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Role of sonic hedgehog in maintaining a pool of proliferating stem cells in the human fetal epidermis

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BACKGROUND: The mammalian epidermis is maintained by the ongoing proliferation of a subpopulation of keratinocytes known as epidermal stem cells. Sonic hedgehog (Shh) can regulate morphogenesis of hair follicles and several types of skin cancer, but the effect of Shh on proliferation of human putative epidermal stem cells (HPESCs) is poorly understood. METHODS AND RESULTS: We first found that Shh, its receptors Patched1 (Ptc1) as well as Smoothened (Smo) and its downstream transcription factor Gli-1 were expressed in the basal layer of human fetal epidermis and freshly sorted HPESCs. Next, treatment of HPESCs with media conditioned by Shh-N-expressing cells promoted cell proliferation, whereas inhibition of Shh by cyclopamine, a specific inhibitor of Shh signalling, had an opposite effect. Interestingly, the mitogenic effect of epidermal growth factor (EGF) on HPESCs was efficiently abolished by cyclopamine. Finally, bone morphogenetic protein 4 (BMP-4), a potential downstream effector of Shh signalling, increased HPESC proliferation in a concentration-dependent manner. CONCLUSIONS: Shh is an important regulator of HPESC proliferation in the basal layer of human fetal epidermis and modulates the cell responsiveness to EGF, which will assist to unravel the mechanisms that regulate stem cell proliferation and neoplasia in the human epidermis.

Key words: BMP-4/EGF/human putative epidermal stem cells/sonic hedgehog

Introduction

The epidermis forms the outer protective layers of the skin, a rapidly renewing tissue undergoing constant regeneration. This renewal capacity of the skin is dependent on proliferation of a subpopulation of keratinocytes known as epidermal stem cells, residing in the epidermal basal layer and the hair follicle bugle (Watt, 2001; Alonso and Fuchs, 2003). It is accepted today that although the epidermal stem cells have unlimited self-renewal capacity, their daughter cells, known as transit amplifying (TA) cells, are destined to undergo terminal differentiation. TA cells divide three to five times before leaving the basal layer, and their progeny move through the suprabasal layers to the tissue surface, where they are shed (Watt, 1998). One protein marker that distinguishes stem cells from TA cells is integrin β1 (Hotchin *et al*., 1995). Stem cells express 2–3 folds higher level of integrin β1 at the surface layer than TA cells, a property used to determine the relative distribution of each keratinocyte subpopulation within the basal layer. We have recently developed a protocol to successfully identify and enrich human epidermal stem cells from the epidermis of aborted human fetuses (Zhou *et al*., 2004). Such a protocol is important, as epidermal stem cells not only play a central role in skin homeostasis and wound repair but are major targets for gene therapy and the site of tumour initiation (Morris, 2000; Owens and Watt, 2003; Perez-Losada and Balmain, 2003). Moreover, it has been shown that mouse epidermal stem cells can produce multiple cell lineages during development, thus being easily accessible and reprogrammed to differentiate to various cell types (Liang and Bickenbach, 2002). However, the molecular mechanisms of epidermal stem cell proliferation and differentiation and their potential role in neoplasia are far from clear.

Sonic hedgehog (Shh) is an important intercellular signalling morphogen, which plays an important role in many developmental stages and stem cell regulation (Goodrich and Scott, 1998; Ingham and McMahon, 2001; McMahon *et al*., 2003; Wetmore, 2003). Recent studies identified involvement of Shh in the proliferation and cell-fate specification of several stem cells such as neural stem cells and mesenchymal stem cells (Palma and Ruiz, 2004; Kondo *et al*., 2005). Shh transduces its signal upon interaction with a protein of the Patched (Ptc) family, the 12-transmembrane molecules that act as receptors for Shh

(Marigo *et al*., 1996; Stone *et al*., 1996). Smoothened (Smo), a 7-transmembrane protein, is a signal transducer that in the absence of Shh interacts with Ptc. This Smo–Ptc interaction represses Smo-signalling activity, therefore acting also as a repressor of Shh signalling. Binding of Shh to its receptor Ptc releases the repression of Smo and transfers the signal by activating transcription factors of the Gli family, which then activate a number of downstream targets of the Shh-signalling pathway. In mammals, Shh signalling is involved with two Ptc receptors (Ptc1 and Ptc2) and at least three Gli proteins (Gli-1, Gli-2 and Gli-3) (Goetz *et al*., 2002).

The roles of Shh signalling in the mammal skin have been investigated previously, especially its important activity in controlling the growth of hair follicle and hair follicle tumours. In mice, overexpression of Shh is sufficient to induce development of basal cell carcinomas (BCC), a tumour originating from undifferentiated pluripotent cells in the skin (Oro *et al*., 1997; Grachtchouk *et al*., 2000, 2003). Transgenic human skin cells expressing Shh show features of BCC (Fan *et al*., 1997), and expression of Gli-1 in basal cells also induces BCC formation (Dahmane *et al*., 1997). Recently, transgenic mouse model supports the concept of a novel role for the Shh signalling in maintaining the epidermal stem cell population (Adolphe *et al*., 2005). Overall, these findings are in line with the notion that Shh has the potential to modulate epidermal stem cell growth, although the exact role of Shh in the growth and development of human putative epidermal stem cells (HPESCs) and TA cells remains unclear. Likewise, whether the pluripotent epidermal cell population is responsive to Shh signalling *in vitro* is unknown. Here, we report that Shh and its downstream signal components are expressed in the human fetal skin and in sorted HPESCs. Shh-N fragment produced by HEK293T cells can stimulate the proliferation of HPESCs. Finally, potential crosstalk between Shh signalling and epidermal growth factor (EGF) in regulating the proliferation of HPESCs as well as the effect of bone morphogenetic protein 4 (BMP-4) on HPESC proliferation is analysed.

Materials and methods

Cell culture

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) with 2 mmol/l L-glutamine, 0.5 mmol/l HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37°C. Fluorescence-activated cell sorter (FACS)-sorted epidermal stem cells were plated on 24-well plates coated with collagen type IV (100 μg/ml, Sigma, St. Louis, MO, USA) in keratinocytes–serum free medium (SFM) medium (Gibco, Grand island, NY, USA) supplemented with 30 μg/ml bovine pituitary extract (Gibco, Grand island, NY, USA), at a density of 1×10^5 cells/well in 5% CO_2 at 37°C.

In situ *hybridization*

All aborted fetuses ($n = 26$) were collected and used under the permission and supervision of the Animal and Medicine Ethical Committee of Institute of Zoology, Chinese Academy of Sciences (CAS). Mid-gestation-aborted normal human fetuses were collected and treated as previously (Zhou *et al*., 2004) and used for *in situ* hybridization (ISH) ($n = 4$) or isolation and enrichment of HPESCs ($n = 26$). The human fetal skins were fixed in Bouin's solution for 12 h, and ISH of the paraffin-embedded tissue sections were carried out as described (Wilkinson and Nieto, 1993). Sense and antisense probes for human Shh, Smo, Ptc1 and Gli-1 labelled with digoxigenin (DIG) were synthesized with T7 and SP6 RNA polymerases (Promega, Madison, WI, USA) and hybridized according to the process provided by the suppliers (Roche, Indianapolis, IN, USA). The results were detected by nitro blue tetrazolium/bromo-chloro-indoryl phosphate (NBT/BCIP) (Promega, Madison WI, USA) and recorded under a microscope (Nikon, Tokyo, Japan).

Isolation of human fetal keratinocytes and enrichment of HPESCs

HPESCs were isolated as reported recently (Zhou *et al*., 2004). Briefly, the basal keratinocytes were isolated after digestion with 0.25% trypsin and 0.02% EDTA overnight at 4°C, and then all keratinocytes from one fetus were immediately stored at –196°C. The basal keratinocytes were prepared for FACS according to the previously described procedure with modifications (Zhou *et al*., 2004). Cultivation or passage of keratinocytes before the sorting process was omitted to avoid activation of gene expression, cell proliferation or cell differentiation. Collected cell populations were used for cell culture or RT–PCR analysis.

RT and semi-quantitative PCR analysis

The expression of Shh-signalling pathway member genes was assayed by semi-quantitative RT–PCR. After cell sorting, $1–5 \times 10^5$ HPESCs or TA cells were collected into Trizol (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted according to the manufacturer's instructions and treated with RNase-free DNase (Roche, Indianapolis, IN, USA) to remove any contaminating genomic DNA. Single-stranded cDNA was synthesized by using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) and Oligo-dT primers according to manufacturer's protocol. Each PCR reaction was carried out in 25 μl of mixture containing 3 μl cDNA, 2.5 μl *Taq* Reaction Buffer, 2 μl of 2.5 mM each dNTP, 0.1 μM sense and antisense primers and 0.5 μl *Taq* DNA polymerase (TaKaRa, Dalian, China). Each PCR reaction was performed as follows: 95°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min followed by 72°C for 7 min. PCR products were detected by electrophoresis on agarose and ethidium bromide staining. The identities of the cDNAs were verified by sequencing.

Sequences of specific primers were as follows:

Integrin α6 (F primer: 5′-aggagtcgcgggatatcttt-3′, R primer: 5′-caggccttctccgtcaaata-3′).
Integrin β1 (F primer: 5′-aatgtttcagtgcagagcc-3′, R primer: 5′-attgggatgatgtcgggac-3′).
EGFR (F primer: 5′-tgccgacgagtacctcatc-3′, R primer: 5′-tggcgcgacccttaggtat-3′).
Shh (F primer: 5′-tgggtgaaagcagagaactc-3′, R primer: 5′-tctcgatcacgtagaagacc-3′).
Smo (F primer: 5′-gttctccatcaagagcaaccac-3′, R primer: 5′-cgattcttgatctcacagtcagg-3′).
Ptc1 (F primer: 5′-cagagaaggcttgtggccac-3′, R primer: 5′-gctcaatgacttccaccttcg-3′).
Gli1 (F primer: 5′-ctcccgaaggacaggtatgtaac-3′, R primer: 5′-ccctactctttaggcactagagttg-3′).
β-actin (F primer: 5′-gtggggcgccccaggcacca-3′, R primer: 5′-cttccttattgtcacgcacgattt-3′).

Transfection and detection of human Shh

The entire human Shh cDNA was amplified from the plasmid phShh (gift from Dr C. Tabin, tabin@rascal.med.harvard.edu), with the primers F (5′-tgaattcgagatggtgctgctggc-3′) and R (5′-aactcgagacagctggacttgacc-3′), and subcloned between the *EcoR*I and the *Xho*I sites of

pcDNA3.1-myc-His to generate recombinant plasmids designated as pcDNA3.1-myc-His-hShh. HKE293T cells were plated onto six-well plates at 60% confluence and then transfected by the plasmid pcDNA3.1-myc-His or pcDNA3.1-myc-His-hShh using LipofectAMINE™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Supernatant from the transfected HEK293T cells was collected 48 h after transfection and precipitated by saturated trichloroacetic acid for Western blot to detect Shh protein. Samples were then boiled and subjected to 8–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blot analysis using anti-Shh amino terminal fragment goat polyclonal antibody (sc-1194, Santa Cruz, Santa Cruz, CA, USA) before incubation with rabbit anti-goat horse-radish peroxidase-conjugated secondary antibody (ZB-2306, Zhongshan Biotechnology, Beijing, China). Protein bands were detected with Western Blotting Luminol Reagent for enhanced chemiluminescence detection (Santa Cruz, Santa Cruz, CA, USA).

Production of conditioned media

Forty-eight hours after transfection with pcDNA3.1-myc-His or pcDNA3.1-myc-His-hShh, HEK293T cells and Shh-producing cells were maintained in keratinocytes–SFM medium supplemented with 30 μg/ml bovine pituitary extract for 12–24 h in preparation of control-conditioned media (Con CM) and Shh-conditioned media (Shh CM).

Immunocytochemistry analysis and flow cytometry detection

After HPESCs were cultured for 72 h, Con CM, Shh CM, cyclopamine (Toronto Research Chemicals, North York, Ontario, Canada), EGF (Sigma, St. Louis, MO, USA) or BMP-4 (R&D Systems, Minneapolis, MN, USA) was added to test the effects on HPESC proliferation. 5′-Bromo-2′-deoxy-Uridine (BrdU) incorporation and immunocytochemistry analysis were performed as followed. To label cells in the S-phase of the cell cycle, we added BrdU (Roche, Indianapolis, IN, USA) to the media at final concentrate of 30 μM for 20 h. The cells were subsequently harvested and fixed in 100% cold methanol for 10 min and then washed three times in phosphate-buffered saline (PBS) before incubating in 2 M HCl for 45 min to denature the DNA. After washing three times in PBS again, the cells were neutralized with 0.1 M $Na_2B_4O_7$ for 30 min and then incubated with anti-BrdU mouse monoclonal antibody (ZM-0013, Zhongshan Biotechnology, Beijing, China) at a dilution of 1:100 in PBS containing 2% bovine serum albumin (BSA) at 4°C overnight. Goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (ZF-0312, Zhongshan Biotechnology, Beijing, China) 1:200 was added, and the cells were incubated at 37°C for 1 h. After washing three times in PBS, the cells were stained with propidium iodide (PI) at a final concentration of 5 μg/ml for 10 min at room temperature and then washed three times with PBS again. Staining was visualized using confocal microscope (Leica, Heidelberg, Germany). Cells were quantified by counting the number of BrdU-positive cells in five to eight independent areas. Each experiment contained three independent cultures.

For flow cytometry detection of cultured cells, cells were collected and washed with 1% BSA in PBS and then incubated in 1% BSA with the FITC-labelled anti-integrin β1 mouse monoclonal antibody (sc-9970 FITC, Santa Cruz, Santa Cruz, CA, USA) for 1 h at 37°C. After washing with 1% BSA three times, cells were filtered with a cell mesh (70 mm). Immediately before analysis, PI (1 mg/ml) was added for viability gating. Cells were kept on ice until the flow cytometry procedure.

Statiatical analysis

Results were presented as the average ± SD of three independent experiments. Statistical differences were evaluated by analysis with *t*-test; values of $P < 0.05$ were accepted as significance.

Results

Expression of Shh signalling in human fetal skin

To determine whether the Shh protein might participate in controlling epidermal cell proliferation, we investigated the expression of several components of the Shh-signalling pathway in the human fetal skin ($n = 4$) using ISH. Figure 1e shows that Shh mRNA was expressed in defined cells of the basal epidermal layer and sebaceous gland of human fetal epidermis. Our *in situ* study also shows that Smo, Ptc1 and Gli-1 mRNAs were expressed in the whole basal layer of the human fetal skin and the sebaceous gland (Figure 1a, c and g). Sense probes gave no signals (Figure 1b, d, f and h). Figure 1i shows the structure of human fetal skin aged ≥20 weeks,

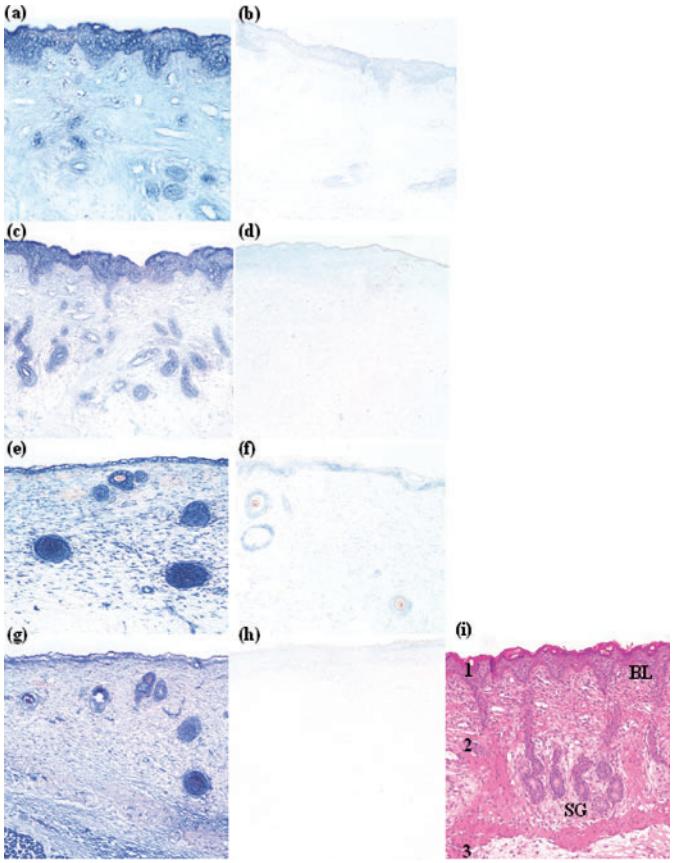

Figure 1. *In situ* detection of sonic hedgehog (Shh) signalling in human fetal epidermis. Expression of Shh, its receptors Smo, Ptc1 and its transcription factor Gli-1 mRNAs was analysed (photographed at ×100). (**a**) Smo, (**c**) Ptc1, (**e**) Shh and (**g**) Gli-1 mRNAs were expressed in the basal layer (BL) and sebaceous gland (SG) of human fetal epidermis. (**b**), (**d**), (**f**) and (**h**) were the control sections of Smo, Ptc1, Shh and Gli-1, respectively. Sense probes gave no signals. (**i**) Histological analysis of the skin of aborted normal human fetus by H&E staining. 1, 2 and 3 represent epidermis, dermis and subcutis, respectively. It shows the structure of human fetal skin aged ≥20 weeks, which has developed into mature status. Paraffin sections of human fetal epidermis were hybridized with DIG-labelled probes specific to human Smo, Ptc1, Shh and Gli-1. Probes were produced by T7 and SP6 RNA polymerases. The results were detected by NBT/5-bromo-Δ-chloro-3-indolyl phosphate (BCIP) and recorded under a microscope. Results shown here are from four independent experiments. Ptc1, patched1; Smo, smoothened.

which has developed into mature status. The presence of Shh, Ptc1, Smo and Gli-1 mRNAs supposes that Shh signalling may play a developmental role in the human fetal skin.

Expression of Shh, Smo, Ptc1 and Gli-1 in sorted HPESCs

Enriched preparations of sorted HPESCs were obtained by FACS analysis as described (Zhou *et al*., 2004), with minor modifications. It is worth indicating that the HPESCs were not cultured before the sorting to avoid any modification in cell phenotype or gene expression. To further explore the potential role of Shh signalling in human fetal basal cells, we determined the expression of the genes encoding Shh, along with other proteins required for Shh signalling, in purified HPESCs. As shown in Figure 2, Shh and its receptors Smo and Ptc1, along with the downstream transcription factor Gli-1, were expressed in HPESCs. Although the apparent expression of Shh mRNA was low, it is premature to decide whether the protein level is low as well. To confirm the reliability of the primers of Shh, the recombinant plasmid pcDNA3.1-myc-His-hShh acts as the positive control. Figure 2 also shows that Smo, Ptc1 and Gli-1 were expressed in TA cells as well. Moreover, these experiments confirm previous reports that HPESCs show higher expression of integrin α_6 and β_1 than TA cells.

Shh-N protein stimulates proliferation of HPESCs

Whether exogenous Shh signalling regulates the proliferation of HPESCs was determined by adding the conditioned media to HPESC culture and monitoring cell proliferation. After transfection, the supernatant from HEK293T cells and Shh-producing cells was harvested and tested whether the transfected HEK293T cells express Shh. Figure 3a shows that conditioned media from Shh-producing HEK293T cells (Shh CM), but not from control HEK293T cells (Con CM), contained a significant amount of Shh-immunoreactive protein, with the expected molecular weight of 19 kDa. When BrdU was added to label the S-phase of HPESCs, Shh-CM significantly increased the percentage of BrdU-positive cells as compared with cells treated with Con CM from control HEK293T cells (Figure 3b, 6.9 ± 6 and 22 ± 13.6%, respectively, $P < 0.05$). We also tested the effect of different concentrations of recombinant Shh-N fragment and found that even at 500 ng/ml, there was no effect on HPESC proliferation (data not shown). Such treatment of HPESCs had no effect on integrin β1 expression (Figure 3c).

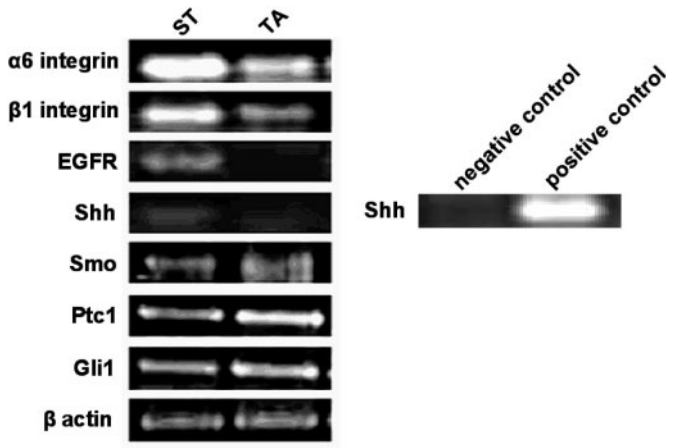

Figure 2. Expression of molecular markers, Shh and its putative receptors and effectors in sorted human putative epidermal stem cells (HPESCs) and TA cells. RT–PCRs were performed on single-stranded cDNA from freshly sorted HPESCs and TA cells. β-actin gene was used as internal control. The apparent expression of Shh mRNA was low in HPESCs. The recombinant plasmid pcDNA3.1-myc-His-hShh acts as the positive control to confirm the reliability of primers of Shh. Receptors Smo and Ptc1, along with the downstream transcription factor Gli-1, were also expressed in HPESCs and TA cells. HPESCs show higher expression of integrin α_6 and β_1 than TA cells. Epidermal growth factor receptor (EGFR) was expressed in HPESCs but not in TA cells. The results shown are from three independent experiments. ST, stem cells; TA, transit amplifying cells; EGFR, epidermal growth factor receptor; Shh, sonic hedgehog; Smo, smoothened; Ptc1, patched1.

Cyclopamine inhibits proliferation of HPESCs and the effect of EGF

To further evaluate whether Shh protein plays a role in the proliferation of HPESCs *in vivo*, we designed experiments to determine the role of endogenous Shh signalling. Cells were treated for 72 h with cyclopamine, a specific inhibitor of Shh signalling (Chen *et al*., 2002), at two different concentrations. Figure 3d shows that incubating HPESCs with cyclopamine decreased the percentage of BrdU-positive cells in a dose-dependent way. When harvested after 72 h of treatment, the viability of the cells was >95% in both the cyclopamine treated and control cultures, implying that the apparent decreased proliferation in the treated cells was not the result of an enhanced cell death. These findings are in line with the notion that endogenous Shh proteins, produced by HPESCs or other cells in the culture, might be involved in regulating proliferation of HPESCs.

It has been shown that EGF is a powerful mitogen for cultured human epidermal keratinocytes (Rheinwald and Green, 1977). In this study, EGF had profound effects on proliferation of HPESCs, increasing the percentage of BrdU-positive cells by 5-fold (Figure 3e). Because cyclopamine largely decreased the proliferating effect of EGF (Figure 3e), EGF and Shh signalling may synergize to regulate proliferation of HPESCs, as discussed.

BMP-4 stimulates proliferation of HPESCs

BMP-4 is an important member of the TGF-β family of growth and morphogenic proteins, and it is also a key downstream effector of Shh signalling (Zhu *et al*., 1999; Bhardwaj *et al*., 2001). To verify whether the mitogenic effect of Shh on HPESCs may involve BMP proteins, we evaluated the effect of BMP-4 on proliferation of HPESCs. Figure 3f shows that recombinant BMP-4 increases the percentage of BrdU-positive cells of HPESCs in a concentration-dependent way.

Discussion

Shh, an important morphogen, is involved in a variety of cellular processes during development and diseases, including cell-fate determination, proliferation, differentiation and survival.

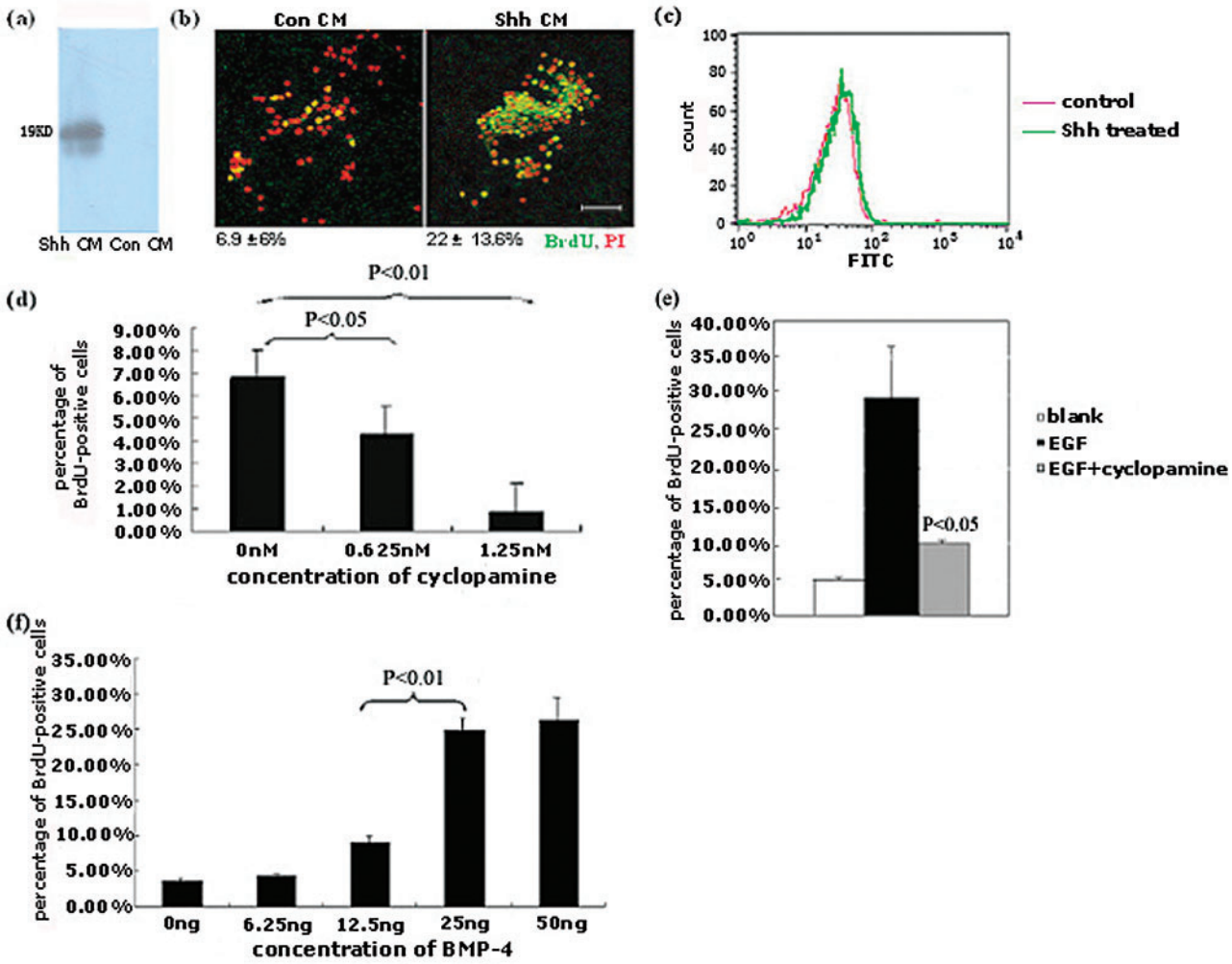

Figure 3. Potential effects of sonic hedgehog (Shh)-signalling pathway on human putative epidermal stem cells (HPESCs). (**a**) Transfection and detection of human Shh. HEK293T cells transfected with pcDNA3.1-myc-His-hShh produced about 19 kDa Shh-N fragment in media (Shh CM) detected by Western blot. Conditioned media from control HEK293T cells (Con CM) did not contain Shh protein. (**b**) Shh-N protein stimulates proliferation of HPESCs. HPESCs were treated with conditioned media from Shh-producing HEK293T cells (Shh CM) or HEK293T cells only transfected by pcDNA3.1-myc-His (Con CM). Shh CM remarkably stimulated the proliferation of HPESCs compared with Con CM. Quantitative measurement of the percentage of BrdU-positive cells was 6.9 ± 6 and 22 ± 13.6% ($P < 0.05$), respectively. Cells were labelled with BrdU 20 h before cytochemistry analysis. Cell nuclei were stained with propidium iodide (PI). (scale bar = 20 μm). (c) Flow cytometry detection of integrin β1 expression. After HPESCs were incubated in media with recombinant Shh-N fragment (Shh treated) or not (control), expression of integrin β1 was assayed by flow cytometry. Result shows that recombinant Shh-N fragment has no effect on integrin β1 expression of HPESCs *in vitro*. (**d**) Cyclopamine inhibits the proliferation of HPESCs. Sorted HPESCs were plated into 24-well plate and treated for 72 h with cyclopamine at two different concentrations. Cyclopamine decreased the percentage of BrdU-positive cells in a dose-dependent way. (**e**) Cyclopamine blocks the proliferation-stimulative effect of epidermal growth factor (EGF) on HPESCs. Before labelled with BrdU, HPESCs were incubated with blank media, EGF and EGF plus cyclopamine, respectively. Quantitative measurements of the percentage of BrdU-positive cells were analysed. Cyclopamine largely decreased the proliferating effect of EGF. (f) Recombinant bone morphogenetic protein 4 (BMP-4) increases the proliferation of HPESCs. When the recombinant BMP-4 protein was directly added to culture medium within 50 ng, it markedly promoted the proliferation of HPESCs in a dose-dependent way. Analyses above were performed three times, and 1000 cells were counted in each case in 5–8 independent areas of microscope. The total fetuses used in the experiments are 26. Error bars indicate the SDs of three independent analyses. Con CM, control conditioned media; Shh CM, Shh conditioned media.

Many studies suggest that Shh signalling might have a potential role in regulating the putative epidermal stem cells of the skin. In an *in vitro* co-culture system, Shh-expressing feeder cells remarkably accelerated the growth of human neonatal foreskin keratinocytes (Kameda *et al*., 2001), implying that the Shh-N fragment released by the feeder cells had a mitogenic effect on human keratinocytes. Also, human keratinocytes transfected with an Shh-expressing vector showed augmented replication and failed to exit from S and G2/M phases in response to calcium-induced terminal differentiation, being the first clear evidence that Shh signalling can directly act at the cell-cycle machinery (Fan and Khavari, 1999; Roy and Ingham, 2002). In *Drosophila*, Hh protein, the homologue of Shh, stimulates proliferation of ovarian somatic cells or epithelial cells, thus acting as a somatic stem cell factor (Forbes *et al*., 1996; Zhang and Kalderon, 2001). Taken together, Shh signalling in mammals regulates epithelial cell proliferation, similarly to its function in *Drosophila* ovary. Our present data demonstrate an

involvement of Shh signalling in the proliferation of HPESCs in human fetal epidermis. To sum up, the gene expression analysis and *in vitro* experiments indicate that Shh signalling plays an important role in the maintenance of a pool of cells with stem cell properties for the proliferation of HPESCs.

The main finding of this work is that Shh signalling represents an endogenous mechanism that regulates proliferation of certain cells in the human fetal epidermis. First, we showed that Shh, its receptors Ptc1, Smo and its downstream transcription factor Gli-1 were expressed in the sebaceous gland and matrix cells of hair follicle (data not shown), being similar to previous observations in the mouse, in which Shh was detected in the sebaceous gland and matrix cells of hair follicle, but not in the basal layer (Bitgood and McMahon, 1995; Gat *et al*., 1998; Wang *et al*., 2000). However, we have also found that in human fetal skin, Shh was additionally expressed in the basal layer, possibly playing a role during the development of the sebaceous gland and hair follicle morphogenesis. Interestingly, the study of Chiang *et al*. (1999) in the mouse has shown that during hair follicle morphogenesis, Shh is required for the normal development to proceed beyond the hair germ stage.

This study also shows that several components of the Shh-signalling pathway are expressed in the FACS-sorted HPESCs and TA cells, indicating that human fetal basal cells are receptive to Shh signalling. However, Shh is weakly or not expressed in the sorted HPESCs or TA cells, respectively. We certainly found that Shh mRNA is highly detected in the basal layer of human fetal epidermis by ISH. So it is more likely that Shh is expressed from neighbouring cells. The exact Shh-secreting cell remains to be investigated. By contrast, it is of interest that the expression level of the various components of the Shh-signalling pathway was not associated with the differentiation state of the cells; HPESCs show higher expression of integrin α_6 than TA cells, whereas Gli-1 and Ptc1 levels were higher in TA cells. The observation that the EGF receptor (EGFR) was expressed in HPESCs, but not in TA cells, further suggests that HPESCs are more primitive cells of the two.

To directly investigate the role of Shh signalling in proliferation of HPESCs, we added cyclopamine to the media. Treatment with cyclopamine effectively decreased proliferation of HPESCs in a dose-dependent manner as measured by BrdU incorporation, showing that HPESCs require Shh signalling for normal proliferation. Although the detected level of endogenous Shh mRNA was low, Shh in HPESC culture plays an important mitogenic role in cell proliferation. In cultured mammalian cells, Shh is produced as a 45 kDa precursor protein that undergoes autocleavage to generate a Shh-N polypeptide and then palmitoylation and addition of a cholesterol molecule (Lee *et al*., 1994; Bumcrot *et al*., 1995; Fietz *et al*., 1995). This final product provides all the known biological activities of the Shh protein (Goetz *et al*., 2002). In this study, recombinant Shh-N derived from bacteria showed no effect on proliferation and differentiation of HPESCs, even at a very high concentration. This initial finding may be related to the widely documented observation that cholesterol and palmitoylation of Shh are important to maintain the full activity of this protein (Goetz *et al*., 2002). However, as Shh-N protein produced by HEK293T cells remarkably enhanced proliferation of HPESCs, we suggest that the biochemical modifications in the Shh-N fragment in eukaryotic cells are very important for the activity of this mitogen in the developing skin. In line with this, in a recent study with an entirely different cell system, Palma and Ruiz (2004) have used much lower concentrations of octyl-modified recombinant Shh-N than the amount of Shh-N used in the past (Wechsler-Reya and Scott, 1999; Zhu *et al*., 1999). It implies that modifications of Shh-N are essential to its appropriate function. But the particular mechanism between modification and function needs to be further investigated. It is also apparent therefore that the fully active Shh-N protein produced by HEK293T cells stimulates the proliferation of HPESCs.

We found that endogenous Shh signalling in HPESCs plays an important role in cell proliferation. Moreover, cyclopamine decreased effectively the proliferating effect of another growth factor, EGF. EGF on its own is not sufficient to cause cell proliferation, which suggests that Shh signalling is possible to be the co-factor of EGF in controlling HPESC proliferation in this system. Co-operative activity between the Shh and EGF pathways was also observed in the developing *Drosophila* (Amin *et al*., 1999) and recently in the mouse brain (Palma *et al*., 2005). Finally, it has been shown here that BMP-4 enhances proliferation of cultured HPESCs in a dose-dependent manner. Co-expression of Shh and components of the BMP signalling was observed in many tissues, including the skin (Bitgood and McMahon, 1995; Morgan *et al*., 1998; Oro and Scott, 1998). Moreover, *in vitro* experiments have shown that Shh induces proliferation of different cells via BMP signalling (Zhu *et al*., 1999; Bhardwaj *et al*., 2001). It remains to be determined whether and how the Shh signalling that regulates proliferation of HPESCs of the human fetal skin interacts with the BMP signalling.

In summary, we have shown that components of the Shh-signalling system are distributed in human fetal skin, and Shh apparently regulates proliferation of basal cells. These results are in line with the notion that Shh signalling controls cell proliferation in the human fetal skin, possibly acting in a niche that maintains an ongoing proliferation of stem (or progenitor) cells. This is consistent with previous findings that Shh and Gli are expressed and regulate cell proliferation in other stem cell niches such as the brain dentate gyrus (Dahmane *et al*., 2001) and the fly ovary (Zhang and Kalderon, 2001; Park *et al*., 2003) and also control stem cell behaviour in the developing neocortex (Palma and Ruiz, 2004). These findings show that Shh is a niche factor regulating the number of stem cells and then contribute to further our understanding of the mechanisms that modulate proliferation of stem cells and neoplasia in the human skin.

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