Expression of Cyclooxygenase Isozymes During Morphogenesis and Cycling of Pelage Hair Follicles in Mouse Skin: Precocious Onset of the First Catagen Phase and Alopecia upon Cyclooxygenase-2 Overexpression

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Cyclooxygenase (COX)-1 and -2 catalyze the key reaction in prostaglandin biosynthesis. Whereas COX-1 is found in most tissues, COX-2, with a few exceptions, is not expressed in normal tissues but becomes transiently induced in the course of inflammatory reactions. In many neoplastic epithelia, COX-2 is constitutively overexpressed. Here we show that COX isozymes are spatiotemporally expressed during morphogenesis of dorsal skin epithelium of NMRI mice. COX-1 and COX-2 mRNA and protein were detected in embryonic and postnatal epidermal tissue by RT-PCR, northern blot, and immunoblot analysis indicating that both isoforms may contribute to prostaglandin production. Being barely detectable in interfollicular epidermis and resting hair follicles of adult mice, COX-2 protein appeared in embryonic skin first in epidermal precursor cells and later on in the basal cells and the peridermal layer of the stratified epidermis. In the course of pelage hair follicle morphogenesis, COX-2 remained expressed in the basal interfollicular compartment and, in addition, became apparent in elongated hair germs and hair pegs and later on in the outer root sheath cells of the distal and proximal hair follicles as well as in basal sebaceous gland cells. During the subsequent synchronous phases of hair cycling, COX-2 expression declined in catagen, was barely detectable in telogen, and was reinduced in the basal outer root sheath and basal sebaceous gland cells of anagen hair follicles. COX-1 immunosignals were detected predominantly in the interfollicular spinous and granular layers of the developing, neonatal, and adult epidermis but not in follicular epithelial cells of developing or cycling hair follicles. Dendritic cells in the interfollicular epidermis and distal hair follicles were also COX-1-positive. Transgenic overexpression of COX-2 under the control of a keratin 5 promoter in basal cells of the interfollicular and follicular epidermis induced a precocious entry into the first catagen stage of postnatal hair follicle cycling and a subsequent disturbance of hair follicle phasing. Furthermore, transgenic mice developed an alopecia. Inhibition of transgenic COX-2 activity by feeding the specific COX-2 inhibitor valdecoxib suppressed the development of alopecia, indicating that COX-2-mediated prostaglandin synthesis is involved in hair follicle biology. ***Keywords: epidermis/transgenic/valdecoxib.***
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Hair follicle morphogenesis is regulated by interactions between epidermal keratinocytes committed to hair-follicle-specific differentiation and a subset of specialized fibroblasts with follicle-inductive properties and committed to form the dermal papilla of the developing hair follicle (Hardy, 1992). These epithelial–mesenchymal interactions finally culminate in the formation of the hair shaft, which is surrounded by the multilayered inner root sheath, the companion layer, and the outer root sheath (ORS), the latter being the outermost concentric layer of epithelial cells (Winter *et al*, 1998; Fuchs and Raghavan, 2002). Once fully developed, hair follicles undergo repeated cycles of regression and involution (catagen), rest (telogen), and renewal (anagen) during the whole life span (Hardy, 1992; Fuchs *et al*, 2001; Stenn and Paus 2001).

Distinct signaling pathways, such as the wnt/β-catenin, sonic hedgehog, bone morphogenic protein, and noggin signaling cascades, control hair follicle morphogenesis, postnatal hair growth, and cycling (Botchkarev *et al*, 1999, 2002; Fuchs *et al*, 2001; Huelsken *et al*, 2001; Fuchs and Raghavan 2002). In addition, distinct growth factors (Stenn and Paus 2001) and prostaglandin (PG) types (Johnstone and Albert, 2002) seem to regulate hair follicle growth and cycling in humans. In fact, stimulation of PGE_2 synthesis is thought to be at least partly involved in the

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Abbreviations: COX, cyclooxygenase; E, embryonic day; EP receptor, prostaglandin E receptor; IFE, interfollicular epidermis; K5, keratin 5; ORS, outer root sheath; P, postnatal day; PG, prostaglandin.

minoxidil-effected induction and prolongation of the anagen phase of normal human hair growth (Michelet *et al*, 1997). Furthermore, inhibitors of PG synthesis including certain nonsteroidal anti-inflammatory drugs have been reported to induce alopecia in humans (Tosti *et al*, 1994; Pillans and Wood, 1995).

PG are mainly synthesized from arachidonic acid, which serves as a major substrate for cyclooxygenases (COX). These enzymes generate the PG endoperoxide PGH_2, which is subsequently converted by prostanoid synthases into the biologically active prostanoids such as the prostaglandins PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxane A_2 in a cell-type-specific manner (Smith *et al*, 2000). There are two COX isozymes, i.e., a constitutive COX-1 and an inducible COX-2. COX-2 expression is stimulated by a wide variety of growth factors, cytokines, and hormones, particularly in the course of inflammatory processes (Wang and Smart, 1999; Smith *et al*, 2000). In adult skin of mice with resting hair follicles, COX-1 was found to be the major isoform expressed in keratinocytes of the interfollicular epidermis (IFE) and the upper part of the hair follicle, whereas COX-2 protein was barely detectable (Scholz *et al*, 1995; Müller-Decker *et al*, 1998) like in other adult tissues under nonpathological conditions (Sorli *et al*, 1998; O'Banion, 1999; Smith *et al*, 1999; Kirschenbaum *et al*, 2000). In contrast, a transient induction of COX-2 protein, but not of COX-1, in the basal cells of the IFE, the ORS, and the sebaceous gland was observed upon treatment of adult mouse skin with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (Scholz *et al*, 1995; Müller-Decker *et al*, 1998). Moreover, upon depilation of adult mouse skin a transient induction of COX-2 mRNA was found in ORS cells of regrowing hair follicles (Torii *et al*, 2002). Upon superficial (Scholz *et al*, 1995) and incisional (Müller-Decker *et al*, 2002a) skin wounding, COX-2 protein was found to be transiently upregulated in the epidermis as well as in the ORS of anagen hair follicles growing out into the healing wound. Furthermore, in heterozygous transgenic mice, a constitutive overexpression of COX-2 driven by the keratin 5 (K5) promoter in the basal cells of the IFE and the pilosebaceous unit led to a delayed emergence of hair shafts and a reduced hair follicle density, as well as to sebaceous gland hyperplasia with concomitant overproduction of sebum (Neufang *et al*, 2001). Alopecia was also described as the gross phenotype of keratin 14 (K14) COX-2 transgenic mice, which was suppressed by treatment with the COX-2-selective inhibitor celecoxib. Neither hair follicle morphogenesis and cycling nor alopecia, however, were histologically analyzed (Bol *et al*, 2002).

Here we describe experiments showing a distinct spatiotemporal epidermal expression pattern of the two COX isozymes during epidermal morphogenesis and hair follicle cycling in the dorsal skin of mice. Moreover, in homozygous transgenic mice, COX-2 overexpression resulting in overshooting PG synthesis severely impaired hair follicles and induced alopecia.

MATERIALS AND METHODS

Materials Goat polyclonal anti-COX-1 (SC-1754), anti-COX-2 (SC-1745), rabbit polyclonal anti-EP2 receptor (SC-20675), goat polyclonal anti-EP3 receptor (SCV-16019), goat polyclonal anti-EP4 receptor (SC-16022), goat polyclonal anti-β-actin antibodies (SC-1616), peroxidase-conjugated goat anti-rabbit IgG, and donkey anti-goat IgG antibodies were purchased from Santa Cruz (Heidelberg, Germany). ELISA BSA and goat serum were purchased from Sigma (Deisenhofen, Germany); rabbit polyclonal anti-EP1 receptor antibody and PGE_2 and $PGF_{2\alpha}$ enzyme immunoassays were from Cayman (Ann Arbor, MI). Aprotinin, leupeptin, and α_2-macroglobulin were from Roche Applied Sciences (Mannheim, Germany).

Animals Experiments were performed with wild-type NMRI mice (outbred strain from RCC, Füllinsdorf, Switzerland) and the homozygous K5 COX-2 transgenic line $675^{+/+}$, generated as described previously (Neufang *et al*, 2001). All mice were kept in the central animal unit of the DKFZ (Heidelberg, Germany) under an artificial day/night rhythm and were fed altromin standard food pellets and water ad libitum if not stated otherwise. For analysis of hair follicle morphogenesis, embryonic and neonatal skin was collected at distinct time points of estimated gestational age (i.e., embryonic days (E) E13, E15, and E18 with the morning on which a vaginal plug was found set E0.5) or at different postnatal ages (postnatal days (P) P1, P4, P8, P11, P12 (hair follicle morphogenesis), P17, P20 (catagen), P21 (telogen), P25, P28, and P29 (1, anagen). In 7-week-old (P49) adult mice with telogen hair follicles in dorsal skin, anagen hair follicles were induced by depilation of hair shafts. Skin was taken for analysis of hair follicles 3–25 d after depilation. Skin biopsies were immediately frozen in liquid nitrogen or embedded in Tissue Tek (Vogel, Gießen, Germany) and processed for immunohistochemistry as described (Müller-Decker *et al*, 1998). All animal experiments have been approved by the Governmental Committee for Animal Experimentation (license 150/97 and 011/02) and by the ethics committee of the DKFZ. To study the effect of a COX-2-selective inhibitor on the transgene-induced phenotype, rodent diet 5010 containing 500 ppm valdecoxib was fed to transgenic mice starting on day 1 after birth for 3 mo by delivering the diet to nursing mice for 4 weeks and later on as regular chow.

RT-PCR and northern blot analysis Total RNA was extracted from pulverized frozen tissues by using RNA Clean (AGS, Heidelberg, Germany), and RT-PCR was performed with the cDNA cycle kit (Invitrogen) as described (Müller-Decker *et al*, 1999). The sense and antisense primers for COX-1 mRNA were 5′-TGCATGTGGCTGTGGATGTCATCAA-3′ and 5′-CACTAAGACAGACCCGTCATCTCCA-3′, respectively, giving rise to a 448-bp PCR product. The amplification conditions for COX-1 were 95°C for 5 min for one cycle and 94°C for 90 s, 63°C for 90 s, and 72°C for 150 s for 40 cycles followed by one cycle for 10 min at 72°C. The primers for COX-2 mRNA were sense 5′-ACTCACTCAGTTTGTTGAGTCATTC-3′ and antisense 5′-TTTGATTAGTACTGTAGGGTTAATG-3′ (583 bp, 94°C/54°C/72°C, 40 cycles), for EP1 receptor were sense 5′-TAGTGTGCAATACGCTCAGCG-3′ and antisense 5′-GAGGTGACTGAAACCACTGTG-3′ (553 bp, 95°C/64°C/72°C, 40 cycles), for EP2 receptor were sense 5′-GACTGCATACCTTCAGCTGTAC-3′ and antisense 5′-TATGGCAAAGACCCAAGGG-3′ (369 bp, 94°C/58°C/72°C, 40 cycles), for EP3 receptor were sense 5′-GGCACGTGGTGCTTCATC-3′ and antisense 5′-GGGATCCAAGATCTGGTTC-3′ (416 bp, 94°C/54°C/72°C, 40 cycles), for EP4 receptor were sense 5′-CTGAACAGCCCGGTGACCATTCC-3′ and antisense 5′-GCCGGCCAGCCGCTTGTCCAC-3′ (363 bp, 95°C/63°C/72°C, 40 cycles), and for β-actin as an internal control were sense 5′-AAACTGGAACGGTGAAGGC-3′ and antisense 5′-GCTGCCTCAACACCTCAAC-3′ (429 bp, 94°C/54°C/72°C, 30 cycles). Signals by potential genomic contamination were ruled out by selection of intron-spanning primers. In addition, samples without cDNA were used as negative controls. PCR was performed at least two times with a single cDNA sample. RT-PCR products were found on sequencing by the ABI prism to be >95% homolog to published sequences. Steady-state mRNA levels of COX-1 and COX-2 in skin were studied as described previously using isozyme-specific cDNA probes (Müller-Decker *et al*, 1995; Scholz *et al*, 1995).

Immunoblot analysis Immunoprecipitation of COX-2 and COX-1 as well as immunoblot analysis from skin were carried out by using isoenzyme-specific antisera samples as described previously (Müller-Decker *et al*, 1998). For immunoblot analysis of prostaglandin E receptors (EP receptors), frozen skin powder was homogenized in a buffer consisting of 50 mM Tris–HCl, pH 7.4, 1% Tween 20, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 2 mM α_2-macroglobulin. Debris was removed by centrifugation in a Beckman GPKR at 2200g for 20 min at 4°C. A total of 120 μg of precipitated and denatured protein was separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene fluoride membranes as described (Müller-Decker *et al*, 1995). Primary antibodies to EP receptors were used at a final dilution of 1:250. The secondary horseradish peroxidase-conjugated anti-IgG antibodies were used at a dilution of 1:2000. Immunodetection was carried out using enhanced chemiluminescence reagent according to the manufacturer's instructions (Amersham Pharmacia, Buckinghamshire, UK). The specificity of the immunosignals was confirmed by complete quenching of the immunosignal upon preadsorption of the antiserum with a 500-fold molar excess of the respective peptide antigen from Santa Cruz or Cayman.

Histochemistry and immunohistochemistry For hematoxylin/eosin staining, skin was fixed in PBS-buffered 4% paraformaldehyde for 16 h before embedding into paraffin. For immunohistochemical staining, 5-μm cryosections were used. COX isozymes were immunodetected as described (Müller-Decker *et al*, 1998; Neufang *et al*, 2001). Primary antibodies were diluted 1:100 (COX-1 and -2), and peroxidase-conjugated

secondary antibodies 1:100 in blocking solution. Development with diaminobenzidine was used for visualization. Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany), dehydrated, and mounted with Eukitt (O. Kindler, Freiburg, Germany). Omission of the primary antibody, or preincubation of the COX-isozyme-selective antibodies with the peptides (500-fold molar excess) used for generation of the antisera did abolish the staining (data not shown). Specimens were examined by an Axioskop 2 microscope connected to an Axiocam camera using Axiovision software (release 2.05, Zeiss, Goettingen, Germany).

Determination of PG concentrations Immediately after excision, biopsies were frozen in liquid nitrogen. Processing of samples and quantitation of PG levels by means of commercially available enzyme immunoassays were carried out as described (Müller-Decker *et al*, 2002b).

Histomorphometry For hair cycle staging, skin cryosections of K5 COX-2/675$^{+/+}$ and of age-matched wild-type mice were stained with hematoxylin/eosin. The percentage of hair follicles in telogen, anagen, and catagen stages were assessed in longitudinally cut hair follicles according to published criteria without considering substages (Müller-Röver *et al*, 2001) on P11, P17, P20, P29, and P49 after birth. Approximately 900 follicles in sections from three wild-type and transgenic mice each were counted by two independent investigators (G.F., K.M.D.). All sections were analyzed at a 100-fold magnification. Statistical differences were evaluated by Student's *t* test. Differences were considered to be significant with p values <0.05.

RESULTS

COX isozyme expression in embryonic and postnatal epidermis and its pilosebaceous appendage Using northern blot and semiquantitative RT-PCR analysis, COX-1 and COX-2 mRNA expression was detected in total mouse embryos at E13 and E15, in skin preparations at E18, and at P1 and P11/12, i.e., the period of hair follicle morphogenesis (**Fig 1*A,B***). Thereafter, mRNA expression of COX-2 declined in postnatal skin with catagen (P17/20) and telogen hair follicles (P21), increased again during the anagen stage (P28), and was barely detectable in adult skin with telogen hair follicles (P49). In contrast, COX-1 mRNA expression was similar through P12 to P21 and slightly increased during the anagen stage (P28) and remained present in adult skin (**Fig 1*A,B***). COX-2 protein was demonstrated in embryonic skin by immunoblot analysis representatively shown for E17 (**Fig 1*C***). Its expression persisted through P8 to P20 but was undetectable in adult skin with resting hair follicles (P49). COX-1 protein expression did not show noticeable alterations during this time period (**Fig 1*C***).

A more detailed immunohistochemical analysis confirmed COX-2 expression in epithelial precursor cells of embryonic epidermis at E13, i.e., prior to the onset of epidermal stratification (**Fig 2*A***). At E15, COX-2 protein was present in the basal cells as well as in the outermost ectodermal periderm layer of the stratified embryonic epidermis, whereas differentiated layers in between were negative (**Fig 2*B***). From E15 to E18, i.e., the time period of maximal induction of pelage hair follicles (Hardy, 1992), COX-2 expression became apparent in elongated hair germs and hair pegs of stages 2 and 3 (Paus *et al*, 1999), whereas dermal papilla cells were negative (**Fig 2*C,C inset***). Upon bulb-like thickening of the hair peg (**Fig 2*C***, *filled arrow*) and differentiation of the bulbous peg cells into matrix cells and progeny thereof, COX-2 expression became restricted to basal ORS and sebaceous gland cells of growing hair follicles (**Fig 2*D***). Up to P12, i.e., over the period of ongoing hair follicle morphogenesis and development of IFE toward the typical three-layered adult phenotype, COX-2 was found in the ORS cells of the distal but also of the proximal hair follicles as well as in basal sebaceous gland cells and in the basal interfollicular compartment. (**Fig 2*D,D inset,E***). During the subsequent synchronous phases of hair cycles, COX-2 expression declined in catagen at P17 (**Fig 2*F***) and was barely detectable in telogen hair follicles at P21 (**Fig 2*G***). COX-2 was reexpressed in ORS cells of early anagen hair follicles at P25 (**Fig 2*H***, ***inset***) as well as in ORS and basal sebaceous gland cells of late anagen hair follicles at P28 (**Fig 2*H, I***). COX-2 protein was barely detectable in IFE and resting hair follicles of adults (P49; Müller-Decker *et al*, 1998). ORS cells of anagen hair follicles induced by depilation (12–18 d after depilation, data not shown) or incisional wound healing in adult skin, however, were COX-2-positive (Müller-Decker *et al*, 2002a).

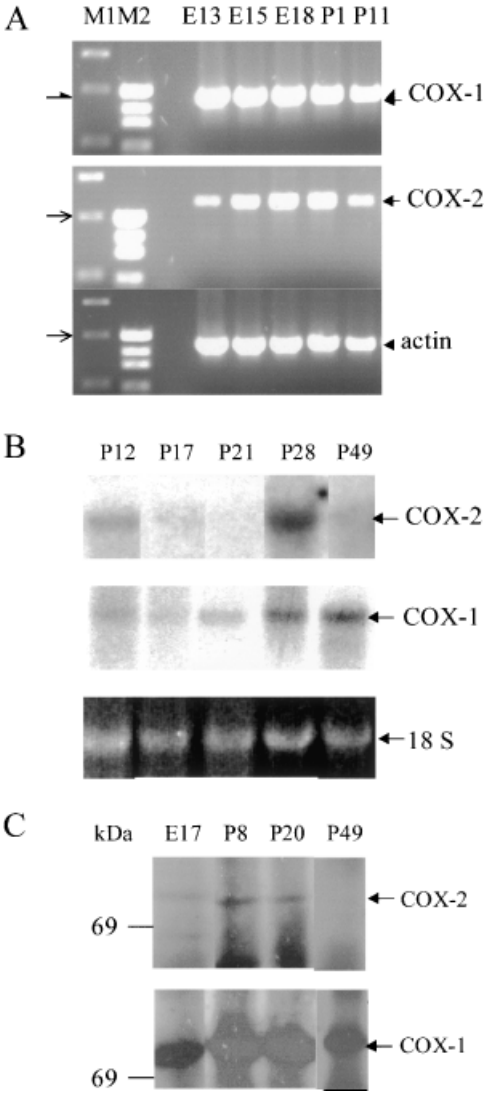

Figure 1. COX isozyme expression in embryonic and neonatal mouse skin. (*A*) One microgram of total RNA isolated from embryos 13 and 15 d after gestation, from embryonic skin at day 18 after gestation (E13–E18), or from postnatal dorsal skin at days 1 (P1) and 11 (P11) after birth was reverse-transcribed and amplified with specific primer sets for COX-1 and COX-2, as described under Materials and Methods. β-Actin was run as an internal control. Markers were MBI Fermentas M1, GeneRuler 1-kb DNA ladder; and M2, pUC19 DNA/*Msp*I; *arrow,* 500-bp fragment. (*B*) Ten micrograms of total RNA isolated from dorsal skin with developing hair follicles at P12, with catagen hair follicles at P17, with telogen hair follicles at P21, with hair follicles in first anagen cycle at P28, and from adult dorsal skin at P49 with telogen hair follicles were subjected to northern blot hybridization using COX isozyme-specific cDNA probes. (*Top*) Hybridization with COX-2 cDNA; (*middle*) hybridization with COX-1 cDNA; (*bottom*) RNA loading control showing ethidium-bromide-stained 18S rRNA signals. (*C*) Immunoblot analysis of COX isozyme expression in skin of E17 embryos and in skin of P8, P20, and P49. *Arrows on right,* COX-1-, COX-2-, β-actin-, and 18S rRNA-specific signals.

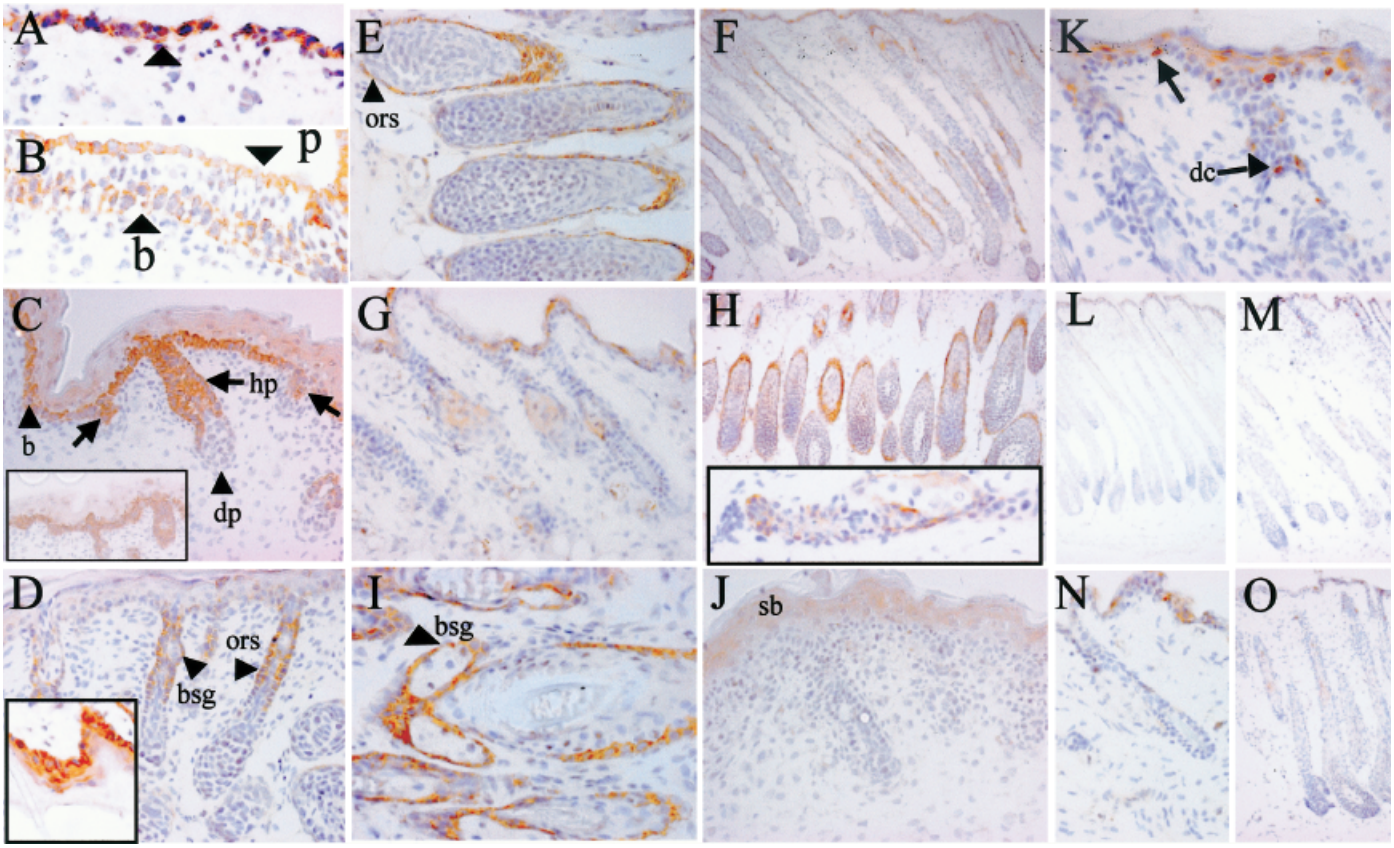

Figure 2. COX isozyme expression during embryonic and neonatal development of mouse epidermis and the pilosebaceous unit as well as during the first postnatal hair cycle. Cryosections of dorsal mouse skin were stained for the presence of COX isozymes by immunohistochemistry using COX-isozyme-selective antibodies and diaminobenzidine as a substrate. (*A–J*) COX-2 stainings of skin from (*A*) E13, (*B*) E15, (*C, including inset*) E18 embryos and (*D*) from 4-d-old, (*D, inset*) 8-d-old, and (*E*) 12-d-old neonatal mice with developing hair follicles; (*F*) of skin with catagen hair follicles from a 17-d-old neonatal mouse; (*G*) of skin with telogen hair follicles from a 21-d-old neonatal mouse; (*H, inset*) of a skin with early anagen at P25; and (*H,I*) late anagen hair follicles from 28-d-old neonatal mice. COX-2 immunosignals were localized to the epithelial precursors of skin (*A, arrow*), to the basal (*B,C, arrow b*), and peridermal (*B, arrow p*) layers of embryonic skin, to basal IFE (*D, inset*), to ORS cells of growing hair follicles (*C,D,E,H,H inset, arrow ors*), and to basal cells of the sebaceous glands (*D,I, arrow bsg*). (*J–O*) COX-1 stainings of skin (*J*) from E18 embryo and (*K,L*) with developing hair follicles from an 8-d-old neonatal, (*M*) with catagen hair follicles from a 17-d-old neonatal, (*N*) with telogen hair follicles from a 21-d-old neonatal, (*O*) with anagen hair follicles from a 28-d-old neonatal mice. COX-1 immunosignals were localized to the suprabasal spinous and granular layers of epidermis (*J,K, sb*), as well as to dendritic cells within IFE and the distal part of the hair follicles (*K,M,N,O, arrow dc*). Original magnifications: ×100 (*L*), ×160 (*F,H,M,O*), ×200 (*C inset*), ×400 (*C–E,G,J,N*), ×630 (*A,B,D inset,H inset,I,K*).

COX-1 showed a different pattern of expression. Immunosignals were detected in spinous and granular keratinocytes of the suprabasal compartment at E15–E18 but not in the developing hair follicle (**Fig 2*J***). Irrespective of hair follicle cycling and epidermal thinning toward the adult phenotype, this pattern of expression was detected in skin through day 28 after birth as well as in skin of 7-week-old mice (**Fig 2*K–O***; Müller-Decker *et al*, 1998). In addition, dendritic cells located interfollicularly and in the distal portion of the hair follicle of developing neonatal (P8–P28, **Fig 2*K, M–O***) and adult skin (data not shown) were COX-1-positive.

COX isozyme expression in embryonic skin (E18) correlated with PGE_2 and $PGF_{2\alpha}$ levels of 2648 ± 535 and 1145 ± 243 pg/mg protein $\pm$ SD ($n = 5$), respectively. In skin of 7-week-old mice the corresponding values were 698 ± 361 and 630 ± 386 pg/mg protein $\pm$ SD ($n = 5$).

PGE_2 signals through EP receptors consisting of four subtypes, EP1, EP2, EP3, and EP4. Using RT-PCR, mRNA of all subtypes was detected from E13 to P11 (**Fig 3*A***). According to immunoblot analysis the four receptors showed a subtype-dependent expression pattern in the course of hair follicle morphogenesis and hair follicle cycling (**Fig 3*B***). EP2 receptor protein was identified in embryonic (E18), postnatal (P1 through P20), and adult skin (P49). EP1 and EP4 immunosignals were also found in all these samples with stronger signals in skin with catagen (P17–P20) and telogen hair follicles (P49). The expression of EP3 receptor protein was restricted to E18 and P1.

Precocious entry into the first hair follicle cycle and alopecia in COX-2 transgenics To determine the effects of COX-2 expression on pelage hair follicles in dorsal skin, transgenic mice were generated expressing a COX-2 transgene under the control of a K5 promoter in basal cells of the IFE and the pilosebaceous appendage of skin (Neufang *et al*, 2001). Homozygous COX-2 overexpression in the skin as well as in the epidermis (**Fig 4*A***) occurred at the expected locations, i.e., in the basal cells of the sebaceous gland and the ORS (**Fig 4*B***) and the IFE (not shown). COX-2 overexpression resulted in highly elevated PGE_2 levels in the skin of adult mice (**Fig 4*C***) and correlated with the sparse hair coat (**Fig 4*E***).

As documented by histological analysis, transgenic overexpression of COX-2 did not alter early stages of postnatal hair follicle morphogenesis as shown for P4 (**Fig 5*A, D***). In transgenic skin at P11, however, mainly catagen follicles were observed, whereas in the skin of age-matched wild-type mice growing hair follicles predominated. This indicates a precocious entry into the catagen phase of the first hair growth cycle in transgenics (**Fig 5*B, E***). Thereafter, the regular hair follicle

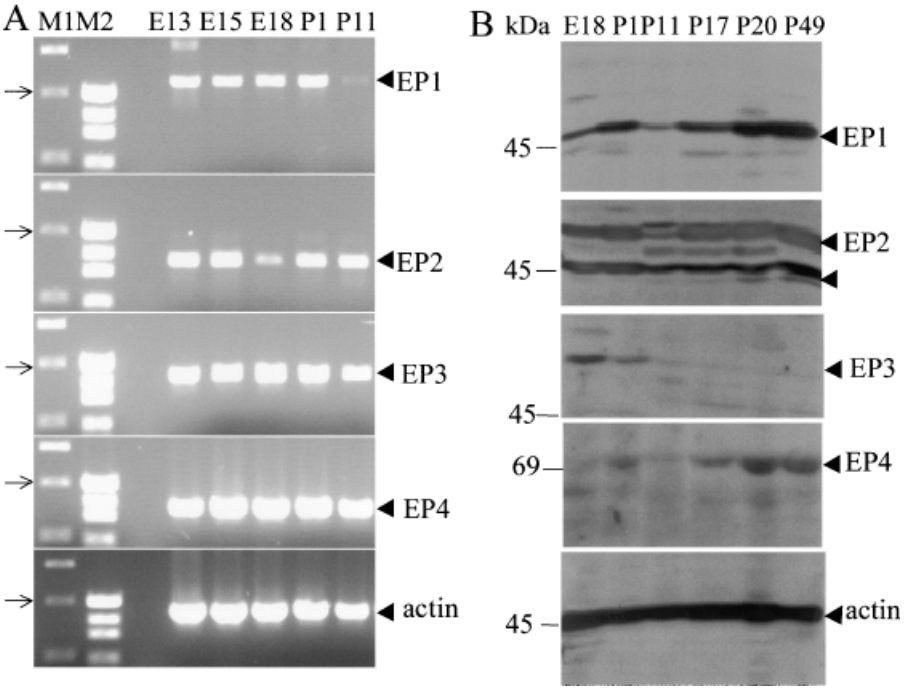

Figure 3. EP receptor expression in embryonic and neonatal mouse skin. (*A*) One microgram of total RNA isolated from E13 and E15 embryos, from embryonic skin at E18, and from postnatal dorsal skin at P1 and P11 was reverse-transcribed and amplified with specific primer sets for EP1—EP4 receptors as described under Materials and Methods. β-Actin was run as an internal control. *Arrows on right,* EP receptor subtype-specific amplicons. Markers were MBI Fermentas M1 and M2; *arrows on left,* 500-bp fragment. (*B*) Immunoblot analysis of EP receptor isoforms EP1, EP2, EP3, and EP4 in skin of E18 embryos and in skin of P1, P11, P17, P20, and P49. Protein loading control showing β-actin signals. *Arrows,* EP receptor subtype-specific immunosignals. Note for EP-2 receptor 45- and approximately 52-kDa signals which are reported to represent partial and fully glycosylated receptor proteins (Konger *et al*, 2002).

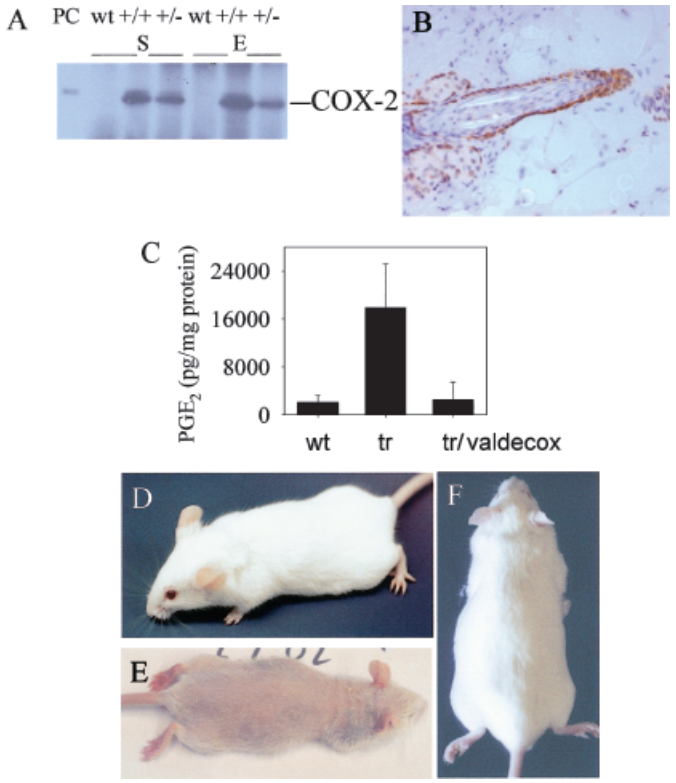

Figure 4. Suppression of the COX-2 transgene-induced PG levels in dorsal mouse skin correlates with suppression of alopecia. (*A*) Expression levels of COX-2 protein in skin (*S*) and epidermis (*E*) of heterozygous (+/–) and homozygous (+/+) transgenic mice compared to age-matched wild-type mice (*wt*). *PC,* COX-2 positive control. (*B*) COX-2 staining of a catagen hair follicle from skin of a 6-mo-old K5 COX-2 transgenic mouse. Note COX-2 expression in basal cells of ORS and sebaceous glands. Original magnification: ×630. (*C*) The levels (mean ± SEM, n = 3) of PGE_2 were determined for the dorsal skin of wild-type NMRI (*wt,* n = 3), K5 COX-2 transgenic mice fed with a control diet (*tr,* n = 3), and K5 COX-2-transgenic mice receiving a valdecoxib-containing diet (*tr/valdecoxib,* n = 3). The gross phenotypes are shown (*D*) for a wild-type, (*E*) for a K5 COX-2-transgenic, and (*F*) for a valdecoxib-treated K5 COX-2-transgenic mouse.

staging in wild-type mice with prevailing catagen hair follicles at P17 (**Fig 5*C***), catagen and telogen follicles at P20 (**Fig 5*G***), predominantly anagen follicles at P29 (**Fig 5*H***), and only telogen follicles at P49 (**Fig 5*I***) was strongly disturbed in COX-2 transgenic skin. The effects of transgenic COX-2 culminated in a strong predominance of catagen hair follicles instead of telogen hair follicles at P49 (**Fig 5*F,J–L***). The quantitative histomorphometric analysis revealed a percentage distribution of catagen, telogen, and anagen hair follicles of 67% to 7% to 26% in skin sections of transgenics compared with 14% to 0% to 86% in wild-type skin at P11 (**Fig 6*A,B***). In wild-type skin, however, 89% of hair follicles had entered the catagen phase not until P17, whereas 41% catagen follicles were found in transgenic skin at this time point. Significant differences in hair follicle staging were also found at P20, P29, and P49. At P49, the latest time point of synchronized hair follicle cycling in wild-type mouse skin, 85% of follicles were telogen and 15% were anagen, whereas 94% of follicles were catagen and 6% anagen in COX-2 transgenic skin (**Fig 6*A,B***).

At the age of 3–10 mo mice had developed an alopecia that was characterized on a histological level by a loss of hair follicles. Only a few hair follicles flanked completely hairless areas of transgenic skin compared to the skin of 10-mo-old wild-type mice (**Fig 5*M–O***). On the other hand, the sparse hair coat visible early after birth is due to a reduced follicle density and a delayed emergence of the hair shafts (Neufang *et al*, 2001). In addition, substantial sebaceous gland hyperplasia became apparent in transgenic skin around P29 (**Fig 5*H,K***).

When transgenic mice were fed the COX-2-selective inhibitor valdecoxib (Talley *et al*, 2000) for 3 mo postnatally, PGE_2 levels in dorsal skin were similar to those of wild-type skin, whereas transgenic littermates fed with a drug-free control diet had highly elevated PGE_2 levels (**Fig 4*C***). Moreover, the transgene-characteristic impairment of the hair coat did not develop under valdecoxib treatment, showing that valdecoxib-caused suppression of COX-2 activity was responsible for this effect (**Fig 4*E,F***).

DISCUSSION

As shown here, the spatiotemporal pattern of COX-1 expression differs from that of COX-2 during embryonic and early postnatal

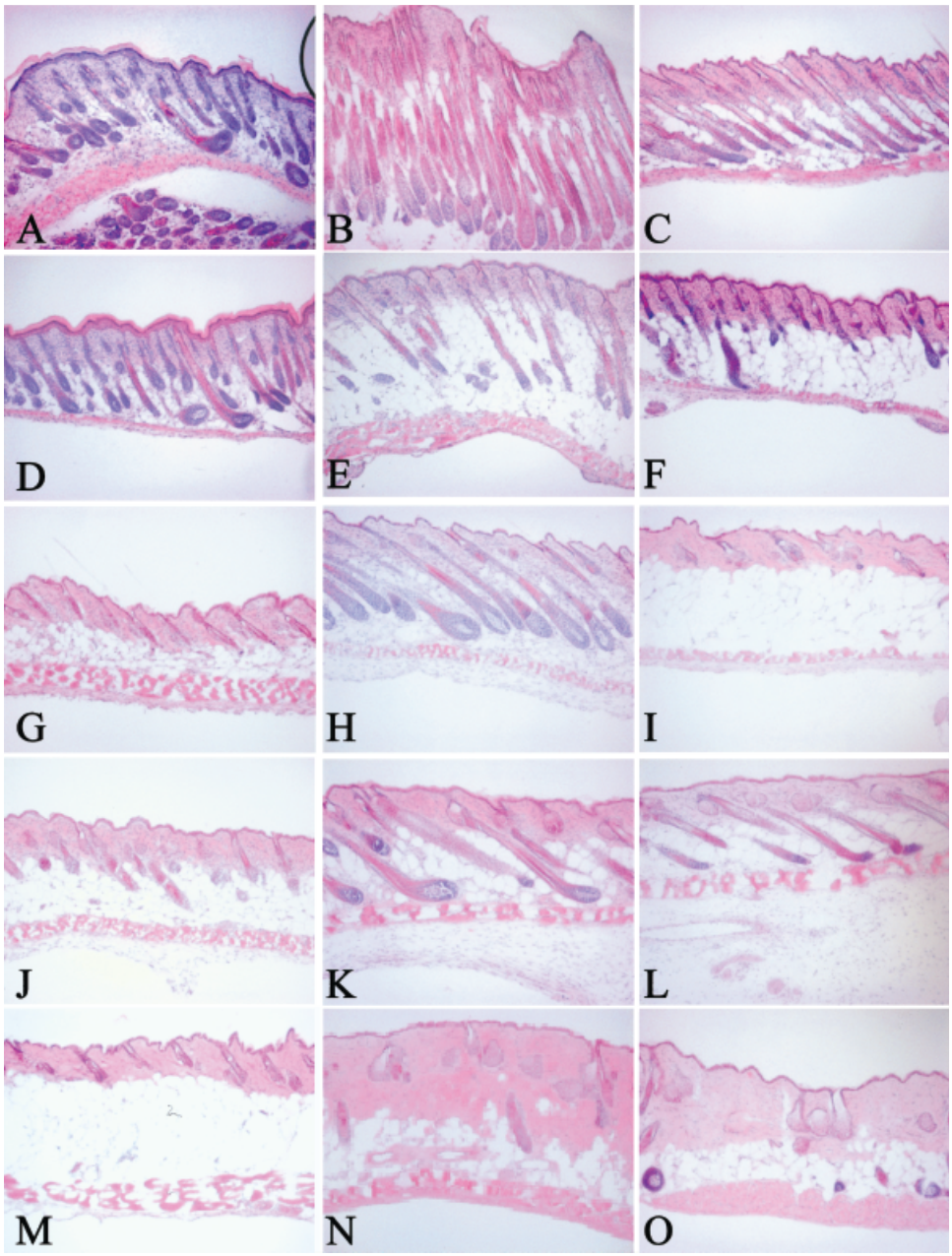

Figure 5. K5 promoter-driven COX-2 overexpression causes a pronounced hair phenotype in dorsal mouse skin. Representative pictures of hematoxylin/eosin-stained longitudinal sections of dorsal skin from 4-d-old (*A,D*), 11-d-old (*B,E*), 17-d-old (*C,F*), 20-d-old (*G,J*), 29-d-old (*H,K*), 7-week-old (*I,L*), 6-mo-old (*N*), and 10-mo-old (*M,O*) wild-type (*A–C,G–I,M*) and homozygous K5 COX-2 transgenic (*D–F,J–L,N,O*) mice. Note the precocious entry of hair follicles into catagen stage at P11 (*E*) and subsequent asynchronous hair follicle cycling (*F,J–L*), hairless areas (*N,O*), and sebaceous gland hyperplasia (*K,L,N,O*) in transgenic skin. Original magnifications: × 100 (*A–O*).

development of mouse epidermis and its pilosebaceous appendage. Whereas keratinocytes of adult skin express COX-1, but not COX-2 (Müller-Decker *et al*, 1998; Goldyne 2000), a strong expression of both isoforms was found in embryonic epidermis. COX-1 expression became detectable upon stratification of the epidermis in spinous and granular layers and persisted until P8–P9, i.e., when IFE started thinning toward the adult phenotype. Afterward throughout life, epidermal COX-1 was restricted to a few individual cells scattered throughout the IFE. Moreover, at about P8, COX-1 protein was found in dendritic cells, which were localized in the IFE and the distal part of the hair follicle. Whether these COX-1-positive cells represent γ/δ-T cell-receptor-positive lymphocytes, which are the predominant immunocytes detectable in early postnatal epidermis, or other dendritic cell types such as Langerhans cells (Paus *et al*, 1998), remains to be clarified. This expression in dendritic cells may indicate a function of COX-1 in skin immune reactions (Uhrade *et al*, 1988) besides its role in normal tissue homeostasis (Tiano *et al*, 2002).

During embryonic development of the epidermis, the onset of COX-2 expression preceded that of COX-1. This observation was not unexpected, since various embryonic tissue types other than skin have been described to express COX-2 (Komhoff *et al*, 1997; Maslinska *et al*, 1999; Kirschenbaum *et al*, 2000; Khan *et al*, 2001). In skin, COX-2 expression appeared first in epithelial precursor cells of the epidermis and continued during stratification. COX-2

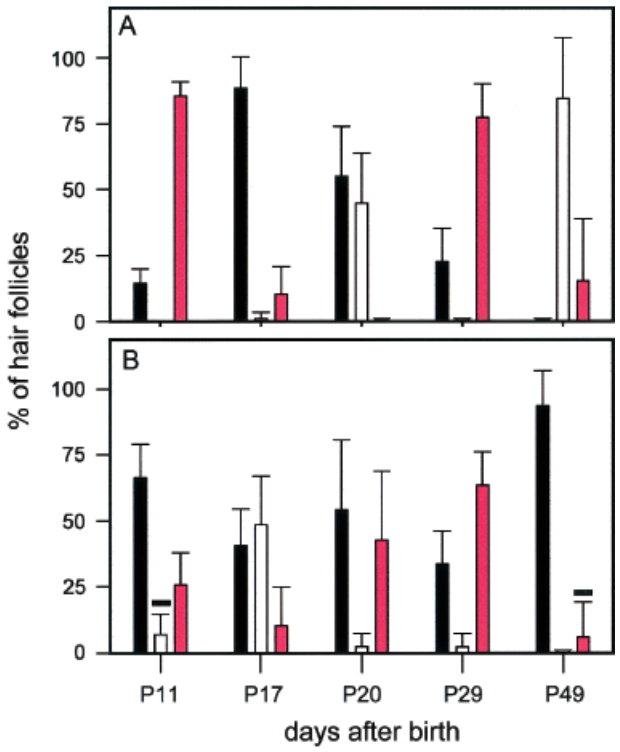

Figure 6. Precocious entry into catagen stage in the skin of homozygous K5 COX-2-transgenic mice. The percentage of hair follicles in catagen (*black bars*), telogen (*white bars*), and anagen (*red bars*) stages were evaluated by quantitative histomorphometry according to published criteria (Müller-Röver *et al*, 2001) in skin sections of wild-type (*A*) and K5 COX-2 transgenic (*B*) mice on P11, P17, P20, P29, and P49. Significant differences ($p < 0.05$–0.0005) were found between individual stages of follicles in skin of K5 COX-2 transgenic versus wild-type mice, except for telogen and anagen follicles at P11 and P49, respectively (indicated by a minus).

was mainly found in basal and peridermal layers. During hair follicle morphogenesis, COX-2 appeared in all cells of elongated hair germs and hair pegs of stages 2 and 3 (Paus *et al*, 1999), whereas dermal papilla cells were negative. In the course of the gradual transition of early-stage hair follicles into fully differentiated follicles after birth, COX-2 expression became restricted to basal ORS and sebaceous gland cells. COX-2 expression declined, when follicles entered catagen, and was barely detectable in telogen follicles. COX-2 protein arose in basal ORS and sebaceous gland cells, however, during the first and subsequent anagen stages. COX-2 was not found in the dermal papilla of adult anagen hair follicles, confirming a previous study (Torii *et al*, 2002). Thus, COX-2 expression and activity were associated with embryonic/postnatal development of IFE and hair follicles, both processes characterized by expansive tissue growth.

This view is in line with the transient upregulation of COX-2 in the course of a regenerative epidermal hyperplasia (Scholz *et al*, 1995; Müller-Decker *et al*, 1998, 2002a). Moreover, constitutive COX-2 overexpression in basal epidermal keratinocytes induced an autopromoted phenotype in dorsal epidermis (Müller-Decker *et al*, 2002b) and an epidermal hyperplasia and dysplasia in tail skin the latter being due to a delay of terminal differentiation rather than to a stimulation of cell proliferation (Neufang *et al*, 2001). Accordingly, knocking-out COX-2 curtailed the 12-*O*-tetradecanoylphorbol-13-acetate-induced hyperplasia that was caused by an accelerated terminal differentiation (Tiano *et al*, 2002). In contrast, the sebaceous gland hyperplasia in COX-2 transgenics was due to a hyperproliferation of basal cells, which finally led to an increased sebum production (Neufang *et al*, 2001).

First evidence for a functional role of COX-2 in hair follicle physiology come from our analysis of hair follicle morphogenesis and cycling in homozygous transgenic mice with K5 promoter-driven COX-2 expression. With respect to the hair follicle, K14, i.e. the obligatory type I partner of K5, is known to be expressed in basal cells of early embryonic epidermis. This basal expression ceases regionally in keratinocytes of the hair placode and the subsequent early hair follicles of stages 2–5. It is, however, specifically restored in the ORS once the hair follicle has reached an almost complete tissue architecture toward the very late stage of gestation and shortly after birth. K5 expression is maintained in ORS during the entire follicle morphogenesis and all stages of the subsequent hair cycles (Kopan and Fuchs, 1989; Winter *et al*, 1998; Pena *et al*, 1999). According to this K5 expression pattern, transgenic COX-2 expression in the ORS is expected to initiate only around birth and to continue throughout life irrespective of follicular cycling. This pattern of transgenic COX-2 expression is unscheduled compared with the expression of endogenous COX-2 and correlated with a precocious entry of growing hair follicles into the first catagen stage after birth. This suggests that aberrant COX-2 expression and activity impair proper hair follicle morphogenesis. A premature entry into the first catagen phase has also been observed in mice with K14-driven neurotrophin-3 or plakoglobin overexpression (Botchkarev *et al*, 1998; Charpentier *et al*, 2000). This early change that is also observed in the skin of COX-2 transgenics appears to be responsible for the subsequent irregular hair follicle phasing. In addition, a premature onset of catagen has been associated with certain hair growth disorders including distinct forms of alopecia (Paus and Cotsarelis, 1999). This may hold true for alopecia development in COX-2 transgenic mice. This assumption is supported by the fact that inhibition of COX-2-dependent PGE_2 synthesis prevented the development of alopecia. At the age of 6 mo, the COX-2-induced alopecia seemed to be of a permanent type characterized by a loss of hair follicles. We did not, however, observe scarring or signs of inflammation or fibrosis being reportedly associated with the development of this type of alopecia (Paus and Cotsarelis, 1999).

It has been shown that PGE_2 acts locally in both an autocrine and a paracrine manner via the specific G-protein-coupled receptors EP1 to EP4 (Narumiya *et al*, 1999). In this study we show a differential expression of PGE_2 receptor proteins. EP1 and EP2 were present in embryonic and postnatal skin. EP3 protein expression was restricted to embryonic skin and P1, whereas EP4 protein increased around P20. According to preliminary results, EP1 to EP3 receptors were similarly expressed in the skin of adult (P49) COX-2 transgenic mice, whereas the EP4 receptor was found to be downregulated. The role of EP4 receptor function for the premature onset of catagen remains to be elucidated. Although the localization of the EP receptor proteins is at present unknown, there is evidence that keratinocytes are able to express all subtypes (Konger *et al*, 1998, 2002; Thompson *et al*, 2001). Recently, EP3 and EP4 transcripts could be located to distinct compartments of anagen hair follicles in depilated adult mouse skin. EP4 mRNA occurred in ORS cells of the hair bulb, whereas EP3 mRNA expression seemed to be restricted to dermal papilla cells (Torii *et al*, 2002). These data suggest that cells of the lower ORS are able to produce and to respond to PGE_2. In addition, COX-2-derived PGE_2 in the ORS may act in a paracrine manner on EP3-containing dermal papilla cells. In this context, it remains to be clarified whether COX-2-derived PGs are components of signaling pathways such as neurotrophin-3 or plakoglobin known to induce precocious catagen entry in transgenic mice (Botchkarev *et al*, 1998; Charpentier *et al*, 2000). Although our data provide evidence for a role of COX-2 expression and activity in hair follicle biology, further studies need to address the molecular mechanism of PG action in various cell types involved in the regulation of hair follicles morphogenesis and cycling, in particular the transition from the anagen to the catagen stage.

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REFERENCES

Bol DK, Rowley B, Ho CP, *et al*: Cyclooxygenase-2 overexpression in the skin of transgenic mice results in suppression of tumor development. *Cancer Res* 62:2516–2521, 2002

Botchkarev VA, Botchkareva NV, Roth V, *et al*: Noggin is a mesenchymally derived stimulator of hair-follicle induction. *Nat Cell Biol* 1:158–164, 1999

Botchkarev VA, Botchkareva NV, Sharov AA, Funa K, Huber O, Gilchrest BA: Modulation of BMB signaling by noggin is required for induction of the secondary (nontylotrich) hair follicles. *J Invest Dermatol* 118:3–10, 2002

Botchkarev VA, Welker P, Albers KM, *et al*: A new role for neurotrophin-3: Involvement in the regulation of hair follicle regression (catagen). *Am J Pathol* 153:785–799, 1998

Charpentier E, Lavker RM, Acquista E, Cowin P: Plakoglobin suppresses epithelial proliferation and hair growth in vivo. *J Cell Biol* 149:503–519, 2000

Fuchs E, Merrill BJ, Jamora C, DasGupta R: At the roots of a never-ending cycle. *Dev Cell* 1:13–25, 2001

Fuchs E, Raghavan S: Getting under the skin of epidermal morphogenesis. *Nat Rev* 3:199–209, 2002

Goldyne ME: Cyclooxygenase isoforms in human skin. *Prostaglandins Other Lipid Med* 63:15–23, 2000

Hardy MH: The secret life of the hair follicle. *Trends Genet* 8:159–166, 1992

Huelsken J, Vogel R, Erdmann B, Costarelis G, Birchmeier W: β-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* 105:533–545, 2001

Johnstone MA, Albert DM: Prostaglandin-induced hair growth. *Surv Ophtamol* 47S1:185–S202, 2002

Khan KN, Stanfield KM, Dannenberg A, Seshan SV, Baergen RN, Baron DA, Soslow RA: Cyclooxygenase-2 expression in the developing human kidney. *Pediatr Dev Pathol* 4:461–466, 2001

Kirschenbaum A, Liotta DR, Yao S, *et al*: Immunohistochemical localization of cyclooxygenase-1 and cyclooxygenase-2 in the human fetal and adult male reproductive tracts. *J Clin Endocrinol Metab* 85:3436–3441, 2000

Komhoff M, Grone HJ, Klein T, Seyberth HW, Nüsing RM: Localization of cyclooxygenase-1 and -2 in adult and fetal human kidney: Implication for renal function. *Am J Physiol* 272:F460–F468, 1997

Konger RL, Malavija R, Pentland AP: Growth regulation of primary human keratinocytes by prostaglandin receptor EP_2 and EP_3 subtypes. *Biochim Biophys Acta* 1401:221–234, 1998

Konger RL, Scott GA, Landt Y, Ladenson JH, Pentland AP: Loss of the EP_2 prostaglandin E_2 receptor in immortalized human keratinocytes results in increased invasiveness and decreased paxillin expression. *Am J Pathol* 161:2065–2078, 2002

Kopan R, Fuchs E: A new look into an old problem. Keratins as tools to investigate determination, morphogenesis, and differentiation in skin. *Genes Dev* 3:1–15, 1989

Maslinska D, Kaliszek A, Opertowska J, Toborowicz J, Deregowski K, Szukiewicz D: Constitutive expression of cyclooxygenase-2 (COX-2) in developing brain: A choriod plexus in human fetuses. *Folia Neuropathol* 37:287–291, 1999

Michelet JF, Commo S, Billoni N, Mahe YF, Bernard BA: Activation of cytoprotective prostaglandin synthase-1 by minoxidil as a possible explanation for its hair growth-stimulating effect. *J Invest Dermatol* 108:205–209, 1997

Müller-Decker K, Hirschner W, Marks F, Fürstenberger G: The effects of cyclooxygenase isozyme inhibition on incisional wound healing in mouse skin. *J Invest Dermatol* 119:1189–1195, 2002a

Müller-Decker K, Neufang G, Berger I, Neumann M, Marks F, Fürstenberger G: Transgenic cyclooxygenase-2 overexpression sensitizes mouse skin for carcinogenesis. *Proc Natl Acad Sci USA* 99:12483–12488, 2002b

Müller-Decker K, Reinerth G, Krieg P, *et al*: Prostaglandin-H-synthase isozyme expression in normal and neoplastic human skin. *Int J Cancer* 82:648–656, 1999

Müller-Decker K, Scholz K, Marks F, Fürstenberger G: Differential expression of prostaglandin H synthase isozymes during multistage carcinogenesis in mouse epidermis. *Mol Carcinog* 12:31–41, 1995

Müller-Decker K, Scholz K, Neufang G, Marks F, Fürstenberger G: Localization of prostaglandin-H synthase-1 and -2 in mouse skin: Implications for cutaneous function. *Exp Cell Res* 242:84–91, 1998

Müller-Röver S, Handjiski B, van der Veen C, *et al*: A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *J Invest Dermatol* 117:3–15, 2001

Narumiya S, Sugimoto Y, Ushikubi F: Prostanoid receptors: Structures, properties, and functions. *Physiol Rev* 79:1193–1226, 1999

Neufang G, Fürstenberger G, Heidt M, Marks F, Müller-Decker K: Abnormal differentiation of epidermis in transgenic mice constitutively expressing cyclooxygenase-2 in skin. *Proc Natl Acad Sci U S A* 98:7629–7634, 2001

O'Banion MK: Cyclooxygenase-2: Molecular biology, pharmacology, and neurobiology. *Crit Rev Neurobiol* 13:45–82, 1999

Paus R, Cotsarelis G: The biology of hair follicles. *N Engl J Med* 341:491–497, 1999

Paus R, Müller-Röver S, Van der Veen C, *et al*: A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. *J Invest Dermatol* 113:523–532, 1999

Paus R, Van der Veen C, Eichmüller S, Kopp T, Hagen E, Müller-Rover S, Hofmann U: Generation and cyclic remodeling of the hair follicle immune system in mice. *J Invest Dermatol* 111:7–18, 1998

Pena JC, Kelekar A, Fuchs EV, Thompson CB: Manipulation of outer root sheath cell survival perturbs the hair-growth cycle. *EMBO J* 18:3596–3603, 1999

Pillans PI, Woods DJ: Drug-associated alopecia. *Int J Dermatol* 34:149–158, 1995

Scholz K, Fürstenberger G, Müller-Decker K, Marks F: Differential expression of prostaglandin H-synthase isoenzymes in normal and activated keratinocytes in vivo and in vitro. *Biochem J* 306:263–269, 1995

Smith WL, Dewitt DL: Garavito RM; Cyclooxygenases: Structural, cellular, and molecular biology. *Annu Rev Biochem* 69:145–182, 2000

Smith TJ, Jennings TA, Sciaky D, Cao HJ: Prostaglandin-endoperoxide H synthase-2 expression in human thyroid epithelium. *J Biol Chem* 274:15622–15632, 1999

Sorli CH, Zhang HJ, Armstrong MB, Rajotte RV, Maclouf J, Robertson RP: Basal expression of cyclooxygenase-2 and nuclear factor-interleukin 6 are dominant and coordinately regulated by interleukin 1 in the pancreatic islet. *Proc Natl Acad Sci USA* 95:1788–1793, 1998

Stenn KS, Paus R: Controls of hair follicle cycling. *Physiol Rev* 81:449–494, 2001

Talley JJ, Brown DL, Carter JS, *et al*: 4-[5-Methyl-3-phenylisoxazol-4-yl]-benzene/sulfonamide valdecoxib: A potent and selective inhibitor of COX-2. *J Med Chem* 43:775–777, 2000

Thompson EJ, Gupta A, Vielhauer GA, Regan JW, Bowden GT: The growth of malignant keratinocytes depends on signaling through the PGE(2) receptor EP1. *Neoplasia* 3:402–410, 2001

Tiano HF, Loftin CD, Akunda J, *et al*: Deficiency of either cyclooxygenase (COX)-1 or COX-2 alters epidermal differentiation and reduces mouse skin tumorigenesis. *Cancer Res* 62:3395–3401, 2002

Torii E, Segi E, Sugimoto Y, Takahashi K, Kabashima K, Ikai K, Ichikawa A: Expression of prostaglandin E2 receptor subtypes in mouse hair follicles. *Biochem Biophys Res Commun* 290:696–700, 2002

Tosti A, Misciali C, Piraccini BM, Peluso AM, Bardazzi F: Drug-induced hair loss and hair growth incidence, management and avoidance. *Drug Safety* 10:310–317, 1994

Urade Y, Ujihara M, Hariguchi Y, Ikai K, Hayaishi O: The major source of endogenous prostaglandin D2 production is likely antigen-presenting cells. Localization of glutathione requiring prostaglandin D synthebase in histiocytes, dendritic, and kupffer cells in various rat tissue. *J Immunol* 143: 2982–2989, 1988

Wang HQ, Smart RC: Overexpression of protein kinase C-α in the epidermis of trans-genic mice results in striking alterations in phorbol-ester induced inflammation and COX-2, MIP-2, and TNF-α expression but not tumor promotion. *J Cell Sci* 112:3497–506, 1999

Winter H, Langbein L, Praetzel S, *et al*: A novel human type II cytokeratin, K6hf, specifically expressed in the companion layer of the hair follicle. *J Invest Dermatol* 111:955–962, 1998