HLF/HIF-2 α is a key factor in retinopathy of prematurity in association with erythropoietin

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An HLF (HIF-1 α -like factor)/HIF-2 α -knockout mouse is embryonic lethal, preventing investigation of HLF function in adult mice. To investigate the role of HLF in adult pathological angiogenesis, we generated HLF-knockdown (*HLF^{kd/kd}*) mice by inserting a neomycin gene sandwiched between two loxP sequences into exon 1 of the *HLF* gene. *HLF^{kd/kd}* mice expressing 80–20% reduction, depending on the tissue, in wild-type HLF mRNA were fertile and apparently normal. Hyperoxia–normoxia treatment, used as a murine model of retinopathy of prematurity (ROP), induced neovascularization in wild-type mice, but not in *HLF^{kd/kd}* mice, whereas prolonged normoxia following hyperoxic treatment caused degeneration of retinal neural layers in *HLF^{kd/kd}* mice due to poor vascularization. Cre-mediated removal of the inserted gene recovered normal HLF expression and retinal neovascularization in *HLF^{kd/kd}* mice. Expression levels of various angiogenic factors revealed that only erythropoietin (Epo) gene expression was significantly affected, in parallel with HLF expression. Together with the results from intraperitoneal injection of Epo into *HLF^{kd/kd}* mouse, this suggests that Epo is one of the target genes of HLF responsible for experimental ROP.

Keywords: erythropoietin/HLF/neovascularization/retinopathy/VEGF

Introduction

Retinal neovascularization is the most common cause of retinopathy of prematurity (ROP), diabetic retinopathy and age-related macular degeneration, which eventually leads

to visual loss (Prost, 1988; Moss *et al.*, 1994). Numerous clinical and experimental observations have indicated that ischemia or hypoxia triggers retinal neovascularization through an excessive production of one or more angiogenic factors. Identification of these factors is an important step toward understanding the mechanism of pathological angiogenesis and development of specific treatments for diseases involving angiogenesis, such as proliferative retinopathy, tumor growth and atherosclerosis. Among multiple factors known to be responsible for retinal neovascularization, many groups have reported vascular endothelial growth factor (VEGF) to be important (Aiello *et al.*, 1995; Alon *et al.*, 1995; Pierce *et al.*, 1995; Seo *et al.*, 1999). These groups have shown that VEGF is upregulated in a mouse model of retinal neovascularization (Pierce *et al.*, 1995) and that overexpression of VEGF in transgenic mice stimulates retinal neovascularization (Okamoto *et al.*, 1997; Tobe *et al.*, 1998). They have also shown that VEGF antagonists or kinase inhibitors of the VEGF receptor prevent retinal neovascularization (Aiello *et al.*, 1995; Seo *et al.*, 1999).

The transcription factors HIF-1 α (hypoxia-inducible factor-1 α) (Wang *et al.*, 1995) and HLF (Ema *et al.*, 1997) [HIF-1 α -like factor, also known as EPAS1 (Tian *et al.*, 1997) and HIF-2 α (Wenger and Gassmann, 1997)] play important roles in embryonic vascularization, and HIF-1 α and HLF also activate the expression of genes such as VEGF (Liu *et al.*, 1995; Forsythe *et al.*, 1996), erythropoietin (Epo) (Wang and Semenza, 1993) and a series of glycolytic enzymes (Firth *et al.*, 1994) in response to ischemic or hypoxic conditions. The two factors are substantially similar in their primary structures and belong to a growing superfamily of transcription factors characterized by structural motifs such as basic helix–loop–helix and PAS (a conserved sequence among Per, Arnt and Sim) domains. Under normoxic conditions, HIF-1 α is negatively regulated by ubiquitylation and proteasomal degradation (Kallio *et al.*, 1999). Prolyl hydroxylation is required for the interaction between HIF-1 α and von Hippel-Lindau (VHL) protein, which plays a critical role in the ubiquitylation of HIF-1 α (Epstein *et al.*, 2001). When the oxygen concentration is reduced, these transcription factors are stabilized and form a heterodimer with another bHLH/PAS protein, Arnt. The heterodimer activates genes encoding angiogenic and hematopoietic factors by binding to the hypoxia responsive element (HRE) in their promoter (Semenza and Wang, 1992; Wang *et al.*, 1995; Ema *et al.*, 1997; Wenger and Gassmann, 1997). However, the modes of expression of HLF and HIF-1 α differ substantially in various tissues of adult mice and during developmental processes (Ema *et al.*, 1997; Jain *et al.*, 1998), indicating that they have their own specific physiological functions *in vivo*.

Gene targeting technology has been utilized to investigate the function of HLF and HIF-1 α , and has revealed that their complete deficiency results in developmental arrest and embryonic lethality. Histopathological analyses of homozygotic mutant embryos showed that HLF deficiency either causes severe vascular defects in both the yolk sac and embryo proper (Peng *et al.*, 2000) or displays pronounced bradycardia due to defective catecholamine production (Tian *et al.*, 1998). In contrast, HIF-1 α -deficient mice manifested neural tube defects and cardiovascular malformations (Iyer *et al.*, 1998; Ryan *et al.*, 1998). Although the functions of these two transcription factors appear distinct, embryonic lethality prevented a detailed analysis of their contribution to angiogenesis in adult animals.

In this study, we generated *HLF* knockdown mice by inserting a neomycin resistance gene, sandwiched between two loxP sequences, into exon 1 of the *HLF* gene that encodes the untranslated region (5'-UTR) of HLF mRNA. In these mice, HLF mRNA was expressed at a much lower level by leaky transcription through a double poly(A) signal of the *neo* gene. These mice are viable and fertile without any evident pathological abnormality. Using these mutant mice, we report the essential roles of HLF in the mouse model of ROP by regulating the expression of the Epo gene.

Results

Targeting of the *HLF* gene by homologous recombination

To investigate the role of HLF in mice, a targeting vector was designed so that the mouse *HLF* gene may be disrupted by inserting a PGK promoter–neomycin gene cassette, which is sandwiched between two loxP sequences, into exon 1 that encodes the 5'-UTR sequence by homologous recombination (Figure 1A). If the double poly(A) signal downstream of the *neo* gene does not work completely, the mRNA encoding HLF in the sequence continuing from the *neo* sequence can be produced because of leaky transcription through the double poly(A) signal at the end of the *neo* gene. The embryonic stem (ES) cell line (clone 144) heterozygous for the recombinant allele (*HLF*^{kd/+}) was established by positive–negative selection (Figure 1B) and used to generate chimeric mice by blastocyst injection. Resulting chimeric mice were crossed with wild-type C57BL/6J mice to obtain mice heterozygous for the mutated *HLF* gene (*HLF*^{kd/+}).

Mice heterozygous for the *HLF*^{kd} gene were phenotypically indistinguishable from their wild-type littermates and were interbred to yield mice homozygous for the mutated allele (*HLF*^{kd/kd}). Genotyping of the newborn mice revealed that the *HLF*^{kd/kd} mice were born in the ratio expected from Mendelian inheritance (Figure 1C and F) and were viable and fertile without any evident histopathological abnormalities. By using real-time quantitative PCR analysis, we have shown that the retinas of *HLF*^{kd/kd} mice expressed HLF mRNA at about one-fifth that of wild type (Figure 1E). To investigate whether the reduced expression of HLF is due to insertion of the *neo* gene containing the double poly(A) signal, we deleted the *neo* gene cassette by mating *HLF*^{kd/kd} mice with AYU-1-

Cre mice that ubiquitously express the Cre protein (Niwa *et al.*, 1993). Genotyping of the resulting offspring revealed that Cre recombinase successfully deleted the *neo* gene from the *HLF*^{kd} gene, leaving one remnant loxP in the first exon (*HLF*^{lox}) (Figure 1D). RT–PCR revealed that *HLF*^{lox/lox} mice recovered a normal level of HLF mRNA expression comparable to that of the wild-type mice (Figure 1E).

Expression of HLF in mice homozygous for the *HLF*^{kd} gene

In *HLF*^{kd/kd} mice, the expression of HLF mRNA was examined in various organs using real-time quantitative PCR (Figure 2A) and was reduced to 20–80%, depending on the organs, of wild type. The reason for this tissue-dependent variability in the reduced expression of HLF mRNA remains unknown. In contrast, the expression of HIF-1 α mRNA in *HLF*^{kd/kd} mice and wild-type mice did not differ in these organs (Figure 2B).

Hemorrhaging was not observed during the embryonic stages in *HLF*^{kd/kd} mice (data not shown), and heart rate did not differ significantly between *HLF*^{kd/kd} and wild-type mice at E18.5 (*HLF*^{kd/kd}: 28.3 ± 2.8 , $n = 3$; wild type: 32.2 ± 6.6 , $n = 5$).

Immunohistochemistry revealed that endothelial cells of the dorsal aorta and sympathetic cells at E10.5 (Figure 2C and F), adrenal gland at E15.5 (Figure 2D and G) and smooth muscle cells (Figure 2E and H) were stained positively with anti-HLF antibody in *HLF*^{kd/kd} mice to a similar extent as in wild-type mice under the normoxic conditions.

Expression pattern of HLF in the mouse model of ROP

Immediately after hyperoxic treatment [postnatal day 12 (P12)–0 h] in the ROP model (Smith *et al.*, 1994) (Figure 3A), eyeballs were removed from the wild-type and *HLF*^{kd/kd} mice. First, expression of HLF and HIF-1 α was examined by western blotting of the eyes of wild-type and *HLF*^{kd/kd} mice at different time points after a shift from hyperoxic to normoxic conditions. Induction of HLF was detected even at 2 h after the normoxia in wild-type mice, while essentially no induction of HLF was observed in *HLF*^{kd/kd} mice during 12 h of normoxia (Figure 3C). Unexpectedly, HIF-1 α expression displayed only a slight variation under hypoxic and normoxic conditions in wild-type mice, or in *HLF*^{kd/kd} mice (Figure 3C).

Secondly, immunohistochemistry was performed to examine HLF and HIF-1 α expression in the retinas of wild-type and *HLF*^{kd/kd} mice. The normoxic treatment following the hypoxic conditions moderately enhanced the expression of HIF-1 α in the inner nuclear layer (INL) and ganglion cell layer (GCL) of both the wild-type and the mutant mice (Figure 3H–K). On the other hand, the expression of HLF was induced in the wild type immediately after shift to normoxia (Figure 3D and E) whereas it was not induced in *HLF*^{kd/kd} mice under subsequent normoxic conditions (Figure 3F and G). Although HLF and HIF-1 α share many biochemical and transcriptional properties (Ema *et al.*, 1997; Wenger and Gassmann, 1997), their expression levels were independently regulated. The *HLF*^{lox/lox} mice recovered enhanced HLF

Fig. 1. Mutation of mouse *HLF* gene. Outline of targeting vector and restriction maps around the first exon (closed box) (A). Arrowheads indicate the sites of primers used to distinguish the wild-type *HLF* gene from the mutant. MC-1-NEO (open box) indicates the neomycin resistance (*NEO*) gene under the control of the MC-1 promoter and TK is the thymidine kinase gene under the control of the herpes simplex virus promoter. The *NEO* gene sandwiched between loxP sequences was inserted into exon 1 at the *Not*I site. DNA blot analysis of DNAs from recombinant ES cells (B) or mouse tails (C). Genomic DNAs were digested with *Sph*I or *Eco*RI, and hybridized with the 5' or 3' external probe, respectively. Genotyping of the *HLF* locus from offspring (D). PCR analyses were carried out with genomic DNAs from offspring generated by mating heterozygous *HLF*^{kd} mice (left panel). PCR analysis of the rescued *HLF*^{lox} gene is also shown (right panel). The inserted *NEO* gene was removed by mating *HLF*^{kd} mice with Cre-transgenic mice. RT-PCR analyses of *HLF* mRNA expression (E). RNAs were prepared from the eyes of wild-type, *HLF*^{kd/kd} and *HLF*^{lox/lox} mice, and subjected to RT-PCR analyses. Real-time quantitative PCR (bottom) was performed, and the amount of *HLF* mRNA from eyeballs of wild-type mice was designated as 1.0. Genotyping of littermates from the intercrosses of *HLF*^{kd/+} heterozygotes 2 weeks after birth (F).

expression under relative hypoxic conditions, as observed in the wild type (data not shown).

Suppression of retinal neovascularization of *HLF*^{kd/kd} mice

Under hyperoxic conditions in the ROP model, retinal capillary obliteration is observed (Smith *et al.*, 1994), and when mice are returned to room air (P12), they are considered to be exposed to relatively hypoxic conditions.

Subsequently, retinal neovascularization occurs in 100% of animals under these conditions with the increase in VEGF mRNA (Pierce *et al.*, 1995). In this ROP model, neovascularization in retinas was also reported to be maximal after 5 days of hyperoxic treatment followed by 5 days of relative hypoxia (P17) (Smith *et al.*, 1994). For reference, when kept under normal conditions, wild-type and *HLF*^{kd/kd} mice showed essentially no difference in their retinal structures at P12 (Figure 4A and B) and

Fig. 2. Comparison of HLF expression between wild-type and *HLF^{kd/kd}* mice. Expression levels of HLF (A) and HIF-1α (B) mRNA were examined under normoxic conditions in various organs (brain, liver, kidney, heart and lung) by real-time quantitative PCR. Immunohistochemistry was undertaken by staining with anti-HLF antibody in the dorsal aorta, adrenal gland and smooth muscle of *HLF^{kd/kd}* (C–E) and wild-type (F–H) mice at E18.5. Note that HLF was similarly expressed in *HLF^{kd/kd}* and wild-type mice in each organ. DA, dorsal aorta; CV, cardinal vein; ST, sympathetic trunk; AG, adrenal gland; K, kidney. Bars indicate 25 μm (F) and 100 μm (G and H).

thereafter (data not shown). However, when wild-type mice were treated in the ROP model, multiple neovascularizations were induced at P17 on the vitreous side of the inner limiting membrane, with prominent neovascular tufts extending into the vitreous body (Figure 4D). In sharp contrast, there was essentially no neovascularization in the *HLF^{kd/kd}* mice (Figure 4E). In total, 250 retinal cross-sections from each experimental group (three animals per group) were counted for neovascular buds to assess the extent of neovascularization, and the number was averaged per cross-section (Figure 4K). It was confirmed that neovascularization was remarkably induced in extent as well as in number in wild-type retinas by relative hypoxic

treatment. Strikingly, in *HLF^{kd/kd}* mice, retinal neovascularization was almost completely suppressed on the inner retinal surface (Figure 4E and K).

To investigate whether the suppressed neovascularization is due to a lowered expression of HLF, the *HLF^{lox/lox}* mice were subjected to the model of ROP. Interestingly, susceptibility to massive proliferative neovascularization, as found in wild-type mice, was recovered in the treated *HLF^{lox/lox}* mice (Figure 4F and K). These results clearly demonstrated that the level of HLF expression is critical for ROP, and that the effects of the reduced expression of HLF cannot be compensated by HIF-1α. In the ROP model, neovascularization was investigated by staining

Fig. 3. Expression of protein and immunohistochemical staining of HLF and HIF-1α in retinas. The time course of the experiment is indicated (A). Samples were taken immediately after 75% O₂ treatment (P12-0 h) and 2 and 12 h (P12-12 h) after the shift from 75% O₂ to normal air. Whole-cell extracts were obtained at the appropriate time point (0, 2 and 12 h) and subjected to SDS-PAGE to detect the expression of HLF and HIF-1α (B). Actin antibody was used for detecting the expression of HLF and HIF-1α protein, and HLF and HIF-1α expressions in wild-type mice at P12-0 h were designated as 1.0 (C). Sections of eyeballs from wild-type (+/+) (D, E, H and I) and *HLF*^{kd/kd} mice (F, G, J and K) at P12-0 h or P12-12 h were incubated with anti-HLF (D-G) and anti-HIF-1α antibody (H-K), and visualized as described in Materials and methods. GCL, ganglion cell layer; ONL, outer nuclear layer; INL, inner nuclear layer.

with an antibody against CD34, a membrane glycoprotein specific for vascular endothelial cells (Young *et al.*, 1995). The formation of neovascular buds was observed on the surface of the GCL of retinas from both wild-type and mutant mice at P12-0 h immediately after shifting from hyperoxic to normoxic conditions, although neovascular buds were clearly fewer in the mutants (Figure 4G and H). After 12 h of normoxic conditions, the neovascular buds were elongated and appeared to infiltrate into the wild-type INL, while in mutant mice they totally disappeared (Figure 4I and J).

Expression of angiogenesis-related genes in the model of ROP

It has been reported that HLF and HIF-1α mRNAs and their proteins are synthesized at almost the same rate under normal O₂ (21%) conditions as they are under hypoxic conditions (Ema *et al.*, 1997). On the other hand, while

their proteins were rapidly degraded by the ubiquitin-proteasome system under normoxic conditions, both HLF and HIF-1α proteins were stabilized and accumulated in response to low oxygen concentrations, leading to the enhanced expression of angiogenesis-related genes such as *VEGF*, *VEGFR-1* (*Flt-1*) (Gerber *et al.*, 1997), *VEGFR-2* (*Flk-1*) (Kappel *et al.*, 1999) and *Tie-2* (Tian *et al.*, 1997). We investigated the expression of such genes by RT-PCR and immunohistochemistry. The results from the RT-PCR analysis of various mRNAs were displayed at 25 and 30 reaction cycles, and real-time quantitative PCR was also performed (Figure 5). RT-PCR analysis using total RNA from the mutant mouse eyeballs confirmed the attenuated expression of HLF mRNA as compared with the wild type, even after relative hypoxia treatment. However, no significant elevation of HIF-1α expression was detected in the wild-type or *HLF*^{kd/kd} mice after relative hypoxia treatment in the real-time quantitative PCR analysis.

Fig. 4. Neovascularization in the retinas of mice with various genotypes of HLF treated in the model of ROP. Frozen retinal sections from wild-type (+/+), *HLF^{kd/kd}* (kd/kd) and Cre-recombinase-expressing *HLF^{kd/kd}* mice (*lox/lox*) were stained with PAS immediately after the shift from hyperoxia to normoxia (P12–0 h) (A–C). Retinas of *HLF^{+/+}*, *HLF^{kd/kd}* and *HLF^{lox/lox}* mice were stained with hematoxylin at P17 (D–F). Arrows indicate numerous clumps of endothelial cells on the surface of the retinas (D and F). Retinas from the *HLF^{+/+}* and *HLF^{kd/kd}* mice at P12–0 h and 12 h after shifting from hyperoxia to normoxia (P12–12 h) were subjected to immunostaining with anti-CD34 antibody (G–J). Arrowheads indicate neovascular buds detected in the GCL (G and H). Note that neovascular buds were elongated and infiltrated into the INL (I). Bar indicates 100 μ m. Quantification of retinal neovascularization of *HLF^{+/+}*, *HLF^{kd/kd}* and *HLF^{lox/lox}* mice at P17 (K). The numbers of neovascular nuclei on the retinal surfaces of *HLF^{+/+}*, *HLF^{kd/kd}* and *HLF^{lox/lox}* mice were counted in 250 sections for each of the treated mice and averaged per section. * $P < 0.01$.

Expression of VEGF mRNA was somewhat upregulated in mice of both genotypes under relative hypoxic conditions as compared with hyperoxic conditions (Figure 5A and E). Immunohistochemistry showed that the protein level of VEGF was significantly elevated by relative hypoxic treatment in both wild-type and *HLF^{kd/kd}* mice (Figure 6A–C). Thus, it was clearly shown that the mode of VEGF expression was not substantially different between wild-type and *HLF^{kd/kd}* mice. Immunostainings of VEGF, Epo, Tie-2 and Flk-1 of *HLF^{kd/kd}* mice at the 0 h point are the same as those of the wild type and deleted from Figure 6.

In sharp contrast, Epo mRNA was enhanced by the relative hypoxia in wild-type mice, while its expression

was markedly attenuated even by the successive normoxic treatment in *HLF^{kd/kd}* mice (Figure 5A and F). This reduced expression of Epo mRNA paralleled that of HLF mRNA in the mutant mice (Figure 5A, C and F). Immunohistochemistry also demonstrated that *HLF^{kd/kd}* mouse retinas were very weakly stained with anti-Epo antibody compared with the wild-type mice under the relative hypoxic conditions (Figure 6D–F). Other angiogenic factors, such as Flk-1, Flt-1, Tie-1, Tie-2, angiopoietin 1 and angiopoietin 2, were subjected to RT-PCR analysis and essentially no significant difference in their expression levels was observed between *HLF^{kd/kd}* and wild-type mice (data not shown). In addition, immunostaining showed that the levels of these factors were

Fig. 5. Expression of angiogenic factors analyzed by RT-PCR. RT-PCR analysis of mRNAs from retinas of *HLF*^{+/+}, *HLF*^{kd/kd} and *HLF*^{lox/lox} mice in the model of ROP (P12–0 h, P12–12 h) was undertaken using specific pairs of primers for HLF, HIF-1α, Epo and two forms of VEGF. The results of the PCR analysis are shown at 25 (A) and 30 cycles (B). G3PDH was used as an internal control. Real-time quantitative PCR analysis was also performed for HLF (C), HIF-1α (D), VEGF (E) and Epo (F) in retinas of *HLF*^{+/+} and *HLF*^{kd/kd} mice kept in the model of ROP (C–F). The relative mRNA level in *HLF*^{+/+} mice at P12–0 h is designated as 1.0.

significantly enhanced under relative hypoxic conditions in *HLF*^{kd/kd} mice as well as in wild-type mice. Immunohistochemistry of VEGF, Flk-1 and Tie-2 displayed different regional expressions, because VEGF is a secretory protein, while Flk-1 and Tie-2 are receptor proteins associated with the plasma membranes of vascular endothelial cells. The expression of VEGF was uniformly enhanced in the GCL and INL of wild-type and mutant retinas exposed to relative hypoxia (Figure 6A–C). In contrast, Tie-2 displayed a rather clustered expression along the extending vascular tubes of wild-type retinas (Figure 6H), while it dispersed rather evenly in *HLF*^{kd/kd} retinas, probably because no vascular formation developed (Figure 6I). Immunostaining using anti-Flk-1 antibody revealed a similar difference in the distribution of Flk-1 between wild-type and *HLF*^{kd/kd} mouse retinas to that of Tie-2 (Figure 6K and L). Epo

receptor (EpoR) was also expressed in the neovascular tubes of wild-type retinas under relative hypoxic conditions (Figure 6M and O), while EpoR immunostaining was eliminated in the presence of EpoR peptide as a control experiment (Figure 6N).

Association of Epo with neovascularization in the ROP

In order to confirm that Epo is involved in the neovascular process, Epo was injected intraperitoneally into *HLF*^{kd/kd} mice during relative hypoxia treatment (Figure 7). Neovascular buds appeared in the GCL at P12–12 h in wild-type mice (Figure 7A and B) regardless of Epo administration, whereas neovascular buds were observed in *HLF*^{kd/kd} mice only when Epo was injected exogenously (Figure 7C and D). Comparison between *HLF*^{kd/kd} mice treated with or without Epo revealed a significant increase in the number of neovascular buds in the GCL of Epo-treated *HLF*^{kd/kd} mice [control phosphate-buffered saline (PBS): 1.3 ± 0.2 ; Epo: 20.4 ± 4.5]. This demonstrated that Epo plays an important role in the formation or maintenance of neovascular buds following a shift from hyperoxic to normoxic conditions. The number of neovascular buds in Epo-injected *HLF*^{kd/kd} mice did not fully recover to the level of wild type, probably due to the same reasons that administered Epo may not work effectively and/or that some factors other than Epo may also be needed for the formation of neovascular buds.

To further clarify the effect of Epo on neovascularization elongating from the GCL towards the INL, EpoR-deficient mice rescued by intercrossing with transgenic mice expressing *EpoR* transgene under the control of the *GATA-1* promoter (*EpoR*^{-/-}:Tg+) (Suzuki *et al.*, 2002) were utilized for the ROP model (Figure 7F–H). In these transgenic mice, the expression of EpoR was only detected in hematopoietic organs, such as the spleen and bone marrow, but not in the retina. The number of vascular tubes reaching into the INL of *EpoR*^{-/-}:Tg+ (Figure 7G and H) at P17 was clearly less than that of the wild type (Figure 7F and H) in which EpoR is expressed normally, suggesting that the Epo/EpoR system is also necessary for neovascularization after relative hypoxic conditions in the retina.

Retinal damage in *HLF*^{kd/kd} mice during relative hypoxic treatment

At P21 in the ROP model, we found degenerative changes in the inner retinal layer of the *HLF*^{kd/kd} mice (Figure 8B), which could not be observed at P12 (Figure 4A and B) or P17 (Figure 4D and E), regardless of the *HLF* genotype. Hematoxylin staining of retinal sections from *HLF*^{kd/kd} mice revealed markedly abnormal retinal structures (Figure 8B), probably resulting from a loss of the INL, as revealed by immunostaining analysis using an anti-vimentin antibody (Figure 8D and E). The expression of vimentin was restricted to the INL, GCL and new vessels of wild-type mice treated with relative hypoxia (Figure 8D). On the other hand, in hypoxia-treated *HLF*^{kd/kd} mice, immunoreactivity against vimentin was observed only in the GCL and the monolayer (Müller cells) immediately above the GCL, showing that the INL cells almost completely disappeared in these mice (Figure 8B and E). TUNEL analyses revealed continual apoptosis in

Fig. 6. Immunohistochemical staining with anti-angiogenic factor antibodies. Frozen retinal sections from *HLF*^{+/+} (A, D, G and J at P12–0 and B, E, H, K, M, N and O at P12–12) and *HLF*^{kd/kd} mice (C, F, I and L at P12–12) treated in the model of ROP were immunostained with antibodies against VEGF (A–C), Epo (D–F), Tie-2 (G–I), Flk-1 (J–L) and EpoR (M–O). Magnified inserts of the enclosed area (M) are also presented in (O). Arrowheads show endothelial tubes expressing EpoR (M and O). Bars indicate 100 μ m (L) and 25 μ m (M and O).

the retinal nuclear layers of the *HLF*^{kd/kd} mice during relative hypoxic treatment. TUNEL analyses of retinas from animals immediately after transfer from hypoxia to normoxia showed frequent induction of apoptosis in both *HLF*^{kd/kd} and wild-type retinas (data not shown). However, while apoptosis in the wild-type and *HLF*^{lox/lox} mice was suppressed along with proliferative neovascularization (Figures 8F and H, 4D and F), it was persistently observed in *HLF*^{kd/kd} mice at P17 (Figure 8G and J). The retinal damage observed in the *HLF*^{kd/kd} mice was caused at least in part by continued apoptosis due to poor neovascularization.

To examine the physiological function of degenerated retinas in visual synaptic transmission *in vivo*, we analyzed single-flash electroretinogram (ERG) patterns of mouse retinas subjected to the model of ROP. The ERG response to a light flash can be divided into three waves: a-wave, b-wave and OPs (oscillatory potentials consisting of three to four wavelets, OP₁–OP₄), arising from photoreceptors, bipolar cells and amacrine/inner plexiform cells, respectively (Harada *et al.*, 1998). ERG analyses revealed that while the wild-type mice subjected to the model of ROP had a normal flash-light response with a-wave, b-wave and OPs, the *HLF*^{kd/kd} mice with the degenerative retinas showed a complete loss of b-wave and OPs, but had a normal a-wave amplitude (Figure 8L). These results indicate that *HLF*^{kd/kd} mice in the model of ROP suffered from functional damage in the neural cells of the INL, but retained functional photoreceptor cells.

Discussion

In order to investigate the functional roles of HLF in adult animals, we produced *HLF*^{kd/kd} mice by inserting the *neo* gene cassette sandwiched between the two loxP sequences into the first exon encoding the 5'-UTR. The double poly(A) addition signals of the inserted *neo* gene did not work completely, such that leaky transcription reduced the synthesis of HLF mRNA to approximately one-fifth of that in the retinas of wild-type mice.

Under normal conditions, *HLF*^{kd/kd} newborn mice grow well with apparently normal retinal structures (data not shown), suggesting that this reduced level of HLF production was not harmful to the animals under normal conditions.

The model of ROP is initiated by hyperoxia-induced obliteration of newly formed blood vessels in the retinas of newborn mice (Alon *et al.*, 1995). Subsequently, the retinas of newborn mice become relatively hypoxic when exposed to normal air, because of obliteration of the retinal vessels and induction of abnormal vasoproliferation (Ozaki *et al.*, 1999). In contrast to wild-type mice, proliferative vascularization was not induced in the retinas of *HLF*^{kd/kd} mice during normoxic conditions preceded by hyperoxic treatment. Although RT-PCR analyses revealed no prominent differences between wild-type and *HLF*^{kd/kd} mice in their expression of the angiogenic factors VEGF, Flt-1, Flk-1, Tie-1 and Tie-2, only Epo mRNA was notably reduced in *HLF*^{kd/kd} mice, in parallel with the attenuated

Fig. 7. Effects of Epo on the formation of neovascularization in retinas. *HLF*^{+/+} and *HLF*^{kd/kd} mice were kept in the model of ROP and examined for the formation of neovascular buds after administration of control PBS (A and C) and Epo (B and D) at P12–24 h. CD34-positive neovascular buds were scored under the microscope (E). **P* < 0.01. After the shift from hyperoxia to normoxia, wild-type (EpoR^{+/+}) mice (F) and EpoR^{-/-}::HG1-EpoR (Tg) mice (G) were examined at P17. The number of neovascular tube formations infiltrated into the INL was counted after the staining with anti-CD34 antibody (H). **P* < 0.05.

expression of HLF (Figure 5A, B and F). In *HLF*^{lox/lox} mice that had the inserted *neo* gene removed by Cre enzyme, the expression of Epo and HLF mRNAs recovered to normal levels, resulting in proliferative neovascularization in response to hypoxic conditions (Figure 4C, F and K). Immunochemical staining of Epo protein displayed similar changes to that of Epo mRNA in wild-type and *HLF*^{lox/lox} mice, suggesting that Epo is one of the target genes of HLF that is responsible for ROP (Figures 5A, B, F and 6E). This suggestion was strongly supported by the results of the ROP experiments showing that intraperitoneal injection of

Epo recovered significant susceptibility in the *HLF*^{kd/kd} mice to proliferative retinopathy and, conversely, that the EpoR-engineered mice lost the susceptibility to retinopathy to a significant level (Figure 7).

Considering that Epo has been reported to function not only as a hematopoietic, but also as an angiogenic factor (Anagnostou *et al.*, 1990; Ribati *et al.*, 1999), it is very likely that its reduced expression is not sufficient to induce proliferative neovascularization in *HLF*^{kd/kd} mice. Needless to say, we cannot rigorously rule out the possibility that factors other than Epo, which are

Fig. 8. Structural and functional alterations induced in retinas from *HLF^{kd/kd}* mice (P21) by prolonged normoxia treatment after hyperoxia. Hematoxylin-stained retinas of *HLF^{+/+}*, *HLF^{kd/kd}* and *HLF^{lox/lox}* mice (P21) exposed to prolonged normoxia treatment following hyperoxia (A–C). Immunohistochemical staining of retinas from *HLF^{+/+}* and *HLF^{kd/kd}* mice (P21) with an anti-vimentin antibody (D and E). Note that loss of the INL from *HLF^{kd/kd}* retinas is presented (B). An arrow shows newly formed blood vessel in the retina (D). TUNEL reactions of retinal sections from *HLF^{+/+}*, *HLF^{kd/kd}* and *HLF^{lox/lox}* mice at P17 (F–H). Magnified inserts of the enclosed areas are also presented (I–K). Retinal sections were subjected to the TUNEL reaction and followed by counterstaining with Nuclear Fast Red. Bars indicate 100 μ m (H) and 25 μ m (K). ERGs of *HLF^{+/+}* and *HLF^{kd/kd}* mice at P21 in the model of ROP (L). Three animals from each experimental group were used.

Table I. Primers used for the PCR

VEGF	5' primer	5'-CTTCCTACAGCACAGCAGATGTGAA-3'
	3' primer	5'-TGGTGAATGGTTAATCGGTCTTTC-3'
VEGF164	5' primer	5'-CTTTACTGCTGTACCTTCACCATGC-3'
	3' primer	5'-AACAAGGCTCACAAGTGATTTCTGG-3'
Epo	5' primer	5'-TGGCACCTGCTGCTTTACTCTCCTT-3'
	3' primer	5'-CTGGAGGCGACATCAATTCCTTCTGAG-3'
EpoR	5' primer	5'-TCTTCACCACCCACAAGGGTAACITCCA-3'
	3' primer	5'-AGATGCCAGAATCGGACACCACAAGGTA-3'
Tie2	5' primer	5'-CGTCAACAAGCATCCTTCCTACCTGCTA-3'
	3' primer	5'-TGCATCCTTCTGGTCCACTACACCTTTC-3'
HLF	5' primer	5'-CAGCTCAGAGCTGAGGAAGG-3'
	3' primer	5'-GTTGTAGACTCACTTGCC-3'
HIF-1 α	5' primer	5'-CAAGATCTCGGCGAACGAAAGAGTCTGA-3'
	3' primer	5'-GAAGCACCTTCCACGTTGCTGACTTGAT-3'
Flt-1	5' primer	5'-TCTTGCTCACCATGGTCAAGC-3'
	3' primer	5'-GTCATGTGCACAAGTTTGGG-3'
Flk-1	5' primer	5'-TCGCTCTGTGGTTCTGCGTG-3'
	3' primer	5'-GGTTTGAAATCGACCCTCGG-3'
G3PDH	5' primer	5'-TGAAGGTCGGTGTGAACGGATTGGC-3'
	3' primer	5'-CATGTAGGCCATGAGGTCCACCAC-3'

specifically regulated by HLF, are involved in proliferative vascularization. Despite its normal expression in *HLF^{kd/kd}* mice, HIF-1 α cannot functionally compensate for HLF in this process, while Epo gene expression is known to be regulated by both HIF-1 α and HLF through the HRE located downstream of the gene by DNA transfection assays (Wanner *et al.*, 2000). It remains to be elucidated why only HLF, but not HIF-1 α , transactivates the expression of the Epo gene in the retinas of ROP.

During prolonged exposure to normoxia following hyperoxic treatment, the structure of the *HLF^{kd/kd}* retinas displayed not only suppression of neovascularization, but also a prominent degenerative change in the INL at P21, probably resulting from prolonged hypoxia due to poor neovascularization. It was recently reported that Epo plays a protective role in neurons against apoptosis in cerebral ischemia (Digicaylioglu and Lipton, 2001; Siren *et al.*, 2001). Indeed, expression of the EpoR was detected in the INL and neovasculature of wild-type mice (Figure 6M and N), suggesting that reduced Epo synthesis in *HLF^{kd/kd}* retinas exposed to relative hypoxia may aggravate degeneration of the INL due to defective neural protection and poor neovascular formation. Localized expression of Epo in the retina is thought to be important in protecting nuclear layers, because Epo concentrations in the blood sera did not differ between *HLF^{kd/kd}* and wild-type mice under normoxic conditions (data not shown).

Single-flash ERG analyses revealed that *HLF^{kd/kd}* mice lost b-wave and OPs mainly derived from bipolar cells and the amacrine/interplexiform during the prolonged relative hypoxic treatment (Figure 8L). In contrast, wild-type mice undergoing the same treatment retained normal neurotransmission of light response, indicating that at least the Epo gene under the control of HLF may play a critical role in protection against degeneration of the INL in the ROP model.

VEGF is thought to represent a major factor triggering neovascularization in the retina of relative hypoxia (Aiello

et al., 1995; Seo *et al.*, 1999). Indeed, VEGF was expressed in the INL and GCL in response to hypoxia, and its receptor Flk-1 was also expressed in the elongated endothelial cells (Figure 6K), confirming that the VEGF/Flk-1 interaction induces proliferation of the retinal vasculature. Although neovascular bud formation was transiently observed immediately after hypoxia treatment, *HLF^{kd/kd}* mice did not develop any vascular formation from these buds during treatment with hypoxia, even with a production of VEGF equivalent to that in wild-type mice (Figures 5E and 6). The results indicated that some factors other than VEGF are also necessary for induction of neovascularization and that Epo may be one of the factors that plays a protective or anti-apoptotic role in endothelial cell buds (Carlini *et al.*, 1999) during neovasclogenesis in the ROP. Therefore, it would be interesting to investigate how Epo and VEGF interplay in the development of neovascularization in response to hypoxia. Recently, inhibitory PAS (IPAS) protein, which acts as a dominant-negative regulator of HIFs, was isolated by Makino *et al.* (2001). IPAS is expressed in response to hypoxia and inhibits the activities of HLF and HIF-1 α in both corneal epithelium and retinal GCL and INL, suggesting a more complicated regulatory mechanism for neovascularization observed in the ROP.

Consequently, in addition to VEGF, Epo was found to be a key factor in the ROP, especially in the development of neovascularization, suggesting a therapeutic possibility of Epo and VEGF inhibitors in the ROP. *HLF^{kd/kd}* mice have turned out to be very useful for investigation of the physiological roles of HLF and might also shed light on the function of HLF that is distinct from that of HIF-1 α in terms of vascularization and angiogenesis under hypoxic conditions.

Materials and methods

HLF gene targeting

A genomic DNA sequence extending from the first exon to 5.1 and 2.7 kb in the 5' and 3' directions, respectively, was cloned from a 129/SV mouse genomic library and used for construction of a targeting vector. A

neomycin resistance gene (*neo*) cassette sandwiched between loxP sequences was inserted into the first exon of the *HLF* gene. The constructed chimeric DNA was then cloned into pUC18. The targeting vector was amplified, linearized by *NotI* digestion and electroporated into E14 cells. The electroporated cells were subjected to double selection in G418 (300 µg/ml) and gancyclovir (1 µg/ml), and amplified. After 10 days, colonies were isolated, expanded and screened for homologous recombination by the PCR method. DNA from an isolated double-resistant clone was further confirmed for homologous recombination by DNA blot analysis. Double-resistant ES cells were injected into C57BL/6J blastocysts that were surgically implanted into the uterus of pseudopregnant ICR foster mothers. Chimeric mice were then intercrossed with C57BL/6J mice and the offspring were genotyped.

Murine model of retinal neovascularization

The murine hyperoxia–normoxia-induced proliferative retinopathy model established by Smith (1994) was used in this study. Briefly, at P7, 20 littermates from each group of *HLF^{exd/kd}* and wild-type mice, along with nursing mothers, were exposed to 75% oxygen for 5 days, and at P12 the animals were returned to room air (21% O₂). Following exposure to room air, a certain number of mice (the number is indicated in parentheses for each time point) were killed at one of the following time points: 0 h (3), 2 h (3), 12 h (3), 24 h (3), 36 h (2), 5 days (P17) (3) and 9 days (P21) (3). An oxygen-enriched atmosphere was obtained by mixing air with oxygen, and oxygen levels were monitored using a polarographic oxygen sensor (Teijin, Tokyo, Japan). Eyeballs were rapidly removed and embedded in an optimal cutting medium (O.C.T. Compound; Sakura, Tokyo, Japan) for histochemical and immunostaining analyses. The treated mice were kept with nursing mothers under normal conditions and eyes were removed at P17 under Nembutal anesthesia for histochemical and immunochemical analyses. Frozen retinal sections were cut at 5 µm for immunohistochemistry and periodic and acid Schiff stain (PAS) and hematoxylin staining.

Immunohistochemistry and electroretinogram analysis

Frozen sections of eyes were immunohistochemically stained using anti-HIF-1α, Epo, Tie-2, VEGF, Flk-1, EpoR (Santa Cruz Biotechnology, Santa Cruz, CA), vimentin (Nichirei, Tokyo, Japan) and CD34 (Young *et al.*, 1995) antibodies. To obtain anti-HLF antibody, two rabbits were immunized with the synthetic 16 amino acids of the C-terminus of mouse HLF (656–671 amino acids), and the resulting bleed was affinity purified (Sawady Technology Inc., Tokyo, Japan). The specificity of the anti-HLF antibody was examined by ELISA and western blot analyses.

The sections were fixed in 4% paraformaldehyde/PBS for 15 min, washed twice with PBS and incubated for 30 min with 10% normal goat serum. The slides were then incubated overnight at 4°C with respective specific antibodies or non-immune serum. After washing with PBS, the slides were further incubated with biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, Inc., Burlingame, CA), followed by streptavidin-conjugated ABC complex (Vector Laboratories) or horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Biosource, Camarillo, CA). Specific staining was visualized by incubation with 3,3'-diaminobenzidine. Counterstaining was carried out using Nuclear Fast Red.

Electroretinogram analysis was performed as described previously (Abe *et al.*, 1999).

RT-PCR

Total RNA was prepared from eyeballs of mice with various genotypes of the *HLF* gene using RNA isolation reagent (Trizol, Invitrogen). cDNAs were synthesized using a RT-PCR kit (Clontech Laboratories) in a reaction solution (20 µl) containing total RNA (1 µl) prepared from eyeballs of mice treated in various ways. An aliquot (1 µl) of synthesized cDNA was amplified (Clontech Laboratories) in a total volume of 20 µl containing 150 mM dNTP, 0.2 U of *Taq* polymerase and 0.12 µg of each pair of primers. The PCR procedure consisted of 25 or 30 cycles of the reaction: 95°C for 30 s and 68°C for 1 min. The PCR products were electrophoretically separated on 2% agarose gels. In addition, cDNA was amplified and quantified using an ABI Prism 7700 Sequence Detector (Parkin Elmer, Foster City, CA). The primers used for the PCR reaction are listed in Table I.

Western blot analysis

Mouse eyeballs were lysed with lysis buffer [10 mM HEPES pH 7.9, 10 mM potassium chloride, 0.1 mM EDTA, 1 mM dithiothreitol, 1% NP-40 and protease inhibitor cocktail (Roche, Mannheim, Germany)]. Whole-cell extracts (100 µg) were resolved in 10% SDS–

polyacrylamide gels and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). For HLF detection, rabbit anti-HLF antibody was used and, for HIF-1α, anti-HIF-1α antibody (H1α67; Lab Vision, Fremont, CA) was used. Detection was performed with HRP-conjugated goat anti-rabbit or rabbit anti-goat immunoglobulins (Biosource) and enhanced chemiluminescence (ECL; Amersham Biosciences, UK). After analysis, goat anti-actin antibody (I-19; Santa Cruz Biotechnology, Santa Cruz, CA) was used to verify equal protein loading and transfer.

TUNEL assay

The TUNEL assay was performed using an *in situ* apoptosis detection kit (TaKaRa, Code No. MK500) as specified by the manufacturer. Frozen eye sections (5 µm) were fixed in 4% paraformaldehyde/PBS for 30 min and washed with PBS for 10 min at room temperature (RT). Slides were treated with 0.3% hydrogen peroxide in methanol for 30 min at RT and incubated with permeabilization buffer for 5 min on ice. Subsequently, the samples were incubated with reaction buffer overnight at 4°C. After several washings with PBS, sections were incubated with anti-FITC–HRP conjugate for 30 min at 37°C and washed with PBS. Staining was performed using diaminobenzidine and samples were counterstained with Nuclear Fast Red.

Epo injection

To investigate the role of Epo in neovascularization, Epo (a generous gift from Chugai Pharmaceutical Co., Tokyo, Japan) was intraperitoneally injected into neonates (5000 IU/kg) (Grimm *et al.*, 2002) at P12–0 h and P12–12 h. The effect of Epo on the formation of neovascular buds was evaluated by scoring the number of buds observed in the GCL at P12–24 h after staining with anti-CD34 antibody.

EpoR^{−/−}:HGF-EPOR (Tg) mice were obtained by crossing EpoR^{+/−}:Tg mice with EpoR^{+/−} mice, as reported previously (Suzuki *et al.*, 2002), and newborn mice were exposed to 75% oxygen for 5 days and subsequently returned to room air. Mice were analyzed at P17 for neovascularization by staining with anti-CD34 antibody, and the number of tube formations reaching the INL were scored under the microscope.

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