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Smad4 loss in mouse keratinocytes leads to increased susceptibility to UV carcinogenesis with reduced Ercc1-mediated DNA repair

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Abstract

Smad4 loss occurs frequently in human skin squamous cell carcinoma (SCC), but it is unknown if this loss increases ultraviolet-induced (UV) carcinogenesis, a major etiological factor in skin cancer. In the present study, mice with keratinocyte-specific *Smad4* deletion (*K14.Smad4*$^{-/-}$) and wildtype (*WT*) littermates were chronically UV-irradiated. Compared to *WT*, *K14.Smad4*$^{-/-}$ mice exhibited increased DNA damage and increased susceptibility to UV-induced skin cancer. Among genes involved in repairing UV-induced DNA damage, *Excision repair cross-complementation group1* (*Ercc1*) mRNA was significantly reduced in UV treated *K14.Smad4*$^{-/-}$ skin compared to *WT* skin. Further analysis revealed that Smad4 loss confers reduced Snail binding to the *Ercc1* regulatory elements, resulting in reduced *Ercc1* transcription. Consistently, transient transfection of *Snai1* into *Smad4*$^{-/-}$ keratinocytes led to increased repair of UV-induced DNA lesions. Transfection of *Ercc1* into *Smad4*$^{-/-}$ keratinocytes restored repair of UV-induced DNA damage. Further, immunostaining revealed that the presence of Smad4 protein is associated with the presence of Snail and Ercc1 proteins in human skin SCC and precancerous actinic keratoses (AK). Collectively, Smad4 loss associated Snail reduction compromises Ercc1-mediated DNA repair, contributing to increased UV-induced skin carcinogenesis. Thus we identified a role for Snail in UV-induced DNA repair.

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Introduction

Solar ultraviolet (UV) radiation is the primary cause of skin cancer. Non-melanoma skin cancer, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), is the most common form of skin cancer (Foster *et al.*, 2008). Solar UV radiation is comprised of UVA (315–400nm), UVB (280–315nm) and UVC (100–280nm), wherein UVB is a strong carcinogen (de Gruijl and Van der Leun, 1994; Narayanan *et al.*, 2010).

The transcription factor Smad4 is a signaling mediator for transforming growth factor β (TGFβ), activin, and bone morphogenetic protein (BMP) (Moustakas *et al.*, 2001). Smad4 down regulation, at the gene level through loss of heterozygosity (LOH) or at the protein level, is frequent in human skin SCCs (Hoot *et al.*, 2008). Mice with keratinocyte-specific *Smad4* deletion develop spontaneous SCCs (Qiao *et al.*, 2006; Yang *et al.*, 2005). We have found that Smad4 loss in the oral mucosa causes functional defects in the Fanconi Anemia (FA) and BRCA1 DNA repair pathways, and spontaneous head and neck SCC (Bornstein *et al.*, 2009). It remains to be determined if Smad4 loss affects repair of other types of DNA damage. Because UV-induced carcinogenesis is primarily initiated by DNA damage, and not all DNA repair genes are in the FA pathway, we sought to determine if Smad4 plays a role in the repair of UV-induced DNA damage and thus Smad4 loss increases susceptibility to UV-induced skin carcinogenesis.

UV radiation causes DNA damage primarily due to generation of cyclobutane pyrimidine dimers (CPD) and pyrimidine pyrimidone (6-4PP) photoproducts. CPD and 6-4PP are repaired primarily by the nucleotide excision repair (NER) pathway (Leibeling *et al.*, 2006). Xeroderma pigmentosum (XP) patients with defective NER exhibit 1,000 fold increased susceptibility to skin cancer (Kraemer *et al.*, 1987). There are seven *XP* genes involved in this pathway, *XPA* through *XPG* (Leibeling *et al.*, 2006). Ercc1 is an endonuclease that, along with XPF, excises a short oligonucleotide encompassing a UV-induced lesion, which is followed by replicative DNA polymerases filling in the gap, using the complementary strand as the template (Nouspikel, 2009). Keratinocyte specific deletion of *Ercc1* in mice causes hypersensitivity to UV-induced skin cancer, demonstrating the critical role of Ercc1 in repairing UV-induced DNA damage (Doig *et al.*, 2006). Genetic mutation or loss of *Ercc1* is rare in humans. In this report, we examined the effect of keratinocyte specific *Smad4* deletion on UV-induced skin carcinogenesis and focused our molecular analysis on repair of UV-induced DNA damage. We provide evidence that Smad4 regulates Ercc1 expression through Snail-mediated transcription, thus *Smad4* deletion contributed to increased susceptibility to UV carcinogenesis.

Results

Epidermal *Smad4* deletion causes increased susceptibility to UV-induced SCC

We generated mice with epidermal specific deletion of *Smad4* by mating *K14.CrePR1* mice with *Smad4* floxed mice ($Smad4^{f/f}$) (Berton *et al.*, 2000). Mice with Cre-mediated *Smad4* deletion in keratinocytes were defined as $K14.Smad4^{-/-}$ mice (n=29). Monogenic *K14CrePR1* and $Smad4^{f/f}$ mice (n=25) with normal Smad4 expression level (Figure 1a), were used as controls, (Figure 1b). All mice are in the C57BL/6 background. Both groups of

mice were shaved and subjected to UVA and UVB irradiation three times a week for 40 weeks. We started with a dose of 1 J/cm^2 of UVA and 0.12 J/cm^2 of UVB, which did not induce erythema in either group. Therefore, we increased UV irradiation to 10 J/cm^2 of UVA and 1.2 J/cm^2 of UVB to induce erythema and gradually reduced UV doses (detailed in Figure S1) to enable long-term UV irradiation without morbidity due to severe UV-induced damage, ending with 4.3 J/cm^2 of UVA and 1.74 J/cm^2 of UVB. Without UV exposure, *K14.Smad4*$^{-/-}$ mice did not develop spontaneous skin tumors, unlike previously reports for *MMTV.Smad4*$^{-/-}$ or *K5.Smad4*$^{-/-}$ mice (Qiao *et al.*, 2006; Yang *et al.*, 2005) presumably due to patchy and weak Cre activity in *K14.CrePR1* mice (Vassar *et al.*, 1989; Wagner *et al.*, 2001; Wang *et al.*, 1997; Zhou *et al.*, 2002). Interestingly, within 10 weeks of UV irradiation, *K14.Smad4*$^{-/-}$ mice started to develop skin tumors, and by 40 weeks, 24 out of 29 mice developed skin tumors (Figure 1b). In contrast, none of the control mice developed tumors during the study period (Figure1b), consistent with previous reports that C57BL/6 mice are extremely resistant to UV carcinogenesis (Strickland and Swartz, 1987). Histological examination revealed that UV-induced *K14.Smad4*$^{-/-}$ tumors were SCCs (Figure 1c).

Smad4$^{-/-}$ keratinocytes exhibit defects in repairing UV-induced DNA damage

We analyzed expression of phosphorylated H2AX (p-H2AX), a marker of DNA damage (Hanasoge and Ljungman, 2007; Marti *et al.*, 2006), in UV-irradiated *WT* skin and *K14.Smad4*$^{-/-}$ skin and tumors. *K14.Smad4*$^{-/-}$ skin and tumors showed a 68% and a 192% increase, respectively, in the number of p-H2AX positive cells compared to *WT* skin (Figure 2a, 2b). This data suggested that DNA repair of UV-induced lesions may be reduced in the skin of *K14.Smad4*$^{-/-}$ mice. To determine if increased DNA damage in UV-irradiated *K14.Smad4*$^{-/-}$ skin reflects inherent defects in DNA repair after Smad4 loss, we assessed levels of spontaneous DNA damage in primary keratinocytes from neonatal *K14.Smad4*$^{-/-}$ and *WT* skin by alkaline comet assay (Figure 2c). *Smad4*$^{-/-}$ keratinocytes exhibit a 16.5% increase in comet tail DNA compared to *WT* keratinocytes, representing higher numbers of double and single strand DNA breaks (DSB and SSB) (Figure 2d).

To focus on UV-induced DNA damage, we measured CPD levels in *WT* and *Smad4*$^{-/-}$ keratinocytes irradiated with 60 mJ/cm^2 of UVB (Figure 2e). Consistent with a previous report (Nguyen *et al.*, 2010), CPD levels within the first 30 minutes reflected the UV dose and there was no statistically significant difference between the two cell types. Afterwards, CPD levels began to decline in *WT* cells presumably due to DNA repair. In contrast, CPD levels remain unchanged in *Smad4*$^{-/-}$ keratinocytes. These results suggest that accumulated DNA damage is a direct effect of Smad4 loss in keratinocytes.

Reduced expression of *Ercc1* mRNA and protein in UV-irradiated *K14.Smad4*$^{-/-}$ skin

To dissect the mechanism underlying reduced DNA repair in *Smad4*$^{-/-}$ keratinocytes, we compared gene expression in *WT* and *Smad4*$^{-/-}$ keratinocytes using a mouse DNA damage signaling pathway RT-PCR array and found reduced expression of *Ercc1*, *Checkpoint homolog* (*Hus1*), *Checkpoint kinase 1* (*Chk1*), *Exonuclease 1* (*Exo1*), *Chromatin assembly factor 1a* (*Chaf1a*) and *Meiotic recombination 11 homolog A* (*Mre11*) genes in *Smad4*$^{-/-}$ cells (Figure 3a). Among DNA repair genes down regulated in *Smad4*$^{-/-}$ keratinocytes, none

are documented direct Smad4 targets (Koinuma *et al.*, 2009). Hence, the effect of Smad4 on these genes requires additional mediators, which could vary among these targets. We began with examination of Ercc1, which has an indispensable role in excisional repair of UV-induced damage (Chipchase and Melton, 2002) and was the most down regulated gene in *Smad4*$^{-/-}$ keratinocytes (Figure 3a). We measured *Ercc1* mRNA levels in UV-irradiated *WT* and *K14.Smad4*$^{-/-}$ skin and observed an 81% reduction of *Ercc1* mRNA level in *Smad4*$^{-/-}$ skin compared to *WT* skin (Figure 3b). Ercc1 immunostaining of UV-irradiated *WT* skin was localized to the nucleus, it was dramatically reduced in *K14.Smad4*$^{-/-}$ epidermis and in *K14.Smad4*$^{-/-}$ SCCs (Figure 3c).

Reduced expression of *Ercc1* mRNA and protein in *K14.Smad4*$^{-/-}$ skin is a result of reduced Snail expression

To further study mechanisms of Ercc1 reduction we searched for putative transcription factor binding sites in the 2kb promoter region of the mouse *Ercc1* gene using P-Match software. We did not find putative Smad4 binding sites, but instead found several putative binding sites (CANNTG) for the transcription factor Snail in the promoter and near the transcription start site (TSS) of *Ercc1* (Figure S4a). Snail, a protein encoded by the *SNAI1* gene, is a direct Smad4 target (Hoot *et al.*, 2008; Peinado *et al.*, 2003) so we investigated the possibility of Smad4 regulation of Ercc1 through Snail. Next, we examined *Snai1* expression level by qRT-PCR in UV-irradiated *WT* and *Smad4*$^{-/-}$ keratinocytes (Figure 4a). *Snai1* mRNA was induced by UV irradiation in both keratinocytes, although the level in *Smad4*$^{-/-}$ keratinocytes continued to be lower than *WT* at all time points (Figure 4a). Similarly, we found an 86% reduction in *Snai1* level in UV-irradiated *K14.Smad4*$^{-/-}$ skin compared to UV-irradiated *WT* skin (Figure 4b). We also examined *Ercc1* mRNA levels in these samples and, consistent with a previous report (Murakami *et al.*, 2001), it was induced by UV irradiation in *WT* and *Smad4*$^{-/-}$ keratinocytes, but the level was 2-fold lower in *Smad4*$^{-/-}$ keratinocytes than in *WT* keratinocytes with or without UV irradiation (Figure S2).

To further test if reduced Snail causes reduced Ercc1 expression in *Smad4*$^{-/-}$ keratinocytes, we overexpressed human Snail in *WT* and *Smad4*$^{-/-}$ keratinocytes and observed that *Ercc1* transcripts were up regulated in *Snai1* transfected *WT* and *Smad4*$^{-/-}$ keratinocytes (Figure 4c). Expression of human *Snai1* mRNA was equivalent in the two cell lines upon transfection (Figure S3). Because Snail has been shown to induce Ercc1 expression in head and neck cancer (Hsu *et al.*, 2010), we examined if Smad4 loss causes reduced Snail binding to *Ercc1* regulatory elements. Using chromatin immunoprecipitation assays, we detected direct Snail binding to the *Ercc1* regulatory region encompassing the putative Snail binding sites at 124–129 bp (Site1, Sup. Figure 4a), 276–281 bp, and 300–305 bp (Site 2, Figure S4a) from the TSS of the mouse *Ercc1* gene. The locations of the Snail binding sites in the mouse *Ercc1* gene are similar to those in the human *Ercc1* gene, where they are important for Snail-mediated transcription (Hsu *et al.*, 2010). Snail binding to the *Ercc1* regulatory region was decreased to 35% at site 1 and 32% at site 2 in *Smad4*$^{-/-}$ keratinocytes prior to UV irradiation (Figure 4d, Figure S4b). In UV-irradiated *WT* keratinocytes, similar to Snail mRNA levels (Figure 4a), Snail binding to both sites was significantly increased at 12 and 24 hours, compared to non-irradiated keratinocytes (Figure 4d). Although Snail binding at

both sites was also increased in UV-irradiated Smad4$^{-/-}$ keratinocytes compared to non-irradiated Smad4$^{-/-}$ keratinocytes, its binding levels were significantly lower than in *WT* keratinocytes at both 12 and 24 hours (Figure 4d). Collectively, these results suggest that reduced Snail levels in *K14.Smad4*$^{-/-}$ skin lead to reduced *Ercc1* transcription.

Restoration of Snail or Ercc1 expression in *Smad4*$^{-/-}$ keratinocytes rescues DNA repair defect

We assessed if Snail restoration in *Smad4*$^{-/-}$ keratinocytes could attenuate defects in repair of UV-induced DNA damage. *WT* and *Smad4*$^{-/-}$ keratinocytes were transfected with a human Snail expression plasmid or empty vector, irradiated with UVB, and CPD level was measured by ELISA at different time points during recovery of these cells. Remarkably, we observed a dramatic decrease in CPD adducts upon *Snai1* transfection in *Smad4*$^{-/-}$ cells at 1 and 3 hour time points following UV irradiation (Figure 4e).

Subsequently, we examined the contribution of reduced Ercc1 expression to the DNA repair defect in *Smad4*$^{-/-}$ cells. Transfection of human Ercc1 expression vector into *Smad4*$^{-/-}$ keratinocytes led to a dramatic reduction of CPD level at 1 and 3 hours following UV irradiation, compared to empty vector transfected keratinocytes (Figure 4f), suggesting that reduced Ercc1 is a major contributor to the DNA repair defect observed in these cells (Figure 4f).

Expression of Smad4 is associated with expression of Snail and Ercc1 proteins in human skin SCC and actinic keratoses (AK)

Data from our mouse model suggest that Smad4 mediated Snail expression leads to Ercc1 expression. If this finding applies to human SCCs, we would expect the presence of Smad4 protein to be associated with the presence of Snail and Ercc1 proteins, and conversely the absence of Smad4 to be associated with the absence of Snail and Ercc1. Immunostaining in a tissue array with 76 skin SCC samples showed Smad4 expression was lost in 47/76 (62%) cases, consistent with our previous report (Hoot *et al.*, 2008), and 43/76 (57%) cases expressed Ercc1 (Figure 5a, b, Table S1). Consistent with our previous report (Hoot *et al.*, 2008), a majority of cases (57/76, 75%) were Snail positive. We observed a statistically significant association between expression of Smad4 and Snail proteins in SCC. Further, most cases positive for Snail (67%) were also positive for Ercc1 and conversely, most cases negative for Snail (74%) were also negative for Ercc1. Finally, we found a statistically significant association between expression of Smad4 and Ercc1 proteins. Most Smad4 positive cases (86%) were also positive for Ercc1 and conversely, the majority of Smad4 negative cases (60%) were Ercc1 negative.

To further determine if the above associations occur in the early stages of skin carcinogenesis thereby suggesting a causal role of *Smad4* loss-mediated DNA damage in skin carcinogenesis, we performed the above immunostaining in 28 human AK samples (Figure 5c, d, Table S1). We found loss of Smad4 protein in 16/28 (57%) AK cases, suggesting Smad4 loss is an early event during UV-induced skin carcinogenesis, similar to our findings in head and neck cancer (Bornstein *et al.*, 2009). Analogous to our above

observations in SCC, we observed statistically significant associations between expression of i) Smad4 and Snail ii) Snail and Ercc1 and iii) Smad4 and Ercc1 proteins.

Discussion

In the present study we examined the contribution of Ercc1 to UV-induced skin cancer. Among molecules related to UV-induced DNA repair, *Ercc1* was the most down regulated in *Smad4*$^{-/-}$ keratinocytes, and transfection of an *Ercc1* plasmid into these cells rescued their defective repair of UV-induced CPDs, suggesting that Ercc1 is an important contributor to the UV-susceptibility of *K14.Smad4*$^{-/-}$ mice. Several other DNA repair genes that are down regulated in *Smad4*$^{-/-}$ keratinocytes have also been reported to regulate DNA damage signaling, cell cycle checkpoints, chromatin assembly after DNA damage, and DSB repair (Chen and Sanchez, 2004; Martini *et al.*, 1998; Niida and Nakanishi, 2006; Olson *et al.*, 2007; Sertic *et al.*, 2011). It remains to be determined whether reduced levels of these genes contribute to the increased susceptibility to UV-induced skin cancer in *K14.Smad4*$^{-/-}$ mice.

Our data suggest that Smad4-dependent *Snai1* expression is one of the major mechanisms inducing *Ercc1* expression during UV carcinogenesis. First, loss of Smad4 led to reduced *Snai1* expression and reduced Snail binding to the *Ercc1* regulatory binding sites; second, Snail activated *Ercc1* expression in keratinocytes. Third, we observed co-expression of Smad4, Snail and Ercc1 proteins in human skin SCCs and precancerous AKs. Similar expression patterns of Snail and Ercc1 have also been observed in head and neck cancer, and bladder cancer (Hsu *et al.*, 2010; Kawashima *et al.*, 2012). Thus, Snail regulated *Ercc1* expression may occur broadly in human cancers.

Snail is an important mediator of epithelial to mesenchymal transition (EMT) and thereby plays key roles in normal morphogenetic movements in the embryo and cancer cell migration promoting metastasis in a variety of tumor types, including skin cancer (Batlle *et al.*, 2000; Cano *et al.*, 2000). Additionally, Snail is shown to promote skin cancer through inducing inflammation (Du *et al.*, 2010). Thus, Snail is considered an oncogenic molecule. Our finding that *Snai1* expression and Snail binding to the Ercc1 promoter and induction of *Ercc1* expression are stimulated by UV-irradiation suggests Snail plays a dual role in UV-induced skin carcinogenesis. On one hand, it promotes Ercc1 mediated DNA repair, thereby inhibiting mutagenesis caused by UV radiation. Consistent with our observation, embryonic fibroblasts, from a Snail transgenic mouse expressing Snail at a level 20% higher than the endogenous level, are more resistant to DNA damaging γ radiation (Perez-Mancera *et al.*, 2005). On the other hand, in later stages of cancer, when cancer cells have accumulated multiple mutations, Snail-induced DNA repair may trigger resistance to DNA damaging therapeutic agents. Supporting this notion, co-expression of Ercc1 and Snail correlates with resistance to the chemotherapeutic agent cisplatin, and poor prognosis in head and neck cancer (Hsu *et al.*, 2010). Similarly, overexpression of Snail in breast cancer cells confers protection against the DNA damaging effect of topoisomerase inhibitor (Kajita *et al.*, 2004).

In summary, we identified a role of Smad4 in regulation of Ercc1 expression through Snail-mediated transcriptional regulation of *Ercc1*. We also ascertained a role of Snail in

promoting repair of UV-induced DNA damage via regulation of *Ercc1* expression. Loss of Smad4 expression in the epidermis leads to a reduced level of Ercc1, resulting in defective DNA repair in the epidermis after UV irradiation. Abrogation of DNA repair is likely to cause accumulation of UV-induced mutations in *K14.Smad4*$^{-/-}$ skin and ultimately lead to carcinogenesis. Our work necessitates further investigation of the stage-specific effects of Smad4, Snail and Ercc1-associated DNA repair in cancer initiation and progression, and its influence on the response of cancer cells to therapies using DNA damaging agents.

Materials and Methods

Mouse model

The generation of *K14.CrePR1* and *Smad4*$^{fl/fl}$ mice has been described previously (Berton *et al.*, 2000; Yang *et al.*, 2002). Bigenic mice *K14.CrePR1*/*Smad4*$^{fl/fl}$ were generated by mating *K14.CrePR1* mice with *Smad4*$^{fl/fl}$ mice. Genotype was determined by PCR as described previously (Berton *et al.*, 2000; Yang *et al.*, 2002). Keratinocyte specific *Smad4* deletion was achieved in *K14.*Cre/*Smad4*$^{fl/fl}$ mice by topical application of RU486 for 5 days, when mice were 8 weeks old (Owens *et al.*, 2008). Animal experiments and care were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver, Anschutz Medical Campus.

UV irradiation of mice—Mice were exposed to UV radiation beginning 3 months of age in a cabinet (Daavlin, Bryan) for 40 weeks and the dosage was increased gradually as depicted in Supplementary Figure 1.

Tumor histology—Skin and tumor samples were fixed in neutral buffered formalin overnight, embedded, sectioned and stained with Hematoxylin and Eosin (H&E) as previously described (Lu *et al.*, 2006). Tumor types were determined by H&E analysis using criteria defined previously (Aldaz *et al.*, 1987).

Immunohistochemistry (IHC) and Immunofluorescence

Immunostaining was performed as previously described (Han *et al.*, 2005). Sections were incubated with an antibody overnight at 4°C as follows: anti-Phospho-Histone2AX (1:100, Cell Signaling, Danvers), anti-Ercc1 (1:100, Santa Cruz Biotechnology, Santa Cruz), anti-Smad4 (1:20, Santa Cruz Biotechnology, Santa Cruz) for mouse sections, anti-Smad4 (1:50, Epitomics, Burlingame) for human tissue sections, anti-Snail (1:50, Abcam, Cambridge). The human skin SCC tumor array SK802 (U.S. Biomax, Rockville) was used. The Department of Dermatopathology, Shanghai Skin Diseases Hospital provided human AK samples as de-identified archived paraffin sections. For IHC, sections were incubated with biotinylated secondary antibodies (1:400) and avidin-peroxidase (Vector Laboratories, Burlingame). Immunostaining was visualized using diaminobenzidine (DAKO, Denmark). For immunofluorescence, Alexa Fluor 488 and 594 labeled secondary antibodies (Life Technologies, Grand Island) were used.

Keratinocyte culture

Primary *WT* and *Smad4*$^{-/-}$ keratinocytes were generated from the above mice as described previously (Han *et al.*, 2006). Keratinocytes in culture were irradiated with 60 mJ/cm^2 of UVB, culture medium (Lonza, Switzerland) was replaced with fresh medium and cells were incubated in a 5% CO_2 incubator for the indicated time period.

Comet assay

Oxiselect Comet assay kit (Cell Biolabs, San Diego) was used according to the manufacturer's instructions. DNA was stained with Vista Green, and quantification of the comet tail moment was done using CASP software.

RNA Extraction and Quantitative PCR

Total RNA was extracted from mouse skin and tumors in Trizol (Life Technologies, Grand Island) as described previously (Hoot *et al.*, 2008). RNA extraction from cultured keratinocytes was performed using the RNAeasy kit (Qiagen, Valencia). Total RNA was reverse transcribed using Superscript III First Strand Synthesis System (Life Technologies, Grand Island) followed by quantitative PCR using SYBR Green master mix (Life Technologies, Grand Island) using primers listed in Supplementary table 2. Alternatively, RT-qPCR was done using primers listed in Supplementary table 3 and Brilliant qRTPCR mix (Agilent technologies, Santa Clara). A mouse DNA damage signaling pathway (SA Biosciences, Valencia) PCR array was used. For each genotype, three to seven samples were analyzed and gene expression was normalized to GAPDH or 18S rRNA.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as previously described (Hoot *et al.*, 2010). Briefly, cultured *WT* and *Smad4*$^{-/-}$ keratinocytes were crosslinked with 1% formaldehyde, genomic DNA was sheared in cell lysate by sonication, immunoprecipitation was performed at 4°C overnight with 4Pg of antibody to Snail (Abcam, Cambridge) or normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz). This was followed by crosslink reversal, DNA purification and quantitative PCR using primers listed in Supplementary Table 4. Enrichment of Snail at the *Ercc1* regulatory region was obtained by normalizing the relative level of the *Ercc1* regulatory region in the output (immunoprecipitated DNA) to the relative level of an intergenic region (lacking *Snail* binding sites) in the output. The relative level of the *Ercc1* regulatory region in the output was determined by normalizing the level of the *Ercc1* regulatory region in the output to that in the input.

Transfection of keratinocytes

WT and *Smad4*$^{-/-}$ keratinocytes were transfected with 5μg of pEGFPC2-SNAI1 (Addgene, 16225) or pEGFP (Lonza, Switzerland) or pCMVFlag*Ercc1* using the Epithelial Cell Nucleofection kit (Lonza) following the Amaxa nucleofector program M-005 (Lonza, Switzerland). Cells were harvested 72 hours post transfection, RNA was extracted and quantitative RTPCR was performed as above.

CPD ELISA

WT and *Smad4*$^{-/-}$ keratinocytes were exposed to UVB radiation at 60mJ/cm^2, and samples were harvested at different time points after treatment. Genomic DNA was extracted using the DNeasy kit (Qiagen, Valencia) and ELISA was performed using the Oxiselect UV-induced DNA damage ELISA kit for CPD quantitation (Cell Biolabs, San Diego).

Statistics

The student's t test was used to analyze data from qRT-PCR, comet assay and CPD ELISA to determine statistical significance of results. Fisher's exact test was used to analyze data from IHC staining of human skin SCC and AK samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

WT	wild type
AK	actinic Keratosis
TSS	transcription start site

References

Aldaz CM, Conti CJ, Klein-Szanto AJ, Slaga TJ. Progressive dysplasia and aneuploidy are hallmarks of mouse skin papillomas: relevance to malignancy. Proceedings of the National Academy of Sciences of the United States of America. 1987; 84:2029–32. [PubMed: 3104907]

Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nature cell biology. 2000; 2:84–9. [PubMed: 10655587]

Berton TR, Wang XJ, Zhou Z, Kellendonk C, Schutz G, Tsai S, et al. Characterization of an inducible, epidermal-specific knockout system: differential expression of lacZ in different Cre reporter mouse strains. Genesis. 2000; 26:160–1. [PubMed: 10686618]

Bornstein S, White R, Malkoski S, Oka M, Han G, Cleaver T, et al. Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. The Journal of clinical investigation. 2009; 119:3408–19. [PubMed: 19841536]

Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nature cell biology. 2000; 2:76–83. [PubMed: 10655586]

Chen Y, Sanchez Y. Chk1 in the DNA damage response: conserved roles from yeasts to mammals. DNA repair. 2004; 3:1025–32. [PubMed: 15279789]

Chipchase MD, Melton DW. The formation of UV-induced chromosome aberrations involves ERCC1 and XPF but not other nucleotide excision repair genes. DNA repair. 2002; 1:335–40. [PubMed: 12509251]

de Gruijl FR, Van der Leun JC. Estimate of the wavelength dependency of ultraviolet carcinogenesis in humans and its relevance to the risk assessment of a stratospheric ozone depletion. Health physics. 1994; 67:319–25. [PubMed: 8083043]

Doig J, Anderson C, Lawrence NJ, Selfridge J, Brownstein DG, Melton DW. Mice with skin-specific DNA repair gene (Ercc1) inactivation are hypersensitive to ultraviolet irradiation-induced skin cancer and show more rapid actinic progression. Oncogene. 2006; 25:6229–38. [PubMed: 16682947]

Du F, Nakamura Y, Tan TL, Lee P, Lee R, Yu B, et al. Expression of snail in epidermal keratinocytes promotes cutaneous inflammation and hyperplasia conducive to tumor formation. Cancer research. 2010; 70:10080–9. [PubMed: 21159631]

Foster PJ, Dunn EA, Karl KE, Snir JA, Nycz CM, Harvey AJ, et al. Cellular magnetic resonance imaging: in vivo imaging of melanoma cells in lymph nodes of mice. Neoplasia. 2008; 10:207–16. [PubMed: 18320065]

Han G, Li AG, Liang YY, Owens P, He W, Lu S, et al. Smad7-induced beta-catenin degradation alters epidermal appendage development. Developmental cell. 2006; 11:301–12. [PubMed: 16950122]

Han G, Lu SL, Li AG, He W, Corless CL, Kulesz-Martin M, et al. Distinct mechanisms of TGF-beta1-mediated epithelial-to-mesenchymal transition and metastasis during skin carcinogenesis. The Journal of clinical investigation. 2005; 115:1714–23. [PubMed: 15937546]

Hanasoge S, Ljungman M. H2AX phosphorylation after UV irradiation is triggered by DNA repair intermediates and is mediated by the ATR kinase. Carcinogenesis. 2007; 28:2298–304. [PubMed: 17615256]

Hoot KE, Lighthall J, Han G, Lu SL, Li A, Ju W, et al. Keratinocyte-specific Smad2 ablation results in increased epithelial-mesenchymal transition during skin cancer formation and progression. The Journal of clinical investigation. 2008; 118:2722–32. [PubMed: 18618014]

Hoot KE, Oka M, Han G, Bottinger E, Zhang Q, Wang XJ. HGF upregulation contributes to angiogenesis in mice with keratinocyte-specific Smad2 deletion. The Journal of clinical investigation. 2010; 120:3606–16. [PubMed: 20852387]

Hsu DS, Lan HY, Huang CH, Tai SK, Chang SY, Tsai TL, et al. Regulation of excision repair cross-complementation group 1 by Snail contributes to cisplatin resistance in head and neck cancer. Clinical cancer research: an official journal of the American Association for Cancer Research. 2010; 16:4561–71. [PubMed: 20823140]

Kajita M, McClinic KN, Wade PA. Aberrant expression of the transcription factors snail and slug alters the response to genotoxic stress. Molecular and cellular biology. 2004; 24:7559–66. [PubMed: 15314165]

Kawashima A, Takayama H, Kawamura N, Doi N, Sato M, Hatano K, et al. Co-expression of ERCC1 and Snail is a prognostic but not predictive factor of cisplatin-based neoadjuvant chemotherapy for bladder cancer. Oncology letters. 2012; 4:15–21. [PubMed: 22807952]

Koinuma D, Tsutsumi S, Kamimura N, Imamura T, Aburatani H, Miyazono K. Promoter-wide analysis of Smad4 binding sites in human epithelial cells. Cancer science. 2009; 100:2133–42. [PubMed: 19686287]

Kraemer KH, Lee MM, Scotto J. Xeroderma pigmentosum. Cutaneous, ocular, and neurologic abnormalities in 830 published cases. Archives of dermatology. 1987; 123:241–50. [PubMed: 3545087]

Leibeling D, Laspe P, Emmert S. Nucleotide excision repair and cancer. Journal of molecular histology. 2006; 37:225–38. [PubMed: 16855787]

Lu SL, Herrington H, Reh D, Weber S, Bornstein S, Wang D, et al. Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. Genes & development. 2006; 20:1331–42. [PubMed: 16702406]

Marti TM, Hefner E, Feeney L, Natale V, Cleaver JE. H2AX phosphorylation within the G1 phase after UV irradiation depends on nucleotide excision repair and not DNA double-strand breaks.

Proceedings of the National Academy of Sciences of the United States of America. 2006; 103:9891–6. [PubMed: 16788066]

Martini E, Roche DM, Marheineke K, Verreault A, Almouzni G. Recruitment of phosphorylated chromatin assembly factor 1 to chromatin after UV irradiation of human cells. The Journal of cell biology. 1998; 143:563–75. [PubMed: 9813080]

Moustakas A, Souchelnytskyi S, Heldin CH. Smad regulation in TGF-beta signal transduction. Journal of cell science. 2001; 114:4359–69. [PubMed: 11792802]

Murakami T, Fujimoto M, Ohtsuki M, Nakagawa H. Expression profiling of cancer-related genes in human keratinocytes following non-lethal ultraviolet B irradiation. Journal of dermatological science. 2001; 27:121–9. [PubMed: 11532376]

Narayanan DL, Saladi RN, Fox JL. Ultraviolet radiation and skin cancer. International journal of dermatology. 2010; 49:978–86. [PubMed: 20883261]

Nguyen TA, Slattery SD, Moon SH, Darlington YF, Lu X, Donehower LA. The oncogenic phosphatase WIP1 negatively regulates nucleotide excision repair. DNA repair. 2010; 9:813–23. [PubMed: 20451471]

Niida H, Nakanishi M. DNA damage checkpoints in mammals. Mutagenesis. 2006; 21:3–9. [PubMed: 16314342]

Nouspikel T. DNA repair in mammalian cells: So DNA repair really is that important? Cellular and molecular life sciences: CMLS. 2009; 66:965–7. [PubMed: 19153660]

Olson E, Nievera CJ, Lee AY, Chen L, Wu X. The Mre11-Rad50-Nbs1 complex acts both upstream and downstream of ataxia telangiectasia mutated and Rad3-related protein (ATR) to regulate the S-phase checkpoint following UV treatment. The Journal of biological chemistry. 2007; 282:22939–52. [PubMed: 17526493]

Owens P, Bazzi H, Engelking E, Han G, Christiano AM, Wang XJ. Smad4-dependent desmoglein-4 expression contributes to hair follicle integrity. Developmental biology. 2008; 322:156–66. [PubMed: 18692037]

Peinado H, Quintanilla M, Cano A. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. The Journal of biological chemistry. 2003; 278:21113–23. [PubMed: 12665527]

Perez-Mancera PA, Perez-Caro M, Gonzalez-Herrero I, Flores T, Orfao A, de Herreros AG, et al. Cancer development induced by graded expression of Snail in mice. Human molecular genetics. 2005; 14:3449–61. [PubMed: 16207734]

Qiao W, Li AG, Owens P, Xu X, Wang XJ, Deng CX. Hair follicle defects and squamous cell carcinoma formation in Smad4 conditional knockout mouse skin. Oncogene. 2006; 25:207–17. [PubMed: 16170355]

Sertic S, Pizzi S, Cloney R, Lehmann AR, Marini F, Plevani P, et al. Human exonuclease 1 connects nucleotide excision repair (NER) processing with checkpoint activation in response to UV irradiation. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108:13647–52. [PubMed: 21808022]

Strickland PT, Swartz RP. Inheritance of susceptibility to phototumorigenesis and persistent hyperplasia in F1 hybrids between SENCAR mice and BALB/c or C57BL/6 mice. Cancer research. 1987; 47:6294–6. [PubMed: 3677078]

Vassar R, Rosenberg M, Ross S, Tyner A, Fuchs E. Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. Proceedings of the National Academy of Sciences of the United States of America. 1989; 86:1563–7. [PubMed: 2466292]

Wagner KU, McAllister K, Ward T, Davis B, Wiseman R, Hennighausen L. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic research. 2001; 10:545–53. [PubMed: 11817542]

Wang X, Zinkel S, Polonsky K, Fuchs E. Transgenic studies with a keratin promoter-driven growth hormone transgene: prospects for gene therapy. Proceedings of the National Academy of Sciences of the United States of America. 1997; 94:219–26. [PubMed: 8990189]

Yang L, Mao C, Teng Y, Li W, Zhang J, Cheng X, et al. Targeted disruption of Smad4 in mouse epidermis results in failure of hair follicle cycling and formation of skin tumors. Cancer research. 2005; 65:8671–8. [PubMed: 16204035]

Yang X, Li C, Herrera PL, Deng CX. Generation of Smad4/Dpc4 conditional knockout mice. Genesis. 2002; 32:80–1. [PubMed: 11857783]

Zhou Z, Wang D, Wang XJ, Roop DR. In utero activation of K5.CrePR1 induces gene deletion. Genesis. 2002; 32:191–2. [PubMed: 11857819]

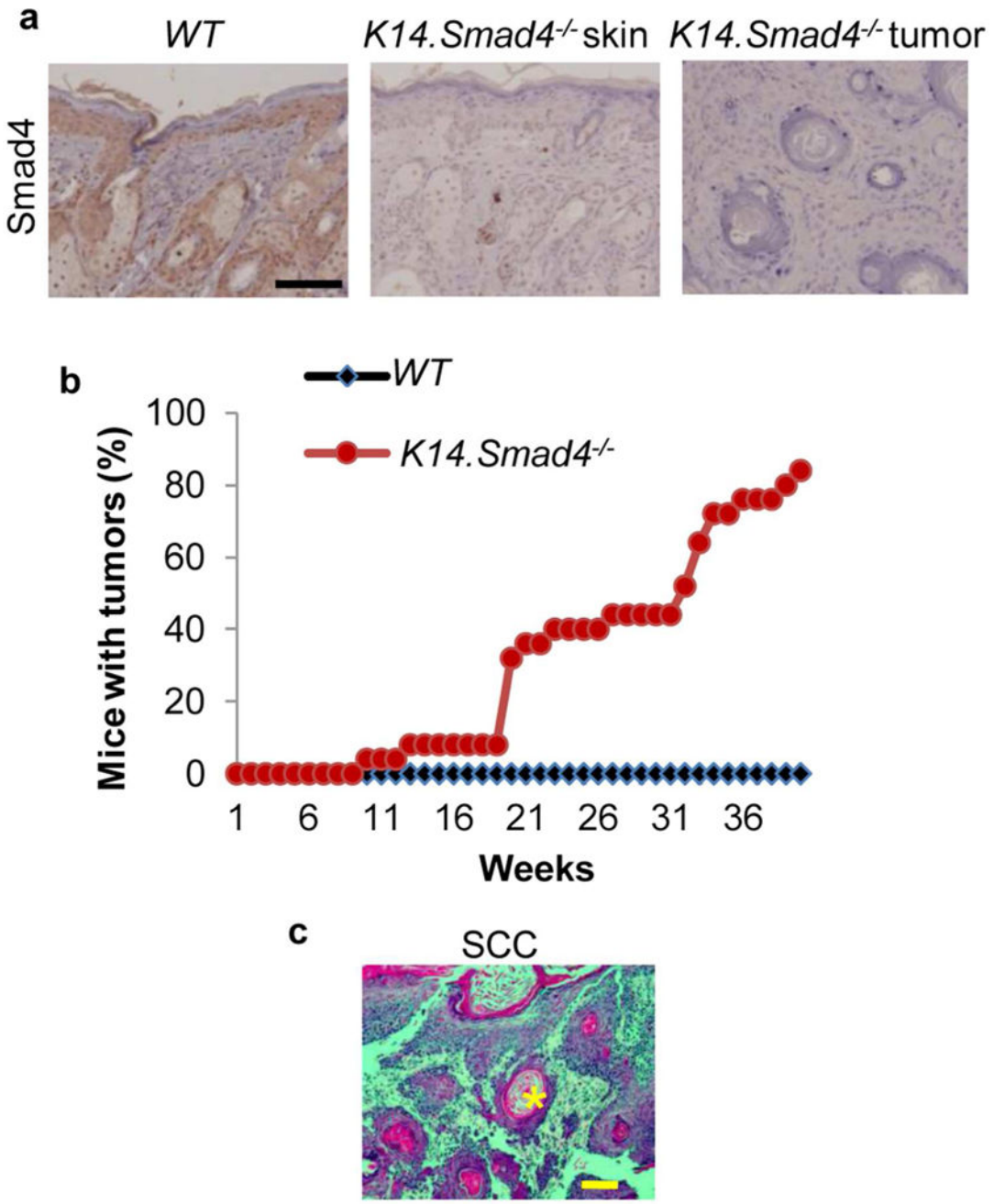

Figure 1. *Smad4*$^{-/-}$ mice developed UV-induced skin tumors

(a) IHC staining of Smad4 in skin samples from UV-irradiated *WT* and *Smad4*$^{-/-}$ mice. (b) Kinetics of tumor formation: data points represent mice that developed tumors, expressed as a percentage of mice with tumors among the total number of mice in that genotype group. (c) H&E staining showing a typical UV-induced *K14.Smad4*$^{-/-}$ SCC morphology. Keratin pearl is highlighted with an asterisk. Scale bars: 100μm.

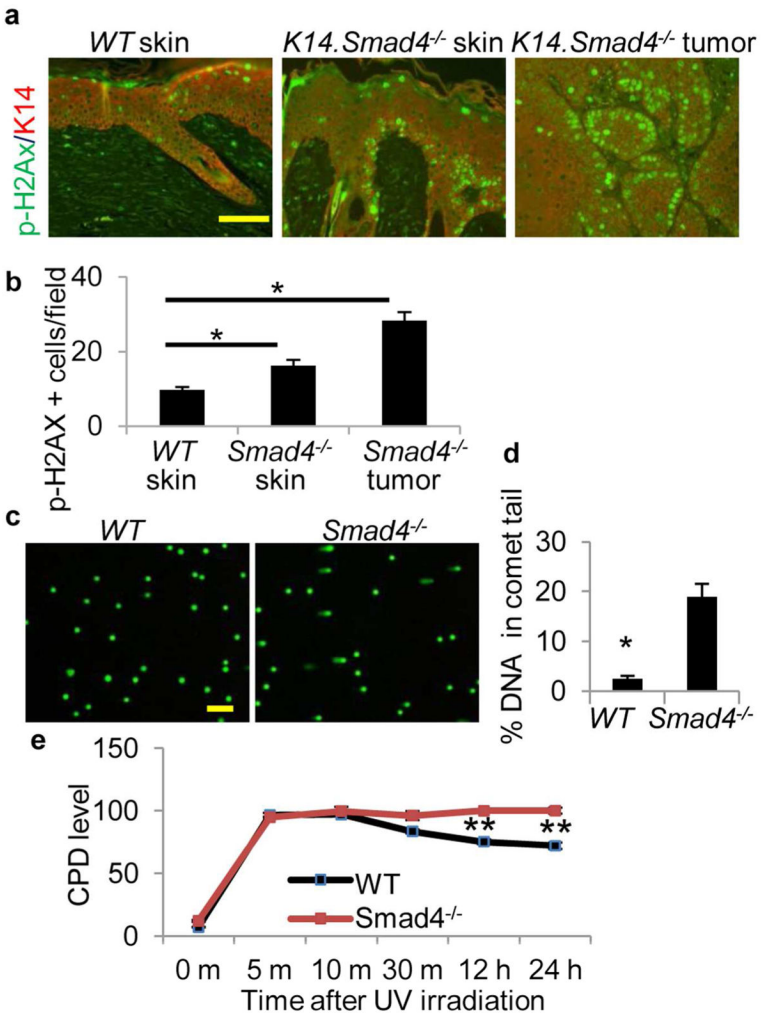

Figure 2. Increased DNA damage in UV-irradiated *K14.Smad4*$^{-/-}$ skin, and reduced repair of CPD in *Smad4*$^{-/-}$ keratinocytes

(a) Immunofluorescence staining of p-H2AX in UV-irradiated mouse skin and tumors. Keratin 14 was used as counterstain. Scale bar is 100μm. (b) Quantification of p-H2AX staining, n=6, 8 and 4 for *WT* skin, *Smad4*$^{-/-}$ skin, *Smad4*$^{-/-}$ tumors, respectively. (c) Alkaline comet assay using *WT* and *Smad4*$^{-/-}$ keratinocytes. (d) Quantification of % DNA in comet tail. n=44. (e) CPD levels in UVB-irradiated *WT* and *Smad4*$^{-/-}$ keratinocytes was measured by ELISA. CPD level in *Smad4*$^{-/-}$ keratinocytes at 10 minutes was set at 100%. P ≥ 0.1 at 5, 10 and 30 minutes, respectively. *denotes p ≤ 0.0002, and ** denotes p ≤ 0.003, n=3. Error bars are S.E.M. (all panels).

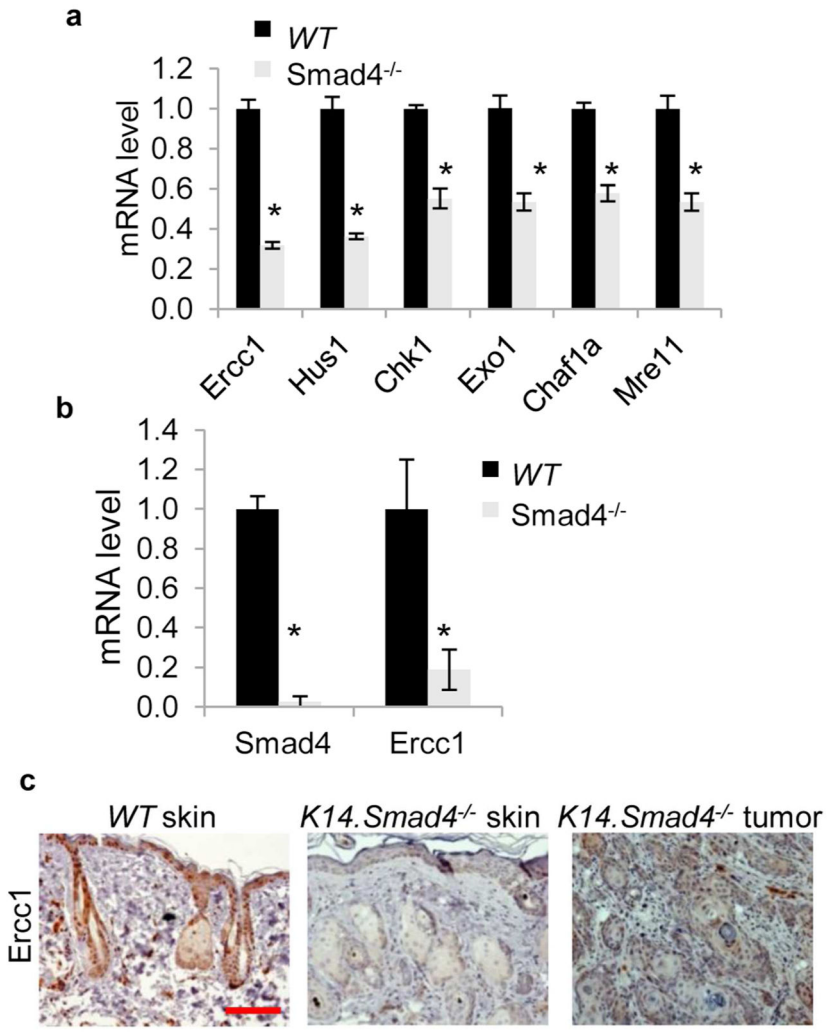

Figure 3. Reduced expression of DNA repair genes in *Smad4*$^{-/-}$ keratinocytes, reduced Ercc1 in *K14.Smad4*$^{-/-}$ skin and tumors

(a) qRT-PCR measured mRNA levels of indicated genes in *WT* and *Smad4*$^{-/-}$ keratinocytes. The mRNA level of each gene in *WT* keratinocytes was set at 1. * p≤ 0.002. Error bars are S.E.M., n=3. (b) mRNA levels of *Smad4* and *Ercc1* in UV-irradiated *WT* and *K14.Smad4*$^{-/-}$ skin. * p≤ 0.02. Error bars are S.E.M., n=3 for *WT* skin and n=7 for *Smad4*−/− skin. (c) Immunohistochemical staining of Ercc1 was performed on UV-irradiated *WT* and *K14.Smad4*$^{-/-}$ skin and tumors.

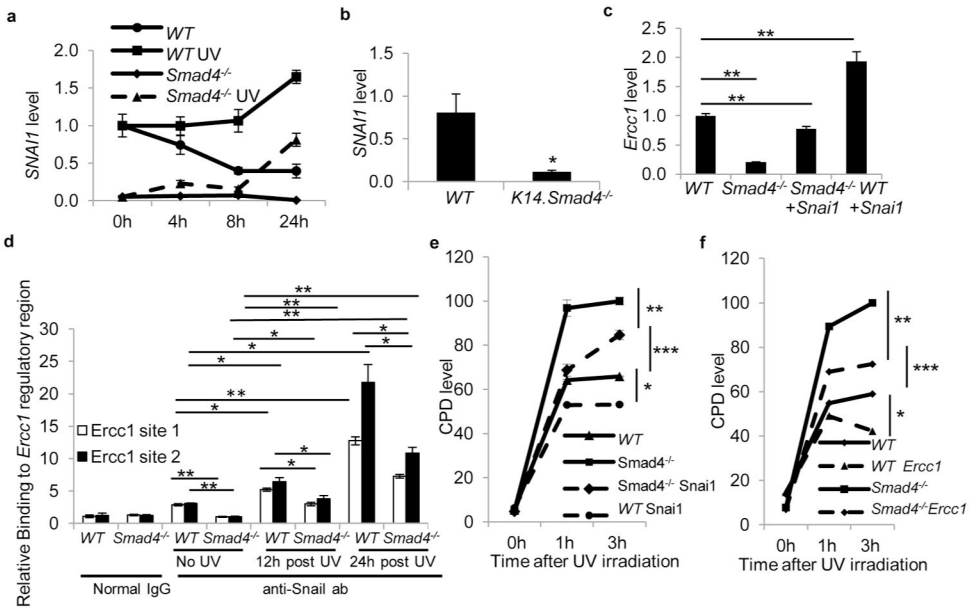

Figure 4. Snail regulates Ercc1 expression, Snail and Ercc1 mediate CPD repair in keratinocytes

(a) *Snai1* mRNA level in UV-irradiated keratinocytes, the level in untreated *WT* keratinocytes was set at 1. (b) *Snai1* mRNA level in UV-irradiated mice skin, the level in UV-irradiated *WT* skin was set at 1, n=4 for *WT*, n=7 for $Smad4^{-/-}$. * denotes p= 0.027 (c) *Ercc1* mRNA level in human *Snai1*-transfected keratinocytes, the level in empty vector transfected *WT* keratinocytes was set at 1. ** denotes p≤ 0.002. (d) Snail Chromatin Immunoprecipitation from *WT* and $Smad4^{-/-}$ keratinocyte extracts with/without UV irradiation. * p<0.03, ** p< 0.005. (e) CPD level in UVB-irradiated *Snai1* transfected keratinocytes *p = $5.09E^{-5}$, ** p=0.005, *** p=0.01. (f) CPD level in UVB-irradiated Ercc1 transfected keratinocytes, *=0.026, **=0.046, *** p=0.10, n=3. Error bars: S.E.M.

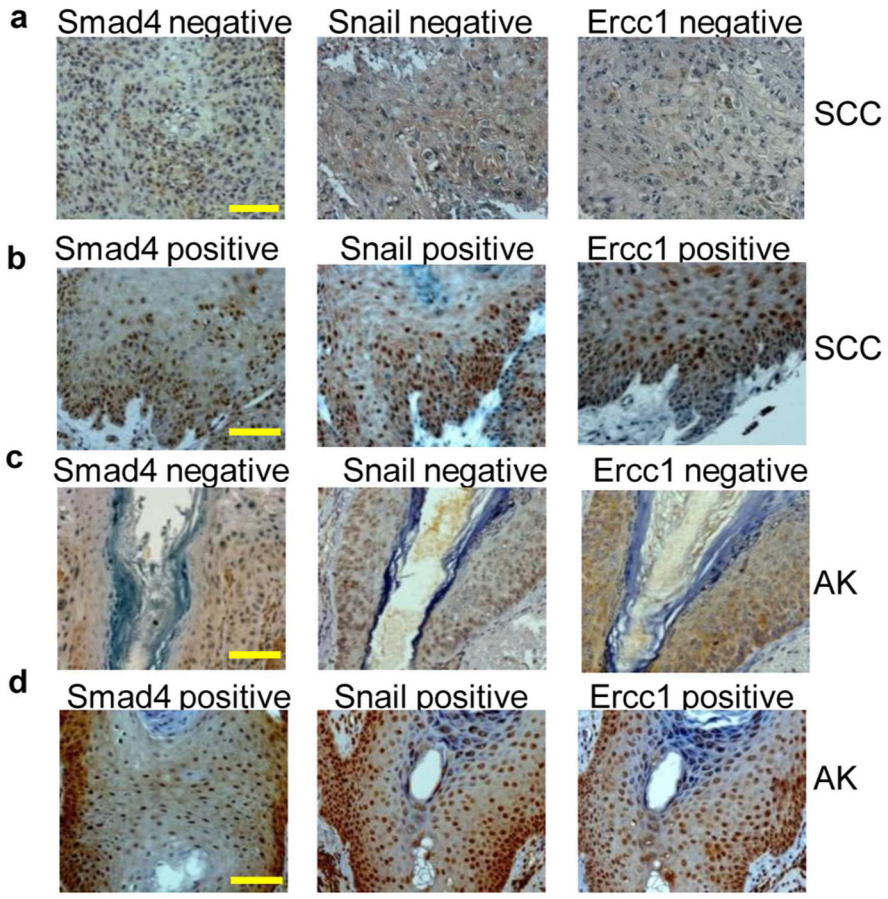

Figure 5. Expression of Smad4 is associated with expression of Snail and Ercc1 proteins in human skin SCC and AK

Smad4, Snail and Ercc1 were stained in a human skin SCC tissue array (a, b) and human actinic keratosis (c, d) Representative images of a Smad4, Snail and Ercc1 triple negative case (a, c) and triple positive case (b, d). Scale bar: 100μm for all panels.