *ski* expression in specific regions such as the gap between the spinal cord and brain staining is more likely due to changes in tissue patterning rather than the general increase in *ski*

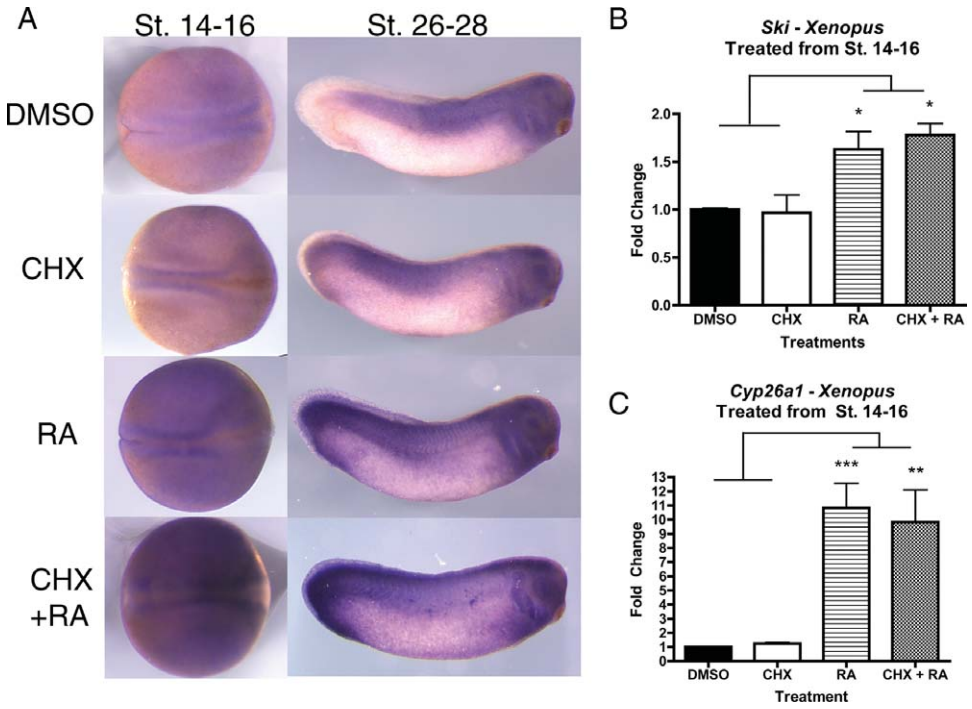

Fig. 5. Retinoic acid (RA) signaling directly alters *ski* expression in vivo. **A:** Whole-mount in situ hybridization of embryos pretreated with 20 min of 1 μM cycloheximide to block translation followed by 1 hr treatment with 1 μM RA. In a dorsal view, embryos treated from St. 14–16 showed increased staining with RA even in the presence of cycloheximide. Embryos treated from St. 26–28 (side view) showed the same result. **B:** Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) of similarly treated embryos between stages 14 and 16 showed that *ski* expression is up-regulated by RA even in the presence of cycloheximide. **C:** qRT-PCR analysis of *cyp26a1*, a known direct target of RA signaling, was used as a positive control for the RA and cycloheximide treatments. Error bars represent standard error of the mean. n=5. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expression. Posteriorization of anterior neural identities is a known result of exposure to RA in *Xenopus* (Sharpe, 1991; Chen et al., 2001).

The changes in *ski* expression that we observed were much smaller than the changes observed for the expression of *cyp26a1*. The greater response by *cyp26a1* may be necessary due to the different mechanisms used to inhibit RA signaling. Cyp26a1 acts to catabolize retinoic acid and may be required at higher levels to have enough activity to reduce RA levels significantly. By acting within the nucleus to disrupt RA signaling, ski may be able to act faster to modulate RA signaling or perhaps be required at relatively low levels. Rip140 (Nrip1) is another nuclear co-repressor that acts in a negative feedback loop to suppress RA signaling (Heim et al., 2007). Rather than simply inhibiting the RA receptor, *Rip140* expression oscillates with time and also plays a role in fine tuning the oscillatory expression of other RA target genes (Heim et al., 2009).

In mice, the loss of *Ski* results in a wide variety of defects including exencephaly, facial clefting, reduced skeletal muscle mass, abnormal skeletogenesis, and ocular defects (Berk et al., 1997; McGannon et al., 2006). While it is tempting to attribute some of these observed defects to excess RA signaling, the ability of Ski to also modulate the activity of other key genes that regulate development, such as TGF-β and BMP signaling (Luo et al., 1999; Takeda et al., 2004), makes it extremely difficult to single out a single pathway. Indeed, the up-regulation of *Ski* by retinoic acid could result in modulation of the activity of other pathways where Ski plays a role, representing a potential direct cross-talk between key developmental pathways. If some of the *Ski* loss of function phenotypes in mice (Berk et al., 1997; Colmenares et al., 2002) can be attributed to an increase in RA signaling, it is clearly different than the phenotype observed when *Cyp26a1* is lost. In *Cyp26a1* knockouts, mice exhibit severe truncations at the posterior end of the embryo in addition to homeotic mutations (Sakai et al., 2001), suggesting that the ability of Ski to suppress RA signaling is not as potent as that of Cyp26a1.

Our experiments demonstrate that, in addition to being able to repress RA signaling, expression of Ski is at least partially regulated by RA. This provides a novel negative feedback loop on RA signaling and a potential mechanism for cross-talk between important developmental pathways. The challenge in future will be to determine the relative roles of these feedback mechanisms in both development and disease.

EXPERIMENTAL PROCEDURES

Embryo Manipulations

Female *Xenopus* females were induced to ovulate by injection of 500–600 IU of human chorionic gonadotropin depending on the size of the animal and eggs were fertilized

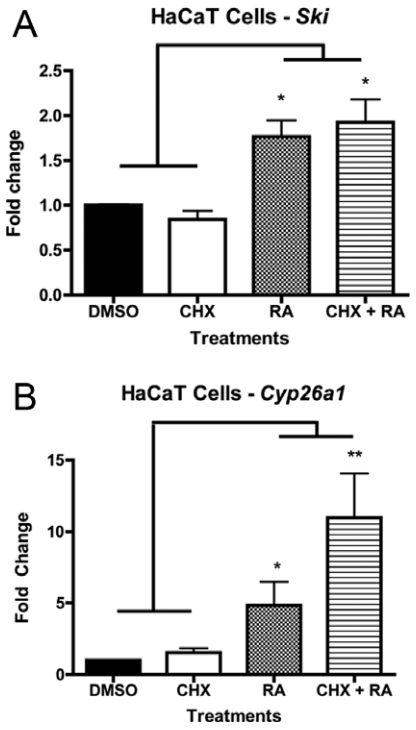

Fig. 6. Retinoic acid (RA) signaling directly regulates *ski* expression in HaCaT cells. **A:** Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) of HaCaT cells treated for 20 min with 1 μM cycloheximide followed by 1 hr with 1 μM RA demonstrates that RA significantly increases ski expression even in the presence of cycloheximide in the same manner as *cyp26a1*, a known direct target of RA signaling (**B**). Error bars represent standard error of the mean. n = 5. $^{*}P < 0.05$, $^{**}P < 0.01$.

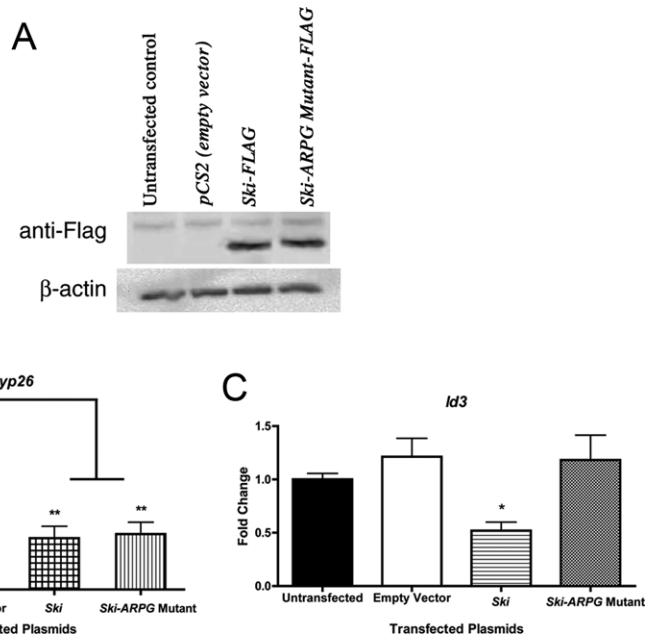

Fig. 7. Overexpression of both *Ski* and the *Ski-APRG* mutant causes decreased expression of *CYP26A1*. **A:** Western blot (anti-FLAG) confirming protein synthesis from the *Ski* and *Ski-APRG* constructs after transfection into HaCaT cells. **B:** Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) quantifying the levels *CYP26A1* mRNA shows a decreased level of expression after transfection with either *Ski* construct. **C:** qRT-PCR of similarly transfected cells quantifying the levels of *ID3* mRNA, a target of bone morphogenetic protein (BMP) signaling, showing that there is decreased levels of expression with *Ski* over expression but not with over expression of the *Ski-APRG* construct. Error bars represent standard error of the mean. mRNA levels were normalized to the *GAPDH* mRNA levels. n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$.

using minced testes in 80% Steinberg's solution. Embryos were dejellied in 2.5% cysteine, pH 8.0, and allowed to develop to the specified stage in 20% Steinberg's solution and embryos were staged according to the Nieuwkoop and Faber staging table (Nieuwkoop and Faber, 1994).

Embryos were treated in 20% Steinberg's solution at various stages using 1 μM all-*trans* retinoic acid (RA) (Sigma), or 1 μM pan-retinoic acid antagonist (RAA) (Allergan 193109; Teng et al., 1997) both diluted from a 1mM stock solution in dimethyl sulfoxide (DMSO). Thus, 1μl/ml DMSO was used as a carrier control. Protein synthesis was inhibited using 1μM cycloheximide (Moreno and Kintner, 2004). When testing the effect of blocking protein synthesis in conjunction with RA treatments, the cycloheximide was added for 20 min before RA addition followed by 1-hr treatments with either DMSO or 1μM all-*trans* RA in the presence of the cycloheximide.

Inhibition of aldh1a2 was done by treatment with either 20 μM diethylaminobenzaldehyde (DEAB) (Cartry et al., 2006) or 120 μM citral (Begemann et al., 2004). Inhibition of cyp26 was done using 50 μM ketoconazole (Lutz et al., 2001). Both inhibitor treatments were from embryonic stage 20 to 28 at which time the embryos were fixed for in situ hybridization. As with the retinoid treatments, the carrier control for the inhibitors was 1 μl/ml DMSO.

In Situ Hybridization

Whole-mount in situ hybridizations were performed according to standard protocols (Harland, 1991) with slight modifications (Deimling and Drysdale, 2009). Antisense riboprobes for *ski* (Amaravadi et al., 1997) and *cpy26a1* (Hollemann et al., 1998) were labeled with digoxigenin-UTP (Roche Diagnostics) without incorporation of radio-labeled nucleotides. BM Purple (Roche Diagnostics) was used as the alkaline phosphate substrate. Embryos were post-fixed in 4% paraformaldehyde for 20 min and endogenous pigment was bleached using 0.5% hydrogen peroxide, 5% formamide, and 0.5% standard saline citrate for several hours. Embryo images were obtained using Northern Eclipse software (Empix Imaging, Mississauga, Canada) on a Leica MZ12 dissecting microscope.

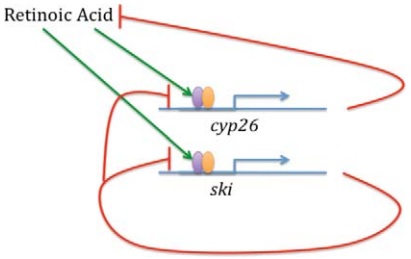

Fig. 8. A proposed model of retinoic acid (RA) signaling negative feedback loops. The best understood feedback loop is when RA directly activates genes that encode enzymes that reduce the level of retinoic acid as exemplified by cyp26a1. Our results suggest that RA can also activate genes such as *ski* allowing ski to act at the level of the RA receptors to attenuate signaling.

Xenopus qRT-PCR

A total of eight embryos were used per treatment group. Embryos were homogenized at earlier stages (< stage 26) using the lysis buffer provided in the Qiagen RNeasy mini Kit according to manufacturer's instructions. Later staged embryos used a homogenization buffer consisting of 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% N-lauroyl-sarcosine, and 0.1 M β-mercaptoethanol. After homogenization, 1/10th of the volume of 2 M sodium acetate (pH 4) was added followed by a 1:1 volume of phenol:chloroform. This was then centrifuged for 5 min at 14K. The aqueous phase was removed, placed in a fresh tube and 2.5× the volume of 70% ethanol was added. This solution was placed in the Qiagen RNeasy spin columns and the RNA purified according to manufacturer's instructions. RNA concentration and quality was then measured using a spectrophotometer and a 1% agarose in TAE gel containing ethidium bromide. cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) with OligodT12–16 primers (Invitrogen) and RNase Out (Invitrogen). *Xenopus* RNA (500 μg) was isolated and used in the RT reaction. Primers for qPCR were designed using Primer3 or published primer sequences were used as cited: *Xenopus ski* - F: TGTGACAAAGGCTGTG AAGC, R: CAGAGCTGTTCCTGG AGGTC, *Xenopus cyp26a1* – F: GCT GCCACGTCCCTCACCTCTT R: GCC GATGCAGCACCTCACTCCA, *Xenopus id3* – F: AAAGCCATCAGCCC AGTG, R: AGTGGCAGACGCTGG TGT (Nichane et al., 2008), *Xenopus hist1h4a* - F: CGGGATAACATTCAG GGTA R: TCCATGGCGGTAACTGTC (Sindelka et al., 2006). In a study of housekeeping genes in *Xenopus*, it was also found that normalization using these genes was problematic (Sindelka et al., 2006) but identified *hist1h4a* as giving the most consistent results other than simply using total RNA. Our tests supported this finding; thus, *hist1h4a* mRNA levels were used to normalize qPCR data.

qPCR was performed according to manufacturer's protocols for Brilliant SYBR Green Master mix (Agilent Stratagene). The denaturation temperature was 94°C, with an annealling temperature of 55°C and elongation at 72°C. All qPCR experiments used a minimum of three ranging to a maximum of five replicates. All statistics were performed with GraphPad Prism4 using a one-way analysis of variance (ANOVA) and a 95% confidence interval with a post hoc Tukey's test.

Cell Culture

Human keratinocyte (HaCaT) cells were grown in 5% fetal bovine serum (FBS) (Wisent) in DMEM, 5% CO_2 at 37°C reaching approximately 75–80% confluence before RNA and protein isolation. HaCaT cells were incubated for 18 hr before treatment with 5% charcoal-stripped serum in DMEM. The cells were then treated using 1 μM all-*trans* RA or 1 μM pan-RA antagonist (Allergan 193109; Teng et al., 1997) dissolved in DMSO. A 1 μl/ml DMSO treatment was used as a carrier control. RNA was isolated using the Qiagen RNeasy mini Kit according to manufacturer's instructions. RNA concentration and quality was measured using a Nanodrop spectrophotometer and also visualized using a 1% agarose in TAE gel containing ethidium bromide. Transfection of plasmids was done using Lipofectamine according to manufacturers' instructions.

cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) with Oligo dT12–16 primers (Invitrogen). Primers for qPCR included *SKI* F: AAAGAGCTCTCCC CACACCT, R: GGGTGTCCACAGT CAGCTTC (designed using Primer3); *CYP26A1* F: TTTGGAGGACACGA AACCAC, R: CAGCATGAATCGGTC AGGAT (Zaitseva et al., 2007); *ID3* F: TGGTTTTCTTTCTCTTTGGGG, R: CGGGAGTAGCAGTGGTTCAT; *GA PDH* F: CATGAGAAGTATGACAAC AGC, R: AGTCCTTCCACGATACCAA AG (Lin et al., 2005).

Using DMEM media without serum in six-well plates (Fisher Scientific), HaCaT cells were transfected with 2 μg plasmid DNA using Lipofectamine (Invitrogen). Cells were assayed after 24 hr. Cells were rinsed twice in cold phosphate buffered saline (PBS) and lysed with RIPA buffer containing 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 4% protease inhibitor cocktail (Sigma). For Western blots of the Flag-tagged Ski products, 100 μg of protein were loaded per lane and separated on a 7.5% polyacrylamide gel followed by transfer to polyvinylidene fluoride (PVDF) membrane. Primary antibodies used for Western blots were Anti-FLAG (ThermoScientific) and anti-β-actin (Santa Cruz). The protein on the membrane then was probed with horseradish peroxidase (HRP) -coupled secondary antibodies and detected using the LumiGLO Chemiluminescent Kit (KPL) on a Versadoc gel documentation system.

To detect endogenous SKI in HaCaT cells after RA exposure, cells were seeded to 6-cm culture dishes at 5×10^5 cells and the following day media was replaced with charcoal-stripped serum-containing media. On the following day, cells were treated with 1 μM RA and total protein was isolated using RIPA lysis buffer containing protease inhibitors. Western blotting was performed on 50 μg total protein using anti-Ski antibody (Kamiya Biomedical, clone G8, 1:1,000 dilution in 5% bovine serum albumin/Tris buffered saline [TBS]-Tween 20) incubated overnight at 4°C followed by detection using anti-mouse IgG-HRP secondary antibody (GE Healthcare). Anti-actin antibody (1:1,000 in 5% skim milk/TBS-Tween 20; Sigma) and anti-rabbit IgG-HRP secondary antibody (GE Healthcare) were used to control for protein loading. Chemiluminescence detection was performed using Luminata Forte Western HRP Substrate (Millipore) and the BioRad ChemiDoc MP Imaging System.

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REFERENCES

Amaravadi LS, Neff AW, Sleeman JP, Smith RC. 1997. Autonomous neural axis formation by ectopic expression of the protooncogene c-ski. Dev Biol 192:392–404.

Barry JB, Leong GM, Church WB, Issa LL, Eisman JA, Gardiner EM. 2003. Interactions of SKIP/NCoA-62, TFIIB, and retinoid X receptor with vitamin D receptor helix H10 residues. J Biol Chem 278:8224–8228.

Begemann G, Marx M, Mebus K, Meyer A, Bastmeyer M. 2004. Beyond the neckless phenotype: influence of reduced retinoic acid signaling on motor neuron development in the zebrafish hindbrain. Dev Biol 271:119–129.

Beland M, Lohnes D. 2005. Chicken ovalbumin upstream promoter-transcription factor members repress retinoic acid-induced Cdx1 expression. J Biol Chem 280:13858–13862.

Berk M, Desai SY, Heyman HC, Colmenares C. 1997. Mice lacking the ski proto-oncogene have defects in neurulation, craniofacial, patterning, and skeletal muscle development. Genes Dev 11:2029–2039.

Botchkarev VA. 2003. Bone morphogenetic proteins and their antagonists in skin and hair follicle biology. J Invest Dermatol 120:36–47.

Boudjelal M, Voorhees JJ, Fisher GJ. 2002. Retinoid signaling is attenuated by proteasome-mediated degradation of retinoid receptors in human keratinocyte HaCaT cells. Exp Cell Res 274:130–137.

Cartry J, Nichane M, Ribes V, Colas A, Riou JF, Pieler T, Dolle P, Bellefroid EJ, Umbhauer M. 2006. Retinoic acid signalling is required for specification of pronephric cell fate. Dev Biol 299:35–51.

Chambers D, Wilson L, Maden M, Lumsden A. 2007. RALDH-independent generation of retinoic acid during vertebrate embryogenesis by CYP1B1. Development 134:1369–1383.

Chen Y, Pollet N, Niehrs C, Pieler T. 2001. Increased XRALDH2 activity has a posteriorizing effect on the central nervous system of Xenopus embryos. Mech Dev 101:91–103.

Colmenares C, Heilstedt HA, Shaffer LG, Schwartz S, Berk M, Murray JC, Stavnezer E. 2002. Loss of the SKI proto-oncogene in individuals affected with 1p36 deletion syndrome is predicted by strain-dependent defects in Ski-/- mice. Nat Genet 30:106–109.

Dahl R, Kieslinger M, Beug H, Hayman MJ. 1998. Transformation of hematopoietic cells by the Ski oncoprotein involves repression of retinoic acid receptor signaling. Proc Natl Acad Sci U S A 95:11187–11192.

de Roos K, Sonneveld E, Compaan B, ten Berge D, Durston AJ, van der Saag PT. 1999. Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and Xenopus laevis embryogenesis. Mech Dev 82:205–211.

Deimling SJ, Drysdale TA. 2009. Retinoic acid regulates anterior-posterior patterning within the lateral plate mesoderm of Xenopus. Mech Dev 126:913–923.

Denissova NG, Liu F. 2004. Repression of endogenous Smad7 by Ski. J Biol Chem 279:28143–28148.

Duester G. 2008. Retinoic acid synthesis and signaling during early organogenesis. Cell 134:921–931.

Feng L, Hernandez RE, Waxman JS, Yelon D, Moens CB. 2010. Dhrs3a regulates retinoic acid biosynthesis through a feedback inhibition mechanism. Dev Biol 338:1–14.

Gosselet FP, Magnaldo T, Culerrier RM, Sarasin A, Ehrhart JC. 2007. BMP2 and BMP6 control p57(Kip2) expression and cell growth arrest/terminal differentiation in normal primary human epidermal keratinocytes. Cell Signal 19:731–739.

Harland RM. 1991. In situ hybridization: an improved whole-mount method for Xenopus embryos. Methods Cell Biol 36:685–695.

Heim KC, Gamsby JJ, Hever MP, Freemantle SJ, Loros JJ, Dunlap JC, Spinella MJ. 2009. Retinoic acid mediates long-paced oscillations in retinoid receptor activity: evidence for a potential role for RIP140. PLoS One 4:e7639.

Heim KC, White KA, Deng D, Tomlinson CR, Moore JH, Freemantle SJ, Spinella MJ. 2007. Selective repression of retinoic acid target genes by RIP140 during induced tumor cell differentiation of pluripotent human embryonal carcinoma cells. Mol Cancer 6:57.

Hollemann T, Chen Y, Grunz H, Pieler T. 1998. Regionalized metabolic activity establishes boundaries of retinoic acid signalling. EMBO J 17:7361–7372.

Hollnagel A, Oehlmann V, Heymer J, Ruther U, Nordheim A. 1999. Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. J Biol Chem 274:19838–19845.

Kang MR, Lee SW, Um E, Kang HT, Hwang ES, Kim EJ, Um SJ. 2010. Reciprocal roles of SIRT1 and SKIP in the regulation of RAR activity: implication in the retinoic acid-induced neuronal differentiation of P19 cells. Nucleic Acids Res 38:822–831.

Kaufman CD, Martinez-Rodriguez G, Hackett PB Jr. 2000. Ectopic expression of c-ski disrupts gastrulation and neural patterning in zebrafish. Mech Dev 95:147–162.

Levy L, Howell M, Das D, Harkin S, Episkopou V, Hill CS. 2007. Arkadia activates Smad3/Smad4-dependent transcription by triggering signal-induced SnoN degradation. Mol Cell Biol 27:6068–6083.

Lin W, Zhang X, Chen Z, Borson N, Voss S, Sanderson S, Murphy L, Wettstein P, Strome SE. 2005. Development and immunophenotyping of squamous cell carcinoma xenografts: tools for translational immunology. Laryngoscope 115:1154–1162.

Luo K. 2003. Negative regulation of BMP signaling by the ski oncoprotein. J Bone Joint Surg Am 85(suppl 3):39–43.

Luo K. 2004. Ski and SnoN: negative regulators of TGF-beta signaling. Curr Opin Genet Dev 14:65–70.

Luo K, Stroschein SL, Wang W, Chen D, Martens E, Zhou S, Zhou Q. 1999. The Ski oncoprotein interacts with the Smad proteins to repress TGFbeta signaling. Genes Dev 13:2196–2206.

Lutz LB, Cole LM, Gupta MK, Kwist KW, Auchus RJ, Hammes SR. 2001. Evidence that androgens are the primary steroids produced by Xenopus laevis ovaries and may signal through the classical androgen receptor to promote oocyte maturation. Proc Natl Acad Sci U S A 98:13728–13733.

Lyons GE, Micales BK, Herr MJ, Horrigan SK, Namciu S, Shardy D, Stavnezer E. 1994. Protooncogene c-ski is expressed in both proliferating and postmitotic neuronal populations. Dev Dyn 201:354–365.

Mark M, Ghyselinck NB, Chambon P. 2006. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu Rev Pharmacol Toxicol 46:451–480.

Massague J, Chen YG. 2000. Controlling TGF-beta signaling. Genes Dev 14:627–644.

McGannon P, Miyazaki Y, Gupta PC, Traboulsi EI, Colmenares C. 2006. Ocular abnormalities in mice lacking the Ski proto-oncogene. Invest Ophthalmol Vis Sci 47:4231–4237.

Moreno TA, Kintner C. 2004. Regulation of segmental patterning by retinoic acid signaling during Xenopus somitogenesis. Dev Cell 6:205–218.

Moutier E, Ye T, Choukrallah MA, Urban S, Osz J, Chatagnon A, Delacroix L, Langer D, Rochel N, Moras D, Benoit G, Davidson I. 2012. Retinoic acid receptors recognize the mouse genome through binding elements with diverse spacing and topology. J Biol Chem 287:26328–26341.

Nichane M, de Croze N, Ren X, Souopgui J, Monsoro-Burq AH, Bellefroid EJ. 2008. Hairy2-Id3 interactions play an essential role in Xenopus neural crest progenitor specification. Dev Biol 322:355–367.

Niederreither K, Dolle P. 2008. Retinoic acid in development: towards an integrated view. Nat Rev Genet 9:541–553.

Niederreither K, McCaffery P, Drager UC, Chambon P, Dolle P. 1997. Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. Mech Dev 62:67–78.

Niederreither K, Subbarayan V, Dolle P, Chambon P. 1999. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat Genet 21:444–448.

Nieuwkoop PD, Faber J. 1994. Normal table of Xenopus laevis (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. New York: Garland Publishing. 252 p. 210 leaves of plates.

Ritter M, Kattmann D, Teichler S, Hartmann O, Samuelsson MK, Burchert A, Bach JP, Kim TD, Berwanger B, Thiede C, Jager R, Ehninger G, Schafer H, Ueki N, Hayman MJ, Eilers M, Neubauer A. 2006. Inhibition of retinoic acid receptor signaling by Ski in acute myeloid leukemia. Leukemia 20:437–443.

Ross AC, Zolfaghari R. 2011. Cytochrome P450s in the regulation of cellular retinoic acid metabolism. Annu Rev Nutr 31:65–87.

Sakai Y, Meno C, Fujii H, Nishino J, Shiratori H, Saijoh Y, Rossant J, Hamada H. 2001. The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo. Genes Dev 15:213–225.

Sharpe CR. 1991. Retinoic acid can mimic endogenous signals involved in transformation of the Xenopus nervous system. Neuron 7:239–247.

Sindelka R, Ferjentsik Z, Jonak J. 2006. Developmental expression profiles of Xenopus laevis reference genes. Dev Dyn 235:754–758.

Sirbu IO, Gresh L, Barra J, Duester G. 2005. Shifting boundaries of retinoic acid activity control hindbrain segmental gene expression. Development 132:2611–2622.

Sleeman JP, Laskey RA. 1993. Xenopus c-ski contains a novel coiled-coil protein domain, and is maternally expressed during development. Oncogene 8:67–77.

Strate I, Min TH, Iliev D, Pera EM. 2009. Retinol dehydrogenase 10 is a feedback regulator of retinoic acid signalling during axis formation and patterning of the central nervous system. Development 136:461–472.

Suzuki H, Yagi K, Kondo M, Kato M, Miyazono K, Miyazawa K. 2004. c-Ski inhibits the TGF-beta signaling pathway through stabilization of inactive Smad complexes on Smad-binding elements. Oncogene 23:5068–5076.

Takeda M, Mizuide M, Oka M, Watabe T, Inoue H, Suzuki H, Fujita T, Imamura T, Miyazono K, Miyazawa K. 2004. Interaction with Smad4 is indispensable for suppression of BMP signaling by c-Ski. Mol Biol Cell 15:963–972.

Teichler S, Schlenk RF, Strauch K, Hagner NM, Ritter M, Neubauer A. 2008. Expression of the nuclear oncogene Ski in patients with acute myeloid leukemia treated with all-trans retinoic acid. Haematologica 93:1105–1107.

Teng M, Duong TT, Johnson AT, Klein ES, Wang L, Khalifa B, Chandraratna RA. 1997. Identification of highly potent retinoic acid receptor alpha-selective antagonists. J Med Chem 40:2445–2451.

Tokitou F, Nomura T, Khan MM, Kaul SC, Wadhwa R, Yasukawa T, Kohno I, Ishii S. 1999. Viral ski inhibits retinoblastoma protein (Rb)-mediated transcriptional repression in a dominant negative fashion. J Biol Chem 274:4485–4488.

Torma H, Rollman O, Vahlquist A. 1999. The vitamin A metabolism and expression of retinoid-binding proteins differ in HaCaT cells and normal human keratinocytes. Arch Dermatol Res 291:339–345.

Wang W, Mariani FV, Harland RM, Luo K. 2000. Ski represses bone morphogenic protein signaling in Xenopus and mammalian cells. Proc Natl Acad Sci U S A 97:14394–14399.

White KA, Yore MM, Warburton SL, Vaseva AV, Rieder E, Freemantle SJ, Spinella MJ. 2003. Negative feedback at the level of nuclear receptor coregulation. Self-limitation of retinoid signaling by RIP140. J Biol Chem 278:43889–43892.

Xu L, Glass CK, Rosenfeld MG. 1999. Coactivator and corepressor complexes in nuclear receptor function. Curr Opin Genet Dev 9:140–147.

Zaitseva M, Vollenhoven BJ, Rogers PA. 2007. Retinoic acid pathway genes show significantly altered expression in uterine fibroids when compared with normal myometrium. Mol Hum Reprod 13:577–585.

Zhang H, Stavnezer E. 2009. Ski regulates muscle terminal differentiation by transcriptional activation of Myog in a complex with Six1 and Eya3. J Biol Chem 284:2867–2879.

Zhao HL, Ueki N, Hayman MJ. 2010. The Ski protein negatively regulates Siah2-mediated HDAC3 degradation. Biochem Biophys Res Commun 399:623–628.

Zhao HL, Ueki N, Marcelain K, Hayman MJ. 2009. The Ski protein can inhibit ligand induced RARalpha and HDAC3 degradation in the retinoic acid signaling pathway. Biochem Biophys Res Commun 383:119–124.