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Hypoxia-inducible Factor 1 Activation from aP2(Fabp4)-Cre Mediated Knockout of Von Hippel-Lindau Gene Leads to Embryonic Lethality

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Abstract

1. VHL protein (pVHL), an E3 ubiquitin ligase from the *Vhl* gene, inhibits the transcriptional activity of HIF-1 α in cells. To gain insight into the HIF-1 α signaling pathway in adipose tissue, we conducted a study to generate fat-specific *Vhl* knockout mice.
2. Cre/loxP technology was used in the knockout study. The mice carrying floxed-*Vhl* alleles were crossed with aP2-Cre mice, in which the Cre gene is driven by the *aP2* (FABP4) gene promoter.
3. The homozygous knockout (*Vhl*^{fl/fl}-Cre) mice exhibited embryonic lethality at E14.5 to E18.5. The homozygous embryos suffered from hemorrhages in the brain and liver. HIF-1 α protein and its target gene protein, vascular endothelial growth factor (VEGF), were increased in the brain and liver. Endothelial proliferation and capillary leakage were observed in the tissues. Heterozygous knockout (*Vhl*^{+/fl}-Cre) mice appeared normal in development, growth and reproductivity.
4. β -galactosidase reporter mice were used in the analysis of tissue-specificity of Cre recombinase in aP2-Cre mice. A strong Cre activity was observed in the dorsal hindbrain region and vertebrae of E12.5 embryos. These results suggest that in the aP2-Cre mice, the recombinase activity is expressed in the central nervous system of the embryos. Central and peripheral hemorrhages are responsible for the embryonic lethality in the homozygous knockout mice.

Keywords

adipose tissue; hypoxia; HIF-1; VHL; aP2-Cre; hemorrhage

Introduction

HIF-1 α is a transcription factor whose activity is induced primarily by hypoxia. In obesity, HIF-1 α activity is enhanced in adipose tissue and involved in the pathogenesis of chronic inflammation (1). Hyperinsulinemia and adipogenesis also contribute to HIF-1 α activation

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in obesity (2). To understand the impact of HIF-1 α activation in adipose tissue, we plan to enhance HIF-1 α activity in a fat-specific manner by knocking out *Vhl*, an inhibitor of HIF-1 α in the oxygen sensing pathway. The von Hippel-Lindau (*Vhl*) gene encodes an E3 ubiquitin ligase that catalyzes ubiquitination of HIF-1 α protein following hydroxylation in the normoxia condition (3). In the presence of hypoxia, the pVHL activity is inhibited leading to HIF-1 α activation. HIF-1 α forms a heterodimer with HIF-1 β to induce transcription of the hypoxia response genes, which include angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (1, 4). In addition, some inflammatory genes are also induced by HIF-1 α , such as macrophage-inhibitory factor (MIF) and transforming growth factor β (TGF- β) (5). In this way, HIF-1 α is likely to contribute to the chronic inflammation in obesity (1). To test this possibility in mice, we would like to enhance HIF-1 α activity in the adipose tissue by inactivation of the *Vhl* gene.

Vhl knockout has been used to investigate HIF-1 α activity in many organs and tissues. Global inactivation of *Vhl* gene leads to embryonic lethality from defective placental angiogenesis (6). *Cre-loxP* strategy has been used to ablate the *Vhl* gene in a tissue-specific manner for the up-regulation of HIF-1 α activity (7–10). In a study of bone regeneration, HIF-1 α activity is enhanced in osteoblasts by crossing *Vhl*^{f/f} mice with the OC-Cre mice (11). The transgenic mice exhibited an increased bone vascularization and strong bone formation in response to distraction osteogenesis. In a liver study, *Vhl*^{f/f} mice were crossed with Alb-Cre mice for liver-specific knockout (9). The *Vhl* inactivation results in severe hepatic steatosis, an increase in vascular cavities and vascularization within the hepatic parenchyma. In mice with *Vhl* inactivation in renal epithelia, erythropoietin and polycythemia expression were enhanced in the renal cyst (8). However, there is no report about *Vhl* deficiency in adipose tissue or brain. It is not clear if *Vhl* inactivation is lethal in mouse embryo.

To investigate HIF-1 α in fat tissue, we inactivated the *Vhl* gene in fat tissue by crossing *Vhl*^{f/f} mice with *aP2-Cre* mice. Unfortunately, the knockout led to embryonic lethality at days E14.5 – E18.5. Our data suggest that the embryonic lethality is a result of hemorrhages in the brain and liver.

Materials and Methods

Generation and genotyping of *Vhl*^{f/f}-Cre embryo

All procedures were performed in accordance with the National Institute of Health guidelines for the care and use of animals and approved by the Institute Animal Care and Use Committee (IACUC) at Pennington Biomedical Research Center. *aP2(Fabp4)-Cre*^{+/+} mice [Stock number: 005069; strain B6.Cg-Tg (Fabp4-cre)1Rev/J], *Vhl*^{f/f} mice (Stock number: 004081; strain C;129S-Vhlh^{tm1Jae}/J) and β -galactosidase reporter mice [Stock number: 007689; strain 129S4/SvJaeSor-Gt (ROSA) 26Sor/J] were obtained from Jackson Laboratory. The *Vhl*^{f/f} mice on the 129 background were back crossed with B6 (C57BL/6J) mice for ten generations to obtain B6 *Vhl*^{f/f} mice. Heterozygous mice (*VHL*^{+/-}-Cre) were obtained by breeding *aP2-Cre*^{+/+} mice with *Vhl*^{f/f} mice. Homozygous mice (*Vhl*^{f/f}-Cre) were obtained from intercrosses of the heterozygous mice. The embryos were collected at different days postcoitum (dpc) (14.5 or 18.5). For *Vhl* genotyping, the forward (5'-CTC AGG TCA TCT TCT GCA ACC-3') and the reverse (5'-TCT GTC TTG GCC TCC TGA GT-3') primers were used for PCR amplification. For Cre genotyping, the forward (5'- TTC CCG CAG AAC CTG AAG ATG TT-3') and the reverse (5'- GTG AAA CAG CAT TGC TGT CAC TT -3') primers were used for PCR amplification.

Cre activity in brain

aP2-Cre^{+/+} mice were bred with β -galactosidase (β -gal) reporter mice to study the tissue specificity of the *aP2* promoter in embryos. Embryos were collected at E12.5 and fixed in 4% glutaraldehyde in buffer B (PBS containing 5 mM EGTA and 2 mM MgCl_2) for 10–30 minutes at room temperature. The fixed embryos were washed three times in buffer C (buffer B plus 0.01% sodium deoxycholate and 0.02% Nonidet P-40) for 30 minutes each time, and then incubated in staining solution (2 mM MgCl_2 , 5 mM EGTA, 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 0.01% Nonidet P-40 and 0.1% X-gal dissolved in PBS) at 37 °C for 7 hours in the dark. After staining, embryos were stored at 4°C in 70% ethanol. Pictures of embryos were taken under a Leica MZ 12 stereomicroscope using a Polaroid 3CCD color camera and Polaroid DMC2 software.

Histological analysis of embryos

Mouse embryos at E18.5 were collected and fixed in neutral buffered formalin or 4% paraformaldehyde for 48 hours. The embryos were embedded in paraffin and sectioned. Histological evaluation was performed on 5- μm slides stained with hematoxylin and eosin (HE). For immunohistochemistry, the sections were deparaffinized in xylenes, dehydrated through graded alcohols, and placed in 0.1% hydrogen peroxide to quench any endogenous peroxidase activity. Slides were pretreated in citrate buffer (pH 6.0) at 95–100 °C for 20 minutes, followed by treatment with 10% normal rabbit serum for 30 minutes to block nonspecific antibody binding. The slides were individually incubated with HIF-1 α (Sigma, H6536) or VEGF monoclonal antibody (Santa Cruz Biotechnology, sc-7269) at 1/200 dilution in a humid chamber at 4°C overnight. Secondary antibody staining was carried out with a biotin-labeled horse anti-mouse antibody 1/200 dilution (Vector Laboratories, catalog number: BA-2000) for 2 hrs at room temperature, followed by incubation with a streptavidin-biotin horseradish peroxidase complex (Vector Laboratories, catalog number: PK-6101). Detection was done using DAB substrate (Vector: peroxide substrate kit, SK-4100) for 10 minutes. Slides were then counterstained with Mayer hematoxylin before dehydration and mounting. We analyzed the expression of HIF-1 α and VEGF by comparing the staining intensities between the different samples with and without the addition of a primary antibody under the same conditions.

Results

aP2-Cre mediated *Vhl* inactivation in embryo

Global disruption of *Vhl* leads to embryo lethality. However, tissue-specific *Vhl* knockout in liver or kidney does not generate the lethality. To investigate the phenotype of fat-specific *Vhl* knockout mice, we first generated *Vhl*-floxed (*Vhl^{f/f}*) mice on the C57BL/6J gene background through 10 generation backcross with B6 mice. Then, we crossed *Vhl^{f/f}* mice with *aP2-Cre* mice (C57BL/6J) and screened offspring for heterozygous *Vhl* inactivation (*Vhl^{f/f}-Cre*). The heterozygous mice were normal in growth and reproductivity, and they survived more than 15 months without evidence of illness. The heterozygous *VHL* KO mice (*Vhl^{f/f}-Cre*) were intercrossed to generate homozygous *Vhl* KO mice (*Vhl^{f/f}-Cre*). We obtained 52 newborn mice in 8 litters at an average of 7 mice/litter. We expected *Vhl* KO mice at a rate of 1/4 according to the genetic rule. However, the genotyping result suggests that there was no homozygous KO mouse (*Vhl^{f/f}-Cre*). There was no evidence of perinatal death in the newborn mice. The results suggest embryonic lethality in the homozygous mice. To test this possibility, embryos were collected at E14.5–E18.5 and analyzed for genotype. In total of 27 embryos, 77% (21) were normal in the body size and 22% (6) appeared smaller (Fig. 1a). Genotyping results suggest that in the normal size embryos, half were heterozygous (*Vhl^{f/f}*, $n = 11$) knockout and half were wild type (*Vhl^{+/+}*, $n = 10$). These rates are similar to those in the newborn mice. The embryos small in size were all homozygous

knockouts (*Vhl^{fl/fl}-Cre*) (Fig. 1, a and b). The result suggests that the complete *Vhl* inactivation inhibits embryo development in the medium to late term of gestation (>14 days). The complete *Vhl* inactivation is responsible for the embryonic lethality.

Hemorrhages in liver and brain of the homozygous embryo

To investigate the cause of embryonic lethality, we conducted histological analyses of the small embryos at E18.5. Interstitial bleeding was observed in homozygous (*Vhl^{fl/fl}-Cre*) embryos for a wide distribution of red blood cells in various tissues (Fig. 2a), suggesting hemorrhage in more than one organ. Upon a close analysis, the hemorrhages were found in the brain and liver under a microscope (Fig. 2, b and c). Additionally, hemorrhages were also observed in the skin of homozygous embryos of 18E (Fig. 1a). These results suggest that hemorrhages may be a primary cause of the embryo death.

Enhanced HIF-1 α , VEGF and CD31 activities in the knockout embryos

The embryo bleeding is likely a result of a defect in the blood vessel structure, a result of HIF-1 α over activation. In the absence of pVHL, HIF-1 α protein is accumulated leading to a high level of the target gene expression, such as VEGF. In addition to stimulating endothelial cell proliferation and differentiation, VEGF increases capillary permeability leading to a high risk of bleeding. To test this possibility in our model, we examined HIF-1 α , VEGF and CD31 proteins in the brain and liver tissues using immunohistostaining. Protein abundance is indicated by the brown color in the slide picture. In the heterozygous embryo, the signals of HIF-1 α , VEGF and CD31 were very weak in the brain and liver (Fig. 3, a and b). In the homozygous, the signals were significantly enhanced for all of these proteins. The results suggest that *Vhl* inactivation enhances HIF-1 α activity and VEGF expression in the brain and liver. VEGF induced the endothelial cell marker CD31 by stimulation of endothelial cell proliferation. This data suggests a molecular basis for the hemorrhages in the two organs.

Cre recombinase activity under the aP2 promoter

The immunohistostaining data suggests that *Vhl* inactivation occurred in the embryonic brain. This is unexpected since the aP2 (also known as Fabp4) promoter is widely used for adipose tissue-specific gene expression in the transgenic studies. To demonstrate if the promoter expresses activity in non-adipose tissues in embryos, we used β -galactosidase reporter mice, in which β -galactosidase (β -gal) is not expressed in the normal condition. In response to Cre-mediated recombination, the gene is activated, and the activity is indicated by a blue color in the embryo after staining with the β -gal substrate. A strong β -gal activity was observed in the hindbrain and spinal cord of the E12 embryo positive in aP2-Cre (Fig. 4). The result suggests that the aP2 promoter exhibits activity in the central nervous system at E12.

Discussion

The 5.4 kb aP2 gene promoter is widely used in fat-specific transgenic studies in mice for both overexpression and gene knockout (12–15). aP2 (Fabp4) is a cytosolic fatty acid chaperone protein found predominantly in mature adipocytes and macrophages (16, 17). In the knockout studies, two different lines of aP2-Cre mice have been used in most published studies. One was developed by Dr. Barbara Kahn's lab at Harvard University (18) and other was made by Dr. Ronald Evans's Lab at the Salk Institute (19). The latter is commercially available from Jackson Laboratory (Stock number: 005069). In both lines, Cre activity is expressed in white and brown adipose tissues in adult mice. There is a third line of aP2-Cre mice reported by Barlow, et al (12). Although this line was the first aP2-Cre model in the literature, it has not been widely used in transgenic studies.

There has been no report in literature about *Vhl* inactivation in the brain. In our study, *aP2-Cre* activity was found in the hindbrain and vertebrae in E12.5 embryos. Our *aP2-Cre* mice were obtained from Jackson Laboratory. Although they were used to inactivate *Vhl* gene in adipose tissue, unexpectedly, the knockout was observed in the brain and liver leading to the hemorrhages. Brain and liver tissues were further investigated for mechanism of hemorrhages. An increase in VEGF expression was found along with a significant elevation in HIF-1 α activity in the organs. A high VEGF level is likely responsible for the embryonic lethality. In a transgenic study of VEGF165 in the brain, the heterozygous transgenic mice are normal in the embryo development, while the homozygous transgenic mice are embryonic lethal (20). The lethality may be a result of high levels of VEGF in the VEGF165 homozygous mice from the gene dose effect. In our study, *Vhl* inactivation may lead to a huge increase in VEGF activity that resembles the condition in VEGF165 homozygous mice. Our study confirms that *Cre* activity is expressed in the embryonic brain in the line of *aP2-Cre* mice made by Evans's laboratory (21). Global *Vhl* inactivation also induces embryonic lethality, but the embryo died from defective placental vasculogenesis (6). Tissue-specific knockout of *Vhl* has been reported in the liver (9), pancreas (7), kidney (8), bone (11) and thymus (10). In these studies, *Vhl* inactivation does not induce the embryonic lethality.

Liver hemorrhages may also contribute to the embryonic lethality. Liver-specific *Vhl* inactivation was made using *Alb-Cre* mice (9). Though the KO mice are not embryonic lethal, they are not comparable to our model. Unlike the *aP2* gene promoter, the *Alb* gene promoter exhibits activity at a relatively late stage during the embryo development. The promoter is only 40% efficient in mice at birth and becomes fully efficient at 42 days after birth (22). We observed that the *aP2* promoter was active in embryo as early as E14. The activity is indicated by *HIF-1 α* and *VEGF* expression in the liver. Since *aP2/Fabp4* gene promoter exhibits activity in macrophages (15, 23), *Vhl* inactivation may occur in macrophages in our model. VEGF elevation in circulation may have contributed to the broad hemorrhages in the brain, liver and skin.

In addition to HIF-1 α , the *Vhl* gene also controls HIF-2 α activity. When *Vhl* activity is absent, HIF-2 α is activated together with HIF-1 α . HIF-2 α shares sequence homology and protein properties with HIF-1 α . HIF-2 α may be involved in the regulation of embryo development as well in our model. Additionally, *Vhl* inactivation may impact p53 and NF- κ B activity (24–26). Contribution of these transcription factors (HIF-2 α , p53 and NF- κ B) to the embryonic lethality cannot be excluded in this study.

The adiponectin (*Acad*) promoter is likely a good alternative to the *aP2* promoter in the control of fat-specific gene expression. Our results suggest that *aP2-Cre* activity may be expressed in multiple tissues including the brain and liver during embryo development. This possibility is in agreement with the observation by Martens, et al for the *aP2* activity in the central nervous system (21). In an early study, *aP2-Cre* activity was found in multiple tissues including the brown adipose tissue, trigeminal ganglia, dorsal root ganglia, cartilage primordia and vertebrae embryos in the mid-gestation stage 9.5 dpc and 15.5 dpc (27). Adiponectin promoter was first used to drive fat-specific *Cre* expression in transgenic mice to knock out *Pten* in 2004 (28). This study was retracted later in 2005. In 2010, Scherer's lab reported an *Acad* promoter that drives gene expression with fat-specificity (29). The promoter contains a 4.9-kb adiponectin promoter plus the exon 1 and two ends of intron 1 of the adiponectin gene. This engineered promoter drives transgene expression specifically to adipocytes with a higher efficiency than that of the native promoter. It controls *Cre* recombinase exclusively in the adipose tissue. This *Acad-Cre* model is different from the *aP2-Cre* model in three ways. First, expression of the transgene is highly restricted to adipocytes. The *Cre* activity was not detected in the stromal vascular fraction of adipose

tissue or peritoneal macrophages (29). This tissue specificity is a major advantage over the *aP2* promoter. Second, the *Acdc* promoter exhibits the same activity in different fat depots, including white and brown fat. In contrast, the *aP2* promoter activity varies considerably cross fat depots. Third, the *Acdc* promoter is weaker than that of the *aP2* promoter. Thus, the *Acdc* promoter is excellent for moderate expression of target genes in fat. *Acdc-Cre* mice are able to generate a fat-specific knockout, but it is better to use a homozygous *Acdc-Cre* for a more efficient knockout. The *aP2* promoter is able to yield a powerful recombination under heterozygous conditions.

In conclusion, we report that *aP2-Cre* induced *Vhl* gene inactivation in the brain and liver during embryonic development. This unexpected activity of *aP2-Cre* led to embryo death at E14–E18. The lethality was associated with hemorrhages in the brain, liver and skin. A high level of VEGF after *Vhl* inactivation is likely responsible for the hemorrhages. Our study suggests that the *aP2-Cre* mice may induce gene recombination in embryos and generate effects in non-adipose tissues.

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Abbreviations

HIF-1α	hypoxia-inducible factor 1 alpha
Vhl	von Hippel-Lindau gene
Fabp4	fatty acid binding protein 4, also called adipose protein 2 (aP2)
Cre	cre-recombinase
Acdc	adiponectin

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(a) Embryonic lethality

(b) PCR genotyping

Fig. 1.

Embryo development and genotyping. (a) Embryos. The upper panel shows 14.5d embryos with placentas. The left one is heterozygous and right one is homozygous KO embryo. The lower panel shows 18.5d embryos. The normal size embryos are wild type ($Vhl^{+/+}$, left) and heterozygous KO ($Vhl^{f/+}-Cre$, right). The small embryo is homozygous KO ($Vhl^{f/f}-Cre$). (b) PCR genotyping result of the 18.5d embryos. The abbreviations stand for wild type (WT), heterozygous (Hetero) and knockout (KO).

(a) Embryo histology

(b) Brain histology

(c) Liver histology

Fig. 2.

Brain and liver histology. Tissue slides were made from the whole embryo of 18.5d and stained in HE. The red blood cells are indicated by arrows in the liver and brain of homozygous KO embryos (*Vhl*^{f/f}-Cre). The wild type (WT) and heterozygous (Hetero) embryos are used in the control. (a) Whole embryo; (b) Liver; (c) Brain.

Fig. 3.

Immunohistostaining. The immunostaining was conducted with unlabeled first antibodies to HIF-1 α , VEGF and CD31. The streptavidin-biotin complex was used in the detection of first antibody signals with DAB substrate. (a) Brain; (b) Liver.

β -gal staining

aP2-Cre positive

aP2-Cre negative

Fig. 4.

Expression of *aP2-Cre* activity in embryos of β -galactosidase reporter mice. The embryos were collected at 12.5d and stained with β -gal substrate. The Cre activity is indicated by the blue color in the positive embryo. The negative embryo (left) exhibits no color after the substrate treatment.