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IV.研究成果の刊行物・別冊

FHL-2 Suppresses VEGF-Induced Phosphatidylinositol 3-Kinase/Akt Activation via Interaction With Sphingosine Kinase-1

Hiroki Hayashi, Hironori Nakagami, Yoichi Takami, Hiroshi Koriyama, Masaki Mori, Katsuto Tamai, Jianxin Sun, Kaori Nagao, Ryuichi Morishita, Yasufumi Kaneda

Objective—In the functional screening of a human heart cDNA library to identify a novel antiangiogenic factor, the prime candidate gene was "four-and-a-half LIM only protein-2" (FHL-2). The goal of this study is to clear the mechanism of antiangiogenic signaling of FHL-2 in endothelial cells (ECs).

Methods and Results—Overexpressed FHL-2 strongly inhibited vascular endothelial growth factor (VEGF)-induced EC migration. In the angiogenic signaling, we focused on sphingosine kinase-1 (SK1), which produces sphingosine-1-phosphate (S1P), a bioactive sphingolipid, as a potent angiogenic mediator in ECs. Immunoprecipitation and immunostaining analysis showed that FHL-2 might bind to SK1. Importantly, overexpression of FHL-2 in ECs inhibited VEGF-induced SK1 activity, phosphatidylinositol 3-kinase activity, and phosphorylation of Akt and eNOS. In contrast, overexpression of FHL-2 had no effect on S1P-induced Akt phosphorylation. Interestingly, VEGF stimulation decreased the binding of FHL-2 and SK1. Depletion of FHL-2 by siRNA increased EC migration accompanied with SK1 and Akt activation, and increased the expression of VEGF receptor-2 which further enhanced VEGF signaling. Furthermore, injection of FHL-2 mRNA into Xenopus embryos resulted in inhibition of vascular network development, assessed by in situ hybridization with endothelial markers.

Conclusions—FHL-2 may regulate phosphatidylinositol 3-kinase/Akt via direct suppression of the SK1-S1P pathway in ECs. (*Arterioscler Thromb Vasc Biol.* 2009;29:909-914.)

Key Words: FHL-2 ■ VEGF ■ sphingosine kinase-1 ■ endothelial cells ■ Akt pathway

A ngiogenesis is critical for organ growth and development, wound healing, and reproduction.[1] Therapeutic modulation of angiogenesis using angiogenic growth factors, such as vascular endothelial growth factor (VEGF), has been proposed for revascularization in ischemic diseases and has investigated in clinical trials.[2] Angiogenesis is ultimately controlled by a balance between endogenous pro- and antiangiogenic molecules, which may include various antiangiogenic peptides, hormone metabolites, and apoptosis modulators.[13]

We recently developed a functional gene screening system with the HVJ-E vector[4] and screened a human heart cDNA library for antiangiogenic genes. One of the candidates was "four and a half LIM domain protein-2" (FHL-2/SLIM3), a member of the LIM-only subclass of the LIM protein superfamily. LIM proteins are defined by the presence of one or more LIM proteins that mediate protein-protein interactions.[5] The members of the FHL subclass of LIM-only proteins consist of four and a half LIM domains, are expressed primarily in cardiac tissues, and mainly function as transcriptional cofactors.[6-10] Heart development and function is normal in FHL-2–deficient mice,[11] but FHL-2 deficient mice develop cardiac hypertrophy in response to β-adrenergic stimulation.[12]

Although FHL-2 is present in a human heart cDNA library, we identified the endogenous expression in vascular endothelial cells (ECs). The goal of the present study was to clarify the role of the FHL-2 gene in angiogenic signaling in ECs.

Materials and Methods

Northern Blotting, Western Blotting, and Immunochemical Staining

Northern blotting, Western blotting and immunochemical staining were performed as previously described.[13] In immunoprecipitation

assay, the interaction between FHL-2 and SK1 was evaluated by immunoblotting with anti-M2 flag antibody (Sigma), anti-HA antibody (Sigma), anti-SK1 antibody (Abgent), and anti-FHL-2 (MBL). Primary antibody used for the immunochemical staining was anti-FHL-2 (Abcam) antibody at 4°C overnight. Corresponding secondary antibodies were labeled with peroxidase (GE healthcare).

In Vivo Xenopus Embryo mRNA Injection Model

Xenopus laevis embryos were generated using standard techniques and staged as previously described.[14] Whole mount in situ hybridization was performed as described previously described,[15] using the DIG RNA Labeling kit (Roche, Basel, Switzerland). To evaluate vascular formation of Xenopus embryos, embryos were hybridized with probes for endothelial marker, Xenopus fli-1, or msr.[16]

Statistical Analysis

All values are expressed as mean±SD. Analysis of variance with subsequent Fisher PLSD test or the unpaired Student t test was used to determine the significance of differences in multiple comparisons. All statistical analysis was performed using Stat-View 5.0 software (SAS. Institute Inc). Values of $P<0.05$ were considered to represent statistical significance.

Results

Expression of FHL-2 in Vascular ECs

We screened a human heart cDNA library using the HVJ-E functional screening system as previously reported to identify the novel antiangiogenic modulators.[4] One candidate of antiangiogenic modulator genes was FHL-2, which is initially identified to be highly expressed in cardiac tissue.[11] Northern blot analysis using human cardiovascular tissue showed that FHL-2 mRNA was detected in the aorta as well as in heart (supplemental Figure IA). Similarly, immunohistochemical staining demonstrated that FHL-2 was expressed in coronary arteries and cardiac myocytes. In the ascending aorta, the expression of FHL-2 was detected both in ECs and smooth muscle cells (SMCs; supplemental Figure IB). Western blot showed that FHL-2 was fairly expressed in human aorta ECs (HAECs) and HASMCs, less in THP-1 (supplemental Figure IC). Several growth factors (ie, vascular endothelial growth factor [VEGF] 25 ng/mL, fibroblast growth factor-2 [FGF2] 25 ng/mL, epidermal growth factor [EGF] 25 ng/mL, VEGF 25 ng/mL +FGF2 25 ng/mL +EGF 25 ng/mL) did not change the expression of FHL-2. However, expression of FHL-2 was slightly decreased by stimulation with tumor necrosis factor-α (TNF-α) 10 ng/mL (supplemental Figure ID).

FHL-2 Attenuates EC Growth and Interacts With SK1 in ECs

FHL-2 was previously identified as an antiproliferative factor.[4] We confirmed that FHL-2 suppressed VEGF-induced cell growth in BAECs by using MTS assay and c-fos promoter assay (supplemental Figure IIA and IIB). FHL-2 also repressed VEGF-induced cell growth in HAECs (supplemental Figure IIC). FHL-2 interacting molecules may elucidate the mechanism of antiangiogenic effect of FHL-2. Importantly, previous studies reported that sphingosine
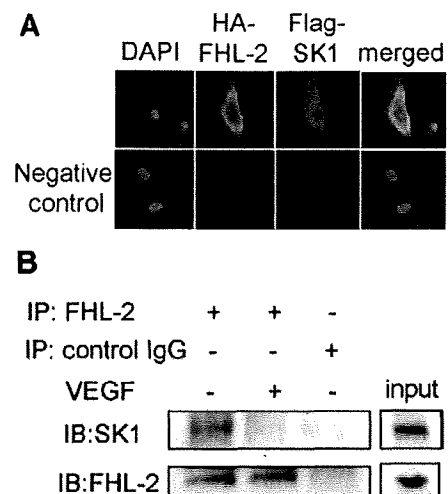

Figure 1. VEGF decreases the interaction between FHL-2 and SK1 in ECs. A, Immunostaining analysis of overexpressed HA-tagged FHL-2 and Flag-tagged SK1 in BAECs (×400 magnification). "DAPI" indicates nuclear staining (blue), "HA-FHL-2" indicates the staining (green) with anti-HA antibody, "Flag-SK1" indicates the staining (red) with anti-Flag antibody and "merged" indicates the staining with both. "Negative control" indidates the staining with nonimmunized IgG. B, Immunoprecipitation in HAECs with or without VEGF treatment. "IP: FHL-2" indicates immunoprecipitation with anti–FHL-2 antibody. "IB: SK1" indicates immunoblot with anti-SK1 antibody. "IB: FHL-2" indicates immunoblot with anti–FHL-2 antibody.

kinase-1 (SK1) bound to FHL-2[17] and produce sphingosine-1-phosphate (S1P), a bioactive sphingolipid that acts as a potent angiogenic mediator in ECs.[18]

Consistent with the previous report in cardiac myocytes,[17] immunostainig analysis in BAECs showed that overexpressed HA-FHL-2 and Flag-SK1 were colocalized (Figure 1A). Immunoprecipitation experiments demonstrated binding of FHL-2 to SK1 in BAECs with overexpressed HA-FHL-2 and Flag-SK1. Interestingly, treatment of BAECs with VEGF 50 ng/mL decreased direct interaction between FHL-2 to SK1 at 5 minutes after stimulation (supplemental Figure IIIA). Moreover, immunoprecipitation showed that endogenous interaction of FHL-2 and SK1 was decreased by stimulation with VEGF 50 ng/mL in HAECs (Figure 1B). However, the stimulation of VEGF 50 ng/mL did not change the expression level of these mRNA and proteins in HAECs (supplemental Figure IIIB and IIIC). These results indicated that FHL-2 interacted with SK1 in ECs.

FHL-2 Suppresses SK1 Activity, Which Regulates VEGF-Induced Akt Phosphorylation and EC Migration

Overexpression of FHL-2 in BAECs strongly suppressed VEGF-induced SK1 activation and S1P production at 5 or 10 minutes after stimulation (Figure 2A). Treatment of HAECs with VEGF or S1P activated Akt phosphorylation, whereas pretreatment with SK1 inhibitor, DMS (dimetylsphingosine), or S1P receptors antagonist, VPC23019,[19] suppressed Akt phosphorylation induced by VEGF (supplemental Figure IVA and IVB). It is known that SK1-S1P signaling strongly contributes to EC migration, which is
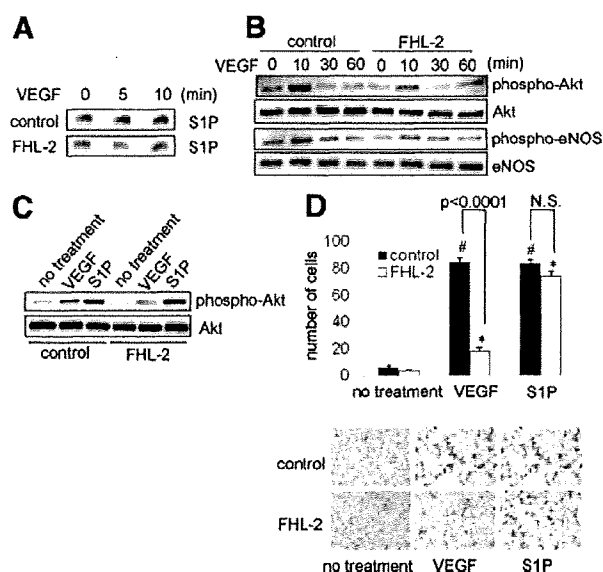
Figure 2. FHL-2 suppresses SK1 activity, which inhibits VEGF-induced phosphatidylinositol 3-kinase/Akt activation and cell migration in ECs. "VEGF"=VEGF 50 ng/mL. "S1P"=sphingosine-1-phosphate 10 μmol/L. "control" indicates overexpressed GFP gene, "FHL-2" indicates overexpressed FHL-2 gene. A, SK1 activity by thin layer chromatography. n=3. B, Phosphorylated or total Akt and eNOS by Western blot. n=3. C, Phosphorylated or total Akt by Western blot. D, Migration assay in BAECs. Upper graph shows the number of cells per field and lower panels show the representative pictures ($\times$200 magnification). #$P<0.0001$ vs control no treatment. *$P<0.0001$ vs FHL-2 no treatment. n=3.
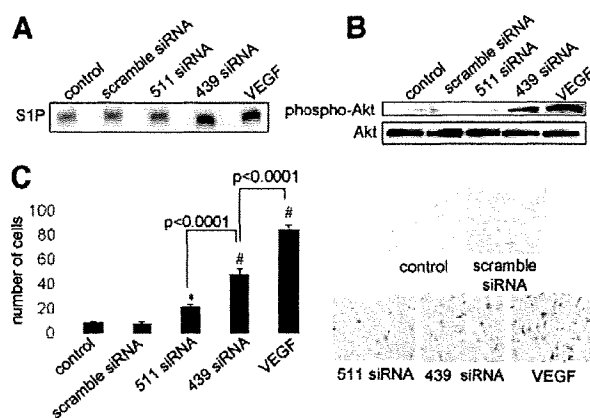

Figure 3. Knockdown of FHL-2 enhances SK1 activity, Akt, and migration in ECs. "control" indicates no transfection and no treatment. "scramble siRNA" indicates transfection with scramble siRNA. "511 siRNA" and "439 siRNA" indicate transfection with FHL-2 siRNA. "VEGF" indicates no transfection and treatment with VEGF 50 ng/mL. A, SK1 activity in BAECs by thin layer chromatography. B, Phosphorylated or total Akt at 48 hours after transfection of siRNA. C, Migration assay in BAECs. Left panel shows the number of cells per field and right panels show the representative pictures ($\times$200 magnification), #$P<0.0001$, *$P<0.005$ vs scramble siRNA, respectively. n=3.

essential for angiogenic processes. DMS or VPC23019 strongly suppressed VEGF-induced migration in HAECs accompanied with Akt phosphorylation (supplemental Figure IVC and IVD). Inversely, S1P-induced cell migration was not inhibited by treatment with DMS (supplemental Figure IVC).

Similarly, overexpression FHL-2 in BAECs inhibited VEGF (50 ng/mL)-induced Akt and eNOS phosphorylation (Figure 2B). Further analysis showed that VEGF-induced Akt phosphorylation was significantly inhibited by overexpressed FHL-2 from 10 minutes to 20 minutes in BAECs (10 minutes: 72% inhibition, 15 minutes: 71% inhibition, 20 minutes: 57% inhibition, compared to control, respectively, supplemental Figure IVE). Overexpressed FHL-2 also attenuated VEGF-induced phosphatidylinositol 3-kinase activity associated with p85 and p110 (supplemental Figure VA). However, S1P-induced Akt phosphorylation was not suppressed by overexpression of FHL-2 in BAECs (Figure 2C). Similarly, VEGF or S1P induced the S1P receptors activity assessed by [^{35}S] GTPγS binding assay, which measures GDP-GTP exchange on the Gαi subunit by coupling of S1P receptors and G proteins. Overexpressed FHL-2 suppressed VEGF-induced S1P receptors activation but not S1P-induced activation (supplemental Figure VB). Moreover, overexpressed FHL-2 strongly inhibited VEGF-induced migration, but not S1P-induced migration in BAECs (Figure 2D) and HAECs (supplemental Figure VC). Scratch wound assay also demonstrated that overexpressed FHL-2 markedly inhibited EC migration (Supplement Figure VD). These results suggested

that FHL-2 strongly suppressed VEGF-induced EC migration, accompanied with the inhibition of Akt and eNOS phosphorylation.

Depletion of FHL-2 Enhances SK1 Activity and EC Growth

To investigate endogenous function of FHL-2 in ECs, we designed 3 different siRNAs for FHL-2 knockdown in ECs. Transfection of FHL-2 439-siRNA strongly reduced FHL-2 expression and 511-siRNA mildly decreased it in ECs (supplemental Figure VIA). Knockdown of FHL-2 expression by transfection of 439-siRNA increased SK1 activity, to a similar level to VEGF treatment, however transfection of 511-siRNA did not (Figure 3A). Transfection of 439-siRNA, but not 511-siRNA, induced Akt phosphorylation (Figure 3B). Similarly, transfection of 439-siRNA, but not 511-siRNA, increased EC migration compared to control or scramble siRNA (Figure 3C), and also increased EC growth compared with control siRNA (119% increase, data not shown). These results indicated that endogenous FHL-2 might have antiangiogenic effect in ECs by regulation of SK1 activity.

Depletion of FHL-2 Enhances the Expression of VEGFR2

We also analyzed the effect of FHL-2 on VEGF receptor activation in VEGF signaling pathway. Overexpressed FHL-2 or pretreatment of SK1 inhibitor, DMS, did not affect the phosphorylation of VEGFR2 (VEGF receptor-2) induced by VEGF or S1P (Figure 4A and supplemental Figure VIB and VIC). However, depletion of FHL-2 enhanced the expression of VEGFR2, accompanied with upregulation of its phosphorylation (Figure 4B). Similarly, depletion of FHL-2 enhanced
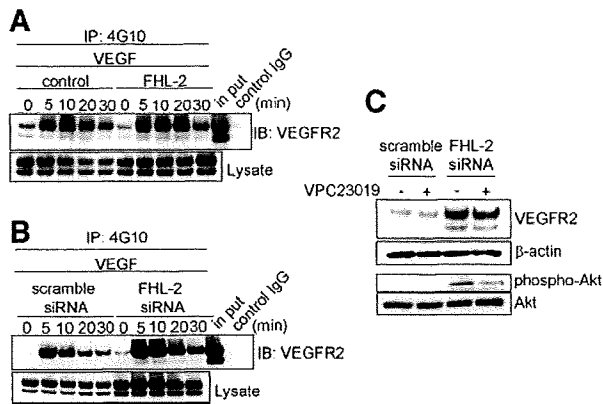
A

B

C

Figure 4. Deletion of FHL-2 enhanced VEGFR2 expression in BAECs. A and B, VEGFR2 activation by immunoprecipitation analysis with antiphoshotyrosine antibody (4G10) and anti-VEGFR2 antibody. C, Western blot of VEGFR2 and phosphorylated Akt with S1P receptors antagonist, VPC23019 (10 μmol/L). n=3. "IP:" indicates immunoprecipitation. "IB:" indicates immunoblot. "in put" indicates loaded cell lysate. "Lysate" indidates immunoblot with anti-VEGFR2 antibody of total protein. "control" indicates overexpressed GFP gene, "FHL-2" indicates overexpressed FHL-2 gene. "scramble siRNA" indicates transfection with scramble siRNA. "FHL-2 siRNA" indicates transfection with siRNA (439) against FHL-2. "VEGF" indicates treatment with VEGF 50 ng/mL. "S1P" indicates treatment with sphingosine-1-phosphate 10 μmol/L.

VEGF-induced phosphorylation of Akt as well as ERK (supplemental Figure VID). We further examined whether depletion of FHL-2 could increase VEGFR2 expression via SK1-S1P pathway. As shown in Figure 4C, depletion of FHL-2 increased the expression of VEGFR2, however cotreatment of the S1P receptors antagonist, VPC23019, did not attenuate it (Figure 4C), which suggests that the regulation of VEGFR2 expression by FHL-2 might be independent of S1P signaling. Importantly, depletion of FHL-2 also increased Akt phosphorylation, phosphatidylinositol 3-kinase activity associated with p85 and p110, S1P receptors activity, which were abolished by cotreatment of S1P receptors antagonist, VPC23019 (Figure 4C and supplemental Figure VIE and VIF). Thus, the activation of phosphatidylinositol 3-kinase/Akt pathway by depletion of FHL-2 may be dependent on SK1-S1P pathway.

FHL-2 Inhibits Vascular Formation in Xenopus Embryos

The SK1-S1P pathway has been implicated in vasculogenesis and vascular maturation.[20–23] FHL-2 mRNA was injected into Xenopus embryos to examine the effect in the development of vascular formation. Expression of FHL-2 and SK1 in Xenopus embryo was verified using RT-PCR analysis (supplemental Figure VII).

To evaluate vascular formation in Xenopus embryos, we examined the expression patterns of endothelial markers, fli-1 and msr. Fli is a member of the ETS-family and shown to be expressed in cranial neural crest cells, angioblasts and endothelial cells of the forming blood vessels.[24] Msr (Mesenchyme-associated serpentine receptor) is a member of the G protein-coupled receptor in Xenopus and homologue to

Table. The Effect of FHL-2 Overexpression on Vascular Formation in Xenopus Embryos

Probe	mRNA	No. of Embryos With AD	No. of Embryos Analyzed	%*
Xfli-1	None	7	77	9
	GFP	5	57	8
	FHL-2	42	70	60
Xmsr	None	9	69	13
	GFP	7	72	9
	FHL-2	32	49	65

AD indicates angiogenic defect. *No. of embryos with AD/No. of embryos analyzed ×100. Xfli-1 indicates Xenopus fli-1. Xmsr indicates Xenopus mesenchyme-associated serpentine receptor; None, no treatment with embryos; GFP, injection of GFP mRNA into embryos; FHL-2, injection of FHL-2 mRNA into embryos. Embryos were analyzed by in situ hybridization with probes for Xenopus endothelial markers.

the human APJ receptor.[25] This gene also traces an endothelial lineage and represents a very early and unique marker in Xenopus of the specification of vascular endothelia.[26] Whole mount in situ hybridization showed a reduced staining for these endothelial markers in stage 32 to 33 embryo injected with FHL-2 mRNA. We quantified this antiangiogenic effect in Xenopus based on the previous report.[27] As shown in the Table, we counted the numbers of embryos showing a reduction in the vitelline vein network (Figure 5, circle) or intersomitic veins (Figure 5, arrowhead), and demonstrated the increased embryos with angiogenic defects by injection of FHL-2 mRNA. These results suggest that injection of FHL-2 resulted in the inhibition of vasculogenesis.

Discussion

The present study demonstrated that FHL-2 inhibited SK1 activity in vascular ECs and that the interaction of FHL-2 with SK1, which was regulated by VEGF, played an important role in the VEGF-induced signaling.

S1P, a bioactive lipid mediator produced by activated-SK1, exerts a variety of actions in many types of cells, including vascular ECs.[28] For example, S1P stimulates proliferation,[28]
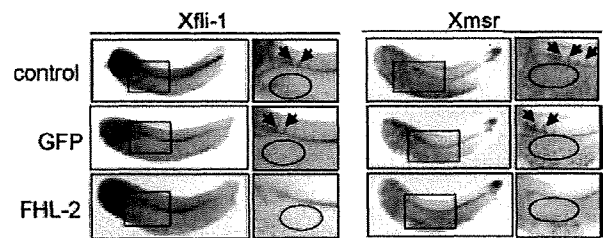

Figure 5. FHL-2 suppresses vascular formation in Xenopus embryos. Whole mount in situ hybridization detection of vascular formation using Xfli-1 and Xmsr probes at stage 32 to 33. "control" indicates no microinjection. "GFP" indicates microinjection of GFP mRNA. "FHL-2" indicates microinjection of FHL-2 mRNA. Left panels indicate whole image of embryo. Right panels indicate strong magnification of squared area of left panel. The staining is reduced in vitelline vein network (circle) or intersomitic veins (arrow head) by microinjection of FHL-2 mRNA.

survival,[29] migration,[29] NO synthesis,[30–32] and angiogenesis[33] in ECs. S1P also promoted EC barrier integrity via S1P receptors ligation and G_α-linked Rho- and Rac GTPase–dependent cytoskeletal rearrangements.[34] Importantly, SK1-S1P signaling during embryonic development is also critical for angiogenesis. Disruption of sphingosine kinase genes in mice resulted in a deficiency of S1P, which severely disturbed angiogenesis, followed by embryonic lethality.[22] Liu et al suggested that S1P receptor is essential for vascular maturation, and that embryonic vascular maturation was incomplete because of a deficiency of vascular SMCs/pericytes.[23] Based on these data, the investigators suggested that vessel coverage by vascular SMCs is directed by the activity of the S1P receptor in ECs. Similarly, our results in in situ hybridization after injection of FHL-2 mRNA into Xenopus embryo showed that FHL-2 significantly inhibited vascular network formation, possibly via inhibition of the SK1-S1P pathway. Until now, 2 subtypes of SK have been identified, SK1 and SK2.[35,36] SK1 upregulates cell survival and prevents cell form apoptosis,[37,38] and SK1 can be the dominant isoform in various organs.[39] Indeed, SK1 is activated by various growth factors, cytokines, antigens, and G-coupled receptor agonists, leading to increase the intracellular S1P, whereas physiological function of SK2 is still unclear.[40–42] On the contrary to SK1, it is reported that function of SK2 inhibits cell growth and promotes apoptosis in vitro.[43,44] SK may have a role of an endogenous modulator that can regulate the fate of cells to either undergo apoptosis or survival.

Unexpectedly, depletion of FHL-2 significantly increased VEGFR2 expression that was not blocked by S1P receptor antagonist. It suggests that this action may not be mediated by extracellular action of S1P on cell-surface S1P receptor. However, it could not still rule out the possibility of intracellular second-messenger functions of S1P independently of S1P receptors,[45] although the direct intracellular target of S1P has not been identified. In addition, FHL-2 is a multifunctional protein with numerous binding targets including receptors and transcriptional factors, which may possibly regulate the transcription of VEGFR2. Furthermore, overexpressed FHL-2 did not affect the VEGFR2 expression, which might suggest the complexity of this regulatory mechanism. Although the mechanism is still unknown in detail, our results demonstrated that FHL-2 might regulate VEGF-induced phosphatidylinositol 3-kinase/Akt activity via SK1-S1P pathway and VEGFR2 expression. In our present study, we identified the expression and function of FHL-2 in ECs. However, it seemed that FHL-2 is expressed not only in ECs but also in SMCs in aorta. Although the function of FHL-2 in SMCs is not fully understood, it has been reported that FHL-2 might contribute the phenotypic switching of SMCs.[46] FHL-2 was upregulated in an SRF-dependent manner during ES cell differentiation and in response to RhoA activation.[9] Importantly, FHL-2 was shown to inhibit serum response factor (SRF)-dependent transcription of SMC-specific genes by competing with myocardin-related transcriptional factors (MRTFs)-A for SRF binding in 293T cells.[9] SRF as well as MRTFs are recognized as liaisons connecting the key pathways of arteriogenesis, ie, Rho pathway with

downstream genes.[47] Therefore, we speculate that FHL-2 in vascular SMCs might contribute the process of atherosclerosis or angiogenesis through suppressing phenotypic switching.

In conclusion, FHL-2 is expressed in vascular ECs, where it may exert VEGF-induced phosphatidylinositol 3-kinase/Akt through inhibition of SK1.

Disclosures

None.

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Molecular therapies for heritable blistering diseases

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Tremendous progress has been made over the past two decades in understanding the molecular genetics of heritable skin diseases. The paradigm for such conditions is epidermolysis bullosa (EB), which comprises a group of heritable blistering disorders caused by mutations in ten genes expressed in the cutaneous basement membrane zone and has high morbidity and mortality. Identification of distinct mutations has improved the diagnosis and subclassification of EB, leading to improvements in disease prognosis, and has provided a basis for prenatal and pre-implantation genetic diagnosis for this disorder. Nevertheless, there is no cure or effective treatment for EB. Here, we review recent exciting developments in the areas of molecular therapies, including gene therapy, protein replacement therapy and bone-marrow-derived stem cell transfer, as potential new avenues to treat EB and other currently intractable heritable skin diseases.

The phenotypic spectrum of heritable skin diseases

Heritable skin diseases comprise a group of disorders with a wide spectrum of phenotypic manifestations [1]. At one end of the spectrum, the skin manifestation can be minor, manifesting, for example, with pigmentary changes, whereas at the other end of the spectrum the cutaneous manifestations can be extremely severe, causing considerable morbidity and mortality. Examples of the latter situation include the most severe forms of epidermolysis bullosa (EB), which manifest with extreme fragility of the skin, often resulting in the early demise of the affected individual within a few days or weeks of birth [2,3]. Considerable progress in understanding the molecular genetics of many of these conditions has been made over the past two decades, with diagnostic and prognostic implications (Box 1).

The cutaneous findings in EB can be associated with extracutaneous manifestations encountered in different subtypes. For example, the patients can have corneal blistering, dental abnormalities (including enamel dysplasia), fragility of the tracheal epithelium, gastrointestinal and urogenital-tract abnormalities, and progressive, late-onset muscular dystrophy. This phenotypic spectrum has led to complex classification schemes riddled with eponyms and there are suggestions that as many as 30 different subtypes of EB exist [4]. Traditionally, however, EB has been divided into three broad categories based on the location of blistering

within the cutaneous basement membrane zone (BMZ; see Glossary) [2] (Table 1) and demonstration of abnormalities in the critical attachment complexes: (i) in the simplex forms (EBS), tissue separation occurs within the basal keratinocytes of the epidermis, the outer layer of the skin; (ii) in the classic junctional forms (JEB), tissue separation occurs within the cutaneous basement membrane that separates epidermis from the underlying dermis; (iii) the dystrophic forms of EB (DEB) depict tissue separation below the dermo-epidermal basement membrane within the upper papillary dermis. In addition, the most recent consensus conference on classification of EB [2] has proposed that additional, rare forms of blistering diseases should be considered to be part of the EB spectrum – these include lethal acantholytic EB [5], plakophilin-deficient skin fragility-ectodermal dysplasia syndrome [6–8] (with suprabasal location of cleavage) and Kindler syndrome [9,10] (with mixed location of blistering).

Here, we focus on the classic forms of EB by discussing the genetic basis of different variants of this disease, summarizing the translational implications of the molecular genetics and, finally, highlighting the progress in molecular therapies for this group of currently intractable blistering disorders.

Molecular genetics of EB

The classic forms of EB simplex are inherited in most cases in an autosomal dominant manner owing to dominant-negative mutations in the keratin 5 *(KRT5)* and keratin

Glossary

Attachment complexes at the cutaneous BMZ: ultrastructurally recognizable structures in the skin, crucial for stable association of the epidermis to the underlying dermis; these include: (i) hemidesmosomes (i.e. protein complexes extending from the intracellular milieu of keratinocytes to the lamina lucida and consisting of $\alpha6\beta4$ integrin, type XVII collagen and plectin); (ii) anchoring filaments, thread-like structures consisting primarily of laminin 332 and traversing the lamina lucida; and (iii) anchoring fibrils, attachment structures composed of type VII collagen and extending from the lower part of the lamina lucida to the upper papillary dermis. These attachment complexes form a continuum of the network required for physiologic stability of the cutaneous BMZ.
Cutaneous basement membrane zone (BMZ): the interface of the two principal layers of the skin, the epidermis (the outer layer) and the dermis (the underlying inner layer), separated by a dermal-epidermal basement membrane consisting of lamina lucida (upper layer) and lamina densa (lower layer).
Epidermolysis bullosa (EB): a heterogeneous group of heritable disorders (Box 1), characterized by separation of the epidermis and the dermis at the cutaneous BMZ upon trauma of varying degrees. Mutations in the genes encoding components of the attachment complexes can result in weakness of the BMZ and manifest as blisters and erosions, which are characteristic of EB [2,3].

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Box 1. Examples of recent advances in molecular genetics of heritable skin diseases

Epidermolysis Bullosa (EB)

- A group of heritable blistering disorders with considerable phenotypic variability, with an overall incidence of 1 in 20 000.
- Over ten distinct genes expressed in the cutaneous basement membrane zone harbor mutations underlying different subtypes [2,3] (Table 1).

Ichthyosis

- A heterogeneous group of scaling disorders with highly variable prognosis. The most common variant, ichthyosis vulgaris, with life-long scaling and dryness of the skin, affects 1 in 250 individuals, whereas harlequin ichthyosis, which is frequently lethal perinatally, has an incidence of 1 in 10^6.
- Ichthyosis vulgaris has semi-dominant inheritance owing to mutations in the filaggrin gene; harlequin ichthyosis with autosomal recessive inheritance is caused by mutations in the ABC transporter gene *ABCA12*; the classic autosomal recessive lamellar ichthyosis is caused by mutations in the transglutaminase 1 gene (*TMG1*), although at least five additional gene loci have been identified; the X-linked variant is caused by steroid sulfatase gene (*STS1*) deficiency [41–45].

Keratinization disorders

- A group of disorders manifesting characteristically with palmo-plantar hyperkeratosis, variably associated with hair, nail and tooth abnormalities, owing to mutations in genes encoding desmosomal cell–cell adhesion molecules.
- Mutations in the desmoplakin (*DSP*) and desmoglein 1 (*DSG1*) genes can result in cardiac manifestations, in addition to cutaneous abnormalities [46,47].

Cutaneous mineralization disorders

- The prototypic diseases, pseudoxanthoma elasticum (PXE) and familial tumoral calcinosis (FTC) are characterized by mineral deposits in the dermis, in addition to the retina and arterial blood vessels in PXE and in subcutaneous periarticular tissues in FTC.
- PXE is caused by mutations in the *ABCC6* gene expressed primarily in the liver and is considered to be a metabolic disorder [43,48].
- Two forms of FTC exist: (i) the hyperphosphatemic variants show elevated serum phosphate levels and are due to defects in the genes regulating renal reabsorption of phosphate (*GALNT3, FGF23, KLOTHO*); and (ii) the normophosphatemic variant is caused by mutations in the *SAMD9* gene, a TNF-α responsive gene of unknown function [49,50].

14 *(KRT14)* genes expressed by keratinocytes exclusively in the basal layer of the epidermis. The junctional forms are inherited in an autosomal recessive fashion and mutations in several genes encoding the components of hemidesmosomes and anchoring filaments, crucial attachment complexes at the dermal–epidermal junction, have been disclosed. Most commonly, mutations in junctional forms of EB reside in the *LAMA3*, *LAMB3* and *LAMC2* genes, which encode the subunit polypeptides of the secreted extracellular glycoprotein laminin 332 [11]. The dystrophic, severely scarring forms of EB arise owing to mutations in the *COL7A1* gene, which encodes type VII collagen, the major, if not the exclusive, component of anchoring fibrils [12]. Type VII collagen is synthesized by both epidermal keratinocytes and dermal fibroblasts.

In general, the severity of skin fragility is dependent on the types and combinations of mutations and their consequences at the mRNA and protein levels [3]. For example, complete absence of laminin 332 as a result of null mutations in any of the three subunit polypeptide genes mentioned earlier results in the most severe phenotype (Herlitz junctional EB), with profound fragility of the skin, leading to demise of the affected individuals usually

within the first year of life. In addition, mutations in the integrin α6β4 subunit genes (*ITGA6* and *ITGB4*) are frequently associated with congenital pyloric atresia, which necessitates perinatal surgery, whereas mutations in the plectin gene *(PLEC1)* can result both in neonatal skin fragility and late-onset muscular dystrophy. In the dystrophic forms of EB, the diagnostic hallmark is an abnormality in the anchoring fibrils, which can be morphologically altered, reduced in number or entirely absent [12]. Because the anchoring fibrils are crucial for the stable association of the dermo–epidermal basement membrane to the underlying dermis, the severity of DEB frequently reflects the degree of abnormalities in anchoring fibrils so that, for example, the absence of type VII collagen manifests with extreme fragility of the skin. This phenotype presents with accompanying scarring, analogous to a third-degree burn, in the recessively inherited form of dystrophic EB (known as generalized RDEB).

Translational implications of the molecular genetics of EB

Distinct mutations in ten different genes underlying the classic forms of EB have been identified in well over 1000

Table 1. Clinical and genetic heterogeneity of EB[a]

EB subtype[b]	Inheritance	Location of blisters	Mutated genes	Altered or missing proteins
Simplex				
• Classic	AD (AR)	Basal layer of epidermis	KRT5, KRT14	Basal keratins
• EB-MD	AR	Basal layer of epidermis	PLEC1	Plectin
Junctional				
• Classic	AR	LL	LAMA3, LAMB3, LAMC2, COL17a1	Laminin 332, type XVII collagen
• EB-PA	AR	Basal layer–LL interface	ITGA6, ITGB4, PLEC1	α6β4 integrin, plectin
Dystrophic				
• Generalized, localized	AD, AR	Sub-lamina densa	COL7A1	Type VII collagen

[a]This classification highlights the most common subtypes of EB (i.e. simplex, junctional and dystrophic), which are differentiated by the location of blister formation within the cutaneous BMZ, as determined by ultrastructural analysis and/or immuno-epitope mapping. Additional extremely rare phenotypes with superficial or mixed locations of blistering have been proposed to belong to the spectrum of EB phenotypes (see main text) [2].
[b]Abbreviations: AD, autosomal dominant; AR, autosomal recessive; EB-MD, epidermolysis bullosa with muscular dystrophy; EB-PA, epidermolysis bullosa with pyloric atresia; LL, lamina lucida.

families [11,12] (Table 1) and comparison of the mutation database with the phenotypic manifestations has enabled the establishment of general genotype–phenotype correlations. Traditionally, EB was divided into three broad categories on the basis of the location of blisters in the skin, as determined by electron microscopic examination or epitope mapping of skin biopsies from the affected individuals [2]. Molecular genetics on EB have now improved the accuracy of diagnosis and subclassification, delivering prognostic improvements [3]. Furthermore, identification of mutations has enabled development of DNA-based prenatal testing for families at risk for recurrence of EB [13,14]. Such testing can be performed from chorionic villus sampling (CVS) as early as the tenth week of gestation, thus providing the parents and healthcare provider with information on the fetal genotype during the first trimester of pregnancy. An extension of DNA-based prenatal testing is pre-implantation genetic diagnosis, which has been established for EB and related blistering disorders [15,16]. Furthermore, an application currently under development for DNA-based prenatal diagnosis entails the examination of fetal cells or free fetal DNA in maternal blood for pathogenic mutations [17]. The benefits of this form of non-invasive prenatal diagnosis include avoidance of complications of invasive procedures, such as the small but clearly increased risk of fetal loss in CVS. Furthermore, an analysis of fetal DNA in the maternal circulation could provide information on the fetal genome as early as the fifth week of gestation [18]. In spite of the impressive progress in the molecular diagnostics of EB over the past two decades, the fact remains that there is no specific or effective treatment currently available for this group of blistering disorders. However, as we show here, recent exciting developments of molecular therapies, including gene therapy, protein replacement therapy and cell-based

therapies, suggest that the era of treatment of EB and other heritable skin disorders is fast approaching.

Prospects for molecular therapies for heritable skin diseases

As indicated earlier, specific molecular defects have been identified in ten distinct genes in the classic variants of EB. In the recessive forms, the majority of the mutations are premature termination-codon-causing mutations resulting in truncation of the newly synthesized proteins. In the dominantly inherited forms of EB, the majority of the mutations are dominant-negative ones, resulting in synthesis of mutated polypeptides that interfere with their wild-type counterparts during the assembly of proteins crucial for stability of the dermal–epidermal junction.

The feasibility of molecular therapies has recently been addressed using preclinical animal models of human EB [19,20]. Specifically, mouse models recapitulating the clinical, genetic, histopathological and ultrastructural features of the most severe junctional and dystrophic forms of EB have been engineered through targeted ablation of laminin 332 genes (*Lama3, Lamc2*) or the type VII collagen gene (*Col7a1*). Several spontaneous mutant animal models for recessively inherited EB have also been identified, including the *Lamb3* mutant mouse with severe cutaneous blistering. In addition, animal models for the dominantly inherited EB simplex have been developed through targeted substitution of crucial amino acid residues in the *keratin 5* gene. Thus, many of these mutant animals serve as excellent model systems to study therapeutic approaches under development for EB.

Gene therapy
Several general strategies have been considered for gene therapy for EB. One of them uses keratinocytes, or isolated

(a) IN VIVO STRATEGY

(b) EX VIVO STRATEGY

(i) Culture keratinocytes from affected patient

(v) Autograft on donor

(iv) Detach as epithelial sheet

(ii) Propagate and transduce cells in culture

(iii) Grow a skin equivalent
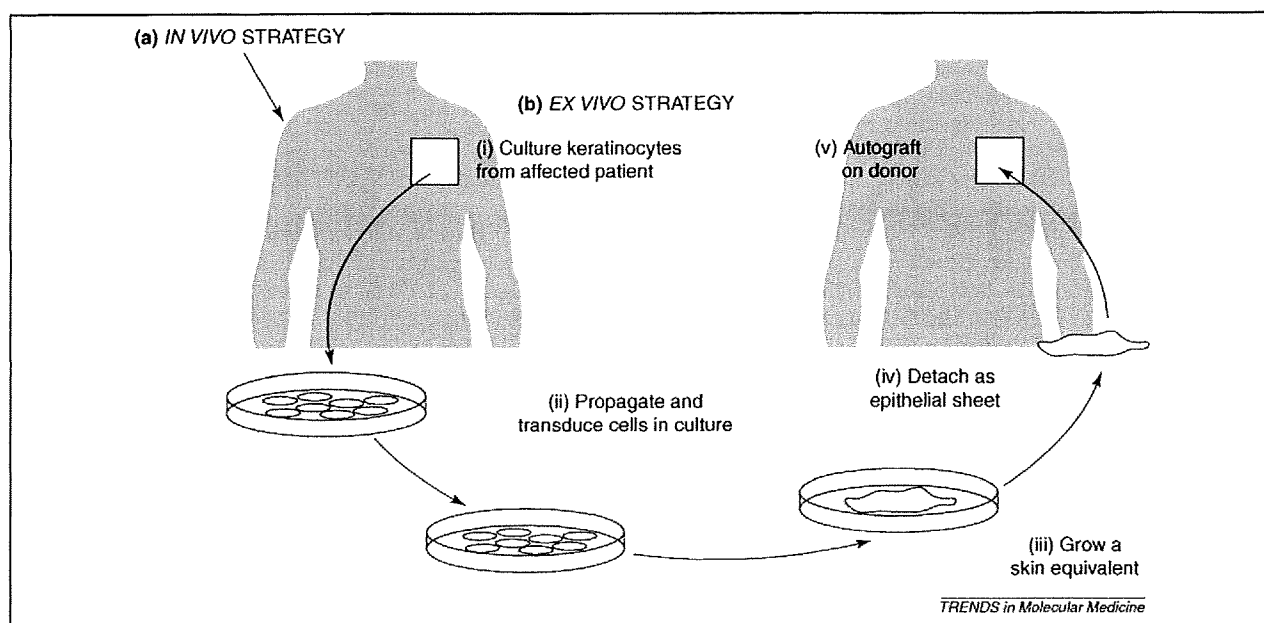
TRENDS in Molecular Medicine

Figure 1. Principles of two primary strategies for cutaneous gene delivery. (a) As an *in vivo* strategy, the genes can be directly delivered into the skin by topical application, intracutaneous injections or by biolistic particle bombardment ('gene gun'). (b) As an *ex vivo* strategy, target cells such as keratinocytes are removed from the skin and propagated in culture. The cells are then transduced with the vector expressing the transgene and the transgenic cells are selected and grown into epithelial sheets that can then be grafted back to the original donor.

subpopulations with stem cell characteristics, in cultures established from the patient's skin, and these cells are then transduced with a cDNA expression construct in a viral vector. Such gene-corrected keratinocytes are grown into epithelial sheets that are grafted back to the donor skin to the site prepared by removal of the keratinocytes *in situ* harboring the mutations (Figure 1) [21]. The feasibility of this approach has already been demonstrated in an Italian study on a patient with relatively mild junctional EB treated with keratinocyte grafts expressing the wild-type *LAMB3* cDNA [22]. A follow-up examination at five years after the grafting has revealed sustained phenotypic reversal and persistence of the skin graft and continued expression of laminin 332 protein (M. DeLuca, personal communication). Furthermore, there is no evidence of immune challenge to the graft and there are no circulating antibodies to the β3 chain of laminin 332. It should be noted that, in this particular case, one of the underlying mutations (E210K) in the *LAMB3* gene enables a low level of expression of the corresponding protein, and the patient's immune system does not recognize the newly synthesized polypeptide as a neoantigen [22]. Although this *ex vivo* keratinocyte gene therapy approach has been successful in treating a small area of skin (a total of ~ 500 cm^2) in one patient, this approach has several potential limitations. In particular, a general concern of the DNA-based, viral-vector-driven gene therapy relates to potential carcinogenesis owing to integration of the vector into the genome in a manner that might conceivably activate proto-oncogenes or inactivate tumor-suppressor genes. Nevertheless, application of such keratinocyte gene therapy to additional patients with junctional EB and for patients with the dystrophic subtypes is currently being contemplated in other medical centers and the general applicability of this approach might become evident soon.

Protein replacement therapy

The concept of protein therapeutics for EB envisages administration of recombinant protein to the skin by topical application, local injection or systemic administration to the circulation. The feasibility of this approach is suggested by recent observations in type VII collagen 'knockout' mice injected intradermally with purified human type VII collagen [23]. The untreated *Col7a1*$^{-/-}$ mice, which recapitulate the cardinal features of RDEB, usually die within the first week of life as a result of extreme fragility of the skin and mucous membranes owing to the lack of type VII collagen and anchoring fibrils [24]. Intradermal injections of recombinant human type VII collagen into these mice significantly prolonged their survival and some mice survived as long as 20–25 weeks (Figure 2a). Examination of the treated mice revealed that the injected collagen homed to the cutaneous basement membrane zone in the areas of blistering and resulted in formation of anchoring fibrils and amelioration of the blistering phenotype (Figure 2b). It is of interest that the mice injected with human type VII collagen developed antibodies recognizing this protein but the antibodies did not react with the mouse protein and were not pathogenic when injected into normal mice [23]. Although these observations do not exclude the possibility that treatment of
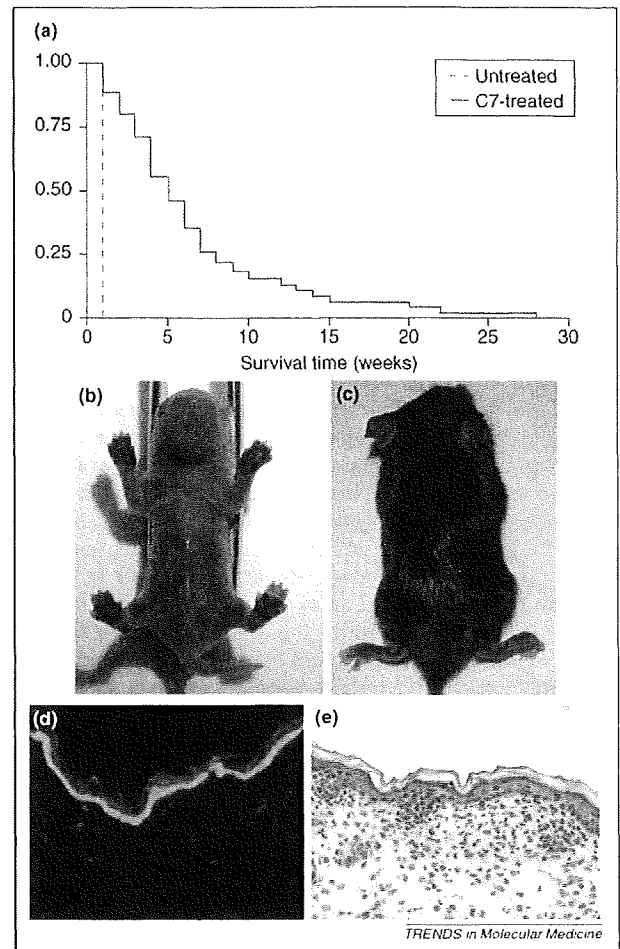

Figure 2. Extended survival of *Col7a1*$^{-/-}$ mice after injection with purified human type VII collagen. (a) Kaplan–Meier curves reflect the fact that untreated mice die within the first few days, whereas mice treated with recombinant protein survive up to a further 20–25 weeks. (b–e). A one-day-old *Col7a1*$^{-/-}$ mouse has hemorrhagic blisters on the paws (b); after injections with purified human type VII collagen (C7), the same mouse survived past 22 weeks (c). Immunofluorescence staining with antibody against type VII collagen demonstrates the presence of the injected protein in the skin at the cutaneous basement membrane zone (d) and a histopathological analysis of the skin reveals stable association of the epidermis and dermis (e). Figure adapted, with permission, from Ref. [23].

Col7a1$^{-/-}$ mice with the mouse type VII collagen – and, by inference, treatment of RDEB patients with human type VII collagen – would not develop pathogenic antibodies, the antibody formation, if shown to be a problem at all, could be blocked, as demonstrated in the mice by administration of an anti-CD40 ligand monoclonal antibody (MR1) that profoundly depresses antibody production [23]. The latter observation highlights the potential of manipulating the innate and adaptive immune systems in the recipient to reduce the immune responses to neoantigens introduced to the patients as part of the molecular therapies, whether in the form of viral vectors or missing gene products [25]. With such refinements, it is conceivable that protein replacement therapy might become an option for treatment of recessive DEB patients in the future and similar work is currently under way for the development of protein replacement strategies for patients with junctional forms of EB owing to absence of laminin 332 [26].

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Cell-based therapies

Recent development of molecular strategies for treatment of EB by cell-based approaches has focused primarily on two different cell types. First, DEB fibroblasts, engineered to overexpress human type VII collagen, have been shown to home to skin wounds and deliver type VII collagen when injected into mice, with subsequent promotion of wound healing [27]. Furthermore, DEB fibroblasts, devoid of type VII collagen expression, were more efficient in producing this protein and forming anchoring fibrils than the corresponding keratinocytes when transduced with type VII collagen cDNA in a retroviral delivery system upon implantation into nude rats [28]. Additional support for the postulate that fibroblast therapy could be feasible for treatment of DEB comes from observations in a hypomorphic mouse that expresses type VII collagen at ∼10% of normal levels [20]. These mice display a phenotype closely resembling human DEB, characterized by cutaneous blistering owing to reduced type VII collagen deposition and paucity of anchoring fibrils in the BMZ. Intradermal injections of fibroblasts cultured from wild-type mice to the hypomorphic mice resulted in local deposition of type VII collagen at the dermal-epidermal junction, accompanied with functional improvement of the blistering phenotype. The increase in type VII collagen deposition at three weeks after the introduction of the fibroblasts to the skin was ∼3.5 fold over the baseline (i.e. ∼25–30% of the wild-type level), suggesting that partial restoration of type VII collagen assembly to anchoring fibrils can be beneficial to patients with EB.

Fibroblast therapy has also been tested by direct injection of these cells to the blistering areas of the skin in patients affected by RDEB. Specifically, autologous or allogeneic fibroblasts were injected intradermally, which resulted in local expression of type VII collagen in the patients' skin and lessening of the tendency to blister [29]. As expected, the autologous cells did not cause major adverse effects and the allogeneic fibroblasts elicited minor inflammation, however, they did not seem to survive in the skin for more than two weeks. Nevertheless, the benefits of cell injections were sustained for at least three months [29]. The mechanism was determined to be sustained cytokine-mediated upregulation of the expression of the mutant type VII collagen gene product in the resident cells in those patients who possessed a residual level of synthetic activity from their mutant alleles, whereas little evidence of the synthesis of normal type VII collagen from the newly introduced cells was noted. Consequently, those patients demonstrating some baseline synthesis of partially functional type VII collagen might benefit from this approach, whereas patients completely lacking type VII collagen gene expression owing to null alleles might not [30]. An associated finding in this study indicated that there seemed to be an improvement in the healing of chronic wounds, a major complication of RDEB. Collectively, injection of cultured fibroblasts from unrelated donors might be useful in improving epidermal–dermal adhesion and in accelerating wound healing in a select subgroup of patients with RDEB.

The cell-based therapy for RDEB has more recently been extended to use bone marrow cell transfer, including stem cells, to the type VII collagen 'knockout' mouse model system. Bone-marrow-derived cells have long been regarded to have a crucial role in the homeostasis of skin, in part through delivery of a variety of inflammatory cells, which are constitutive at low levels in normal skin. More recently, however, it has become clear that the plasticity of bone marrow stem cells enables their differentiation into cell types responsible not only for skin maintenance but also for rebuilding skin structures after injury [31,32]. For example, bone marrow cells expressing green fluorescent protein (GFP) that were transplanted into non-GFP mice revealed trafficking and homing of bone-marrow-derived cells to both wounded and non-wounded skin [33]. Wounding of the skin also stimulated the engraftment of these cells into skin and facilitated their differentiation into cells, such as fibroblasts, aiding in regeneration of damaged tissues. These observations illustrate the potential of bone marrow to serve as a valuable source of stem cells for the skin.

Two recent studies used GFP-expressing mice as the source of bone marrow cells for transplantation of RDEB mice, thus enabling the investigators to trace the donor cells in the skin and other tissues. In one study, various isolated subpopulations of cells within the source bone marrow were tested in $Col7a1^{-/-}$ mice by injecting the mice at birth or within a few days of birth, and survival of the mice beyond three weeks was monitored as a robust sign of amelioration of the blistering phenotype [34]. A specific subpopulation of bone-marrow-derived cells, positive for signaling lymphocytic activation molecule (SLAM/SLAMF1) family receptor (CD150$^+$/CD48$^-$), extended the survival of some animals beyond three weeks. The surviving animals also showed evidence of engraftment of the GFP-positive donor cells in the skin, production of type VII collagen and healing of skin blisters.

Another study has demonstrated successful engraftment of GFP-positive bone marrow cells in the skin after embryonic bone marrow cell transfer [35] (Figure 3). These cells also showed evidence of differentiation towards fibroblastic phenotypes and expression profiles, including deposition of type VII collagen. The embryonic bone marrow cell transfer also ameliorated the severity of the dystrophic EB phenotype at birth and the treated mice had an extended survival of up to several weeks (Figure 3a). An intriguing observation in this study was that the mice subjected to embryonic bone marrow cell transfer became tolerant to GFP and subsequent grafting of GFP-expressing skin did not induce the production of antibodies against GFP [35]. This situation enabled survival of the graft in GFP–bone-marrow-treated mice, whereas the graft in control mice was rejected before six weeks (Figure 3b).

Collectively, these preclinical studies attest to the possibility that bone-marrow-derived cells can serve as a source of dermal cells, such as fibroblasts, for regeneration of damaged skin in heritable skin diseases.

Clinical perspective

The preclinical studies using $Col7a1^{-/-}$ mice as an animal model for EB constitute a 'proof-of-principle' in support of the possibility that allogeneic hematopoietic stem cell transplantation, either from bone marrow or from umbili-
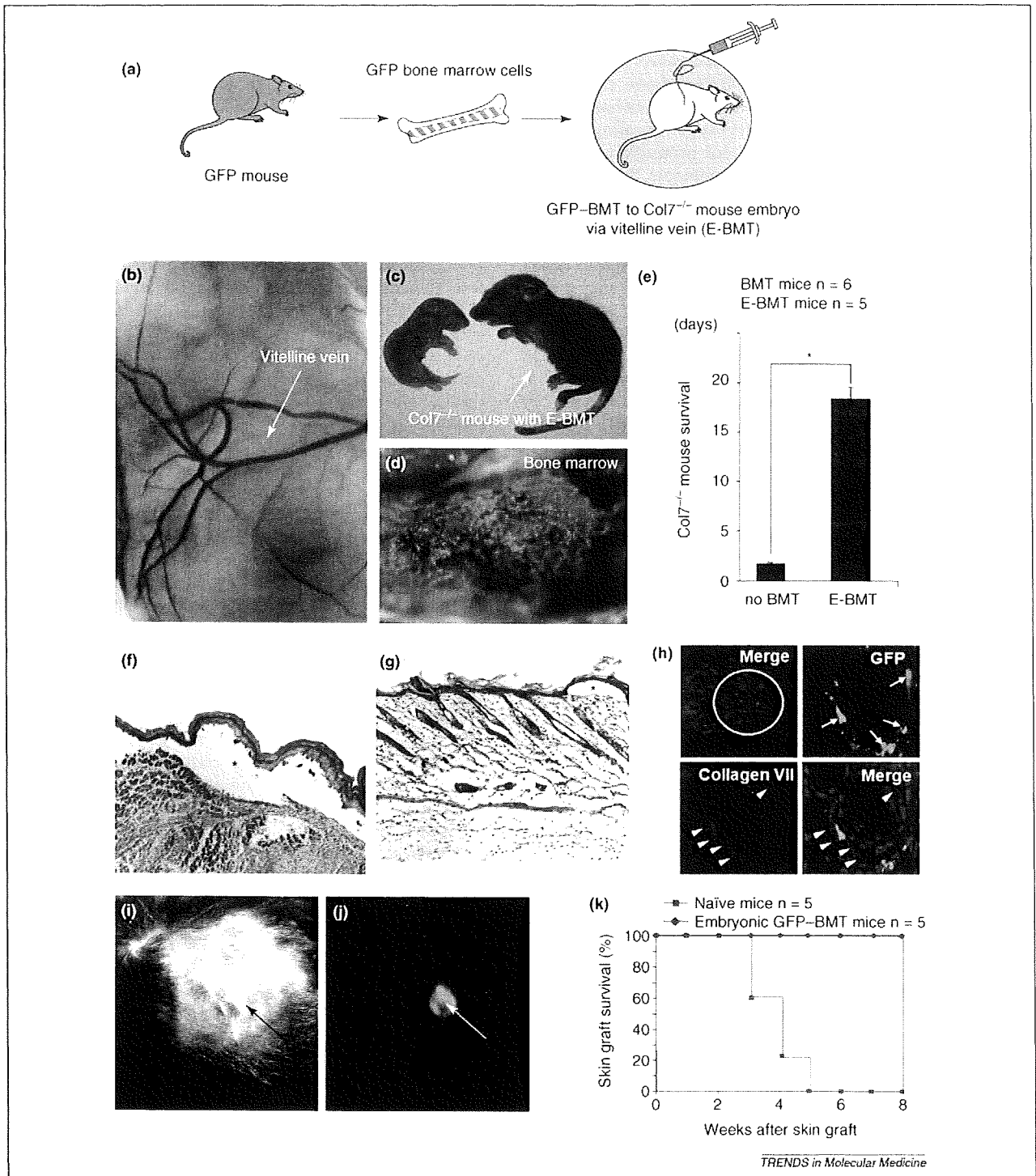
Figure 3. Embryonic bone-marrow transfer (E-BMT) into fetal circulation ameliorates recessive dystrophic epidermolysis bullosa (RDEB) by providing fibroblasts to the skin and by inducing tolerance. (a,b) Transgenic mice expressing GFP were used as donors of bone marrow cells that were injected into the fetus through the vitelline vein of the mother carrying $Col7a1^{-/-}$ pups. (c,e) The untreated $Col7a1^{-/-}$ mice died within the first few days postpartum, whereas those mice that received embryonic bone marrow transfer survived for up to three weeks. (d) The E-BMT-treated mice demonstrated bone marrow chimerism, as detected by GFP fluorescence. Examination of the skin revealed the presence of GFP-positive (green) fibroblastic cells that demonstrated expression of type VII collagen (red); these cells were frequently observed in close proximity to the hair follicles (h). Histopathology of the newborn $Col7a1^{-/-}$ mice demonstrated ready separation of epidermis from the underlying dermis (f), whereas the corresponding mice treated with E-BMT showed the presence of microblisters only (g) (asterisks indicate blisters). Induction of tolerance to GFP by E-BMT (i–k). Skin from GFP-positive transgenic mice was engrafted onto seven-week-old mice with or without prior treatment by embryonic GFP-bone marrow transfer; skin grafts were visualized either under normal (black arrow) (i) or under fluorescent (white arrow) (j) light. The grafts placed on naïve mice without treatment were rejected within five weeks of grafting, whereas grafts placed on mice treated previously with E-BMT persisted beyond eight weeks of grafting (k). Thus, tolerization of the embryonic mice by bone marrow transfer offers an avenue to prevent antibody formation after treatment at a later age. Figure adapted, with permission, from Ref. [35].

Box 2. Outstanding questions

- What are the most suitable preclinical models for testing of molecular therapies for epidermolysis bullosa? Do transgenic mice appropriately recapitulate the features of the human disease?
- What are the most beneficial approaches of molecular therapies for treatment of heritable blistering diseases? Which strategies have the lowest risk–benefit ratio in different forms of the disease?
- If there is a risk of carcinogenesis in gene therapy, owing to potential random integration of viral constructs, can close surveillance enable timely removal of the skin tissue undergoing malignant transformation?
- Does the treatment of patients with RDEB with human recombinant type VII collagen result in formation of antibodies against type VII collagen and, if so, are these antibodies pathogenic?
- Can the immunosuppressive preparation of patients serving as recipients of bone marrow transfer be modified to lessen the morbidity and avoid the mortality associated with standard bone marrow transfer procedures?
- What is the potential role and feasibility of embryonic bone marrow transfer in human pregnancies carrying an affected fetus?
- What are the immune barriers to successful introduction of viral vectors, transgenes and autologous cells to the patients' skin? Can manipulation of innate and adaptive immune systems prevent or dampen the immune response?
- What are the efficiencies of exogenously introduced proteins for assembling into the supramolecular organizations of the skin, and what is the half-life of such components in the skin?
- What is the capacity of good manufacturing practice (GMP) production to generate type VII collagen or other proteins for lifelong treatment of affected individuals?
- Are the approaches contemplated so far for treatment of autosomal recessive diseases also applicable to autosomal dominant variants of EB? In particular, will strategies such as silencing of the mutant allele by siRNA, RNA transplicing or antisense oligomer technology counteract diseases owing to dominant-negative mutations?
- What are the best approaches for the treatment of extracutaneous manifestations frequently associated with cutaneous findings encountered in patients with epidermolysis bullosa?
- What are the costs of the different strategies for molecular therapies?

cal cord blood, could be an option for treatment of human RDEB. Indeed, in 2007, a one-year-old male with severe RDEB was infused with cells derived from the bone marrow of an HLA-matched older sibling donor, after standard myeloablative preparation [36]. Subsequent examination of the skin by immunofluorescence and electron microscopy has documented sustained expression of type VII collagen and assembly of anchoring fibrils, with gradual decrease in blister formation [36]. Three additional patients have entered similar trials and the overall outcome of these interventions is still pending. However, one of the patients died of cardiomyopathy during the myeloablative conditioning before bone marrow infusion and another patient died from complications after mismatched umbilical cord blood transfer [36]. Thus, optimistically, these early observations suggest that transfer of bone marrow stem cell populations could provide a means to correct the basement membrane defect in patients with RDEB and perhaps in other genetic skin diseases characterized by compromised integrity of the skin. At the same time, it should be noted that intense myeloablative conditioning routinely used for preparation of recipients for bone marrow transplant is associated with considerable morbidity and even mortality owing to susceptibility to infections, cytokine storm and

graft-versus-host disease. For these reasons, strategies for reduced-intensity conditioning, which can have less complications during the conditioning phase, and non-myeloablative allogeneic stem cell transplantation have been developed both for malignant and non-malignant diseases [37]. In non-malignant conditions, such as RDEB, this strategy could well provide enough immunosuppression to promote engraftment of the stem cells and permit correction of the underlying genetic defect. This kind of reduced-intensity conditioning can be combined with the use of umbilical cord blood as the source of pluripotent stem cells with capacity to differentiate into different lineages, including cutaneous cells [38]. In fact, such reduced intensity conditioning strategies are being contemplated for the treatment of patients with different variants of EB [39]. It is clear, however, that these strategies need to be refined in experimental settings in specialized centers before these modalities can be recommended for the treatment of patient populations at large (Box 2).

As discussed here, several complementary avenues are currently being pursued towards treatment of patients with EB, including gene therapy, protein replacement and cell therapy approaches. It is clear that the information emanating from these studies will be helpful to ongoing efforts to find a cure for other heritable and acquired skin diseases as well. Furthermore, crucial insight into the mechanisms of pathology can be obtained from genetic observations, as for example with revertant mosaicism, a phenomenon noted particularly in the junctional forms of EB [40]. Nevertheless, although most of these studies are still at the preclinical level, some of them, such as bone-marrow-derived stem cell therapy, have already entered the clinical arena. Which of these approaches, if any, will be successful in providing amelioration and perhaps a cure for EB and other heritable skin disorders in the future remains to be proven by carefully controlled clinical trials.

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