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Epidermal hyperproliferation in mice lacking fatty acid transport protein 4 (FATP4) involves ectopic EGF receptor and STAT3 signaling

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ABSTRACT

Fatty acid transport protein (FATP) 4 is one of a family of six FATPs that facilitate long- and very long-chain fatty acid uptake. Mice lacking FATP4 are born with tight, thick skin and a defective epidermal barrier; they die neonatally due to dehydration and restricted movements. Both the skin phenotype and the lethality are rescued by transgene-driven expression of FATP4 solely in suprabasal keratinocytes. Here we show that *Fatp4* mutants exhibit epidermal hyperplasia resulting from an increased number of proliferating suprabasal cells. In addition, barrier formation initiates precociously but never progresses to completion. To investigate possible mechanisms whereby *Fatp4* influences skin development, we identified misregulated genes in *Fatp4* mutants. Remarkably, three members of the epidermal growth factor (EGF) family (*Ereg*, *Areg*, and *Epgn*) showed increased expression that was associated with elevated epidermal activation of the EGF receptor (EGFR) and STAT3, a downstream effector of EGFR signaling. Both Tyrphostin AG1478, an EGFR tyrosine kinase inhibitor, and curcumin, an inhibitor of both STAT3 and EGFR, attenuated STAT3 activation/nuclear translocation, reduced skin thickening, and partially suppressed the barrier abnormalities. These data identify FATP4 activity as negatively influencing EGFR activation and the resulting STAT3 signaling during normal skin development. These findings have important implications for understanding the pathogenesis of ichthyosis prematurity syndrome, a disease recently shown to be caused by FATP4 mutations.

Introduction

The mature mammalian skin is a stratified epithelium derived from the embryonic ectoderm; it is the first line of defense against mechanical and chemical trauma. The skin also functions as a barrier to block the entry of microorganisms and to prevent the loss of body hydration. During skin morphogenesis, several signaling pathways participate in a series of inductive events that guide the separation of the hair lineage from the epidermal lineage and promote epidermal differentiation and stratification (Fuchs and Raghavan, 2002; Millar, 2002).

Epidermal stratification begins when proliferative basal cells migrate suprabasally and exit the cell cycle, beginning a series of steps culminating in cornification (Elias and Jackson, 1996). The first event is keratinization, the switch in intermediate filaments from a keratins 5/14-containing network to a keratins 1/10-containing network, permitting cells in the spinous layer to form a rigid cytoskeleton. Secondly, keratohyalin granules containing profilaggrin are synthesized to facilitate the assembly of keratin bundles, forming the granular layer. Thirdly, involucrin (IVL), loricrin, and other proteins are cross-linked by transglutaminase, forming an insoluble cornified envelope beneath the cell membrane. Finally, lipid-enriched lamellar bodies secrete their contents into the intercellular spaces of the cornified layer, completing the formation of a water-resistant barrier. This progression from a proliferating basal cell to a terminally differentiated, cornified squamous cell occurs continuously throughout life, as skin is a constantly regenerating organ (Fuchs and Raghavan, 2002).

We previously identified an autosomal recessive mouse mutation called *wrinkle free* (Moulson et al., 2003). Homozygous mutants are born with taut and shiny skin, a thickened epidermis, a defective skin barrier, and sparse hair follicles; neonates die due to dehydration and restricted movements. By positional cloning, we found the mutation to be caused by a spontaneous retrotransposon insertion into *Slc27a4*, the gene encoding fatty acid transport protein (FATP) 4. One targeted mutation in *Slc27a4* (here referred to as *Fatp4* for simplicity) shows an identical phenotype (Herrmann et al., 2003), and another was reported to cause very early embryonic lethality (Gimeno et al., 2003).

FATP4 is one of a family of six transmembrane proteins that facilitate long- and very long-chain fatty acid uptake. FATP4 exhibits acyl-CoA synthetase (ACS) activity and has been proposed to facilitate uptake of fatty acids indirectly by mediating their esterification to CoA

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(Hall et al., 2005; Herrmann et al., 2001), in contrast to the direct fatty acid transport functions identified for other FATPs, such as FATP1 (Richards et al., 2006; Schaffer and Lodish, 1994). FATP4 is widely expressed, suggesting roles in many organs (Herrmann et al., 2001; Moulson et al., 2003). In skin FATP4 is normally detected in basal and suprabasal keratinocytes (MHL and JHM, unpublished data), with the strongest expression in the granular layer of the epidermis (Moulson et al., 2007). Suprabasal keratinocyte expression of a FATP4 transgene in the epidermis rescues the neonatal lethality and ameliorates the skin phenotype of *Fapt4* mutant mice, indicating crucial, skin-intrinsic roles for FATP4 in the development of skin and its appendages (Moulson et al., 2007).

The recent identification of *Fatp4* mutations in patients with ichthyosis prematurity syndrome (Klar et al., 2009) makes an understanding of the mechanism whereby the absence of FATP4 causes the wrinkle free phenotype in mice an especially important goal. Here we characterized the skin abnormalities in *Fatp4*$^{-/-}$ mice at the cellular level and found an increased number of proliferating keratinocytes in the suprabasal layer. To identify possible mechanisms that link the lack of FATP4 to specific defects in the development of skin and its appendages, we carried out microarray analyses with embryonic skin RNAs and found upregulation of several EGF family ligands in *Fatp4* mutant skin, together with increased activation of STAT3, a downstream effector of the EGF receptor (EGFR) signaling pathway. Furthermore, pharmacological blockade of EGFR and STAT3 activation suppressed epidermal hyperplasia in *Fatp4* mutants, and this correlated with reduced hyperproliferation. These data indicate that the lack of FATP4 creates an environment, presumably via direct effects on lipid metabolism and homeostasis, that promotes epidermal proliferation via overactivation of the EGFR and the downstream STAT3 signaling pathways.

Materials and methods

Mice and skin barrier assays

Fatp4 mutant and transgenic mice have been previously described (Moulson et al., 2007; Moulson et al., 2003). Embryonic day (E) 15.5 to E17.5 embryos were dissected from pregnant females, with the morning when the copulation plug was observed considered E0.5. For inward permeability assays, embryos were stained in the dark at 37 °C overnight in X-Gal solution (1 mg/ml X-Gal, 3 mM $K_4Fe(CN)_6$, 3 mM $K_3Fe(CN)_6$, 1.3 mM $MgCl_2$, 0.1 M NaH_2PO_4) at pH 4.5 as described (Hardman et al., 1998). In some experiments embryos were incubated in a series of ascending and then descending concentrations of methanol, equilibrated in phosphate buffered saline (PBS), and stained briefly in 1% toluidine blue in water followed by destaining in PBS (Hardman et al., 1998). Stained samples were fixed in 4% paraformaldehyde in PBS at room temperature for 1 h to overnight. For outward transepidermal water loss (TEWL) assays, embryos were rinsed in PBS, blotted gently with a Kimwipe, and air-dried for 5 min. The water loss through the dorsal or lateral skin was measured using a Vapometer (Delfin Technologies, Kuopio, Finland) with the sensor chamber attached to a nail adaptor.

Immunohistochemistry

Embryos were fixed at room temperature for 2 to 3 h in 4% paraformaldehyde in PBS. To increase the penetration of fixative, E15.5 or older embryos were decapitated, and the abdominal cavity was exposed. Fixed embryos at E14.5 were cut into halves along the dorsal midline, embedded in paraffin, and sectioned parasagittally at 5 μm. For fixed embryos at older stages, the dorsal skin was collected for paraffin embedding.

Immunohistochemical analyses were performed using the peroxidase Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with DAB as a chromogen (Pierce, Rockford, IL) as described (Lin et al., 2000), with some modifications. To retrieve antigens, rehydrated sections were boiled in 10 mM citrate buffer (pH 6), Trilogy (Cell Marque, Rocklin, CA), or 1 mM EDTA (pH 8). To block endogenous biotin and avidin, the Avidin/Biotin blocking kit (Vector Laboratories) was used before adding primary antibodies. The antigen retrieval method and dilutions of primary antibodies used were as follows: EDTA and 1:50 for phospho-STAT3 (Tyr705); EDTA and 1:100 with tyramide amplification for phospho-EGFR (Tyr1068); citrate and 1:100 for phospho-p44/42 MAP kinase (Thr202/Tyr204); citrate and 1:100 for phospho-p38 MAPK (Thr180/Tyr182); citrate and 1:100 for phospho-SAPK/JNK (Thr183/Tyr185) (all antibodies were from Cell Signaling Technology, Beverly, MA); Trilogy and 1:100 with tyramide amplification for JAK2 (phospho Y1007 + Y1008) (Abcam, Cambridge, UK); 1:1,000 for keratin 6 (Covance, Princeton, NJ). Sections were counterstained with hematoxylin and mounted.

Double immunofluorescence analysis of Ki67 (Novocastra Laboratories, Newcastle upon Tyne, UK) and laminin γ1 (Millipore, Danvers, MA) was performed on paraformaldehyde-fixed, frozen sections of tissue embedded in OCT (Sakura Finetek, Torrance, CA). Both primary antibodies were applied at 1:1,000, and signals were detected using Alexa 488-conjugated anti-rabbit and Alexa 594-conjugated anti-rat, respectively, with Hoechst 33258 as a counterstain. The number of Ki67-positive suprabasal cells present in a field encompassing 100 Ki67-positive basal cells was used as a measurement of suprabasal cell hyperproliferation (Hansen et al., 2000). A total of at least 100 Ki67-positive basal cells were counted in each sample. Proliferation of basal cells was assayed by determining the percentage of Ki67-positive cells in the basal layer in a population of at least 100 Hoechst 33258-positive basal cells for each sample.

In situ hybridization

Embryos at various stages were fixed and paraffin-embedded as described above, except that the fixation was performed at 4 °C overnight. In situ hybridization with digoxigenin-UTP labeled riboprobes was performed as described (Moulson et al., 2007), with hybridization temperatures ranging from 55 to 60 °C.

Riboprobes were synthesized from either linearized plasmids or PCR amplicons using DIG RNA labeling mix (Roche, Mannheim, Germany) and RNA polymerase Plus (Ambion, Austin, TX), and purified by NucAway spin columns (Ambion) following the manufacturers' instructions. For some of the genes examined, both antisense and sense riboprobes were tested on samples to confirm their specificities. The plasmids contained cDNAs from: *Areg* (clone ID 3597695), *Ereg* (clone ID 5325124), *Epgn* (clone ID 8734042), *Mmp3* (clone ID 3962288), *Ccl12* (clone ID 1548072), *Ifi202b* (clone ID 4945974; all above are IMAGE clones obtained from OpenBiosystesm, Huntsville, AL), and *Il24* (clone ID RZPDp981C12223D; from imaGenes, Berlin, Germany). The PCR amplicons were generated using the cDNAs reverse-transcribed from RNA with Superscript III reverse transcriptase (Invitrogen). Primers used to make PCR amplicons were as follows, with T7 and T3 promoter sequence underlined: *Klk7*: 5′ TATAATACGACTCACTATAGGGGAGTGCAAGAAGGTGTACAAG 3′ and 5′ GAAATTAACCCTCACTAAAGGGTGGAGGAAAGGTAAAGCCAG 3′, *Dusp6*: 5′ TATAATACGACTCACTATAGGGGGATCACTGGAGCCAAAAC 3′ and 5′ GAAATTAACCCTCACTAAAGGGGGAACTGAAGGAATGGGGAC 3′.

RNA isolation and microarray analyses

Total RNAs were isolated from the dorsal skin of embryos at E15.5 using a tissue homogenizer (Powergen 125, Fisher Scientific, Pittsburgh, PA) and the RNeasy Fibrous Tissue Mini kit with an in-column DNase treatment (Qiagen, Chatsworth, CA) following the manufacturer's instructions. Purified RNAs were qualitatively assessed by RNA LabChip (Agilent, Palo Alto, CA) and quantified