# Slug (Snai2) Expression during Skin and Hair Follicle Development

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## TO THE EDITOR

Our previous investigations showed that Slug (Snai2), a member of the Snail family of developmental transcription factors, is expressed in unperturbed adult murine epidermis, where it regulates a wide variety of gene targets (Newkirk *et al.*, 2008b). Slug expression is induced by a number of growth factors and environmental stimuli (Hudson *et al.*, 2007; Kusewitt *et al.*, 2009). Slug enhances cutaneous wound reepithelialization, skin tumor progression, and the sunburn response (Savagner *et al.*, 2005; Newkirk *et al.*, 2007, 2008a; Hudson *et al.*, 2009). The present studies document Slug expression in embryonic and neonatal epidermis and hair follicles, and significant alterations in hair growth kinetics in Slug knockout mice during the first postnatal hair cycle, indicating a further contribution of Slug to the maintenance of skin homeostasis.

Microwave antigen retrieval was performed in citrate buffer on deparaffinized E10–E17 CD1 mouse embryo sections (Zyagen, San Diego, CA). Slides were incubated overnight at 4 °C in monoclonal rabbit anti-Slug antibody (#9585, Cell Signaling, Danvers, MA) diluted 1:50, treated with Biocare Rabbit on Rodent HRP-Polymer (Biocare Medical, Concord, CA) and 3,3′-diaminobenzidine chromagen, then counterstained with hematoxylin. Of the many anti-Slug antibodies we have tested, only this antibody consistently gives single bands of appropriate size on protein isolated from HaCaT human epidermal cells (data not shown). Keratin 14 immunostaining was performed similarly, applying first the primary antibody (Covance, Princeton, NJ) at a dilution of 1:500 for 30 minutes at room temperature, and then Envision Plus labeled polymer anti-rabbit-HRP (DAKO, Carpinteria, CA) for 30 minutes.

In embryonic tissues, Slug staining was exclusively nuclear. On E10 and E11, Slug was expressed in scattered cells of the single-layered epidermis and in most of the underlying primitive mesenchymal cells, a time when keratin 14 immunoreactivity was seen in only a few cells (Figure 1). At the beginning of periderm formation at E12, when strong keratin 14 immunoreactivity was first observed, Slug was expressed in essentially all skin epithelial cells and in the underlying mesenchyme. As the epithelium underwent stratification, Slug and keratin 14 expression was progressively confined to basal keratinocytes, with Slug localization occurring earlier. Slug staining was observed in progressively fewer dermal cells as primitive mesenchymal cells matured. However, a substantial number of dermal cells continued to express Slug. In the placode, hair germ, and peg stages of hair follicle development, Slug expression was prominent in the thickened and invaginating epithelium, but was absent from the underlying mesenchymal cells that ultimately form the dermal papillae (Figure 1).

In pigmented Slug-lacZ 129 mice, the Slug locus was inactivated by an in-frame insertion of the *β-galactosidase* gene into the zinc finger coding region of the *Slug* gene (Jiang *et al.*, 1998). Mice homozygous for the Slug-lacZ allele are functional Slug knockout animals, but heterozygous Slug knockout mice are phenotypically normal. Daily examination of newborn mice (Figure 2a) revealed that darkening of the skin occurred in all wild-type and heterozygous Slug knockout mice by postnatal days 2–3; however, darkening of the skin of Slug knockout neonates was not seen until postnatal days 4–7. In wild-type and heterozygous knockout mice, hair emergence occurred on postnatal days 5–7, but in knockout mice the emergence was delayed until days 8–10. Differences between knockout and wild-type/heterozygous mice were highly significant for both skin darkening and hair emergence ($P<10^{-7}$ using the log-rank statistic). Skin darkening in pigmented neonatal mice occurs during early hair follicle growth and hair shafts emerge from follicles during mid to late follicle maturation (Muller-Rover *et al.*, 2001). Thus, our findings suggest delayed hair follicle development in neonatal Slug knockout mice.

Immunohistochemical analysis for Slug in 129 wild-type mice showed that Slug was expressed in many, but not all, basal keratinocytes at birth. As previously reported for adult epidermis (Parent *et al.*, 2004), Slug-expressing keratinocytes were clustered around hair follicles. In developing follicles, Slug was stably expressed in the developing external root sheath, hair matrix cells, and some mesenchymal cells of the dermal papilla (Figure 2b). Most interfollicular epidermal cells were Slug-positive at 3 days after birth, but staining progressively declined. At 18 days after birth, follicles in catagen expressed little or no Slug, and Slug expression was also absent from the interfollicular epidermis. Early telogen follicles and interfollicular epidermis showed no Slug immunoreactivity. Localization of Slug was confirmed by immunohistochemical analysis for

*Abbreviation: DAB, 3,3′-diaminobenzidine*

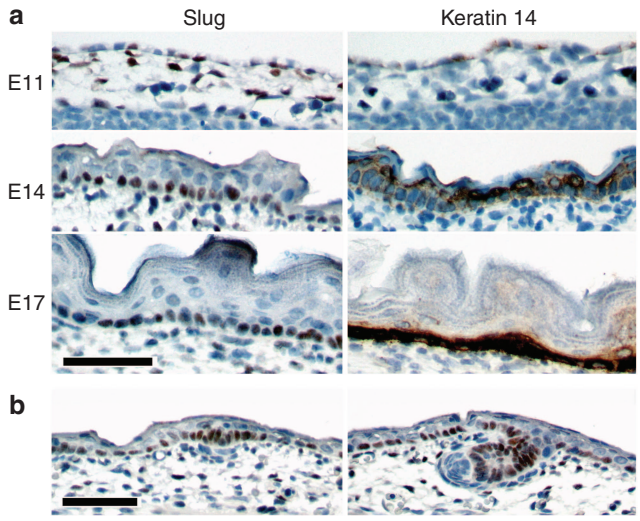


**Figure 1. Expression of Slug in embryonic epidermis and hair follicles.** Slug was localized to the nuclei of epithelial cells in the developing epidermis and hair follicles of the embryo. (**a**) Both Slug and keratin 14 expression in the developing epidermis was progressively restricted to basal keratinocytes (bar = 50 μm). (**b**) Slug was prominently expressed in placodes and hair pegs of the developing hair follicle on E14. Note that Slug was not expressed in mesenchymal precursors of the dermal papilla (bar = 50 μm).

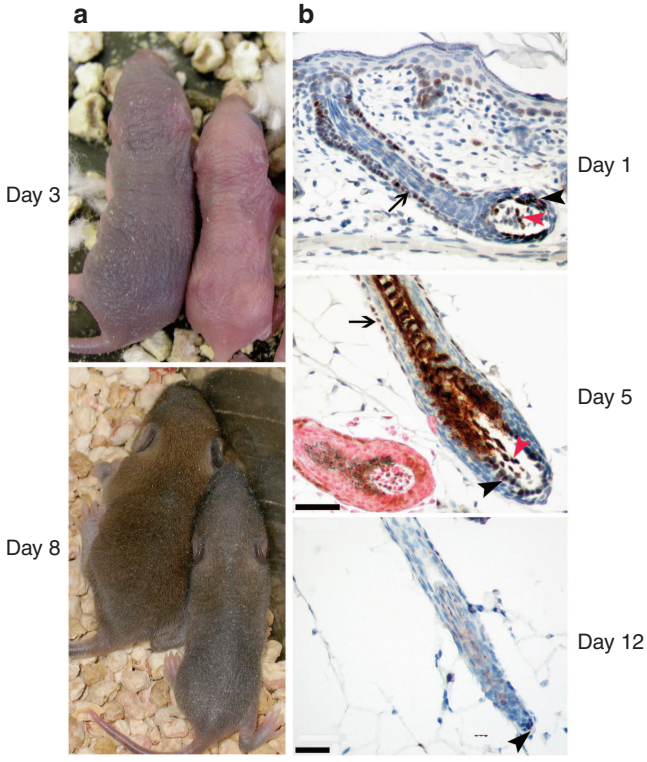


**Figure 2. Slug expression in the neonate.** (**a**) The upper panel shows 3-day-old littermates. The skin of the wild-type or Slug-lacZ heterozygote on the left had already darkened, whereas that of the Slug knockout on the right remained unpigmented. In the lower panel, 8-day-old littermates are shown. Although the wild-type or Slug-lacZ heterozygote on the left was fully haired, hair had emerged only in the shoulder region of the Slug knockout. (**b**) Throughout follicle development and into very early catagen, Slug expression was evident in the basal layer of the interfollicular epithelium and the external root sheath (arrows), which is continuous with the basal epithelium, in hair matrix cells (black arrowheads), and in mesenchymal cells of the dermal papilla (red arrowheads). Slug staining in cells of the hair matrix and dermal papilla, which was exclusively nuclear, can readily be distinguished from melanin pigment located in the cytoplasm in the middle panel. The inset shows a similar pattern as revealed by staining for β-galactosidase in a Slug-lacZ-expressing mouse (bar = 50 μm). During catagen, shown in the lower panel, Slug expression disappeared from the regressing hair bulb (black arrowhead) and external root sheath (bar = 40 μm).

Slug in tissues of mice expressing the Slug–β-galactosidase fusion protein (Figure 2b, inset). Staining was performed on frozen sections using an affinity-purified rabbit anti-β-galactosidase (#RGAL-45, Immunology Consultants Laboratory, Newberg, OR) as recommended by the supplier.

Immunohistochemical and *in situ* hybridization studies by Jamora *et al.* (2005) indicated that expression of the closely related Snail family transcription factor Snail (Snai1) in the epidermis is limited to hair bud cells during the period E15.5 days to birth. No staining of mesodermal skin cells was reported by these investigators. In contrast, Franci *et al.* (2006) detected Snail expression only in mesenchymal cells of the skin, particularly in the mesenchymal cells clustered below the hair buds of embryonic skin and in the dermal papillae of adult hair follicles. The discrepancy between the two studies has not been resolved; however, it does appear that the patterns of expression of Slug and Snail in the epidermis differ substantially.

The pelage of adult Slug knockout animals appeared normal; thus, Slug was not required for hair follicle development and maintenance. However, Slug knockout mice showed significantly delayed onset of postnatal hair growth, and Slug expression was modulated temporally and spatially in embryonic and postnatal hair follicles. These findings indicate a modest but distinct role for Slug in the hair cycle.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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# Effect of Aging on DNA Excision/Synthesis Repair Capacities of Human Skin Fibroblasts

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## TO THE EDITOR

A key factor in the skin aging process is the cumulative effects of chronological aging and environmental-based assaults. Endogenous cellular oxidative processes generate reactive oxygen species and reactive polyunsaturated fatty acid derivatives (Lindahl, 1993; Marnett and Plastaras, 2001). These attacks on DNA cause substantial base and sugar damage, and the persistence of such lesions leads to mutations and genome instability. Skin may also suffer from chronic exposure to sun; UV radiation causes oxidative DNA damage and induces photoproducts (mainly cyclobutane pyrimidine dimers and 6-4 photoproducts (Moriwaki and Takahashi, 2008)). Age-related accumulation of somatic damage can thus be worsened by sun exposure, leading to an increased incidence of skin disorders and dramatic acceleration of skin aging (Niedernhofer, 2008).

Mammalian cells have evolved several DNA-repair pathways to remove all the categories of DNA base lesions, relying in particular on DNA excision mechanisms. One of these, nucleotide excision repair, removes bulky adducts and is thus an essential mechanism for correcting UV-induced DNA damage (Sarasin, 1999). The base excision repair pathway corrects small base modifications such as oxidized and alkylated bases (Almeida and Sobol, 2007).

The importance of repair mechanisms is demonstrated by the hazardous consequences of genetic defects in DNA repair (Friedberg, 2001), but investigating DNA repair with respect to aging remains a challenge. This is due to the complexity of the underlying repair mechanisms as well as to the varying approaches in terms of assays and end points measured (Vijg, 2008).

To better understand the relationship between aging and DNA repair, we took advantage of our newly developed multiplexed excision/synthesis assay (Millau *et al.*, 2008) to examine simultaneously, using nuclear extracts, the base excision repair and nucleotide excision repair capacities of human primary fibroblasts derived from healthy donors of different ages. In addition, we investigated changes in DNA repair attributed to chronic sun exposure.

A total of 33 healthy Caucasian women were recruited by the Dermscan Group (Lyon, France). Biopsy removal was performed in accordance with the Declaration of Helsinki Principles Guidelines after approval for the study had been given by a medical ethics committee and written consent obtained from the donors. The volunteers were classified into three groups by age (group 1: mean age = 25 years, range 20–33, $n = 9$; group 2: mean age = 46 years, range 40–50, $n = 9$; group 3: mean age = 65 years, range 61–68, $n = 15$). All subjects were nonsmokers, had phototype II or III skin, declared no excessive exposure to sun or UVA, had no cutaneous pathology, and were not receiving medical treatment. Fibroblast cultures were established from outgrowth of two 3 mm punches taken on the volar forearm (photoexposed area) and the upper inner arm (photoprotected area). Cells were harvested during the exponential phase of growth and stored frozen in liquid nitrogen at passage 5. Nuclear extracts were prepared as described by Millau *et al.* (2008). For each sample, excision/synthesis repair reactions were run for 2.5 hours at 30 °C at a final protein concentration of 0.15 mg ml$^{-1}$, along