Targeted Disruption of the Protein Kinase SGK3/CISK Impairs Postnatal Hair Follicle Development

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Submitted January 14, 2004; Revised June 10, 2004; Accepted June 29, 2004
Monitoring Editor: Richard Assoian

Members of the serum- and glucocorticoid-regulated kinase (SGK) family are important mediators of growth factor and hormone signaling that, like their close relatives in the Akt family, are regulated by lipid products of phosphatidylinositol-3-kinase. SGK3 has been implicated in the control of cell survival and regulation of ion channel activity in cultured cells. To begin to dissect the *in vivo* functions of SGK3, we generated and characterized *Sgk3* null mice. These mice are viable and fertile, and in contrast to mice lacking SGK1 or Akt2, respectively, display normal sodium handling and glucose tolerance. However, although normal at birth, by postpartum day 4 they have begun to display an unexpected defect in hair follicle morphogenesis. The abnormality in hair follicle development is preceded by a defect in proliferation and nuclear accumulation of β -catenin in hair bulb keratinocytes. Furthermore, in cultured keratinocytes, heterologous expression of SGK3 potently modulates activation of β -catenin/Lef-1-mediated gene transcription. These data establish a role for SGK3 in normal postnatal hair follicle development, possibly involving effects on β -catenin/Lef-1-mediated gene transcription.

INTRODUCTION

The serum- and glucocorticoid-regulated kinase (SGK) family of serine-threonine kinases is comprised of three isoforms that share >75% identity in their kinase domains, are closely related to the Akt kinases (the SGK1 and Akt1 catalytic domains share >50% identity and >60% similarity), and are activated by phosphatidylinositol-3-kinase (PI3K) (Lang and Cohen, 2001). SGK3 (also termed CISK and SGK1) was cloned by homology with SGK1 (Dai *et al.*, 1999; Kobayashi *et al.*, 1999) and independently by expression cloning as a factor that promoted interleukin (IL)-3-independent growth in cultured hematopoietic cells (Liu *et al.*, 2000). It is expressed in a variety of tissues (Dai *et al.*, 1999; Kobayashi *et al.*, 1999) and shares phosphorylation substrates with other members of the SGK/Akt family *in vitro* (Kobayashi *et al.*, 1999; Liu *et al.*, 2000; Dai *et al.*, 2002). Uniquely within the SGK/Akt family, SGK3 bears an amino-terminal Phox homology (PX) domain that targets SGK3 to endosomal membranes via its interaction with phosphoinositides (Virbasius

et al., 2001; Xu *et al.*, 2001), where it has been colocalized with epidermal growth factor receptor (EGFR) (Xu *et al.*, 2001). Like the Akts, the SGKs are phosphorylated and activated by the PI3K effector phosphoinositide-dependent kinase-1 (PDK1) *in vitro* (Kobayashi and Cohen, 1999; Kobayashi *et al.*, 1999). Via this pathway insulin, insulin-like growth factor-I, IL-3, and epidermal growth factor (EGF) activate SGK3 (Liu *et al.*, 2000; Virbasius *et al.*, 2001). SGK1, the most extensively studied of the three isoforms, is also regulated by insulin and EGF and plays a key role in the regulation of epithelial sodium transport (Pearce, 2001; Wulff *et al.*, 2002). Of the six Akt/SGK family members, three have been knocked out in mice: Akt1, Akt2, and SGK1 (Chen *et al.*, 2001; Cho *et al.*, 2001a,b; Wulff *et al.*, 2002; Garofalo *et al.*, 2003). All three null strains have distinct, surprisingly mild phenotypes, in contrast to an Akt1/Akt2 double null (the only double knockout [KO] characterized at this time), which has a severe phenotype that is not a simple superposition of the individual KO phenotypes, suggesting overlapping functions. Importantly, in all cases the phenotypes of the null mice were either only partially predicted by, or were counter to, predictions based on *in vitro* studies. To begin to elucidate the *in vivo* actions of SGK3, we generated and characterized mice bearing targeted disruption of the *Sgk3* locus.

MATERIALS AND METHODS

Construction of Targeting Vector and Generation of *Sgk3* KO Mice

All animal experiments were conducted following institutional Committee on Animal Research Committee approval. The targeting strategy for disruption

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04-01-0027. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-01-0027.

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Abbreviations used: E, embryonic day; ENaC, epithelial sodium channel; GSK, glycogen synthase kinase; IRS, inner root sheath; KO, knockout; NHK, newborn human keratinocyte; ORS, outer root sheath; P, postpartum day; PDK1, phosphoinositide-dependent kinase-1; PX, Phox homology; SGK, serum- and glucocorticoid-regulated kinase.

of the *Sgk3* gene involved removing parts of exons 10, which contains the ATP-binding site necessary for the catalytic activity of SGK3, and 11, deleting intron 10, and introducing an in-frame STOP codon into exon 11. Plasmid pNTK loxp (gift from Dr. S. Coughlin, University of California, San Francisco, CA) was used to generate the targeting vector. Two mouse genomic fragments, containing exons 8–11 and exons 10–17, were amplified from 129 $\times$ 1/SvJ DNA by polymerase chain reaction (PCR) and cloned into pCR4-TOPO and pCR-XL-TOPO (both from Invitrogen, Carlsbad, CA), respectively, and characterized by restriction enzymes. The exon 8–11-containing construct was used as a template in a second round of PCR to generate a 2.6-kb exon 8–10 fragment with a *Bam*HI site added to the 5' end and an *Mfe*I site added to the 3' end. This fragment was used as the short arm and was inserted into the *Bam*HI/*Mfe*I sites of the targeting vector. The 10-kb-long arm fragment was generated by using the exon 10–17-containing construct as a template in a second round of PCR. A *Clal* site was added to the 5' end, and a STOP codon was added before the start of exon 11; a *Xho*I site was added to the 3' end. This fragment was inserted into the *Clal*/*Xho*I sites of the targeting vector. The targeting vector was linearized by digestion with *Xho*I and electroporated into RW-4 embryonic stem cells (derived from 129 $\times$ 1/SvJ mice). G418- and gancyclovir-resistant clones were initially screened by PCR using oligonucleotide primers located inside and outside the targeted locus to confirm homologous recombination. Two positive clones were expanded and their genomic DNA analyzed by Southern blot analysis after digestion by *Mfe*I. An external probe (a 2-kb restriction fragment lying between exons 1 and 7) was used to verify correct targeting. The two positive clones were injected into C57BL/6 blastocysts and transferred into pseudopregnant females. Chimeric males, identified by their agouti coat color, were mated with C57BL/6 females. To generate mice homozygous for the targeted allele, the resulting *Sgk3*^{+/−} males and females were interbred.

PCR Analysis of Genotype and Sex

Genomic DNA was prepared from tail biopsies by overnight digestion in 500 μ l of proteinase K/STE (1% SDS, 50 mM Tris-Cl, pH 8.0, 0.5M NaCl, 1 mM EDTA) (0.5 mg/ml). Digests were diluted 1:100 and used directly in PCR reactions with the forward primer 5'-CTTCTTGCAAAACGGAAACTG-GATG3' and the reverse primer 5'-CCCCTCCATTAACAAAATCCAGAAC 3'. Reactions were performed using LA *Taq* (TaKaRa; Otsu, Shiga, Japan) with the conditions 94°C 1 min; 94°C 30 s, 62°C 45 s, 68°C 7 min for 14 cycles, and then increased extension by 15 s/cycle; final extension of 72°C for 15 min. PCR products were resolved on 1% agarose gels. The wild-type (WT) allele PCR product was 0.2 kb; that for the mutated allele was 1.9 kb. Sexing of newborn mice was performed by PCR as described previously (McClive and Sinclair, 2001).

Northern Blot Analyses

Total RNA was isolated using STAT-60 reagent (Tel-Test Inc., Friendswood, TX). Eight micrograms of RNA was resolved by formaldehyde-agarose gel electrophoresis, transferred to Hybond-NX membrane (Amersham Biosciences, Piscataway, NJ) and probed with a fragment spanning the entire *Sgk3* open reading frame. After autoradiographic exposure, the membrane was stripped and reprobed multiple times by using fragments corresponding to the *Sgk1*, *Sgk2*, *Akt1*, *Akt2* (to assess compensatory induction), and *cyclophilin* (loading control) coding sequences.

Western Blot Analysis

Western blot analysis was performed as described previously (Chen *et al.*, 1999). Protein extracts from WT and *Sgk3* KO mice were used for Western blot analysis by using an SGK antibody (gift from Dr. G. Firestone, University of California, Berkeley, CA) that cross-reacts with SGK1, SGK2, and SGK3. To distinguish between the three SGK isoforms, SGK1, SGK2, and SGK3 proteins synthesized in a coupled reticulocyte system (Promega, Madison, WI) were analyzed on the same blot; this also confirmed the expected size for the SGK3 protein (56.4 kDa).

Histological Studies

To analyze skin morphology, dorsal skin was biopsied and fixed overnight in 10% neutral buffered formalin (Fisher Scientific, Hampton, NH). Samples were dehydrated, paraffin-embedded, and sectioned (6 μ m). For basic morphology, sections were deparaffinized and stained with hematoxylin and eosin; samples were taken from heterozygote and *Sgk3* KO littermates to obtain sufficient numbers.

In Situ Hybridization

Localization of *Sgk3* mRNA expression in dorsal skin was determined using 6- μ m-longitudinal sections (paraffin-embedded) of skin from *Sgk3*^{+/−} mice by in situ hybridization, as described previously (Etchevers *et al.*, 2001), except sections were not treated with proteinase K, and after hybridization, slides were washed twice in 50% formamide, 1 $\times$ SSC, 0.1% Tween 20. Postpartum day 1 (P1), P3, and P4 sections were exposed to substrate for 9 d; P5 sections were exposed for 6 d. Sections were counterstained with nuclear fast red (Sigma-Aldrich, St. Louis, MO), dehydrated and permanently mounted.

Proliferating Cell Nuclear Antigen (PCNA) and β -Catenin Immunohistochemistry, and Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL)

Immunohistochemistry was performed on 6- μ m-longitudinal sections (paraffin-embedded) from *Sgk3* heterozygote and KO mice by using the M.O.M. immunohistochemistry kit, and alkaline phosphatase detection system in conjunction with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (all from Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol, except with incubation times of 60 min for primary antibody, 20 min for secondary antibody, and 20 min for ABC. Dilutions of 1:100 of anti-mouse PCNA antibody (Novocastra Laboratories, Newcastle-On-Tyne, United Kingdom), 1:50 of anti-mouse β -catenin antibody (BD Transduction Laboratories, Lexington, KY) were used. BCIP/NBT detection substrate was incubated for 20 to 80 min, to achieve optimal staining. Sections were counterstained with Nuclear Fast Red, dehydrated, and permanently mounted. TUNEL was performed using the DeadEnd colorimetric TUNEL system (Promega, Madison, WI) according to the manufacturer's protocol; sections were dehydrated and permanently mounted.

Cell Culture and [³H]Thymidine Incorporation in Mouse Primary Keratinocytes

Primary mouse keratinocytes were prepared from P4 heterozygote and KO mice by using a protocol developed in our laboratory. Briefly, culture flasks or plates first were coated with a collagen/fibronectin mix containing 0.01 mg/ml fibronectin, 0.032 mg/ml type 1 collagen and 0.1 mg/ml bovine serum albumin dissolved in mouse keratinocyte media (EMEM with EBSS; Cambrex Bio Science Walkersville, Walkersville, MD). Dispersed keratinocytes were collected by centrifugation and the cells then were resuspended in mouse keratinocyte media (EMEM with EBSS; Cambrex Bio Science Walkersville) containing 10% chelexed fetal bovine serum (FBS), penicillin/streptomycin/amphotericin, and 0.025 mM Ca²⁺. EGF (50 ng/ml) was added 5 h after initial plating, to allow optimum keratinocyte attachment. Primary keratinocytes (2 $\times$ 10⁵) from *Sgk3* heterozygote or KO mice were seeded on six-well plates and incubated for 24 h, followed by pulsing for 8 h with [³H]thymidine (1 mCi/well). Cultures were washed twice with phosphate-buffered saline, and reactions were terminated using ice-cold 5% trichloroacetic acid (TCA). Cells were washed with an additional volume of 5% TCA and with distilled water and were solubilized in 0.3 M NaOH for 10 min at 50°C. The amount of [³H]thymidine incorporated into DNA was determined by liquid scintillation counting.

Transient Transfection Assays

HaCaT cells (generously provided by Dr. N. Fusenig, German Cancer Research Center, Heidelberg, Germany) were maintained in DMEM supplemented with 10% FBS. Newborn human primary keratinocytes were maintained in medium 154CF supplemented with human keratinocyte growth supplement and 70 μ M CaCl₂ (Cascade Biologics, Portland, OR). The mouse SGK3 open reading frame was subcloned into pCDNA3 (SGK3), and expression vectors for kinase-dead (K191M, which prevents ATP-binding to the SGK3 catalytic site) and constitutively active (S486D, which substitutes an acidic residue that mimics phosphorylation by PDK1) forms of SGK3 were generated by PCR-based mutagenesis. The FKHL1-responsive promoter plasmid (FHRE) and FKHL1 expression plasmids were provided by Dr. M. Greenberg (Harvard Medical School, Boston, MA). The reporter consisting of seven Lef-1 response elements driving luciferase (p⁷ Lef-fos Luc) and a Lef-1 expression vector were provided by Dr. R. Grosschedl (University of Munich, Munich, Germany). For transient transfections, low passage HaCaT cells or primary keratinocytes at passage 3 or 4 were seeded 24 h before transfection at a density of 2 $\times$ 10⁵ cells/well in six-well plates. Transient transfections were performed using the TransIT reagent (Mirus, Madison, WI) according to the manufacturer's protocol. For Lef-1 experiments in HaCaT cells, 100 ng of reporter, 10 ng of Lef-1 and 5, and 10 or 25 ng of mutant SGK3 were used. Twenty-four hours after transfection, serum-free medium was added; after a further 18 h, fresh serum-free medium with 50 μ M LY294002 or dimethyl sulfoxide (vehicle) was added. Cells were harvested after a further 6 h and freeze-thaw lysed in 100 μ l of 0.25 M Tris, pH 7.6. For human foreskin keratinocyte (NHK) cells, 100 ng of reporter, 100 ng of Lef-1, and 100 ng of mutant SGK3 were used. For FHRE experiments, 100 ng of FHRE, 300 ng of FKHL1, and 25 ng of WT or mutant SGKs were used. For EGF treatment, 24 h posttransfection, insulin-free medium lacking EGF, or with 50 ng/ml EGF or transforming growth factor (TGF)- α , were added and cells harvested 24 h later. Luciferase activity of 5 μ l of lysate was assayed using Promega Luciferin Reagent and normalized to total protein levels, which were measured by adding 100 μ l of Bio-Rad protein assay dye (Bio-Rad, Hercules, CA) to 5 μ l of lysate and measuring in a plate reader. Transfections were performed at least three times.

Weight and Growth Analyses

To assess for disturbances of weight and growth, several analyses were performed. Newborn mice from 7 litters were weighed within 18 h of birth,

sacrificed and tails harvested, and genotype and sex determined by PCR as described. 10 d old mice from 7 litters were categorized according to whether they displayed the hair phenotype, and weighed; WT and heterozygote mice were not distinguished, and sex was not determined. Despite not distinguishing between sexes, this analysis is valid, because male and female mice of the same genetic background, including the B6;129 background, do not differ significantly in body weight at P10; body weights of male and female mice only begin to diverge after weaning (P21) (Rhees and Atchley, 2000; Lupu *et al.*, 2001). Thus, differences in the numbers of male or female mice between genotypes will not skew the data. To calculate the relative weight of *Sgk3* KO mice, their weights were expressed relative to the mean weights of WT and heterozygote mice (set to 100%). The relative weights of *Sgk3* KO mice were calculated for seven litters and meaned. For growth curves from 3 to 8 wk of age, mice from 15 litters (including those weighed at P10) were sexed, genotyped by PCR, and weaned at 3 wk of age. Weights were measured weekly from 3 to 8 wk of age.

Intraperitoneal Glucose Tolerance Test

WT and *Sgk3* KO littermates of the same sex were fasted overnight (16 h) and then injected intraperitoneally with 1 mg/g of body weight D-glucose [10% (wt/vol) stock solution in phosphate-buffered saline]. Blood samples were collected from the transversely sectioned tip of the tail, and whole blood glucose was measured using a Glucometer Elite (Bayer, Leverkusen, Germany) at 0 min (just before glucose injection), and at 15-, 30-, 60-, 90-, 120-, 180-, and 240-min intervals after the glucose load.

Sodium Balance Studies

Six each of wild-type and *SGK3*^{-/-} mice (3 mo of age), were kept in metabolic cages for an acclimatization period of 4 d with free access to water and food (standard diet C1314, 0.2% Na; Altromin, Lage, Germany). Urine was collected every 24 h for 7 d and stored at -20°C. From the second day of urine collection, the standard diet was changed to a low sodium diet (C1036, 0.015% Na; Altromin). After 4 d, the mice were returned to the standard diet. Blood was drawn, from the orbital plexus before and after 4 d on the low sodium diet, and serum was separated by centrifugation and stored at -20°C. Serum aldosterone was measured with a commercial RIA-kit (Aldosterone RIA, Diagnostic Systems Laboratories, Webster, TX) according to the manufacturer's protocol. Urinary sodium concentration was determined using colorimetric analysis (Advia 1650; Bayer). Urinary 24-h excretion of sodium was calculated using the daily urine volume.

Statistical Analyses

All data were analyzed using the Statview 4.5 software package. For growth curves, glucose tolerance tests and sodium balance studies, a repeated measure analysis of variance (ANOVA) was performed.

RESULTS

Generation of *Sgk3* KO Mice

The targeting strategy for disruption of the *Sgk3* gene involved removing parts of exons 9 (which contains the ATP-binding site necessary for the catalytic activity of SGK3) and 10, deleting intron 9, and introducing an in-frame STOP codon into exon 10 (Figure 1A). Offspring from matings of *Sgk3*^{+/-} mice were genotyped by PCR (Figure 1B); examination of 39 litters from *Sgk3*^{+/-} matings showed a normal representation of *Sgk3* KO mice (*Sgk3*^{+/-} [27%], *Sgk3*^{+/-} [51%], and *Sgk3*^{-/-} [22%] mice; *p* > 0.3, chi-square test), with a normal sex ratio. Absence of *Sgk3* mRNA and protein in homozygous mice was confirmed by Northern and Western blot, respectively (Figure 1, C and D). Northern blot analysis of tissues from 12-wk-old WT mice revealed moderate-to-high expression levels of *Sgk3* mRNA in heart, kidney, liver, lung, skeletal muscle (quadriceps), and thymus, with lower expression in adrenal gland, brain, skin, spleen, and fat (Figure 1C; unpublished data). These data are consistent with previous reports of widespread but variable levels of expression of *Sgk3* mRNA in human tissues (Dai *et al.*, 1999; Kobayashi *et al.*, 1999). In *Sgk3* KO mice, no obvious compensatory increases in the expression of *Sgk1*, *Sgk2*, *Akt1*, or *Akt2* mRNA were detected in a panel of tissues (unpublished data), as assessed by Northern blot analysis. Both male and female *Sgk3* KO mice were fertile.

Mice Lacking SGK3 Are Viable but Display a Defect in Postnatal Hair Follicle Development

Sgk3 KO mice seemed normal at birth, but by P10 clearly displayed sparse hair growth relative to WT littermates (Figure 2A); heterozygotes were indistinguishable from WT mice. This initial abnormality persisted for at least 4 wk (Figure 2B); however, as the *Sgk3* KO mice increased in age, the hair became increasingly thick (Figure 2C). At all ages, *Sgk3* KO mice displayed wavy coat fur and curly vibrissae that resembled those of mice with spontaneously or engineered mutations in the EGF/TGF- α and β -catenin signaling pathways (see below) (Luetteke *et al.*, 1993, 1994; Mann *et al.*, 1993; Murillas *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995; Hansen *et al.*, 1997; Gat *et al.*, 1998; DasGupta and Fuchs, 1999; Fuchs *et al.*, 2001; Huelsken *et al.*, 2001; Andl *et al.*, 2002).

Hair growth in rodents and humans is cyclic and proceeds through proliferative (anagen), regressive (catagen), and quiescent (telogen) phases (Muller-Rover *et al.*, 2001). In mice, hair follicle development (also called morphogenesis) begins at embryonic day 14.5 (E14.5) with placode formation and is completed at P16 with termination of the first growth phase (first anagen), at which time large numbers of hair follicles reside deep in the subcutis (Muller-Rover *et al.*, 2001). By P19, catagen (characterized by hair bulb involution and a reduction in hair follicle length) is advanced, and by P22 the first hair cycle has finished and follicles are in telogen (characterized by thinning of the subcutis, and follicles which reside entirely in the dermis and lack an inner root sheath [IRS]). The second anagen phase begins at approximately P26: the dermal papilla becomes enlarged, the hair bulb and IRS reform and a new hair shaft begins to develop (Muller-Rover *et al.*, 2001). To determine at what stage hair follicle morphogenesis becomes abnormal in *Sgk3* KO mice, dorsal skin was harvested at various time points between E15.5 and P30 from KO mice and heterozygous littermates, sectioned, and examined by light microscopy. At all time points examined, the hair follicles of heterozygous mice underwent appropriately-timed development (Muller-Rover *et al.*, 2001) and were indistinguishable from WT mice (Figure 3, A-E; unpublished data). *Sgk3* KO embryos displayed normal early hair follicle development, as indicated by the presence of placodes and germs at E15.5 (unpublished data), and nascent follicles at P1 (Figure 3A) and P3 (unpublished data) that morphologically resembled those of wild-type animals. By P4, a clear defect in morphogenesis was emerging, characterized by a failure of the hair bulb to enlarge and migrate deep into the subcutis (Figure 3B). This defect became more pronounced by P5 (Figure 3C) and persisted through follicle morphogenesis (i.e., at P7, P10, and P14; unpublished data), and beyond. At P19, when heterozygotes were in late catagen (Figure 3D) and at P22, when they had progressed fully into telogen, the *Sgk3* KO animals were still consolidating their first anagen (Figure 3E). Morphologically, the developing hair follicles in knock-out mice were disorganized, lacking the uniform orientation observed in WT and heterozygote mice. The cells of the outer root sheath (ORS) were disorganized (Figure 3F), and the layers of the inner root sheath were reduced, as was the hair shaft. These defects are the most likely cause of the wavy hair growth observed in *Sgk3* KO mice. The early hair abnormality in *Sgk3* KO mice thus seems to be a combination of impaired follicle progression through the first hair cycle and abnormal follicle organization. Although adult mice also display the hair follicle defect (i.e., wavy hair) observed in young mice (Figure 2C), further studies are required to

Figure 1. Targeted disruption of the *Sgk3* allele. (A) Targeting strategy. S represents the introduced in-frame STOP codon. The locations of genotyping PCR primers and predicted PCR product sizes for WT and mutant alleles are indicated by small arrows. The short bar between exons 1 and 7 indicates the DNA fragment used as a DNA probe for Southern blot analysis of embryonic stem clones. (B) Genotyping of mice by PCR. Genomic DNA extracted from tails was analyzed by PCR as described in *Materials and Methods*. The predicted amplicon size derived from the WT allele is 0.2 kb, whereas that from the disrupted *Sgk3* allele is 1.9 kb. (C) Northern blot analysis of total RNA from WT (+/+), and homozygous (-/-) mice. Total RNA (8 μ g) from a panel of tissues, including kidney, lung, and thymus, was analyzed by Northern blot by using a DNA probe specific for *Sgk3*. The same blot was stripped and reprobed for *Sgk1*, *Sgk2*, *Akt1*, and *Akt2* mRNA to assess for compensatory induction (unpublished data), and for cyclophilin (Cyclo) as a loading control. (D) Western blot analysis of protein extracts from kidney and liver from WT (+/+) and homozygous (-/-) mice by using an SGK antibody that cross-reacts with SGK1, SGK2, and SGK3. In vitro-transcribed SGK1, SGK2, and SGK3 were analyzed on the same blot to confirm the identity of each isoform (unpublished data). The predicted size of the SGK3 protein was 56.4 kDa; the SGK2-specific band is shown to confirm equal loading.

determine whether this is also accompanied by defects in hair cycling. It is also notable that histological abnormalities were confined to the follicles themselves; interfollicular epidermis seemed normal, and the progressive thickening of the dermis and subcutaneous fat that accompanies entry into anagen in wild-type and heterozygous mice proceeded normally in *Sgk3* KO mice (Figure 3). Together with the SGK3 expression pattern (described below), these observations suggest that the defect in *Sgk3* KO animals is intrinsic to the follicular keratinocytes.

Sgk3 mRNA Is Discretely Expressed in the Hair Follicle

To begin to understand the cellular basis of the effect of SGK3 in regulating hair follicle morphogenesis, *Sgk3* mRNA expression was localized by in situ hybridization in dorsal skin sections from early postnatal heterozygote animals. SGK3 showed a highly restricted pattern of expression in skin: At P1 and P3, SGK3 expression was relatively low, and only apparent in the root sheath of a small number of hair follicles (Figure 4; unpublished data). By P4, expression was increasing and becoming particularly intense in a subset of

root sheath cells (possibly isolated to the basal layer of the ORS), with lower level expression in matrix cells (which give rise to the IRS and precursors of the hair shaft). At P5, intense expression was seen in root sheath (seeming to encompass both outer and inner root sheath) and in some matrix cells, with lower level expression seen in numerous matrix cells of the hair bulb (Figure 4). At no time point was *Sgk3* mRNA expressed appreciably in interfollicular epidermis, nor was it expressed in nonepithelial cells of the skin or underlying subcutis.

Defective Proliferation and Normal Apoptotic Index in *Sgk3* KO Hair Bulbs and Keratinocytes

To begin to dissect the mechanism underlying the hair follicle morphogenesis defect, PCNA immunolocalization was performed to assess follicular cell proliferation. In hair bulbs of heterozygotes, PCNA-positive keratinocytes progressively increased from P1 to P5 (Figure 5A). This increase was delayed and blunted in the *Sgk3* KO mice. At P1, there was no apparent difference in PCNA staining between heterozygotes and KOs (Figure 5A), but at P3 a difference was

Figure 2. Targeted disruption of the *Sgk3* allele results in impaired postnatal hair growth. Heterozygote (shown on the left of each panel) and *Sgk3* KO (shown on the right of each panel) littermates were photographed at P10 (during hair follicle morphogenesis) (A), P28 (B), and 7 mo of age (C). Photographs shown represent typical appearance at each age for both sexes.

beginning to emerge (Figure 5A). By P4, nuclear PCNA staining was strikingly lower in the hair bulbs of *Sgk3* KOs (Figure 5A); in heterozygote hair bulbs, 57.4% of keratinocytes were PCNA positive compared with 10.9% in KO hair bulbs ($p < 0.001$; $n = 10$). At P5, this difference persisted, but it was less striking due to a late increase in PCNA-positive keratinocytes in the KOs (Figure 5A) (67.1% PCNA-positive nuclei in heterozygotes vs. 32.7% in KOs; $p < 0.001$; $n = 10$). At all ages, there was a high rate of proliferation in the interfollicular epidermis (unpublished data), and the ORS (Figure 5A), with no obvious differences between genotypes.

In vitro proliferation assays performed on primary keratinocytes prepared from P4 *Sgk3* heterozygote and KO mice revealed that *Sgk3* KO keratinocytes display 39.1% less [3 H]thymidine incorporation ($16,295 \pm 2657$ [S.D.] vs. $26,748 \pm 3078$ cpm-incorporated [3 H]thymidine after 8 h; $p < 0.02$; $n = 4$). TUNEL staining revealed no difference in apoptotic rates between heterozygote and *Sgk3* KO hair follicles at P4 (unpublished data) or P5 (Figure 5B), suggesting that increased apoptosis is not a cause of the hair follicle defect. Together, these observations suggest that the hair growth defect in *Sgk3* KO mice is due at least in part to a failure in induction of hair bulb keratinocyte proliferation.

Defective Nuclear Accumulation of β -Catenin in *Sgk3* KO Hair Bulbs

Several previous observations have implicated the Wnt/ β -catenin pathway in hair follicle development and cycling (Gat *et al.*, 1998; Fuchs *et al.*, 2001; Huelsken *et al.*, 2001; Andl *et al.*, 2002). Notably, selective expression of stabilized β -catenin in the ORS results in unchecked follicular proliferation (Gat *et al.*, 1998), whereas conditional timed disruption of β -catenin expression in hair follicles blocks further hair follicle development or cycling (Huelsken *et al.*, 2001). Furthermore, transgenic mice carrying a β -catenin/Lef-1-dependent reporter (TOPGAL) demonstrate reporter expression during hair follicle development that overlaps the *Sgk3* expression found in the present study (Figure 4; DasGupta and Fuchs, 1999). In view of these observations, we were interested in examining the timing and pattern of β -catenin expression in the follicles of *Sgk3* null mice in the early postnatal period. Hence, we performed immunohistochemistry by using an anti- β -catenin antibody on skin sections from P1 to P5 in KO and heterozygous mice (Figure 5C). In interfollicular epidermis, most of the β -catenin was associated with the plasma membrane, and there was no difference between heterozygous and KO animals at any time point. In the follicular keratinocytes of P1 animals, most of the β -catenin staining was associated with plasma membrane or cytoplasm (Figure 5C), and although there was detectable nuclear accumulation of β -catenin in some cells, there was no clear difference between heterozygote and *Sgk3* KO mice. By P3, however, nuclear β -catenin had begun to increase in root sheath and hair bulb keratinocytes of heterozygous but not in KO mice (Figure 5C). The difference increased progressively and by P5 there were substantially fewer β -catenin-positive nuclei in the lower root sheath and matrix cells of KO mice: 24.6% of the nuclei in the hair bulbs of *Sgk3* KO mice displayed nuclear accumulation of β -catenin in contrast to 42.8% of nuclei in heterozygotes ($p = 0.001$; $n = 10$). It is notable that the proliferative defect and failure of β -catenin nuclear localization occurred at approximately the same stage of development (P3-P4) and hence temporal precedence could not be determined.

SGK3 Modulates β -Catenin/Lef-1- and FKHRL1-mediated Gene Transcription in Cultured Keratinocytes

Mutations in both growth factor-regulated PI3K-dependent pathways (Luetteke *et al.*, 1993, 1994; Mann *et al.*, 1993; Murillas *et al.*, 1995; Sibilio and Wagner, 1995; Threadgill *et al.*, 1995; Hansen *et al.*, 1997), and in the Wnt- β -catenin/Lef1 (Gat *et al.*, 1998; DasGupta and Fuchs, 1999; Fuchs *et al.*, 2001; Huelsken *et al.*, 2001; Andl *et al.*, 2002) pathway have been associated with hair development abnormalities that resemble the SGK3 KO in many respects (see *Discussion* and below). Growth factors have well characterized inhibitory effects on forkhead-mediated gene transcription (Jackson *et al.*, 2000), and recent evidence has suggested that EGF in par-

Figure 3. Abnormal hair growth in *Sgk3* KO mice is due to a defect in hair follicle morphogenesis. Dorsal skin was harvested from P1 (A), P4 (B), P5 (C), P19 (D), P22 (Het) (E), and *Sgk3* KO littermates (KO). Lettering on C indicates layers of skin: D, dermis; E, epidermis; S, subcutis; and P, panniculus carnosus. P5 *Sgk3* KO hair follicles show differentiation defects (F) as reflected by an expanded outer root sheath (O) and reduced inner root sheath (I) and hair shaft (HS). Paraffin-embedded skin sections (6 μ m) were prepared and stained with hematoxylin and eosin and examined by light microscopy. A representative section from each time point and genotype is shown. Bars, 200 μ m (A–E) and 10 μ m (F).

ticular also may regulate β -catenin activities (Muller *et al.*, 2002; Lu *et al.*, 2003). To begin to define the potential signaling roles of SGK3 in these pathways, we examined the effects of transfected SGK3 on β -catenin/Lef-1 and forkhead reporters in transfected keratinocytes.

Figure 4. *Sgk3* mRNA expression increases to high levels by P5 in the root sheath and hair bulb. In situ hybridization was performed on 6- μ m-longitudinal sections of dorsal skin from P1, P3, P4, and P5 skin from *Sgk3*^{+/+} mice as described, followed by nuclear fast red counterstaining. Expression levels were undetectable to low at P1 (unpublished data). *Sgk3* mRNA expression (purple staining) increases from P3 to P5. *Sgk3* mRNA is expressed punctately in the root sheath and in matrix cells of the hair bulb. Hair follicle structures identified on P3 section: HS, hair shaft; DP, dermal papilla; MX, matrix cells; PR, precursor cells. Bar, 25 μ m. Note that melanin granules stain dark brown.

We looked first at SGK3 effects on the activity of a β -catenin/Lef-1 reporter (p⁷ Lef-fos Luc) (Giese and Grosschedl, 1993) in HaCaT cells, an immortalized line derived from human keratinocytes (Boukamp *et al.*, 1988). As demonstrated in other cell lines (Giese and Grosschedl, 1993), reporter activity was strongly Lef-1 dependent (Figure 6A). Interestingly, whereas a kinase-dead mutant of SGK3 (SGK3/K191M) inhibited reporter activity in a dose-dependent manner, a constitutively active mutant (SGK3/S486D) strongly stimulated reporter activity (Figure 6A). The inhibitory effect of the kinase-dead mutant is consistent with dominant-negative activity, which is not surprising in view of the fact that HaCaT cells express substantial levels of SGK3 (unpublished data). The PI3K inhibitor LY294002 also markedly blunted Lef-1-dependent reporter activity, and this effect was completely reversed by coexpression of SGK3/S486D (Figure 6A), suggesting that SGK3 acts downstream of PI3K.

Although HaCaT cells demonstrated robust PI3K-dependent reporter activity, growth factors (EGF and TGF- α , in particular) had no effect. Unfortunately, repeated attempts to culture and transfect keratinocytes from *Sgk3* KO mice were unsuccessful, possibly due to their low proliferative rate. We therefore next examined β -catenin/Lef-1 reporter activity in primary cultures of newborn NHK cells. In these cells, reporter activity was stimulated by EGF (Figure 6B), as well as by TGF- α , which also acts through the EGFR (unpublished data). Like HaCaT cells, NHK cells express endogenous SGK3 (unpublished data), and both basal and EGF-stimulated Lef-1 reporter activity was inhibited by SGK3/K191M (Figure 6B); SGK3/S486D stimulated reporter

Figure 5. Failure of induction of proliferation and β -catenin nuclear accumulation, but normal cell survival in $Sgk3^{-/-}$ hair bulbs. (A) PCNA-positive keratinocytes progressively increased in number from P1 to P5 in $Sgk3$ heterozygote (Het) hair bulbs but not in $Sgk3$ KO hair bulbs. Immunolocalization revealed no significant differences in proliferation at P1 or P3 in $Sgk3$ KO bulbs, but this difference diminished by P5. Arrowhead indicates example of PCNA-positive cell. (B) TUNEL staining revealed an absence of apoptosis in P5 heterozygote and $Sgk3$ KO hair follicles. Negative ($-TdT$) and positive ($+DNaseI$) controls are shown. Arrowhead on positive control indicates an example of an apoptotic nucleus; black granules are melanin. (C) β -Catenin-positive keratinocytes progressively increased in number from P1 to P5 in $Sgk3$ heterozygote hair bulbs but not in $Sgk3$ KO hair bulbs. At P1 no obvious differences in nuclear β -catenin were apparent, but by P3 $Sgk3$ KO bulbs displayed an emerging failure to accumulate nuclear β -catenin. By P4, deep-lying hair bulbs displayed markedly reduced accumulation; this difference persisted at P5. Arrowhead indicates example of cell displaying nuclear β -catenin. Bars, P1 bar represents 25 μm on P1 and P3 sections, P4 bar represents 25 μm on P4 and P5 sections (A); 200 μm (B); P1 bar represents 50 μm on P1 and P3 sections, P4 bar represents 50 μm on P4 and P5 sections (C).

activity, mimicking the stimulatory effect of EGF. Furthermore, a reversal of LY294002 inhibition of reporter activity by SGK3/S486D also was seen in NHK cells (unpublished data). Together, these data suggest that SGK3 mediates EGF/TGF- α activation of β -catenin/Lef-1-dependent transcription in NHK cells.

Finally, we examined the response of a forkhead-dependent reporter gene in NHK cells cotransfected with SGK3 and an expression vector for the forkhead transcription factor FKHL1. Consistent with previous results in 293T cells

(Xu *et al.*, 2001), EGF inhibited FHRE activity, an effect that was mimicked by SGK3/S486D but not by wild-type SGK3, unless activated by EGF (Figure 6C).

***Sgk3* KO Mice Display a Transient Growth Defect, Normal Glucose Disposal, and Normal Sodium Homeostasis**

Although SGK3 is expressed in most if not all tissues, histological analysis revealed no gross abnormalities in tissues other than skin, possibly due to overlapping functions with

Figure 6. SGK3 activates β -catenin/Lef-1-mediated, and inhibits FKHRL1-mediated gene transcription in cultured keratinocytes. (A) Immortalized keratinocytes (HaCaT cells) were transfected with a reporter containing seven Lef-1 binding sites driving *luciferase* and a Lef-1 expression plasmid (Basal). The effects of cotransfected SGK3 mutants on Basal Lef-1-mediated reporter activity were determined. Kinase-dead SGK3/K191M (K191M) inhibited, whereas constitutively active SGK3/S486D (S486D) stimulated reporter activity. The PI3K inhibitor LY294002 reduced basal reporter activity (Basal + LY), but 25 ng of S486D overcame this effect (S486D + LY). (B) Primary human keratinocytes (NHK cells) were transfected with Lef-1 reporter and Lef-1 expression plasmid (Basal). The effect on Basal activity (open bars) of 24-h incubation with 50 ng/ml EGF was determined (filled bars). Similar effects of K191M or S486D on reporter activity to those seen in HaCaT cells were seen, and S486D mimicked EGF activation of reporter activity. (C) In primary keratinocytes, activity of a reporter containing a FKHRL1-binding site driving *luciferase* (FHRE) was stimulated by cotransfecting FKHRL1 (FK), and the effects of cotransfected nonactivated WT (WT), EGF-activated WT (WT+E), or constitutively active SGK3 (S486D) on FKHRL1-mediated stimulation of FHRE were determined. In A–C, luciferase activity was normalized to total protein and represented as mean activity $\pm$ SEM relative to basal activity (A), basal activity in the absence of EGF (B), and FHRE (C). In A, * $p < 0.01$ decreased activity relative to basal; $^{\dagger}p < 0.01$ increased activity relative to basal; in B, * $p < 0.05$ and ** $p < 0.01$, relative to basal lacking EGF; $^{\dagger}p < 0.01$, relative to control, + EGF; in C, * $p < 0.01$, relative to FHRE activity in the presence of FKHRL1 (FK); t tests.

Table 1. *Sgk3* KO mice display normal birth weights

Sex	Genotype	Mean weight (g $\pm$ S.D.) (n)
Male	<i>Sgk3</i> ^{+/+}	1.51 $\pm$ 0.15 (10)
Male	<i>Sgk3</i> ^{-/-}	1.38 $\pm$ 0.13 (7)
Female	<i>Sgk3</i> ^{+/+}	1.49 $\pm$ 0.15 (9)
Female	<i>Sgk3</i> ^{-/-}	1.42 $\pm$ 0.03 (4)

other SGK/Akt family members. *Akt1* KO mice weigh 20–25% less than WT mice from birth to at least 14 mo of age (Chen *et al.*, 2001; Cho *et al.*, 2001b); *Akt2* KO mice display insulin resistance, a diabetic phenotype, and a mild growth defect (Cho *et al.*, 2001a; Garofalo *et al.*, 2003), whereas *Sgk1* KO mice display a sodium wasting phenotype (Wulff *et al.*, 2002). We therefore assessed birth weights and growth characteristics of *Sgk3* KO mice and examined their response to a glucose challenge or sodium restriction.

Birth weights of *Sgk3* KO mice did not significantly differ from WT or heterozygous littermate weights (Table 1). However, by P10, the relative weight of *Sgk3* KO mice (identified by fur appearance, but not distinguished with respect to sex), was 10% less than WT/heterozygous littermates (t test; $p < 0.001$). From 3 to 6 wk of age, reduced body weight was discernible in male *Sgk3* KO mice, but it had normalized by 7 wk of age (repeated measures ANOVA; $p < 0.001$) (Figure 7A). In contrast, female *Sgk3* KO mice grew more rapidly than WT female mice over the same time course (repeated measures ANOVA; $p = 0.02$). Both of these effects were, however, mild. This mild transient growth abnormality of *Sgk3* KO mice is statistically significant, but qualitatively different from that of *Akt1* KO mice, which demonstrate substantial growth retardation throughout life. The subtle growth defect might reflect compensatory effects of Akt1. Furthermore, unlike *Akt2* KO mice, glucose tolerance in 8- to 10-wk-old *Sgk3* KO mice was indistinguishable from that of WT (Figure 7B). *Sgk3* KO mice also exhibited normal sodium balance and aldosterone induction when placed on a low sodium diet (Figure 7, C and D), in contrast to *Sgk1* KO mice. Hence, it seems that SGK3 is not required for normal glucose metabolism or renal sodium reabsorption or that its lack is compensated for by other SGK/Akt isoforms.

DISCUSSION

SGK3 Proliferation and Signaling during Postnatal Hair Follicle Development

Although considerable progress has been made in understanding prenatal development in general, and hair follicle development, in particular, substantially less is known about postnatal events. The data presented here demonstrate that although dispensable for embryonic hair follicle development, SGK3 is unexpectedly required for normal postnatal follicle development. By P5, *Sgk3* KO hair follicles display multiple abnormalities, including reduced hair bulb size (Figure 3D), disorganized ORS, and greatly reduced IRS and hair shaft (Figure 3F). Furthermore, an abnormality in nuclear β -catenin accumulation and a proliferative defect were apparent in P3–4 animals (Figure 5), both of which preceded gross histological changes (Figure 3). Thus, the hair follicle defect seems to be at least in part due to a failure of induction of proliferation in maturing hair follicles. The proximal cause of the proliferative defect cannot be discerned with certainty at this time because the abnormality in

Figure 7. *Sgk3* KO mice display a mild transient growth defect, but normal glucose tolerance and sodium-restriction response. (A) Growth is mildly perturbed in *Sgk3* KO mice. Male and female mice from 15 litters were weighed at weekly intervals from 3 to 8 wk of age. Data represent means at each age $\pm$ SEM (closed circles, male WT; open circles, male homozygotes; closed squares, female WT; open squares, female homozygotes); $n = 10$ –17. Repeated measures ANOVA revealed significant differences between the growth rates of both male and female *Sgk3* KO mice relative to WT. (B) *Sgk3* KO mice display normal glucose homeostasis. Plasma glucose levels were measured at the indicated times in 8- to 10-wk-old mice fasted and subjected to an intraperitoneal glucose load (1g/kg body weight). Total blood was collected from the transected tip of the tail and glucose measured using a glucometer. Data are represented as mean $\pm$ SEM (closed circles, male WT; open circles, male homozygotes; closed squares, female WT; and open squares, female homozygotes); $n = 10$ mice for each genotype and sex. (C) *Sgk3* KO mice display normal sodium balance when placed on a low sodium diet. Male wild-type and *Sgk3* KO mice (3 mo old) were acclimatized to metabolic cages and basal urinary sodium was measured. The diet was changed from standard (0.2% Na) to low sodium (0.015% Na) for 4 d and then switched back to standard diet. Urinary sodium excretion in response to this challenge was determined on a daily basis throughout. Data are represented as mean $\pm$ SEM (closed squares, WT; and open squares, homozygotes); $n = 6$ mice for each genotype. (D) Normal induction of aldosterone secretion in response to sodium restriction in *Sgk3* KO mice. The mice described in C had blood drawn before, and after 4 d on the low sodium diet. Serum aldosterone was measured with a commercial RIA-kit (closed bars, WT; and open bars, homozygotes). Data are represented as mean $\pm$ SEM; $n = 6$ mice for each genotype.

β -catenin localization did not clearly precede the proliferation defect. Furthermore, SGK3 seems to mediate effects on both β -catenin/Lef-1 and forkhead-dependent gene transcription (Figure 6). It also should be noted that in addition to proliferation, SGK3 might influence cell migration and differentiation.

It is notable that although no member of the SGK/Akt family has previously been identified as a primary mediator of hair follicle development, growth factor receptors that signal through PI3K have been strongly implicated in follicle development (Luetke et al., 1994; Murillas et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Together, our present data suggest that SGK3 may play a role in linking PI3K signaling (possibly through EGFR-dependent activation of this pathway) to the regulation of both forkhead (traditional pathway) and β -catenin (alternate pathway). At this point, it is unclear whether defects in β -catenin nuclear localization precede defects in cellular proliferation in the follicles of *Sgk3* KO mice, although β -catenin has been shown to have proliferative effects in other contexts (Giles et al., 2003). Moreover, in cell culture experiments, a constitutively active mutant increased the activity of a β -catenin/Lef-1-driven reporter in the absence of EGF or TGF- α (both of which act through EGFR), whereas a dominant negative SGK3 mutant inhibited basal and EGF-activated reporter activity (Figure 6, A and B). In contrast, constitutively active SGK3 inhibited FKHRL1-mediated gene transcription in the absence of the growth factors (Figure 6C). Because forkhead transcription factors are potent inhibitors of cell proliferation (Burgering and Medema, 2003), their inhibition would be expected to contribute to a proliferative state.

The phenotypes of several mutant mouse models are also consistent with a role for SGK3 in mediating EGFR effects on keratinocytes through forkhead and β -catenin: between P2 and P5, EGFR expression in hair follicles increases dramatically (Hansen et al., 1997), and *Egfr* KO mice display a hair growth phenotype with significant similarities to the *Sgk3* KO described here (Sibilia and Wagner, 1995; Threadgill et al., 1995; Hansen et al., 1997), as does the Wa-2 mouse, which is due to a spontaneously arising *Egfr* hypomorphic allele (Luetke et al., 1994). Similarly, both the TGF- α KO and spontaneously occurring Wa-1 mouse have hair growth abnormalities with features resembling the *Sgk3* KO mice (Luetke et al., 1993; Mann et al., 1993). Moreover, keratinocyte-specific deletion of phosphatase and tensin homolog on chromosome 10 results in acceleration of hair follicle morphogenesis (Suzuki et al., 2003), and keratinocyte-specific overexpression of β -catenin causes de novo follicle development (Gat et al., 1998).

Inhibition of glycogen synthase kinase (GSK)3- β through the Wnt pathway results in stabilization of free cytoplasmic β -catenin, which is subsequently translocated to the nucleus where it regulates gene transcription. Interestingly, although growth factors (acting through PI3K and SGK/Akt) also inhibit GSK3- β , it is widely believed that this inhibition is restricted from impacting on β -catenin stability (Biondi and Nebreda, 2003). However, there is evidence to support the idea that in some contexts this is not the case. Thus, EGF induction of β -catenin signaling under conditions where it stimulates cell motility (Muller et al., 2002), and Akt stabilization of β -catenin (Fukumoto et al., 2001) have both been reported in vitro. Our data lend further in vivo support to this idea.

It should further be noted that the disorganized ORS, and reduced hair bulb, IRS, and hair shaft size observed in *Sgk3* KO hair follicles suggest that SGK3 might play a role in mediating signals that control cell migration, which also

plays an important role in hair follicle morphogenesis and adult hair cycling (Oshima et al., 2001).

The precise substrates of SGK3 and its point of action in regulating β -catenin expression and nuclear accumulation remain to be resolved. Although it seems likely that effects on GSK3- β that influence β -catenin activation of gene transcription will be implicated, other components of the Wnt pathway and endosomal recycling pathways for activated EGFR also could be involved (Cozier et al., 2002), as well as effects on forkhead family-regulated transcriptional events. Another intriguing possibility is that SGK3 alters cell proliferation by altering the activity of the epithelial sodium channel (ENaC) (Friedrich et al., 2003). ENaC is expressed in keratinocytes (Mauro et al., 2002) and can alter ionic milieu, cell volume, and proliferative state (Lang et al., 2003).

Possible Redundancy between SGK3 and Other Members of the SGK/Akt Family

Given the widespread expression pattern of SGK3, it is worth noting the lack of gross abnormalities in tissues other than skin in *Sgk3* KO mice. Similarly, *Sgk1*, *Akt1*, and *Akt2* knockout mice have relatively mild phenotypes, which differ substantially from one another (Chen et al., 2001; Cho et al., 2001a,b; Wulff et al., 2002; Garofalo et al., 2003). The phenotype of *Akt1/Akt2* KO mice is distinct from and substantially more severe than either single gene deletion, suggesting partial redundancy between these two family members (Peng et al., 2003). Thus, it seems likely that partial redundancy accounts for the relatively benign features of *Sgk3* gene disruption, possibly due to overlap in substrate specificity (Kobayashi et al., 1999; Liu et al., 2000; Dai et al., 2002) and phosphoinositide binding. Interestingly, the PX domain, present in SGK3 (Liu et al., 2000) and possibly in SGK1 (Helms et al., 2003), and the PH domain, present in Akt1 and Akt2 have partially overlapping phosphoinositide subtype binding (Virbasius et al., 2001; Xu et al., 2001). Full elucidation of the developmental and physiological roles of SGK3, as well as the mechanisms underlying its actions, will require characterization of animals with *Sgk3* deletion in conjunction with deletion of other SGK/Akt genes.

ACKNOWLEDGMENTS

We appreciate helpful assistance from S. Teglund and the Karolinska Center for Transgene Technologies for embryonic stem cell work and generation of chimeric mice; H. Ordanza with animal care and glucose tolerance tests; D. Kusewitt (Ohio State University Mouse Phenotyping Service, Columbus, OH) for multiple-tissue histological analysis and blood values; L. Prentice for skin section preparation; S. Pennypacker for providing NHK and primary mouse keratinocyte cells; E.-H. Choi for expert assistance with [³H]thymidine incorporation assays; and A. Oro and G. Barsh for helpful discussions and critical review of the manuscript. This work was supported by American Heart Association Western States Postdoctoral Fellowship 0225080Y (to J.A.M.), National Institutes of Health grant AR44341 (to T.M.), Deutsche Forschungsgemeinschaft (La 315/4-6) (to F.L.), and National Institutes of Health grant DK56695 (to D.P.).

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