Sox9 is a β-catenin-regulated transcription factor that enhances the colony-forming activity of squamous cell carcinoma cells

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Abstract. Squamous cell carcinoma (SCC) is a common skin cancer, of which the incidence is relatively high, ranking second among the non-melanoma skin cancers. It is known that numerous intracellular signal regulators are involved in the pathogenesis of SCC. The Wnt/β-catenin signaling pathway serves an important role in cancer development. However, the downstream effectors of β-catenin remain to be clearly elucidated yet. The present study investigated the functional importance of Wnt/β-catenin signaling in cutaneous SCC. β-catenin expression was reduced using recombinant adenovirus expressing specific microRNA (miR). Knockdown of β-catenin resulted in a marked reduction of the colony-forming activity of the SCC cells, SCC12. In an attempt to identify the β-catenin downstream genes, it was found that Sox9 was regulated by β-catenin in SCC12 cells. Overexpression of a constitutively active form of β-catenin led to the induction of Sox9, while knockdown of β-catenin resulted in downregulation of Sox9. When the expression of Sox9 was reduced using specific miR, colony-forming activity of the SCC12 cells was significantly reduced. When Sox9 was overexpressed in cells where β-catenin was knocked down, it partially restored the colony-forming potential. Taken together, the present results suggested that Sox9 is a β-catenin downstream transcription factor and is positively involved in SCC development.

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Introduction

Squamous cell carcinoma (SCC) is a common skin cancer originating from the upper layers of skin epidermis. The incidence of SCC is relatively high, ranking second among the non-melanoma skin cancers (1). Numerous factors are implicated in the pathogenesis of SCC, of which ultraviolet (UV) radiation is regarded as the most important environmental risk factor. UV radiation induces DNA damage and mutations of numerous susceptible genes, including the tumor suppressor, p53 (2). In addition, several intracellular signal regulators are involved in cancer development and progression. Examples include epidermal growth factor receptor, nuclear factor-κ-light-chain-enhancer of activated B cells and the Wnt/β-catenin signaling pathways (3-7).

In canonical Wnt/β-catenin signaling, binding of Wnt ligands to their cognate membrane receptors leads to the inactivation of the β-catenin degradation complex, resulting in the stabilization of cytoplasmic β-catenin. Once accumulated, β-catenin locates to the nucleus and interacts with the Lef-1/TCF family of DNA-binding proteins to generate a functional transcription factor complex (8). The pivotal role of Wnt/β-catenin signaling in cancer development has been previously described (9-11). The importance of β-catenin in skin cancer is supported by the fact that epidermis-specific ablation of the β-catenin gene results in the loss of cancer initiating cells and complete tumor regression (12). In addition, a previous genetic study showed that chromosome loci harboring Wnt signaling genes are frequently amplified in SCC (13), indicating a potential importance of Wnt/β-catenin signaling in cutaneous SCC.

Although the pivotal role of Wnt/β-catenin signaling in SCC is well recognized, the downstream effectors of β-catenin remain to be clearly elucidated. The present study identified Sox9 as a β-catenin-regulated gene and demonstrated that Sox9 has a potential role in the regulation of SCC cells.

Materials and methods

Cell culture. The SCC12 human squamous cell carcinoma cell line was provided by Professor Tae-Jin Yoon (Gyeongsang

National University, Jinju, South Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Human keratinocytes were isolated from skin specimens then immortalized using a recombinant retrovirus expressing Simian virus 40 large T antigen (SV40T), as described in a previous report (14). SV40T-transformed human epidermal keratinocytes (SV-HEK) were maintained in keratinocyte-serum free medium (Thermo Fisher Scientific, Inc.), supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Thermo Fisher Scientific, Inc.) (14). Human dermal fibroblasts were primary cultured and maintained in DMEM, supplemented with 10% FBS. Human melanocytes were cultured in Medium 254 (Cascade Biologics, Portland, OR, USA) and human melanocyte growth supplement (Cascade Biologics) (15,16). Cells were maintained at 37˚C in an atmosphere 5% CO_2 and 90% relative humidity.

Immunohistochemistry. All human skin samples were obtained under the written informed consent of donors, in accordance with the Ethical Committee approval process of the Institutional Review Board of Chungnam National University School of Medicine. Paraffin sections of skin specimens were dewaxed, rehydrated and washed three times with phosphate-buffered saline (PBS). The tissue sections were subsequently incubated with proteinase K (Dako, Carpinteria, CA, USA) for 5 min at 37˚C, and treated with H_2O_2 for 10 min at room temperature. The tissue sections were blocked in 0.1% Tween-20 and 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 30 min. Following blocking, the tissue sections were incubated at 4˚C with the appropriate primary antibodies as follows: Rabbit polyclonal anti-β-catenin (1:100 dilution; cat. no. sc-7199; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA); mouse monoclonal anti-Sox9 (1:200 dilution; cat. no. ab76997; Abcam, Cambridge, MA, USA) overnight. Subsequently, the tissue sections were incubated with the following peroxidase-conjugated secondary antibodies (dilution, 1:1,000): Horseradish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit immunoglobulin (Ig; cat. no. P0448) and HRP-conjugated polyclonal rabbit anti-mouse Ig (cat. no. P0161; both Dako), visualized using 3,3'-diaminobenzidine solution from the Chemmate envision detection kit (Dako) and photographed under a Diaphot inverted microscope (Nikon Corporation, Tokyo, Japan).

Western blot analysis. The cells were lysed in Proprep solution (Intron, Daejeon, Korea). The total protein was quantified using a bicinchoninic acid protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL, USA). The samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (100 V for 1 h) and transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Inc.). The membranes were then blocked using 5% skimmed milk in Tris-buffered saline and Tween-20 (Sigma-Aldrich) for 1 h at room temperature, and incubated (for 1 h at room temperature) with mouse monoclonal anti-Sox9 (1:200 dilution), rabbit polyclonal anti-β-catenin (1:100 dilution) and mouse monoclonal anti-β-actin [1:1,000 dilution (cat. no. A2228), Sigma-Aldrich] primary antibodies. Following primary antibody incubation, the membranes were incubated for 1 h at room temperature with the following secondary antibodies: Rabbit anti-mouse IgG (cat. no. ab97046) and goat anti-rabbit IgG (cat. no. .ab6721; both Abcam) and were visualized by enhanced chemiluminescence (Intron).

Production of recombinant adenovirus. The recombinant adenovirus expressing an 87-amino acid N-terminally truncated β-catenin (Ad/ΔN87-β-cat) or Sox9 (Ad/Sox9) were previously described (17,18). For knockdown experiments, the recombinant adenovirus expressing microRNA (miR) was generated. The target sequences for β-catenin and Sox9 were designed using BLOCK-iT™ RNAi Designer (Thermo Fisher Scientific, Inc.). The double-stranded DNA oligonucleotides were synthesized and cloned into the parental vector, pcDNA6.2-GW (Thermo Fisher Scientific, Inc.). In this vector system, the miR sequence for the target gene is located downstream of emerald green fluorescent protein (EmGFP) coding sequence, allowing the identification of miR expressing cells by observing GFP under an IX71 fluorescent microscope (Olympus Corporation, Tokyo, Japan). This vector was termed pcDNA6.2-GW/EmGFP-miR. The expression cassette for miR was moved into the pENT/CMV vector and then adenovirus was generated. The miR sequences were as follows: β-catenin, top strand: 5'-TGCTGTCTGCATGCCCTCATC TAATGGTTTTGGCCACTGACTGACCATTAGATGGGC ATGCAGA-3' and bottom strand: 5'-CCTGTCTGCATGCC CATCTAATGGTCAGTCAGTGGCCAAAACCATTAGAT GAGGGCATGCAGAC-3'; Sox9, top strand: 5'-TGCTGTGTT CTTGCTGGAGCCGTTGAGTTTTGGCCACTGACTGAC TCAACGGCCAGCAAGAACA-3' and bottom strand: 5'-CCT GTGTTCTTGCTGGCCGTTGAGTCAGTCAGTGGCCAA AACTCAACGGCTCCAGCAAGAACA-3'C. For adenovirus transduction, SCC12 cells were incubated with 10 multiplicity of infection of adenovirus for 6 h at 37˚C in an atmosphere 5% CO_2 and 90% relative humidity. The cells were replenished with fresh medium and incubated for a further 2 days.

Colony forming assay. The cells were trypsinized and counted using a hemocytometer. Following counting, ~1,000 cells were resuspended in DMEM, supplemented with 10% FBS, and were seeded into 100 mm culture dishes. The cells were incubated for 2-3 weeks and stained with crystal violet (Sigma-Alrdrich).

Statistical analysis. The data were statistically analyzed using one-way analysis of variance with SPSS software (v22.0; IBM SPSS, Chicago, IL, USA). $P<0.01$ was considered to indicate a statistically significant difference.

Results

In order to elucidate the importance of β-catenin in cutaneous SCC, the present study first determined the expression of β-catenin by immunohistochemistry. In normal skin, β-catenin immunoreactivity was observed in all epidermal layers, with a characteristic membrane staining pattern. In SCC, very intense immunostaining of β-catenin was observed, and notably, many of the SCC cells exhibited nuclear staining of β-catenin (Fig. 1A). These results suggested a fundamental role of β-catenin signaling in cutaneous SCC. The relative expression level of β-catenin in SCC12 cell line and skin-comprising

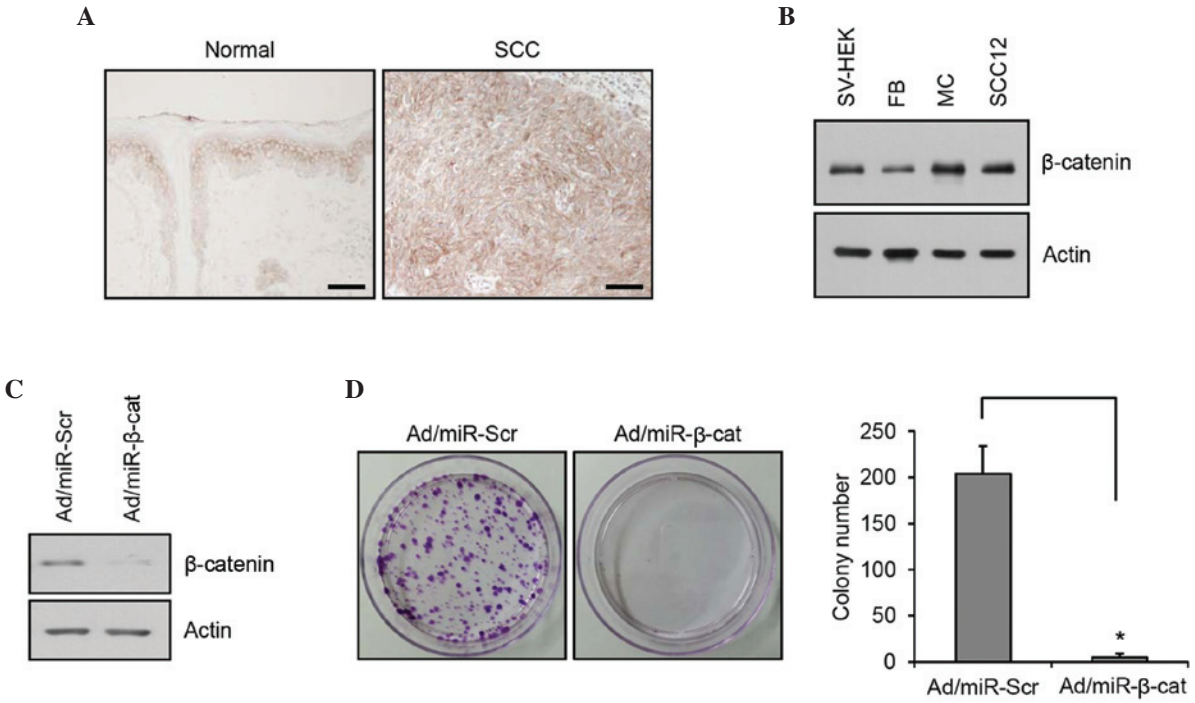

Figure 1. Expression and role of β-catenin in SCC. (A) Immunohistochemistry analysis of β-catenin expression in normal skin and cutaneous SCC. In SCC, intense immunoreactivity was observed in both the membrane and nuclei (scale bar, 100 μm). (B) The protein expression of β-catenin in cultured skin cells was determined by western blotting. Actin was used as a loading control. (C) The SCC12 cells were transduced with Ad/miR-β-cat. Western blotting revealed effective knockdown of β-catenin. Ad/miR-Scr was used for negative control and actin was used as a loading control. (D) A colony-forming assay was performed after Ad transduction. The SCC12 cells were cultured for 2 days, treated with trypsin/EDTA, re-suspended and seeded at a low concentration. The cells were cultured for 2 weeks and stained with crystal violet. Colonies were counted. The data are expressed as the mean± standard deviation (n=3; *P<0.01). SCC, squamous cell carcinoma; SV-sHEK, simian virus 40 large T antigen-transformed human epidermal keratinocytes; FB, dermal fibroblasts; MC, melanocytes; miR, microRNA; Ad, adenovirus; Scr, scrambled; β-cat, β-catenin.

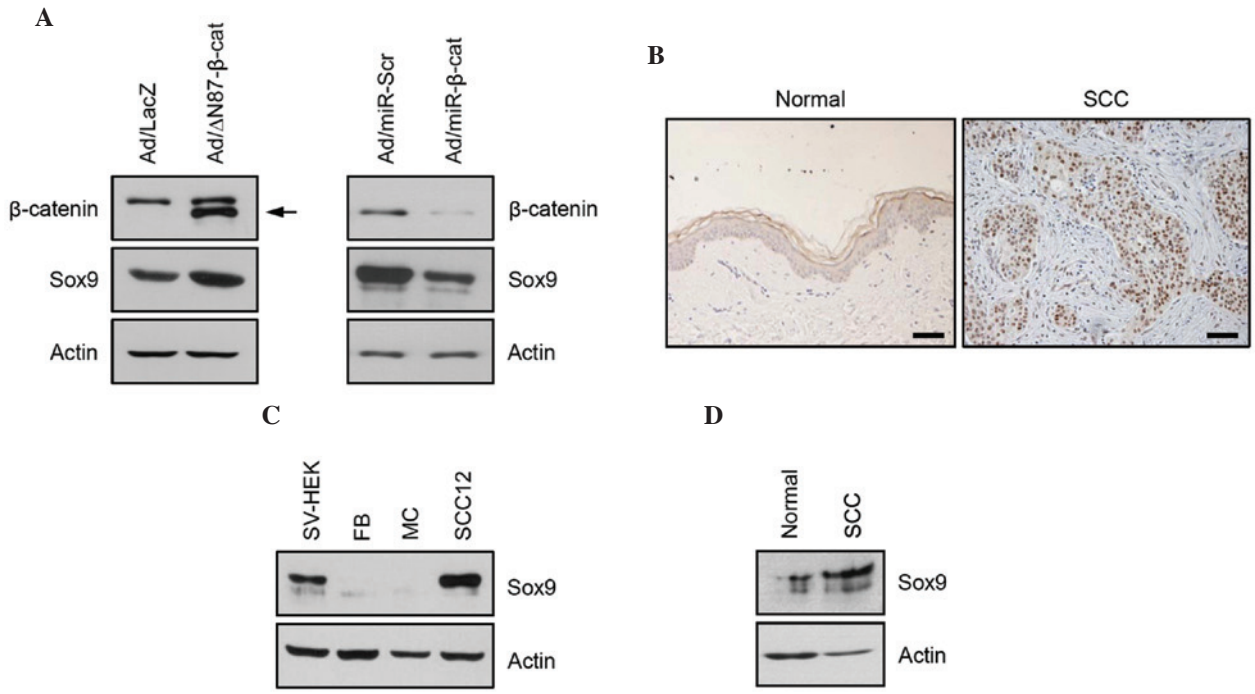

Figure 2. Identification of Sox9 as a β-catenin-regulated gene in SCC12 cells. (A) SCC12 cells were transduced with Ad expressing a constitutively active form of β-catenin (ΔN87) and/or miR specific for β-catenin. Exogenous N-terminal truncated β-catenin (arrow) was detected. Following the activation and/or repression of β-catenin, the expression of Sox9 was determined by western blotting. Ad expressing LacZ and/or miR-scr were used as negative controls. Actin was used as a loading control. (B) Immunohistochemical analysis of the expression of Sox9 in normal skin and cutaneous SCC tissues. In SCC, intense immunoreactivity is seen in the nucleus (scale bar, 100 μm). (C) The expression of Sox9 in normal skin tissue and SCC tissue was confirmed by western blotting. Actin was used as a loading control. (D) The expression levels of Sox9 in cultured skin cells were confirmed by western blotting. Actin was used as a loading control. Ad, adenovirus; miR, microRNA; scr, scrambled; SCC, squamous cell carcinoma; SV-sHEK, simian virus 40 large T antigen-transformed human epidermal keratinocytes; FB, dermal fibroblasts; MC, melanocytes.

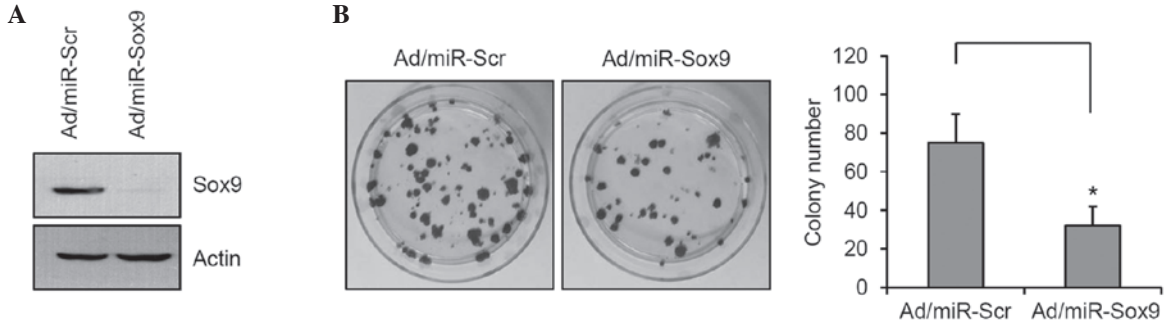

Figure 3. Decreased colony-forming activity in SCC1 cells as a result of Sox9. (A) SCC12 cells were transduced with Ad expressing miR-Sox9. Western blotting demonstrated effective knockdown of Sox9. (B) Following Ad transduction, a colony-forming assay was performed. Knockdown of Sox9 decreased colony-forming activity of SCC12 cells. Colonies were counted. The data are expressed as the mean± standard deviation (n=3; *P<0.01). Ad, adenovirus; miR, microRNA.

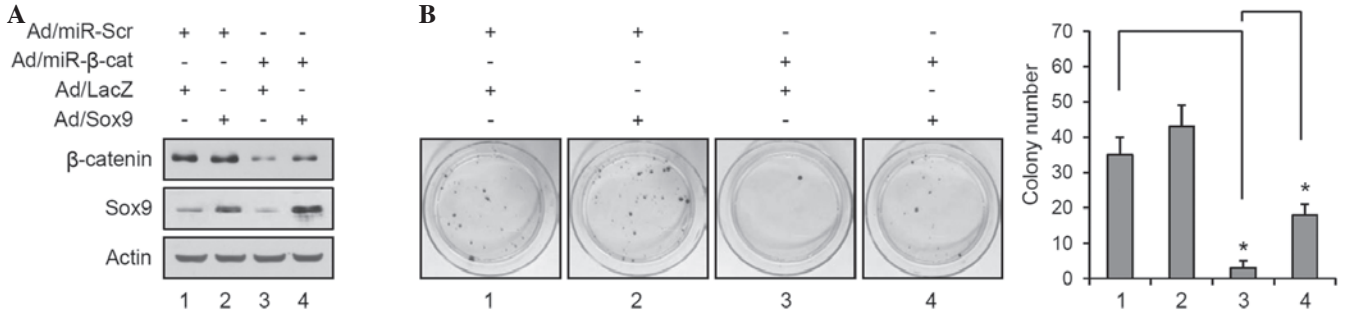

Figure 4. Restoration of colony-forming activity of β-cat-knockdown cells by Sox9. (A) SCC12 cells were co-transduced with Ad expressing miR specific for β-cat and Sox9-overexpressing adenovirus. The expression levels of β-cat and Sox9 were detected by western blotting. (B) Following Ad transduction, colony-forming assays were performed. Overexpression of Sox9 restored the colony-forming activity of β-cat-knockdown cells. The colonies were counted. The data are expressed as the mean ± standard deviation (n=3; *P<0.01). Ad, adenovirus; miR, microRNA; β-cat, β-catenin; Scr, scrambled.

cells, including SV-HEK, fibroblasts and melanocytes, was determined. Although it was not significant, the total β-catenin level was marginally increased in the SCC12 cells compared with the keratinocytes (Fig. 1B). To confirm the potential role of β-catenin, clonogenic assays were performed as an *in vitro* tumorigenic test (19). Following transduction of recombinant adenovirus expressing miR specific for β-catenin, gene knockdown was confirmed (Fig. 1C). Knockdown of β-catenin markedly reduced the colony-forming potential of SCC12 cells (Fig. 1D), supporting the pivotal role of β-catenin in the tumorigenesis of SCC.

The present study next attempted to identify the genes regulated by β-catenin. To this end, the present study transduced the recombinant adenovirus expressing N-terminal 87-amino acid truncated β-catenin (Ad/ΔN87-β-cat), a constitutively active form of β-catenin (20). It was found that Sox9 was induced by the overexpression of the constitutively active form of β-catenin. Conversely, knockdown of β-catenin resulted in downregulation of Sox9 (Fig. 2A). These results suggested that Sox9 is a β-catenin-regulated gene in SCC cells and has a potential role in tumorigenesis. Therefore, the expression of Sox9 was next determined in cutaneous SCC. Immunohistochemistry and western blotting showed that the expression of Sox9 was increased in cutaneous SCC tissue compared with normal skin tissue (Fig. 2B and C). Consistent with these data, the relative protein expression level of Sox9 was significantly increased in SCC12 cells compared with keratinocytes cultured *in vitro* (Fig. 2D).

The expression of Sox9 was reduced in SCC12 cells using the recombinant adenovirus expressing miR specific for Sox9 (Fig. 3A). The colony-forming potential of SCC12 cells was determined, and the data showed that knockdown of Sox9 significantly decreased the colony number (Fig. 3B). These results suggested that Sox9 is a potential transcription factor that can increase tumorigenicity of SCC cells.

To further confirm the association between β-catenin and Sox9, β-catenin levels were reduced and the expression of Sox9 was simultaneously increased using the recombinant adenovirus (Fig. 4A). Overexpression of Sox9 led to a slight increase of colony number in the control miR transduced group, however, it was not significant (lane 1 vs. 2; Fig. 4B). Consistent with previous data, knockdown of β-catenin resulted in a marked decrease of colony formation; however overexpression of Sox9 partially restored the colony-forming potential (lane 3 vs. 4; Fig. 4B). These results strengthened the notion that Sox9 is a β-catenin-downstream transcription factor and is involved in the development of SCC.

Discussion

In the present study, it was found that Sox9 was regulated by β-catenin in SCC12 cells. When β-catenin signaling was activated by the overexpression of stabilized β-catenin (ΔN87-β-Cat), Sox9 expression was increased. By contrast, knockdown of β-catenin using specific miR resulted in the downregulation of Sox9. Sox9 demonstrated tumorigenicity in

terms of colony-forming activity . Therefore, the present data suggested that β-catenin-regulated Sox9 is one of the effector molecules that can positively affect the development of SCC.

Sox9 is a member of high mobility group box transcription factor family and serves a critical role in various biological events, including embryonic development, cell fate determination and lineage commitment (21,22). Germline mutation for Sox9 causes campomelic dysplasia, a disorder characterized by numerous skeletal abnormalities, defects in central nervous system and XY sex reversal (23,24). A potential role of Sox9 for cancer development has been previously recognized. For example, Sox9 supports breast tumor cell proliferation and directly contributes to the poor clinical outcomes associated with invasive breast cancer (25,26). In other examples, high levels of Sox9 were detected in colorectal cancer and Sox9 exhibits several properties, including the ability to promote proliferation, inhibit senescence and collaborate with other oncogenes in neoplastic transformation (27,28). Based on these findings, it is plausible that Sox9 can also affect cutaneous SCC development. The present study demonstrated that Sox9 was increased in cutaneous SCC, and that Sox9 enhanced the colony-forming activity of SCC12 cells. These data supported the notion that Sox9 is an important pro-oncogenic protein in SCC development.

The putative association between β-catenin and Sox9 has been demonstrated several times in other systems. For example, the Wnt signaling pathway promotes chondrocyte differentiation in a Sox9-dependent manner (29). Another example shows that Sox9 protein is expressed in the intestinal epithelium in a pattern characteristic of Wnt/β-catenin targets. Additionally, inhibition of β-catenin signaling by the overexpression of dominant negative TCF4 resulted in a marked decrease of Sox9 in human colon carcinoma cells (30). Finally, conditional inactivation of adenomatous polyposis coli, thereby activating Wnt/β-catenin signaling in mouse colon, is associated with the induction of Sox9 expression and initiation of crypt budding (31). In the present study, activation and inactivation of β-catenin signaling clearly affected Sox9 expression in a positive manner. Therefore, the present study suggested that Sox9 is an authentic β-catenin-downstream transcription factor and exerts its effect as a positive regulator for cutaneous SCC.

In conclusion, the present study demonstrated that Sox9 is a functional downstream effector of β-catenin in cutaneous SCC. These findings provided novel insights into the association between β-catenin and Sox9 in SCC, and may assist with developing novel therapeutic targets for skin cancer in the future.

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