Effect of transgenic overexpression of BMP antagonist noggin on chemically-induced skin carcinogenesis

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Abstract. To study the role of BMP signaling pathway in the development of skin tumors, we employed two-stage chemical carcinogenesis protocol on K14-Noggin mice expressing BMP antagonist noggin in the epidermis. A comparative analysis showed an earlier appearance and a significant increase in the number of skin papillomas in K14-Noggin mice compared to the wild-type control (FVB). In contrast to control mice, the transgenic K14-Noggin mice also developed squamous cell carcinoma with local metastasis of malignant cells into the dermis of the skin. The results of this work indicate the tumor suppressive role of BMP signaling pathway in the skin epithelium.

1. Introduction
The skin is the largest organ in the body and is essential for the survival of all vertebrates. Skin protects the body from water loss, temperature change, radiation, trauma, and infections, and it allows the body to perceive the environment through tactile sense. Bone morphogenetic proteins (BMPs) and their antagonists, such as noggin, play an essential role in skin development, homeostasis and regeneration by regulating cell proliferation, differentiation and apoptosis [1-4]. Recent studies also showed that inhibition of BMP signalling pathway in the skin epithelium stimulates wound healing, but also leads to the development of benign hair follicle-derived tumours [5-6]. However, the role of BMP pathway in chemically induced skin carcinogenesis remains unclear. Here, we investigate the effect of BMP inhibition on the development of skin tumours in mice using a cutaneous two-stage chemical carcinogenesis protocol [7].

2. Research methods
All animal works were performed under the approval of PPL 40/2989 license at the University of Bradford.
Bradford. Mice had free access to food (standard rodent diet) and tap water. Transgenic (TG) K14-Noggin mice used in this study were provided by Dr. P Overbeek (Baylor College of Medicine, USA). These TG mice were generated on FVB background using transgenic construct, which drives expression of mouse noggin gene under the control of human K14 promoter. FVB mice served as a control wild-type (WT) group and were purchased from Charles River (United Kingdom).

For chemical carcinogenesis, skin tumours were induced using a carcinogen 7,12-dimethylbenz[a]anthracen (DMBA) (Sigma-Aldrich) and a tumour promoter 12-tetradecanoin-phorbol-13-acetate (TPA) (Sigma-Aldrich). Back skin of 8-week-old female TG and WT mice (n=5 for each mouse strain) was shaved and treated with a single dose of DMBA (250 µg/ml) followed by twice weekly application of TPA (40 µg/ml) for 20 weeks. Tumour progression was observed up to 25 weeks. Skin samples were collected from TG and WT mice, snap-frozen in liquid nitrogen and embedded into Tissue-Tek (Sakura, USA). Skin cryosections were processed for morphological and immunofluorescent detection of cell proliferation and differentiation markers. For the morphological analysis of skin sections histochemical staining with hematoxylin together with detection of alkaline phosphatase (H&AP) activity were performed, as previously published [8].

Briefly, cryosections (9 µm) were fixed in 4% solution of paraformaldehyde (PFA) for 10 minutes at room temperature (RT), and incubated in developing solution (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM MgCl2, 0.005% Naphtol ASBI phosphate, 0.5% DMF) for 15 mins. Sections were counterstained with haematoxylin.

Immunofluorescent analyses were performed as previously described [9]. Briefly, cryosections (9 µm) were fixed in 4% PFA (10 mins at RT). Sections were initially preincubated in 5% bovine serum albumin (BSA)/0.1% Triton X100 to block non-specific binding, following by overnight incubation with primary antibodies at +4°C. The following antibodies were used: rabbit anti-Ki67 (DAKO, 1:1000 dilution) and rabbit anti-Loricrin (R&D systems, 1:150 dilution). Sections were then incubated with TRITC-labelled secondary antibody (Jackson ImmunoResearch) for 1 hour at +37°C. Cell nuclei were visualized with 4’6-Diamidino-2- phenylindol (DAPI).

Image preparation and analyses were performed by using bright-field and fluorescent microscope (Nikon), in combination with SPOT digital camera and image analysis software (Diagnostic Instruments).

3. Results
K14-Noggin mice showed a marked increase in epidermal thickness in the interfollicular epidermis (IFE). A morphometric analysis revealed a two-fold increase in epidermal thickness in the TG mice compared to WT mice (32.4 um versus 15.2 um, p<0.01) (figure 1 A-C).

To further characterise the observed epidermal hyperplastic changes, we analysed the expression of markers of proliferation and differentiation. Immunofluorescent detection of the Ki67 protein, which accumulates in actively proliferating cells, revealed a marked increase in the number of Ki67-positive cells in K14-Noggin epidermis versus WT mice (figure 1D-F). Quantitative analysis showed at least a two-fold increase in a number of Ki67-positive cells in the basal layer of TG skin compared to WT mice (figure 1D). Moreover, Ki67 expression was also seen in some suprabasal keratinocytes in the TG epidermis (figure 1F, arrows).

In WT epidermis, expression of loricrin, a marker of terminally differentiated keratinocytes, was detected in the outermost layers of the epidermis (figure 1G). Similar pattern of the loricrin expression was observed in TG skin (figure 1H). These data suggest that the process of keratinocytes differentiation was not affected by overexpression of Noggin in the skin. Instead, Noggin-induced BMP suppression resulted in the stimulation of cell proliferation in the IFE.
Figure 1. Hyperplastic changes in the K14-Noggin epidermis. (A, B) H&AP staining shows increased thickness of the epidermis in TG skin (B) compared to WT (A) (arrow indicates an area of dysplasia); (C) Epidermal thickness in TG and WT skin; (D) Number of Ki67-positive cells in the basal layer of WT and TG epidermis; (E, F) Ki67 expression in the epidermis. Note increased Ki67-positive cells in TG skin (F), compared to WT mice (E) (arrows indicate Ki67-positive suprabasal cells); (G, H) Loricrin expression in WT (G) and TG (H) epidermis. Scale bars, 50 um.
Despite the epidermal hyperplasia, no spontaneous tumours arising from IFE were detected in K14-Noggin mice during year of observation. To investigate if the skin of K14-Noggin mice is more susceptible to tumour development, we induced skin tumours by topical applications of skin carcinogen DMBA and TPA. A single dose of DMBA application followed by twice-weekly TPA treatment for 20 weeks resulted in development of skin papillomas in both WT and TG skin (figure 2A and 2B). However, in TG skin the first tumours emerged as early as 6 weeks after DMBA treatment. In contrast, WT mice developed papillomas much later by 11 weeks of the treatment (figure 2C).

![Image of skin tumours](image)

Figure 2. Chemically induced tumours in K14-Noggin skin (A, B) Papilloma development in WT (A) and TG (B) skin; (C) The tumours emerged much earlier and a greater amount in the TG skin compared to WT mice; (D) Squamous cell carcinoma (arrows) in K14-Noggin mice after application of the DMBA/TPA protocol [note numerous foci of invasion into the dermis (arrowheads)]. Scale bar, 200 um.

Moreover, weekly monitoring of mice revealed a marked difference in the number of tumours between TG and WT mice; there was a 5-fold increase in the total number of skin tumours in TG compared to WT mice by the end of the experiment (figure 2C). Histological examination of skin tumours in WT mice did not show any signs of malignancy. In contrast, atypical cells with abnormal keratinization were observed in the tumours of TG mice suggesting the development of squamous cell carcinoma (SCC) with metastases of malignant cells into the dermis (figure 2D).
4. Conclusion
Thus, inhibition of the BMP signalling leads to epidermal hyperplastic changes with increased cell proliferation. Furthermore, noggin overexpression greatly enhances the skin susceptibility to chemical carcinogenesis and promotes malignant transformation of benign tumours into SCCs.

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