Krt6a-Cre Transgenic Mice Direct *LoxP*-Mediated Recombination to the Companion Cell Layer of the Hair Follicle and Following Induction by Retinoic Acid to the Interfollicular Epidermis

To the Editor:

The keratins are a diverse group of structural proteins, which contribute to the intermediate filament cytoskeleton and are important in the maintenance of epithelial structure and integrity (Fuchs and Cleveland, 1998). Whereas the keratins are structurally similar, they play a diverse role in many tissues and their expression patterns are often uniquely restricted. Keratins are generally expressed as coordinate pairs of type I and type II proteins, which differ by virtue of their biochemical properties. All studies were approved by the University of Queensland Animal Ethics Committee. Seven mouse keratin 6 isoforms that have been identified to date and shown be encoded by independent

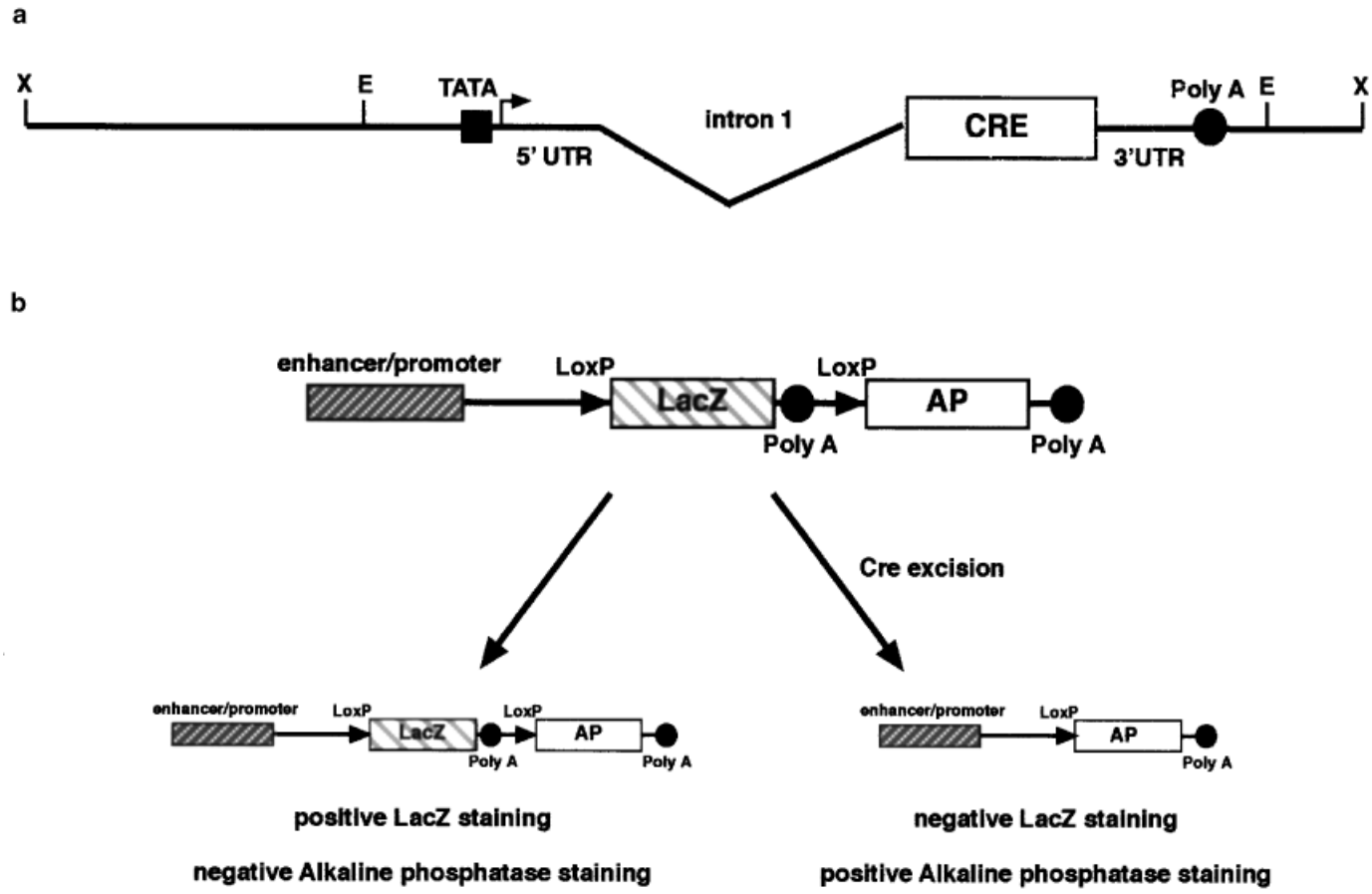

Figure 1
Diagrammatic representation the *Krt6a*-Cre transgene and the Z/AP reporter construct. (*A*) The keratinocyte-specific expression vector is derived from the regulatory sequences of the mouse *Krt6a* gene (Rothnagel and Roop unpublished data; Mahony *et al*, 2000). The vector contains 6.5 kb of upstream sequences, the first intron and 1.5 kb of downstream sequences. The Cre cDNA (Sauer and Henderson, 1989) was inserted into the *Pme*I site of the polylinker of the *Krt6a* transgene. The resultant transgene was released from the plasmid backbone by *Xho*1 digestion and injected into the pronuclei of fertilized CBA/C57Bl6J embryos using standard techniques (Hogan *et al*, 1994). Offspring were screened for the presence of the transgene by both Southern blot and polymerase chain reaction using primers within the *Cre* gene (Cre5 5′-CTGTTTCACTATCCAGGTTAC-3′ and Cre4 5′-GATATCTCACGTACTGAC GGT-3′). Transgenic animals were crossed with heterozygous Z/AP reporter mice (Lobe *et al*, 1999) and offspring were genotyped by polymerase chain reaction using Cre5 and Cre4 primers and LacZ staining of tail tips (Lobe *et al*, 1999). (*B*) Diagrammatic representation of the reporter gene excision before and after Cre excision. The Z/AP reporter mouse (Lobe *et al*, 1999) contains the transgene with the LacZ gene flanked by loxP sites followed by the alkaline phosphatase gene. Prior to Cre excision the tissues would stain positive for LacZ expression, but negative for alkaline phosphatase. Tissues double transgenic for Z/AP and *Krt6a*-Cre would excise the LacZ gene and switch to expression of alkaline phosphatase.

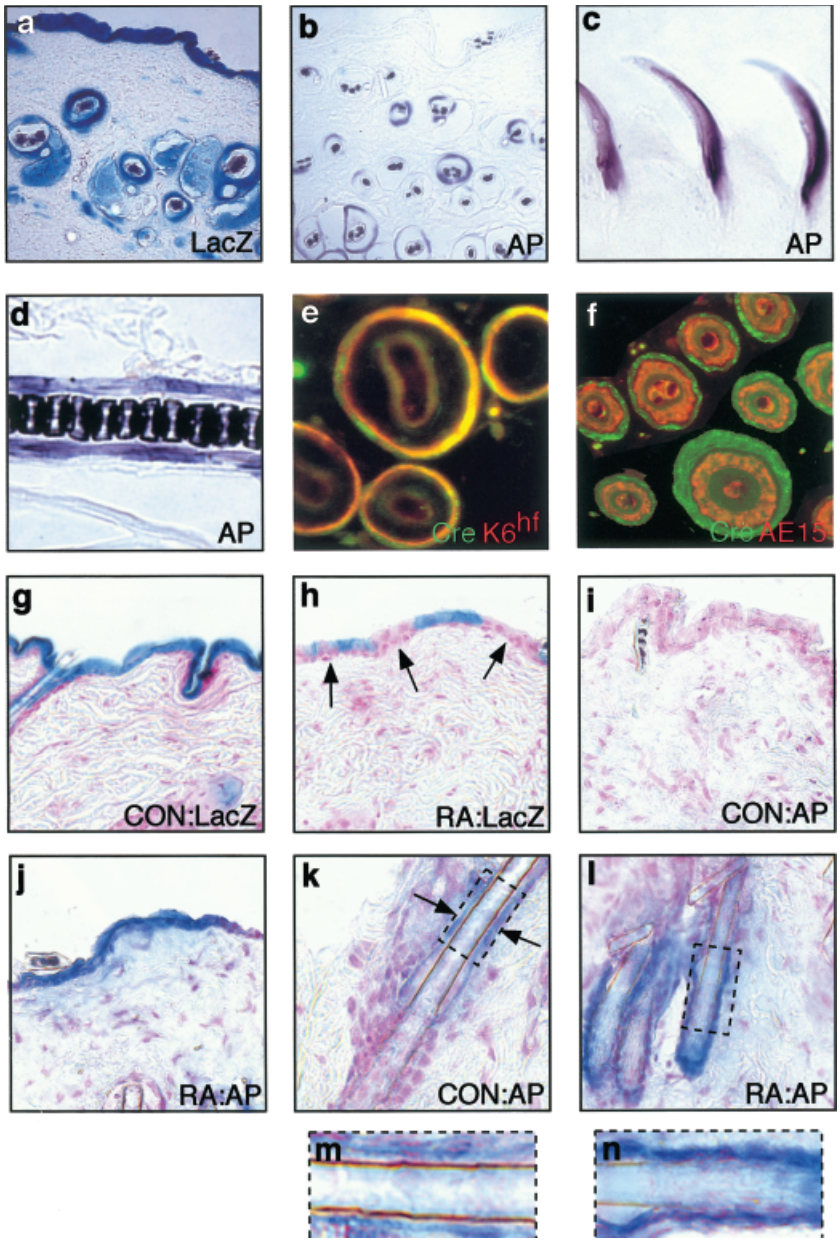

Figure 2
Reporter expression in skin of double-transgenic Z/AP/*Krt6a*-Cre mice. Back skin sections stained for LacZ expression (*A*) or alkaline phosphatase activity (*B*) indicating that the *Krt6a*-Cre transgene demonstrated specific deletion in a portion of the hair follicle. High magnification (× 100) of positive alkaline phosphatase activity demonstrated in the tongue (*C*) and the companion cell layer of the hair follicle (*D*). Tissues of double transgenics were mounted in OCT, cryosectioned, and stained for LacZ and alkaline phosphatase activity using published protocols (Lobe *et al*, 1999). Merged confocal images of double immunofluorescence-labeled double-transgenic mice Z/AP/*Krt6a*-Cre (*E*) anti-Cre (green) and Bax-1 (red) demonstrating *Krt6a*-Cre expression in the companion cell layer and (*F*) anti-Cre (green) and anti-AE15 (red) demonstrating that *Krt6a*-Cre is not expressed in the inner root sheath. Immunofluorescence analysis on 4% PFA-fixed paraffin-embedded sections using antibodies against trichohyalin (AE15, O'guin *et al*, 1992), $K6^{hf}$ (Bax-1, Winter *et al*, 1998), and Cre (Novagen, Madison, WI). Induction of *Krt6a*-Cre excision after treatment with RA. LacZ expression in untreated (*G*) or RA-treated (*H*) Z/AP/*Krt6a*-Cre back skin (original magnification × 40). Alkaline phosphatase expression in untreated (*I*) or RA-treated (*J*) Z/AP/*Krt6a*-Cre back skin (original magnification × 40) demonstrating induction of Cre excision in the epidermis upon RA treatment. Alkaline phosphatase expression in untreated (original magnification: (*K*) × 60; (*M*) × 100) or RA-treated Z/AP/*Krt6a*-Cre hair follicles (original magnification: (*L*) × 60; (*N*) × 100). Shaved adult double Z/AP/*Krt6a*-Cre mice were challenged with 30 μg of RA in 200 μL of acetone (500 nM), which was applied once topically to the back skin. Skin biopsies were taken 24 h postapplication and processed for alkaline phosphatase and LacZ activity as described above. Control animals were treated with acetone alone.

genes clustered on chromosome 15 (Takahashi *et al*, 1998; Rothnagel *et al*, 1999; Aoki *et al*, 2001; Wojcik *et al*, 2001). The most characterized isoform (K6a) exhibits both constitutive and inducible expression. Analysis of the murine *Krt6a* promoter in transgenic mice has determined that the mouse gene is constitutively expressed in various stratified epithelia, including footpad epidermis, nail bed, oral mucosa, tongue, and the companion cell layer of the hair follicle (Rothnagel *et al*, 1999; Mahony *et al*, 2000). In addition, inducible transcription of the *Krt6a* promoter in response to external chemical challenge with phorbol esters and all *trans*-retinoic acid (RA) has been demonstrated (Rothnagel *et al*, 1999; Mahony *et al*, 2000). This induction results in *Krt6a* expression in the outer root sheath of the follicle and throughout the epidermis, including the basal cell layer.

The Cre-LoxP recombination system is being increasingly utilized to induce tissue-specific knockout of target genes in the mouse (Orban *et al*, 1992). The system allows the study of genes where global homozygous loss of function in null animals is lethal, and it permits the study of targeted, and often inducible, gene inactivation in a particular tissue. The specific expression of Cre from a characterized promoter results in the knockout of a gene that is surrounded by LoxP sites in a particular tissue delimited by the activity of that promoter. We have generated a transgenic mouse that expresses Cre recombinase under the control of the mouse *Krt6a* promoter as a tool to study the genes involved in the development and maintenance of the hair follicle and other K6a expressing tissues. The Cre recombinase cDNA (Sauer and Henderson, 1989) was cloned into the *Pme*1 site of the *Krt6a* expression vector (Rothnagel and Roop unpublished data; Mahony *et al*, 2000) (Fig 1*A*). We have assayed the effectiveness of this promoter in mediating Cre excision by crossing *Krt6a*-Cre transgenic animals with a Z/AP reporter mouse line. The Z/AP reporter mouse (Lobe *et al*, 1999) uses a double-reporter system to determine the precise cell type of Cre

excision. Cells that do not express Cre recombinase stain positively for LacZ expression. Whereas cells where Cre-mediated excision has occurred, LacZ expression is replaced by alkaline phosphatase expression (Fig 1*B*). With the Z/AP reporter it is possible to differentiate between expression of Cre and its ability to excise target sequences in the genome. LacZ expression in the Z/AP/*Krt6a*-Cre double transgenics is observed only in a limited region of the hair follicle and the epidermis (Fig 2*A*). The dermis is mostly devoid of LacZ expression, as it is largely comprised of extracellular matrix and fibroblasts, which do not express the reporter construct. Alkaline phosphatase expression in the Z/AP/*Krt6a*-Cre double transgenics is detected in the hair follicles (Fig 2*B*,*D*) and also in the rugae of the tongue (Fig 2*C*). To determine the specific layer of the hair follicle expressing *Krt6a*-Cre colocalization of the companion cell-specific $K6^{hf}$ (Bax-1; Winter *et al*, 1998) and Cre demonstrated that *Krt6a*-Cre is expressed in the companion cell layer (Fig 2*E*). In addition, the lack of colocalization of Cre with the AE15 antibody, which stains trichohyalin, an inner root sheath marker (O'Guin *et al*, 1992), demonstrated that *Krt6a*-Cre was not expressed in the inner root sheath (Fig 2*F*). The companion cell layer is a discrete population of cells located between the inner and outer root sheaths (Rothnagel and Roop, 1995). Recombination of the reporter was not observed in the cells of the sebaceous gland, cortex or medulla cells of the hair follicle. Moreover, alkaline phosphatase expression was not observed in the interfollicular epidermis of uninduced *Krt6a*-Cre skin (Fig 2*B*).

To determine whether recombination could be induced in other epidermal skin types, we treated double Z/AP/*Krt6a*-Cre mice with RA. Previous studies have demonstrated induction of the *Krt6a* promoter in interfollicular basal and outer root sheath cells (Rothnagel *et al*, 1999; Mahony *et al*, 2000). In control Z/AP/*Krt6a*-Cre skin sections treated with acetone alone, LacZ expression was observed throughout the epidermis (Fig 2*G*) and epidermal alkaline phosphatase staining was absent (Fig 2*I*) indicating that Cre excision had not occurred in the epidermis. Upon treatment with RA, a loss of LacZ staining (Fig 2*H*) concurrent with alkaline phosphatase expression (Fig 2*J*) was detected in the epidermis indicating successful induction of the promoter and subsequent *loxP*-mediated excision. In addition, alkaline phosphatase staining was expanded in the outer root sheath of the hair follicle (Fig 2*L*) after RA treatment when compared with the hair follicles of the control skin sections (Fig 2*K*).

These results indicate that the *Krt6a* promoter can be successfully used to drive recombination in a discrete population of hair follicle cells, known as the companion cell layer. One of the major advantages of using the *Krt6a* promoter to drive Cre expression is the ability to activate recombination in interfollicular basal and outer root sheath cells upon treatment of *Krt6a*-Cre mice with RA. These *Krt6a*-Cre mice should provide an interesting and informative tool for studying the effects of various genes on follicle development. Targeted deletion of genes such as those involved in the hedgehog and Wnt signaling pathways, which are involved in follicle specification and development can be selectively inactivated using these mice. The inducible nature of the promoter will also allow discrimination of gene function in the follicle against cells of the interfollicular epidermis. The generation of these animals will benefit the study of homozygous inactivation of tumor suppressor genes in the hair follicle and epidermis (including potential stem cells residing in the basal cell layer), and in the study of genes involved in hair follicle development and hair growth.

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