The RAIG Family Member, GPRC5D, Is Associated with Hard-Keratinized Structures

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***R*etinoic *a*cid-*i*nducible *g*ene-1 was originally identified as an orphan G-*p*rotein *c*oupled *r*eceptor induced by retinoic acid. Three highly homologous oGPCR (GPRC5B, GPRC5C, and GPRC5D) have since been classified into the RAIG1 family. We describe here, the unique tissue distribution of GPRC5D and its mechanism of expression. Hybridization *in situ* has shown that GPRC5D is expressed in differentiating cells that produce hard keratin, including cortical cells of the hair shaft, the keratogenous zone of the nail, and in a central region of the filiform papillae of the tongue. The GPRC5D transcript is expressed in hair follicles during mid- and late anagen, and catagen but not at telogen and early anagen phases. The differentiation-inducer, all-*trans* retinoic acid, induces GPRC5D expression in cultured hair bulb cells. Because the tissue distribution of GPRC5D indicates a relationship with hard keratins that constitute the major structural proteins of hard epithelial tissues, we investigated the effect of GPRC5D on acid hard keratins. Analyses of cultured cells showed that transient overexpression resulted in suppression of Ha3 and stimulation of Ha4 hair keratin gene expression. The expression was maintained in the hair follicles of *whn*-deficient (nude) mice, suggesting that this gene is regulated by a signal pathway different from that of hair keratin synthesis. Collectively, these data provide a framework for understanding the molecular mechanisms of GPRC5D function in hard keratinization.**

Key words: cortex/G protein-coupled receptor/hair bulb cell/keratinization/retinoic acid
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Retinoic acid (RA) is a transcriptionally active metabolite of vitamin A (retinol) that can cause striking changes in various structures and functions. It activates two families of nuclear retinoid receptors (retinoid X receptors (RXR) and retinoic acid receptors (RAR)) that have the potential to regulate the expression of many genes (Darwiche *et al*, 1995; Reichrath *et al*, 1995; Chen *et al*, 1996). The mechanisms underlying such regulation, however, are not well understood (Wei, 2003).

RA can also affect hair follicles (Terezakis and Bazzano, 1988; Bazzano *et al*, 1993; Bergfeld, 1998; Kochhar *et al*, 1998). Several RA receptors are expressed in hair follicles and analyses using a retinoid receptor antagonist have shown that deficient receptor function can inhibit hair growth (Viallet and Dhouailly, 1994; Billoni *et al*, 1997; Kochhar *et al*, 1998). The life of a hair follicle can be histologically divided into anagen (growth), catagen (regression), and telogen (resting) phases (Chase *et al*, 1951; Stenn and Paus, 2001). Analyses of mouse and human hair follicles have suggested that RA prolong the anagen and shorten the telogen phase, but the signal cascade that proceeds from nuclear receptors to pharmacologic effects and the regulation mechanism remain unclear (Bazzano *et al*, 1993; Bergfeld, 1998).

Abbreviations: GPCR, G-protein coupled receptor; RA, retinoic acid; ATRA, all-*trans* retinoic acid; HBC, hair bulb cells; AIG1, *r*etinoic *a*cid *i*nducible GPCR 1

Hard (hair) keratin genes are the most useful for investigating this signal cascade. They are mainly expressed as major structural proteins in "hard" epithelial tissues (hair follicles, nail and oral filiform papillae) and classified into two major sequence types (acidic (Ha) and basic (neutral; Hb) keratins) (Langbein *et al*, 1999, 2001). The pairwise regulation of Ha and Hb keratin genes reflects the composition of keratin polymers that form a 10 nm interfilament cytoskeletal network in epithelial cells. Their expression contributes to the formation of hair shafts and other hard epithelial tissues. For example, during hair shaft formation, the soft, initially nonkeratinized cells of the cortex assume a longitudinal form as they are pressed upwards into the funnel-like mold of the root sheath by the pressure of cell division at the base of the hair. During this process, keratins are synthesized, accumulated, and oriented along the longitudinal axis of the cells (and also of the hair fibers themselves), which intensifies the hair fiber structure (Stenn and Paus, 2001). Therefore, they are key molecular markers of differentiation events in hard epithelial tissues.

We isolated an orphan GPCR (now called GPRC5B) using a new method (Inoue *et al*, 2000). According to amino acid sequence homology, this gene is now classified in the orphan GPCR family, RAIG1 (*r*etinoic *a*cid *i*nducible *g*ene 1 or *r*etinoic *a*cid *i*nducible GPCR 1) family, which consists of four genes (RAIG1, GPRC5B, GPRC5C, and GPRC5D) (Brauner-Osborne and Krogsgaard-Larsen, 2000; Robbins *et al*, 2000; Brauner-Osborne *et al*, 2001). RAIG1 was first isolated using differential display as an orphan GPCR

(G-*p*rotein *c*oupled *r*eceptor), the expression of which was induced by all-*trans* retinoic acid (ATRA) in human head and neck squamous cell carcinoma (HNSCC) (Cheng and Lotan, 1998). GPCR constitute the largest family of 7-transmembrane receptors in the mammalian genome and play fundamental roles in cellular functions (Bockaert and Pin, 1999). To investigate the shared features of the RAIG1 family of genes, we focused on the function of individual RAIG1 genes, and their effects on cell growth and differentiation. This study shows the unique tissue distribution of GPRC5D and its relationship with the regulation of hard keratinization (differentiation in hard epithelial tissues). We initially investigated GPRC5D expression by reverse transcription–polymerase chain reaction (reverse transcription–PCR) and *in situ* hybridization. The profiles of hair follicles were investigated during the hair cycle of dorsal skin synchronized by depilation. We analyzed the upregulation of GPRC5D by ATRA in cultured hair bulb cells (HBC). The dorsal skin of whn-deficient (nude) mice was also examined by *in situ* hybridization to determine the regulation of GPRC5D expression. Furthermore, we examined whether GPRC5D expression is related to the regulation of hard keratin gene expression.

Results

Expression of GPRC5D The reverse transcription–PCR analysis showed that GPRC5D was expressed only in the skin (Fig 1*a*). To identify which cells expressed GPRC5D, we assayed sections of the dorsal skin by *in situ* hybridization. As shown in Fig 1(*b–d*), GPRC5D was expressed in the differentiating hair shaft but not in the outer root sheath, inner root sheath, or follicular papilla. Even in the hair matrix, which is a progenitor of the hair shaft, GPRC5D was undetectable. Moreover, other skin structures such as the epidermis and sebaceous glands were also negative (see also Fig 3). The hair shaft consists of three layers (cuticle, cortex, and medulla). For detailed examination, *in situ* hybridization was performed using vibrissae derived from ICR mice to avoid melanin pigment (Fig 1*e–g*). The GPRC5D anti-sense specific signal was observed in the cortex layer, but not in the cuticle or medulla layer of the hair shaft. This expression profile resembled those of some hard keratins, which are expressed in other hard-keratinized tissues. Therefore, we also examined tissue sections of the nail and tongue by *in situ* hybridization. GPRC5D was expressed in the upper part of the nail matrix, the keratogenous zone of the nail (Fig 2*a–c*), and in central regions of filiform papillae on the dorsal surface of the tongue (Fig 2*d–f*). Significant levels of GPRC5D were not expressed in the heart, lung, kidney, diaphragm, adipose tissue, eyeball, lymph node, spleen, thymus, pancreas, submaxillary gland, adrenal gland, esophagus, stomach, small and large intestine, liver, cholecyst, urinary bladder, testis, seminal vesicle, epididymis, spinal cord, and brain according to *in situ* hybridization (data not shown).

GPRC5D expression during the hair cycle We monitored GPRC5D expression during the hair cycle synchronized by depilation in dorsal skin (Fig 3). At the early phase of anagen, when a new follicle was invaginating from the skin surface but the hair shaft had not yet formed, GPRC5D expression was absent in sections of dorsal skin (Fig 3*a,b*). During the middle (Fig 3*c,d*) and late (Fig 3*e,f*) phases of anagen, GPRC5D was expressed exclusively in cortical cells of the hair shaft. During the catagen phase (Fig 3*g,h*), GPRC5D expression continued in the cortical cells with

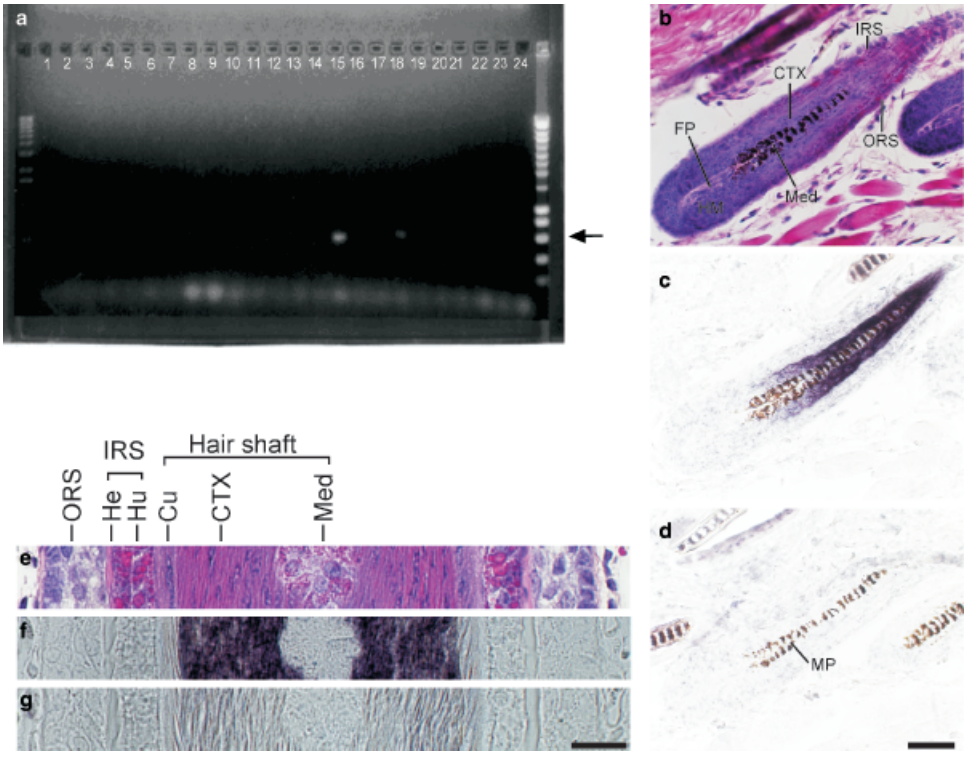

Figure 1
Tissue distribution of mouse GPRC5D. Reverse transcription–PCR analysis (*a*). *Lanes: 1*, brain; *2*, thymus; *3*, heart; *4*, liver; *5*, kidney; *6*, stomach; *7*, spleen; *8*, small intestine; *9*, muscle; *10*, adrenal gland; *11*, lung; *12*, ovary; *13*, testis; *14*, uterus; *15*, skin; *16*, prostate gland; *17*, embryo e8.5; *18*, virgin breast; *19*, embryo e9.5; *20*, pregnant breast; *21*, embryo e12.5; *22*, lactating breast; *23*, embryo e19.5; *24*, involuting breast. The *arrow* indicates a 545 bp PCR candidate product derived from GPRC5D. *In situ* hybridization of GPRC5D or hematoxylin–eosin-stained serial sections from C57BL/6 mouse dorsal skin (*b–d*) or vibrissae of ICR mice (*e–g*). Hematoxylin–eosin staining (b,e); *in situ* hybridization with GPRC5D anti-sense probe (*c,f*); with sense probe (*d,g*). GPRC5D-anti-sense-specific signal (black) exclusively located in differentiating cortical cells (CTX). Cu, cuticle; HM, hair matrix; IRS, inner root sheath; Medical, medulla; He, Henle's layer; Hu, Huxley's layer; MP, melanin pigment; ORS, outer root sheath; FP, follicular papilla. *Scale bars* = 20 μm.

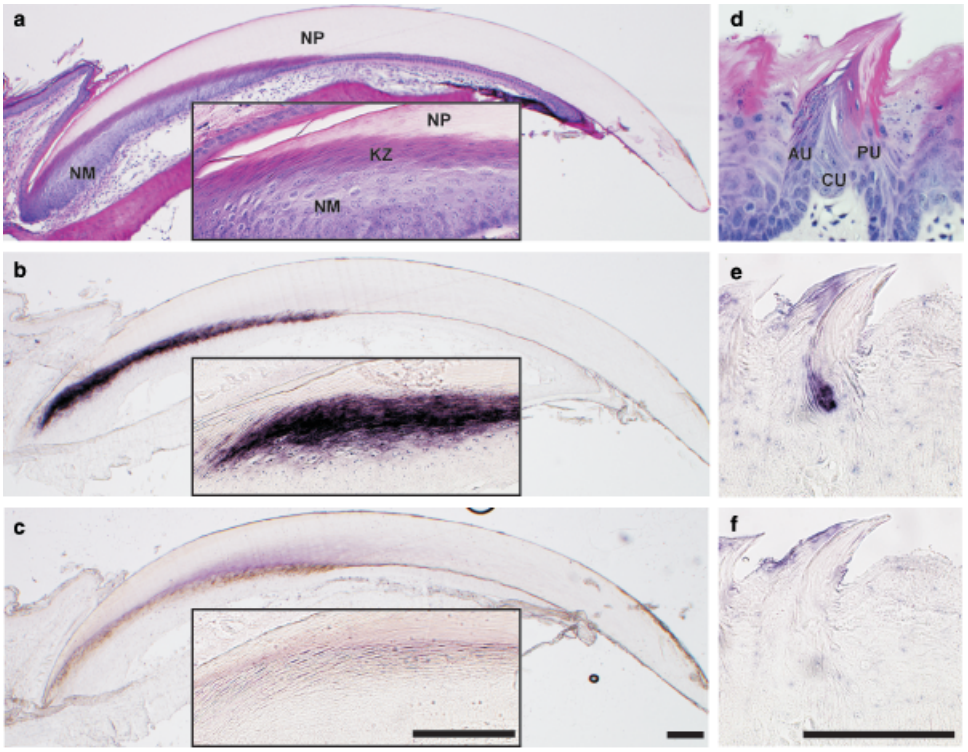

Figure 2
GPRC5D mRNA expression in hard keratinized structures. *In situ* hybridization with GPRC5D anti-sense probe (*b,e*) or GPRC5D sense probe (*c,f*) or hematoxylin–eosin staining (*a,d*) of serial sections prepared from nails (*a–c*) and tongue (*d–f*) of a C57BL/6 mouse. Insets in *a–c* indicate higher magnifications of the root of nail. GPRC5D anti-sense-specific signals are present in the upper matrix (NM) and keratogenous zone (KZ) of nail and a central unit of the filiform papilla on dorsal tongue surface. NP, nail plate; AU, anterior unit; PU, posterior unit. *Scale bars* = 100 μm.

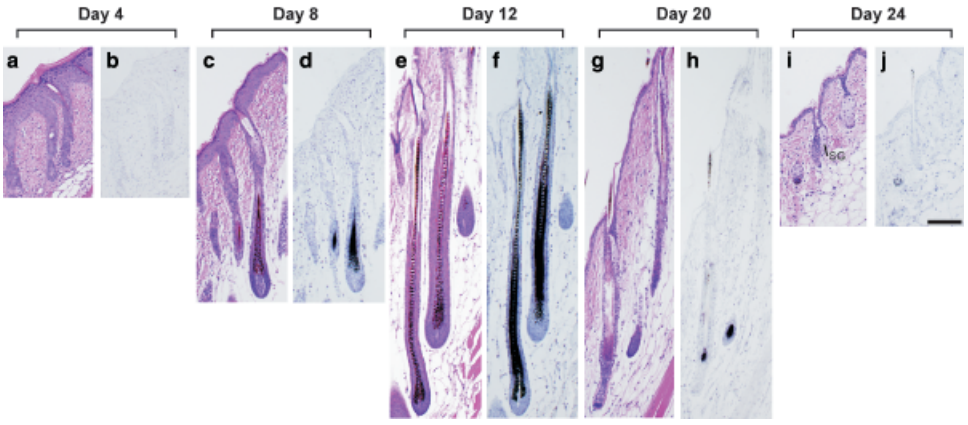

Figure 3
Oscillation of GPRC5D mRNA expression during the hair cycle. *In situ* hybridization with GPRC5D anti-sense probe (*b,d,f,h,j*; all specimens are counterstained with toluidine blue) or hematoxylin–eosin (*a,c,e,g,i*) staining of C57BL/6 dorsal skin at each hair cycle phase induced by depilation. Days on top indicate days after depilation (day 4, anagen II/III; day 8, anagen IV; day 12, anagen V/VI; day 20, catagen; day 24, telogen). SG, sebaceous gland. *Scale bar* = 100 μm.

regression of the hair root. GPRC5D expression was undetectable at the telogen phase, however, when the hair shaft was not produced (Fig 3*i,j*).

Effect of ATRA on GPRC5D and hard keratins Although GPRC5D is a predicted RA-inducible member of the RAIG1 family, ATRA does not induce it in a human small lung carcinoma cell line (Brauner-Osborne *et al*, 2001). Our *in situ* hybridization data indicated that GPRC5D is expressed in the differentiating (mature) (cortex of the hair shaft), but not in the premature region (matrix) of the hair follicle. Northern blots revealed significant expression in hair follicles from which we prepared HBC, but not in cultured HBC (Fig 4*a*). We therefore designed TaqMan probes to investigate the ATRA induction of GPRC5D in HBC. The results showed that ATRA induced significant amounts of GPRC5D expression (Fig 4*b*).

We also examined the expression of four major acidic hard keratins (Ha1–4). Results obtained using the TaqMan probes suggested that Ha1, Ha3, and Ha4 were induced by adding ATRA to the growth medium (Fig 4*b*).

Relationship between expression of GPRC5D and Ha3 *In situ* hybridization data showed that the expression profiles of GPRC5D and Ha3 were similar (Bertolino *et al*, 1990; Winter *et al*, 1994; Meier *et al*, 1999). Therefore, to investigate whether both genes are regulated by the same transcription signal pathway, we examined their expression in nude mice that lack the *whn* transcription factor (Forkhead/Winged–Helix transcription factor) activity. *In situ* hybridization (Fig 5*a–f*) and real-time quantitative PCR (Fig 5*g,h*) using samples of dorsal skin showed that the expression of Ha3 was significantly reduced to 10% to 20%, whereas that of GPRC5D was not.

Effects of exogenous expression of GPRC5D in HBC We examined the effects of GPRC5D on the expression of hard keratins and on the metabolic activity. GPRC5D was

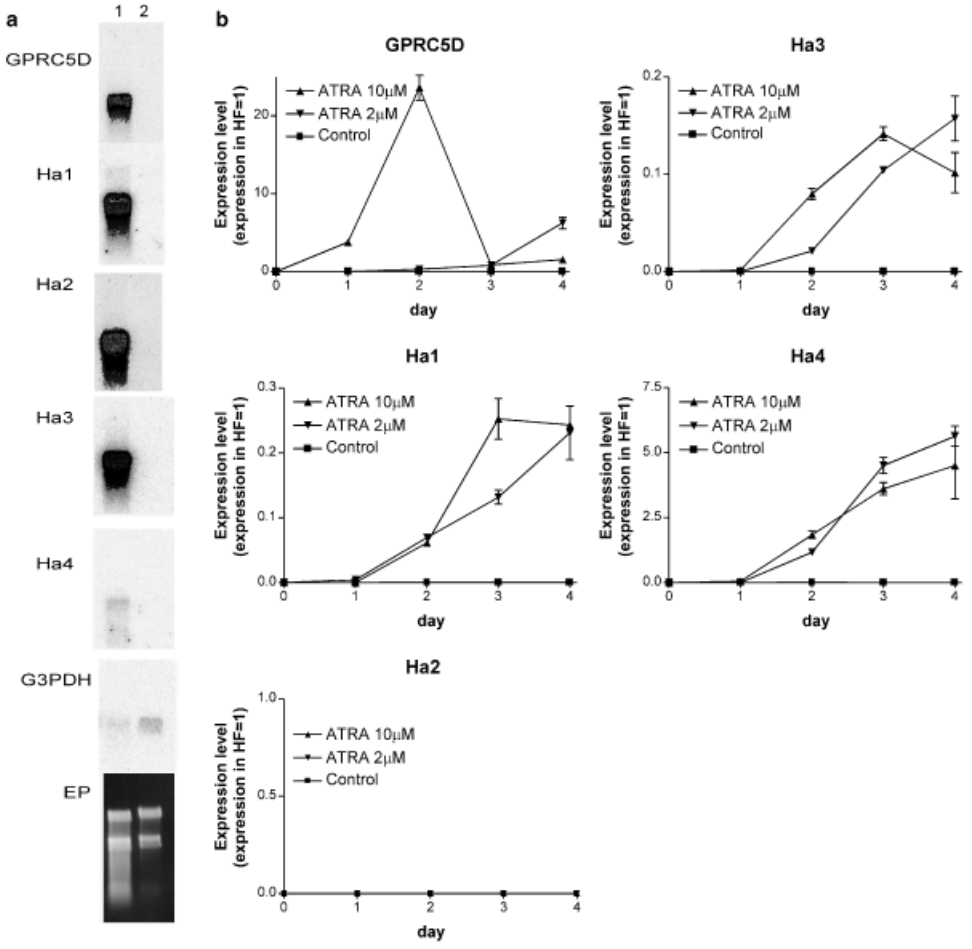

Figure 4
Upregulation by ATRA. (*a*) Northern blots of GPRC5D, Ha1, 2, 3, and 4. *Lane 1*, original hair follicles; *lane 2*, cultured HBC (day 0). EP indicates EtBr-stained agarose gel after electrophoresis before capillary transfer to Hybond N^+ membrane for northern blotting. (b) Time course of mRNA expression determined by reverse transcription and real-time quantitative PCR (TaqMan) in HBC cultured with ATRA (+/–).

transiently expressed in HBC in the presence of ATRA. The time course of the metabolic activities and expression levels of Ha3 and Ha4 in HBC are shown in Fig 6(*a*), Fig 6(*b*), and Fig 6(*d*), respectively. These results suggest that exogenous GPRC5D reduced the metabolic activity and expression of Ha3, but induced the expression of Ha4 in HBC. On day 3 after transfection, the expression levels were significantly affected ($p < 0.01$) by the transient expression of GPRC5D (Fig 6*c,e*). On the other hand, Ha1 and Ha2 seemed unaffected (data not shown).

Discussion

GPRC5D might be a member of the RAIG1 family. RAIG1 is an orphan GPCR family of type C GPCR (family 3) (Cheng and Lotan, 1998; Brauner-Osborne and Krogsgaard-Larsen, 2000; Brauner-Osborne *et al*, 2001). Type C GPCR include metabotropic glutamate, Ca^{2+} sensing and pheromone receptors and have a long N-terminal extracellular domain that can directly bind ligands (Bockaert and Pin, 1999); however, the RAIG1 family members have short chains and the ligand(s) has not been identified (Robbins *et al*, 2000). Studies have indicated that RAIG 1 functions are involved with tissue differentiation and maturation through ATRA-induced expression.

In situ hybridization showed that the expression of GPRC5D was restricted to "differentiating" tissues that express hard keratins. Within hair follicles (including eyelash follicles, vibrissae, etc.; data not shown), GPRC5D was found only in the cortex that contains abundant hair fibers (Fig 1), suggesting that its expression is orchestrated with the hair cycle. In fact, GPRC5D was identified only in cortical cells at all phases of the hair cycle (Fig 2) and only around the keratogenous zones of the nail and tongue (Fig 3). Recent reverse transcription–PCR analyses have found that GPRC5D mRNA is widely expressed in the human peripheral system and that this is not induced by ATRA (Brauner-Osborne *et al*, 2001); however, we could not reproduce these results using *in situ* hybridization and reverse transcription–PCR in mouse tissues (no ubiquitous expression). Why the tissue distribution and ATRA-inducibility of GPRC5D differs is unclear but may involve species and cell differences. Moreover, low levels of a GPCR cannot function as a signal-transducing receptor even when endogenously expressed (Kawamata *et al*, 2003). Therefore, GPRC5D might be involved in signal transduction only in hard keratinized tissues.

We discovered that GPRC5D expression in HBC could be induced by ATRA like other RAIG1 family genes (Fig 4*b*) (Cheng and Lotan, 1998; Brauner-Osborne and Krogsgaard-Larsen, 2000). We showed that 2 μM ATRA induced expression in a time-dependent manner (day 1, not detected; day 2, about 0.3-fold; day 3, 0.8-fold; day 4, 6.2-fold); however, the expression peaked 2 d after adding 10 μM ATRA and then decreased (Fig 4*b*). The expression

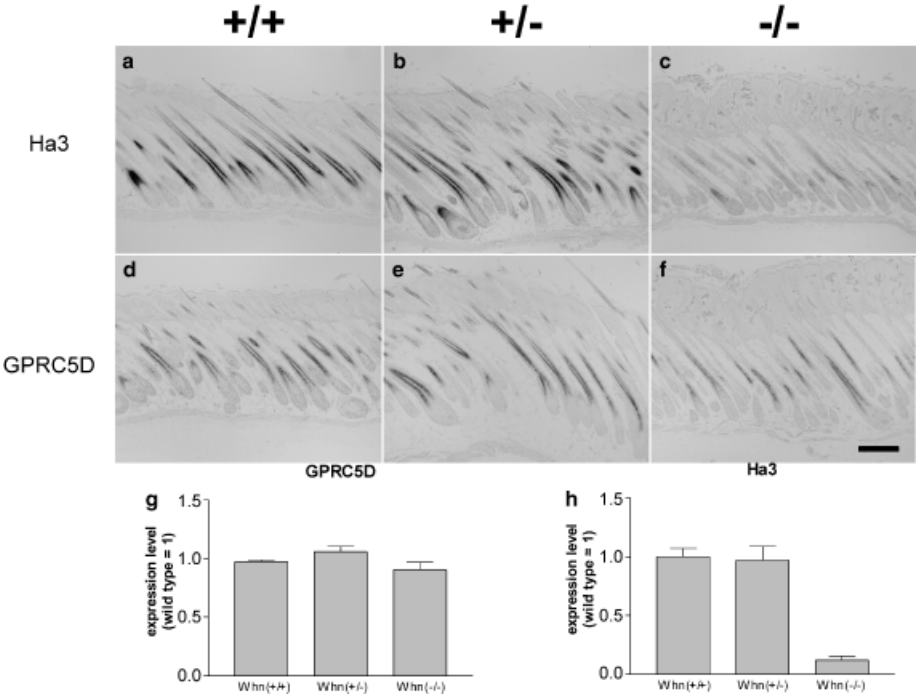

Figure 5
Expression of GPRC5D in nude (*whn*) mice. Sections of dorsal skin of *whn* +/+, +/– (+/nu) and –/– (nu/nu) mice. Anti-sense RNA probes were of Ha3 (*a–c*) and GPRC5D (*d–f*). *Scale bar* = 200 μm. Quantitation of GPRC5D (*g*) and Ha3 (*h*) expression. RNA isolated from excised dorsal skin were analyzed by reverse transcription and real-time quantitative PCR (TaqMan). The expression levels of each gene in each sample (n = 3) were determined in comparison with that of wild-type (+/+) (= 1).

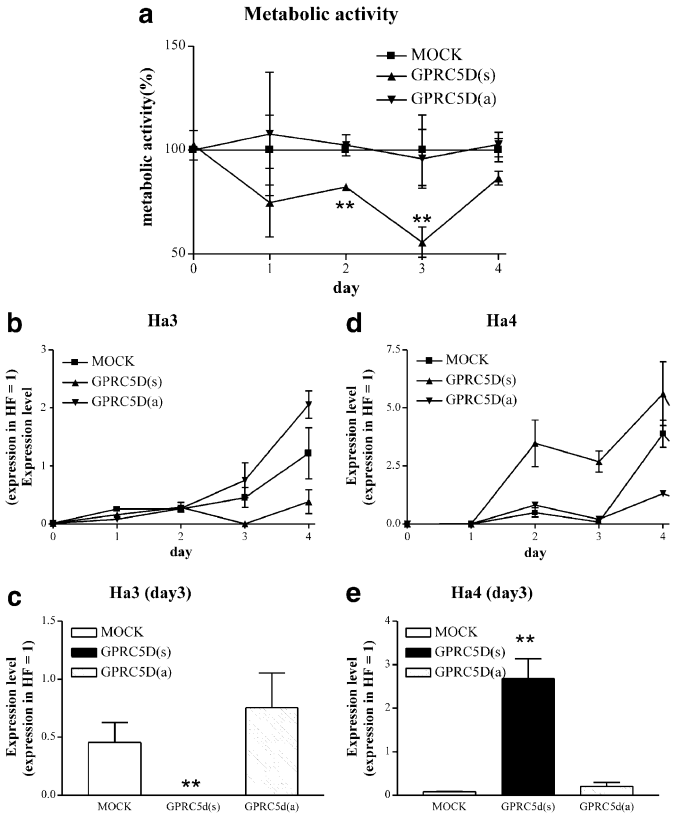

Figure 6
Effects of transient expression of GPRC5D in HBC. Metabolic activities of HBC and real-time quantitative PCR using RNA from HBC exogenously transfected with GPRC5D (sense (s) or anti-sense (a)) or mock transfected in the presence of 1 μM ATRA. Metabolic activities and the expression levels of Ha3 and Ha4 were monitored over 4 d after transfection (*a,b,d*). Graphs of expression levels at day 3 after transfection and addition of ATRA (*c,e*). Results are expressed as mean ± SEM (n = 5) of HBC data. $^{**}p < 0.01$ significance of difference between GPRC5D- and mock-transfected cells.

threshold was considerably higher than that in the original hair follicles (about 23-fold). The hair matrix cells (not expressing GPRC5D) are proliferating and differentiating into the cortical cells (expressing GPRC5D). When the rate of proliferation is higher than that of differentiation, the cortex continues to grow. On the other hand, when the rate of differentiation exceeds that of proliferation, the growth was stopped because of depletion of stem cells (= matrix cells). If GPRC5D is a differentiating marker in HBC analysis, its higher expression level might indicate considerably accelerated differentiation by ATRA, the rate of which exceeds that of proliferation. A high concentration of RA is toxic (Beard *et al*, 2001). Retinoic acids are used to treat alopecia, and are used as a depilatory. Therefore, this transient induction may be due to excessive concentrations of ATRA and parallel the phenomena when ATRA is used as a depilatory.

TaqMan probe analysis also suggested that ATRA could induce some types of hard keratin genes in HBC (Fig 4*b*); however, 10 μM ATRA did not induce the transient expression of these genes like that of GPRC5D. In addition, among Ha1, Ha3, and Ha4, only the expression of Ha4 was induced more than that in original hair follicles (Ha1, about 0.1–0.3 fold; Ha3, 0.1–0.2 fold; Ha4, 3.5–4.5 fold at 3 d after adding ATRA). Whether the mechanism of their induction is direct through RAR remains unknown. Additional studies are necessary to determine this. Indirect upregulation or downregulation of (soft) keratin genes by RA in the epidermis has been reported (Virtanen *et al*, 2000). Like soft keratin genes, hard keratin genes can be regulated by several factors in hard epithelial tissue, which has a mosaic of differentiation programs. For example, the signal transduction of RA induction activates the β-catenin/LEF-1 signal pathway, which could contribute to the upregulation of hard keratin genes (Zhou *et al*, 1995; DasGupta and Fuchs, 1999;

Liu *et al*, 2002). We therefore speculate that upregulation of hard keratins by ATRA might not be a direct effect via the retinoid receptors because we could not find any significant recognition sequences for RAR or RXR around the promoter regions of several mouse Ha gene loci (data not shown).

In situ hybridization and real-time quantitative PCR suggested that the expression of GPRC5D was not affected in the *Whn* –/– animals, whereas Ha3 was downregulated (Fig 5). *Whn* regulates the expression of several keratin genes, particularly of Ha3 (Meier *et al*, 1999; Schlake *et al*, 2000; Baxter and Brissette, 2002), and *Whn* –/– mice did not express (Ha3) or had reduced expression (Ha1 and 4) of hard keratins (Meier *et al*, 1999). Real-time quantitative PCR showed that expression of Ha3 was reduced to 10% to 20% in BALB/C nude mice, whereas that of GPRC5D was not (Fig 5*g,h*). This indicates that GPRC5D signaling of hair keratin gene regulation is either upstream of whn in a sequential pathway or operates independently of whn in a parallel pathway. The cortical cells at the bottom of hair follicles are pressed upwards in a funnel-like mold of the root sheath by cell division pressure. In this process, keratins are synthesized and oriented, so the time course and distribution of expression are synchronized. The expression of *whn* regulating Ha3 was reported to precede Ha3 expression (Meier *et al*, 1999) and if GPRC5D signaling could be upstream of *whn*, GPRC5D expression should precede that of *whn* (and Ha3). *In situ* hybridization data showed that Ha3 and GPRC5D are expressed in almost the same region of the cortex (Fig 5*a,d*). Accordingly, these data support the hypothesis that GPRC5D is regulated by a parallel pathway different from the main sequential process even though it could affect hard keratin synthesis.

Our data showed that the expression of some hard keratin genes could be affected by exogenous GPRC5D expressed in the presence of ATRA (Fig 6*b–e*). GPRC5D is a member of the GPCR family and that must require ligands for activation. Ligands for the RAIG1 family have not yet been identified; however, several GPCR with known or unknown ligands can maintain activities without binding to specific ligands (Leurs *et al*, 1998; Lee *et al*, 2000; Audinot *et al*, 2001; Seifert and Wenzel-Seifert, 2002; Teitler *et al*, 2002). Thus, activation of GPRC5D could depend only on its expression level and its constitutive activity without a specific ligand might affect the expression of hair keratin genes. Hard keratin expression in hair follicles is stringently regulated in a zone-dependent manner (Bertolino *et al*, 1990; Langbein *et al*, 1999, 2001; Meier *et al*, 1999). Among the four major Ha genes, Ha1, Ha3, and Ha4 are sequentially expressed in the cortical cells, in the order of Ha1, Ha3, and Ha4, whereas, Ha2 is expressed only in the cuticle; however, the mechanism of their regulation remains unclear. Several transcription factors (Whn, LEF-1, HOXC13) regulate their expression through direct interaction with promoter elements but these are "common" regulators and the complexity of the expression profiles of hard keratin genes has yet to be explained (van Genderen *et al*, 1994; Zhou *et al*, 1995; Godwin and Capecchi, 1999; Meier *et al*, 1999; Schlake *et al*, 2000; Jave-Suarez *et al*, 2002). Our data therefore indicate that signal transduction via GPRC5D could play an important part in modulating the order of their expression.

In addition, GPRC5D overexpression affected not only the expression of hard keratins but also reduced the metabolic activities of HBC (Fig 6*a*). Electron micrographs of cross-sections have shown that the nuclear and ribosomal material of the cortical cells in metabolically active hair follicles is decomposed and deprived of water when completely filled with keratins at the end of protein synthesis (Forslind and Swanbeck, 1966; Jones, 1996). The expression of each hard keratin gene in cortical cells is regulated during this differentiation process. Ha1, Ha3, and Ha4 expression are sequentially induced in the cortex (Bertolino *et al*, 1990; Langbein *et al*, 1999, 2001; Meier *et al*, 1999). According to HBC analysis, ATRA induced more Ha4 than Ha1 and Ha3 expression (Fig 4*b*), and exogenous expression of GPRC5D also induced Ha4 (and reduced Ha3 expression) (Fig 6*b–e*). If these phenomena in HBC parallel those of the hair follicle, then there could be the transcription signal cascade "RA–GPRC5D–Ha4", and GPRC5D could play a more direct role on a sequential pathway of differentiation of cortical cells (especially on a part of terminal differentiation) than on the regulation of hard keratin itself. This is because GPRC5D (a member of GPCR) may affect the cell by regulating intracellular signal molecules (cyclic adenosine monophosphate, $[Ca^{2+}]i$, etc.) via G proteins (Bockaert and Pin, 1999; Josefsson, 1999; Lapinsh *et al*, 2002). Some activated GPCR induce apoptosis or cell cycle arrest (Ren *et al*, 1997; Dale *et al*, 2000; Teijeiro *et al*, 2002). Soma *et al* (1998) recently found TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling)-positive cells in the keratogenous zone at anagen and proposed that apoptosis-like cell death is related to terminal differentiation in the epithelial component of the hair follicles. The results of our *in situ* hybridization indicated that this region of TUNEL-positive cells is similar to that of GPRC5D expression. To investigate whether GPRC5D is related to this apoptosis-like death, HBC that exogenously expressed GPRC5D were examined using TUNEL staining and caspase assays; however, we could not identify a significant relationship between GPRC5D and apoptosis (data not shown). Additional analysis or different approaches may be required to determine the effect of GPRC5D on hair cells themselves such as an examination of cell cycle arrest.

In summary, we characterized GPRC5D with respect to its relationship to hard keratins and suggest that it is involved in the regulation of keratin synthesis in hard epithelial tissues. GPRC5D is located on chromosome 12p13.3 (human) and on chromosome 6 G1 (mouse). Sparse hair on the scalp is a feature of trisomy 12p syndrome (Chrzanowska and Fryns, 1989; Takakuwa *et al*, 1997). Raunch *et al* (1996) proposed that the smallest duplications of 12 (p13.2pter) and 12 (p13.1p13.33) produce the trisomy 12p syndrome. Therefore, the trisomy of chromosome 12p may cause sparse hair via excessive expression of GPRC5D located on chromosome 12p13.3. The relationship between GPRC5D and genetic hair disorders such as this requires investigation. In addition, further analyses of GPRC5D will provide clues not only to the mechanism of keratin synthesis in hard epithelial tissues, but also to the function of other members of the RAIG1 family.

Materials and Methods

Animals We prepared HBC from C3H/HeN mice and used C57BL/6J (or N), ICR, and BALB/C (+/+, +/nu, nu/nu) mice for *in situ* hybridization. The mice (purchased from SLC, Shizouka, Japan; CLEA, Tokyo, Japan; and Charles River Japan, Inc., Kanagawa, Japan) were housed communally with 12 h light periods and given access to water and mouse chow ad libitum.

Isolation of mouse GPRC5D cDNA We isolated a novel oGPCR (now termed GPRC5B) using a new method (Inoue *et al*, 2000) and found a homologous gene in mouse expressed sequence tag. Using this sequence as a probe, mouse GPRC5D cDNA (GenBank accession no. AB099816) was isolated by conventional means from a mouse skin cDNA library (Stratagene, catalog no. 937313, West Cedar Creek, TX).

Reverse transcription–PCR of GPRC5D The mouse Rapid Scan Gene Expression Panel (Origene, Rockville, MD) was subjected to PCR for GPRC5D using AmpliTaq Gold (Applied Biosystems, Tokyo, Japan). GPRC5D F1 (5′-CAA ACT GCC CCT GTT CGC TAC TTC-3′) and GPRC5D R1 (5′-GAC CTG TAG AGT ATG CTC AGC TCA-3′) were the primers. The PCR conditions consisted of first denaturation at 94°C for 10 min followed by 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, followed by a final elongation at 72°C for 10 min.

Northern hybridization The probes for northern hybridization were amplified by PCR using the following primers:

mouse Ha1, F1 (5′-CCC TGC CCC TTG CAC AC-3′) and R1 (5′-CCA CTG GCA CTT CAG AGT CCT C-3′); nucleotides 1254 to 1270 and 1555 to 1534 in GenBank accession no. NM_010659, respectively;

mouse Ha2, F1 (5′-CTT AAC AAG CAG GTG GCC ACA AGC T-3′) and R1 (5′-CTT CTA CCC AAG GAA ATA GAG GAG G-3′); nucleotides 785 to 809 and 1464 to 1440 in GenBank accession no. NM_010665, respectively;

mouse Ha3, F1 (5′-ATC ATC GAG CTG AGA CGC ACA GTC A-3′) and R1 (5′-AAA ATA CCT CGT GTG GCC CCT TCA C-3′); nucleotides 571 to 595 and 1221 to 1197 in GenBank accession no. X75650, respectively;

mouse Ha4, F1 (5′-CTG AAC TGA TTC CCT TTT GAA GAT G-3′) and R1 (5′-AGA AGA AAG GAA GGC AGG AAA-3′), nucleotides 1240 to 1264 and 1538 to 1518 in GenBank accession no. NM_027563, respectively.

Primers were designed according to reported sequences (Bertolino *et al*, 1990; Winter *et al*, 1994; Meier *et al*, 1999).

The probe for GPRC5D was prepared using GPRC5D F1 and R1 as described above.

Amplified PCR fragments purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) were used as probes for northern hybridization. Total RNA (5 μg) derived from hair follicles and cultured HBC were analyzed by northern hybridization using a standard protocol.

***In situ* hybridization** We synthesized digoxigenin-labeled RNA probes using mouse GPRC5D full-length cDNA (nucleotides 1–1207 in GenBank accession no. AB099816) and a digoxigenin RNA labeling kit (SP6/T7) according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, Indiana). Hybridization probes for Ha3 were also synthesized using a partial cDNA fragment (nucleotides 725–1221 in GenBank accession no. NM_027563).

Mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde in phosphate buffer. Tissues were postfixed for 12 to 24 h with 4% paraformaldehyde in phosphate buffer, embedded in paraffin, and then 5 μm sections were mounted on MAS-coated slide glass (Matsunami, Osaka, Japan). In some experiments, ICR mice were used to avoid melanin pigment. We investigated the relationship between *whn* and GPRC5D using BALB/C (6 d, +/+, +/nu, nu/nu) mice.

Tissue sections were immersed in 0.2 M HCl for 30 min, digested with 3 μg proteinase K per mL for 15 min at 37°C and fixed once again with 4% paraformaldehyde. Hybridization proceeded for 18 h at 50°C in hybridization solution (40% formamide, 0.6 M NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM ethylenediamine tetraacetic acid, 1 × Denhardt's solution, 250 μg per mL tRNA, 125 μg salmon sperm DNA per mL, 10% dextran sulfate, and 200 ng per mL of each digoxigenin-labeled probe). After digestion with 20 μg RNase A per mL (Sigma, St Louis, Missouri) for 30 min at 37°C and a wash with 0.2 × sodium citrate/chloride buffer at 55°C, the sections were immunolabeled with anti-digoxigenin alkaline-phosphatase conjugate (Roche Diagnostics). Dark purple signals were developed using a mixture of NBT/BCPI (Roche Diagnostics). In some experiments, specimens were counterstained with toluidine blue.

Hair cycle induction A synchronized anagen phase was induced in the dorsal skin of C57BL/6 female mice by depilation as described (Paus *et al*, 1990). The dorsal skin was removed 4, 8, 12, 20, or 24 d after depilation to prepare paraffin sections. The phase of the hair cycle was determined histologically (Chase *et al*, 1951).

Preparation and analysis of HBC Mouse HBC were prepared from newborn mice on day 4 and cultured using the modified protocol as described in blend medium (HuMedia-KG2:EpiLife-KG2 at a ratio of 1:1; Kurabo, Osaka, Japan) with 10 μM minoxidil sulfate (Sigma) instead of K-GM (Kurabo) (Takahashi *et al*, 1998; Shirai *et al*, 2001). Mouse skin was dissected from newborn mice and digested with 20 μg dispase per mL (Invitrogen) in Dulbecco minimal Eagle's medium containing 10% fetal bovine serum at 4°C for 16 h. The epidermis was peeled off and the dermal pieces containing hair follicles were washed with PBS and digested with collagenase (Invitrogen, 0.25% in PBS) at 37°C for 1 h. After washing with PBS, HBC were prepared from the purified hair follicles (= original hair follicles, see Fig 4*a*, lane 1) by trypsin treatment, incubated for 18 h with Dulbecco minimal Eagle's medium containing 10% fetal bovine serum and then cultured for 24 h in blend medium at 37°C in an atmosphere of 5% CO_2 and 95% air. The HBC were seeded on to collagen-coated microplates (24 well, Asahi Techno Glass, Chiba, Japan, $1-2 \times 10^5$ cells per well) and studied after briefly washing the cells with PBS (day 0).

Transfection of GPRC5D into HBC Full-length mouse GPRC5D cDNA was subcloned into pcDNA3 (Invitrogen). The expression vectors containing GPRC5D cDNA (sense and anti-sense (nucleotides 1–1207 in GenBank accession no. AB099816)) were extracted and purified using an EndoFree Plasmid kit (Qiagen) according to the manufacturers' instructions. The DNA were transfected into the HBC using the Lipofectamine Plus Reagent package and Opti-MEM (Invitrogen) according to standard protocols. Plasmid DNA (0.4 μg), plus reagent (4 μL) and Lipofectamine reagent (1 μL) were added to wells containing HBC. Cultured (adherent) HBC in the 24-well microplates were briefly washed twice with Opti-MEM before transfection with DNA. After 3 h at 37°C, the HBC were washed twice with blend medium, and then cultured in fresh medium containing 1 μM ATRA.

AlamarBlue Assay (detection of metabolic activity) HBC were subjected to the AlamarBlue assay according to the manufacturer's protocol (BioSource International, Camarillo, CA). After adding an amount of AlamarBlue equal to 5% of the culture volume and incubation for 4 h at 37°C, fluorescence was measured with excitation at 530 nm and emission at 580 nm using a CytoFluor multiwell plate reader (PerSeptive Biosystems, Foster City, CA). The metabolic activity of each sample was calculated by comparison with mock-transfected samples (100%) at each same day.

Real-time quantitative PCR (TaqMan probe analysis) RNA were prepared from whole HBC in culture medium using Isogen LS (Wako, Osaka, Japan). The RNA were additionally purified using

RNeasy 96 kit after treatment with DNase (Qiagen). Purified RNA samples were reverse transcribed using an RNA PCR kit Ver.2.1 (Takara, Tokyo, Japan), followed by TaqMan probe analysis. TaqMan PCR reactions were performed in duplicate using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) in a reaction volume of 30 μL, containing 1 × Universal master mix, 300 nM of forward and reverse primers, 100 nM fluorescent probe (FAM labeled), 100 nM of forward and reverse Ribosomal RNA primers, 400 nM Ribosomal RNA probe (TaqMan Ribosomal RNA Control Reagents VIC probes, Applied Biosystems), and 1 μL of each reverse transcription–PCR sample. The temperature cycling program consisted of a 2 min initial incubation at 50°C followed by 10 min at 95°C and 45 PCR cycles of 15 s at 95°C and 1 min at 62°C.

Probe and primer sequences were designed using Primer Express 1.5a (Applied Biosystems) as follows:

GPRC5D TaqMan probe, 5′-FAM CGGAAGGTTCAGGACTG-CAGCCA-3′-TAMRA;
forward primer, 5′-TCCTGGCATTTCTGTTCCTCAT-3′;
reverse primer, 5′-GAGTGGGAAGCACGTTCCA-3′;
Ha1 TaqMan probe, 5′-FAM CAATGCATGCGGCAAGCCCATT-3′-TAMRA;
forward primer, 5′-GCAACCCCTGTGCCACA-3′;
reverse primer, 5′-ATTGGAGACGCAGGGCC-3′;
Ha2 TaqMan probe, 5′-FAM CCAGCAACCGCCGGCCA-3′-TAMRA;
forward primer, 5′-CTACTTGTCCAGCTCTTGCCAA-3′;
reverse primer, 5′-CATGGAGCTAGAGATGCAACCTG-3′;
Ha3 TaqMan probe, 5′-FAM AGGATCCTGGACGAACT-GACCCTCTGC-3′-TAMRA;
forward primer, 5′-AGACCTCAATGGCCTGCG-3′;
reverse primer, 5′-ACCTGTGCCTCCAGATCAGACT-3′;
Ha4 TaqMan probe, 5′-FAM AGTTGCTGTGGACCTTGCGG-CAG-3′-TAMRA;
forward primer, 5′-CGCCACCACCAATGCTAGT-3′;
reverse primer, 5′-GTTCAGTTAACAGCAACGCTTTGA-3′.

The amount of RNA extracted from the HBC was normalized by that of the extracted ribosomal RNA. A standard curve was generated for each gene by simultaneously amplifying known amounts of the first strand cDNA derived from the original hair follicles (Fig 4*a*, lane 1). The expression level of each gene in the samples was calculated by comparison with that in the hair follicles (considered as 1).

Statistical analysis Statistical analysis was performed with the nonparametric Mann–Whitney test. Statistical significance was established at p less than 0.01.

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