Vascular defects and liver damage by the acute inactivation of the *VHL* gene during mouse embryogenesis

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Inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene leads to the development of central nervous system hemangioblastomas, pheochromocytomas and renal cell carcinomas. The biological role of the *VHL* gene during development is poorly understood because of early lethality of *VHL*-null embryos. To overcome early embryo lethality observed in the conventional knockout mouse, we introduced a tamoxifen-inducible *Cre* (*CreERTM*) transgene for the stage specific inactivation of the *VHL* gene. Acute tamoxifen-induced inactivation of the *VHL* gene at E10.5 resulted in embryonic lethality between E14.5 and E15.0 with extensive hemorrhage and necrosis, while littermate controls showed normal development. Examination of the *VHL*-inactivated embryos between E10.5 and E14.5 revealed dilated blood vessels, hemorrhage and necrotizing liver damage. Concomitant with severe hemorrhage and abnormal vasculature at E15.0, blood circulation in the yolk sac was impaired in the *VHL*-inactivated embryos, which may be the cause of embryo death. Placental development looked normal before embryo death (E14.5); however, at E16.5 following embryo death, we observed reduced growth of the placental labyrinthine layer. Inactivation of the *VHL* gene resulted in hypoxia-inducible factor (HIF)-1 α stabilization and induction of its target genes, *VEGF* and *CAIX*, in mouse embryonic fibroblasts (MEFs). In addition, we observed lactate overproduction and acidification of culture media by the inactivation of the *VHL* gene. Thus, by using a novel conditional *VHL* knockout mouse model, we could show that the *VHL* gene plays an important role in the developing vasculature and liver during embryogenesis through regulation of HIF-1 α and its target genes. This mouse model will be useful for the screening of anti-HIF or anti-VEGF drugs *in vivo*. Additionally, this acute *VHL* inactivation system may provide a useful tool for the *in vivo* study of genes that cause early embryonic lethality.

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von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome, which is transmitted in an autosomal dominant manner and affects approximately 1 in 36 000 individuals.¹ The *VHL* gene was mapped to 3p25 and isolated by positional cloning.^{1,2} *VHL* germline mutations are responsible for the development of highly vascular tumors including hemangioblastomas of the retina and central nervous system, pheochromocytomas and renal cell carcinomas (RCC). pVHL is a component of an

E3 ubiquitin ligase complex that recognizes and degrades the alpha subunits of hypoxia-inducible factor (HIF) under normoxic conditions.^{3,4} Type 1, type 2A and type 2B *VHL* mutations, which disrupt regulation of HIF, lead to the accumulation of HIF under normoxic conditions.⁵ HIF target genes, such as vascular endothelial growth factor (*VEGF*), Glucose transporter 1 (*Glut-1*) and carbonic anhydrase IX (*CAIX*) are upregulated in response to the inactivation of the HIF regulatory activity of pVHL.^{6–8} It is likely that the HIF regulatory activity of pVHL is related to the development of hemangioblastomas and RCC in VHL patients. However, type 2C mutations in the *VHL* gene, which are responsible for pheochromocytoma development, retain the HIF regulatory activity, but are defective in

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promoting fibronectin matrix assembly.⁹ Recently pVHL has also been shown to bind microtubules and stabilize their structure.¹⁰ This activity is disrupted only in the type 2A *VHL* mutants that have diminished but not defective HIF regulatory activity. These biochemical analyses of pVHL function provide possible molecular clues to explain the phenotype-genotype correlations evident in *VHL* disease.

HIF regulation under normoxic condition is the most well-described function of pVHL. Oxygen is essential for oxidative phosphorylation, which satisfies the high energy demand of multicellular organisms. Cells maintain oxygen homeostasis and also generate necessary energy metabolites. HIF is a transcription factor that plays an essential role in modulating both cellular and systemic responses to changes in oxygen concentration. It regulates the expression of genes involved in angiogenesis, hematopoiesis, pH regulation and energy metabolism (glucose transporter and glycolytic enzymes). *VHL* inactivation results in oxygen-independent accumulation of HIF and thus, upregulation of its target genes under normoxic conditions. It has been demonstrated that HIF and its target genes are important for tumor progression.¹¹

Several *VHL*-knockout (KO) mouse models have been developed to investigate the biological role of pVHL. Vascular hepatic tumors developed in the heterozygous *VHL*-KO mice and also in β -actin-promoter driven Cre-mediated conditional *VHL*-KO mice.^{12,13} Upregulation of HIFs and their target genes were reported in these mouse models. Additional roles for pVHL in different organs have been suggested through the use of Cre-mediated *VHL* inactivation. *VHL* inactivation resulted in defects in spermatogenesis, thymus cell survival and bone development due to reduced cell proliferation and/or increased cell death.^{13–15} Contrary to expectation, *VHL*-inactivation did not increase cell proliferation in somatic and germ cells. Teratomas derived from *VHL* null embryonic stem (ES) cells displayed a highly vascular phenotype but showed severe reduction of tumor volume compared with heterozygous null ES cells.¹⁶ Reintroduction of *VHL* into *VHL* null ES cells restored tumor growth, so that the tumor volume was comparable to that of *VHL* heterozygous null ES cells. Specifically this growth disadvantage of teratomas was shown to be related to *VHL* type 2A mutations.¹⁷ Thus, it is likely that additional mutations in unknown gene(s), which can facilitate cell growth, might be necessary for the development of tumors in *VHL* patients.

VHL homozygous null mouse embryos died *in utero* at E10.5 to E12.5 due to defects in placental vasculogenesis.¹⁸ As a result of early embryo lethality, little is known about the role of the *VHL* gene in embryo development. Here, we explored the functions of the *VHL* gene during embryogenesis by inactivating the *VHL* gene at mid-gestation. We used an inducible system in which tamoxifen induced

Cre recombinase (CreERTM)¹⁹ to inactivate the floxed *VHL* gene in an acute manner during embryo development. *VHL* inactivation occurred in a mosaic pattern and produced vascular defects and liver damage in the *VHL* inactivated embryos.

Materials and methods

Generation and Genotyping of *VHL*^{f/d}/CreERTM Embryos

Previously, we generated mice harboring floxed (*f*) and deleted (*d*) *VHL* alleles on a C57BL6/129 mixed background.¹³ *VHL*^{d/+} mice were crossed with mice carrying a CreERTM transgene obtained from Dr Corinne Lobe (University of Toronto) to produce *VHL*^{d/+}/CreERTM mice. *VHL*^{d/+}/CreERTM mice used in this study were either *VHL*^{d/+}/CreER^{TM(tg/0)} or *VHL*^{d/+}/CreER^{TM(tg/tg)} since they were maintained by backcrossing with either the CreER^{TM(tg/0)} or CreER^{TM(tg/tg)} transgenic mice. *VHL*^{d/+}/CreERTM mice were crossed with *VHL*^{f/f} mice to produce *VHL*^{f/d}/CreERTM (*VHL*^{f/d}/CreER^{TM(tg/0)}) embryos, in which the floxed *VHL* allele could be excised by tamoxifen induced Cre recombinase activity. Tamoxifen dissolved in corn oil was injected intraperitoneally (0, 0.1, 1, 2, 4 and 8 mg) into pregnant females (25–30 g) at mid-gestation. Embryos and their placentas were collected 2–6 days after tamoxifen injection, photographed and fixed in neutral formalin for histological examination. For genotyping, genomic DNAs were isolated from the yolk sacs after proteinase K digestion. For *VHL* genotyping, three primers were used to amplify wild type (244 bp PCR product), floxed (277 bp PCR product) and deleted (161 bp PCR product) *VHL* alleles; P1 5'-GGCAAG CACTCTGTCACTGTTC-3', which is specific to genomic sequence upstream of the 5'-loxP sequence; P2 5'-GGCTATGGCATTGGGTGAGCTA-3', which is upstream of 3'-loxP sequence; P3 5'-CACC AGGACTCCTCTCGGACAT-3', which is downstream of 3'-loxP sequence. For Cre genotyping, the forward (5'-GCAACATTTGGGCCAGCTAAAC-3') and the reverse (5'-CCGGCATCAACGTTTCTTTTTC-3') primers were used for PCR amplification. DNAs isolated from embryos and their placentas were used for Southern blot analysis using a PCR-generated 250 bp probe specific to sequence upstream of the 5' loxP site of *VHL* as described.¹³ Briefly, genomic DNAs were digested with *Hind*III restriction enzyme, electrophoresed in 0.8% agarose, blotted onto a HybondN+ membrane and probed with the ³²P-labeled probe.

Evaluation of Cre Activity in the CreERTM Transgenic Embryos

Tamoxifen induced Cre activity was evaluated in embryos from crosses of the CreERTM transgenic mice with *ROSA26* reporter mice,²⁰ in which

excision of the floxed neomycin cassette by Cre recombinase allows *lacZ* gene expression. Tamoxifen (2 mg/30 g mouse) was injected into pregnant females at E10.5 and embryos were harvested at E13.5 or E14.5. β -galactosidase activity was measured *in situ* in whole embryos, in dissected organs or in frozen sections using standard staining techniques. Tissue sections were counterstained with nuclear fast red (Sigma).

Generation of MEFs and Cell Culture

Mouse embryonic fibroblasts (MEFs) were generated from E13.5 mouse embryos. Embryos were harvested, minced and trypsinized (0.25% trypsin) for 10–20 min at 37°C. Tissue culture media (DMEM containing 10% fetal bovine serum and penicillin/streptomycin) was added to cell suspensions and pipetted up and down for further disaggregation. Cell suspensions were cultured in T75 flasks containing 10 ml media in a CO₂ incubator at 37°C. Confluent cultures were trypsinized and replated onto two 150 mm dishes. After 3 days of culture, cells were trypsinized and resuspended in DMEM containing 10% FBS and 10% DMSO for liquid nitrogen storage. Frozen vials were subsequently thawed and passaged every 3 days. Cells were seeded at 7.5×10^5 cells per 100 mm dish or equivalent. Early passage MEFs (P3–P10) were used for the experiments. 4-hydroxy tamoxifen (4OHT) dissolved in ethanol was used to induce CreERTM activity during MEF culture.

Measurement of Lactate Production

MEFs were plated on two 12-well plates (3.5×10^4 cells/well), and 4OHT (0, 5, 20 and 100 nM) was added to the culture media 1 day after plating. On the fourth day, media was changed and 4OHT was added again. On the seventh day, cell numbers were counted in one of the two replicative plates. Culture media in the second plate was replaced with fresh media and 1 day later 50 μ l of media was collected for lactate measurement. Lactate concentration in the culture was measured as described with minor modifications.²¹ Briefly, 5 μ l of culture media was added to the reaction mixture containing lactate oxidase, peroxidase and substrate, ABTS (Sigma). After 10 min of reaction at room temperature, absorbance was measured at 405 nm. Lactate production was normalized to the cell number.

Isolation of RNAs from MEFs and Quantitative RT-PCR

Total RNAs were isolated from MEFs using Trizol reagent (Invitrogen) according to the manufacturer's instruction. To remove genomic DNA contamina-

tion, RNAs were digested with DNase I for 1 h at 37°C followed by heat denaturation at 70°C for 20 min. Total RNAs (2.5 μ g) were primed with 100 ng random primers and reverse-transcribed by Superscript II reverse transcriptase (Invitrogen) at 42°C for 1 h. The same reactions were performed without reverse transcriptase to generate negative controls. The following PCR primers were generated by using Primer 3 software:²² *VEGF* forward, 5'-CAGGCTGCTGTAACGATGAA-3'; *VEGF* reverse, 5'-TATGTGCTGGCTTTGGTGAG-3'; *VHL* forward, 5'-TGTGCCATCCCTCAATGTCCG-3'; *VHL* reverse, 5'-AGGCTCCGCACAACTGAAG-3'; β -actin forward, 5'-GACAGGATGCAGAAGGAGATTACTG-3'; β -actin reverse, 5'-GCTGATCCACATCTGCTGGAA-3'; *CAIX* forward, 5'-CCTCTCCCGGAAGTGAAGCC TAT-3'; *CAIX* reverse, 5'-TGTTCTGAGCCTGGGT GATCTG-3'. Quantitative RT-PCR was performed with SYBR-Green reagent (Applied Biosystems) with the ABI PRISM sequence detection system (Applied Biosystems) following the manufacturer's instruction. All reactions were run in triplicate using the β -actin gene as an internal control since β -actin mRNA levels do not change across the *VHL* genotypes. The relative level of a particular gene expression was evaluated according to the function described previously.²³ All expression levels reported were expressed relative to the expression levels determined for *VHL*^{f/+} MEFs.

Immunoblots

MEFs incubated with tamoxifen were harvested and lysed in RIPA buffer. Cell lysates were resolved by 8–16% SDS PAGE and blotted onto PVDF membrane. Equal protein loading was confirmed by Ponceau S staining. Antibodies for HIF-1 α , HIF-2 α (Novus biologicals Inc.) and pVHL (Santa Cruz Biotechnology Inc.) were used for Western-blot analysis. Immunoblots were processed by ECL (Pierce) according to the manufacturer's instruction.

Phenotype Evaluation and Histopathology

Embryos and placentas were dissected at E12.5–E18.5 and gross morphological changes were examined under a dissection microscope. They were fixed in 10% formalin, embedded in paraffin, and 5 μ m sections were prepared. The sections were deparaffinized and rehydrated through an ethanol dilution series into water. Sections were stained with hematoxylin and eosin.

Results

Evaluation of Tamoxifen Toxicity and Tamoxifen-Induced Excision of the Floxed Sequence

To inactivate the *VHL* gene during embryogenesis, we used mice carrying the CreERTM transgene, in

which introduction of tamoxifen induces Cre recombinase to excise the floxed gene. *CreERTM* transgenic mice exhibit highest Cre activity in embryos from pregnant females injected with 8 mg of tamoxifen, although some toxicity to the mother was observed.¹⁹ To avoid any harmful effects of tamoxifen, we determined the least toxic concentration of tamoxifen that was still effective in inducing Cre recombination. We evaluated tamoxifen toxicity in embryos by injecting 1, 2, 4 or 8 mg of tamoxifen into the pregnant female (25–30 g) intraperitoneally at E10.5 and evaluating gross morphological changes in the embryos at E13.5. We found that 8 mg of tamoxifen caused hemorrhage in every embryo examined (8/8). However, 1, 2 and 4 mg of tamoxifen induced morphological changes in only 1/20 embryos (data not shown). We selected 2 mg tamoxifen as the maximum dose that produced no morphologic changes.

We asked whether 2 mg of tamoxifen (per 25–30 g body weight) could excise the floxed gene sequence in the embryos by crossing *CreERTM* mice with *ROSA26* reporter mice and measuring β -galactosidase staining in the embryos as a measure of Cre recombinase activity. *CreERTM/ROSA26* double transgenic embryos exhibited positive *lacZ* staining while their *ROSA26* embryo littermates showed negative staining (Figure 1a and b). *LacZ* positive staining was observed in a mosaic pattern in most of the tissues and organs to varying degrees (Figure 1c, d and e). In the heart, liver and brain, approximately 50, 10 and 20% of the cells, respectively, were positive for *lacZ*, indicating that the efficiency of deletion may be 10–50% in these embryonic organs. We also examined Cre recombinase activity in the placenta. *CreERTM/ROSA26* double transgenic placentas exhibited *lacZ* positive staining primarily in the chorionic plate and the umbilical vessels (Figure 1f). However, a limited number of cells in the labyrinthine layer showed *lacZ* staining. *CreERTM/ROSA26* placentas from the corn oil injected (no Tam) female did not show any *lacZ* positive staining (Figure 1g). Depending upon the Cre transgene expression and the accessibility to tamoxifen, the Cre-mediated excision rate may be different for different organs. Based on these results, we decided to inject 2 mg of tamoxifen into pregnant females to induce *CreERTM*-mediated excision of the floxed *VHL* gene.

Acute Inactivation of the *VHL* Gene Leads to Embryo Lethality

Previously, we developed mice harboring either a floxed or a deleted *VHL* allele by using genetically altered murine embryonic stem cells.¹³ The floxed *VHL* allele encodes the wild-type *VHL* protein but the deleted *VHL* allele does not produce functional protein.¹³ In this study, we generated *VHL^{d/+}/CreERTM* mice and crossed them with

VHL^{f/f} mice to produce *VHL^{f/d}/CreERTM* embryos. Based on the *CreERTM/ROSA26* results, tamoxifen treatment was predicted to produce mosaically distributed *VHL*-inactivated (*VHL^{d/d}*) cells in the *VHL^{f/d}/CreERTM* embryos. In order to inactivate *VHL* during the mid-gestational stage, we injected 2 mg of tamoxifen into pregnant females at E10.5. At 6 days after tamoxifen injection (E16.5), embryos were collected and their morphological changes were noted. All the *VHL^{f/d}/CreERTM* embryos were dead (8/8, Figure 2a), while their littermate controls showed normal development (7/7, Figure 2b). *VHL* inactivated embryos (*VHL^{f/d}/CreERTM*) were smaller than their littermates and were pale. The diameter of the *VHL^{f/d}/CreERTM* placenta was comparable to that of *VHL^{f/+}/CreERTM* (Figure 2a and b). However, the thickness of the *VHL^{f/d}/CreERTM* placenta, especially the labyrinthine layer, was reduced compared with littermate controls (Figure 2d and f). In addition, dilated blood vessels and spongiotrophoblast cells were frequently observed in the labyrinthine layer of the *VHL^{f/d}/CreERTM* placenta (Figure 2g).

We measured the efficiency of *VHL* inactivation in the embryos and placentas by Southern blot analysis of genomic DNAs extracted from these tissues. We detected the deleted *VHL* allele in the *VHL^{f/+}/CreERTM* embryos at about one third the band intensity of the floxed allele band (Figure 2i). By comparing the band intensity of the floxed *VHL* allele and the deleted *VHL* allele in the *VHL^{f/d}/CreERTM* embryos, we determined that on average about 25% of the floxed alleles were converted to deleted alleles (Figure 2j), supporting a mosaic pattern of *VHL* inactivation in the embryos. On the other hand, the deleted *VHL* allele was barely detectable in the *VHL^{f/+}/CreERTM* placenta suggesting that *VHL* inactivation occurred at a much lower frequency in placentas compared with embryos (Figure 2i).

Hemorrhage and Liver Necrosis in the *VHL*-Inactivated Embryos

To ascertain the cause of embryonic death, 64 embryos from a total of seven pregnant females were collected at different time points between E12.5 and E15.5 following tamoxifen-induced *VHL* inactivation at E10.5. Morphological changes in the *VHL^{f/d}/CreERTM* embryos were observed from E13.5 (Figure 3a) and were distinct at E14.5 (Figure 3b), while littermate controls, including *VHL^{f/+}/CreERTM* embryos, showed normal development (Figure 3e–h). Hemorrhage was found in the embryos at E14.5 and became extensive afterwards. All the affected embryos (12/12, 100%) died between E14.5 and E15.5 with severe hemorrhage in the dorsolateral region and extensive necrosis in the body (Figure 3c and d), while most of littermates showed normal development (22/24, 92%). The head and limbs of the affected embryos turned pale

Figure 1 Tamoxifen-induced Cre excision of floxed allele in *CreERTM/Rosa26* embryos. *LacZ* staining of the double transgenic *CreERTM/Rosa26* embryos from a pregnant female injected with 2 mg tamoxifen is shown. Tamoxifen was injected at E10.5 and embryos were collected at E13.5 or E14.5. Positive *lacZ* staining was found in *CreERTM/Rosa26* embryos (a), but not in *Rosa26* littermate embryos (b). *LacZ* staining of the brain (c), heart (d), and liver (e) of the *CreERTM/Rosa26* embryo. Mosaic pattern of *lacZ* staining represents mosaic deletion of floxed sequences by Cre recombinase. *LacZ* staining of the *CreERTM/Rosa26* placentas from a tamoxifen treated female (f) and from an untreated female (no Tam, g). *LacZ* positive staining was seen in the chorionic plate of the placenta and the umbilical vessels (arrow). A mosaic pattern of *lacZ* staining was also seen in the yolk sac (asterisks). However, *lacZ* staining in the labyrinthine layer was limited. Placentas derived from corn oil injected females exhibited no *lacZ* positive staining. Tissue sections were counterstained with nuclear fast red.

Figure 2 Embryonic lethality and abnormal placental development by the acute and mosaic inactivation of the *VHL* gene. Acute Cre-mediated excision of the *VHL* floxed allele by the administration of tamoxifen at E10.5 resulted in lethality of the embryos at E16.5. (a) All the *VHL*-inactivated *VHL*^{f/d}/CreERTM embryos (8/8, from two litters) were found dead 6 days after tamoxifen administration, but (b) their littermate *VHL*^{f/+}/CreERTM, *VHL*^{f/d}, and *VHL*^{f/+} controls (7/7, from two litters) showed normal development. (c) Histological analysis of the *VHL* inactivated embryos showed extensive necrosis in the brain and other parts of the body. The placentas of the *VHL* inactivated embryos (f) showed a thinner labyrinthine layer (La) compared with their littermate controls (d). The labyrinthine layer of the *VHL*-inactivated placentas often showed dilated blood vessels (arrow heads) and spongiotrophoblast cells (asterisks) (g), whereas the controls showed well-organized vasculature (e). PCR genotyping of the DNAs from embryos and placentas (h) at E15.5. (*) indicates genotyping using tail DNA from a *VHL*^{f/d} mouse. Southern blot analysis of *Hind*III digested mouse embryo and placental genomic DNA using a *VHL* probe (i and j). The *VHL* deleted allele (12.4 kb) was observed in the *VHL*^{f/+}/CreERTM embryo treated with tamoxifen (i, lane 2), but was not detected in the *VHL*^{f/+}/CreERTM placenta (i, lane 4). Tail genomic DNA from a *VHL*^{f/d} mouse (i, lane 5) was used as a control. Decrease of floxed (*flox*) and increase of deleted (*del*) allele intensities were observed in tamoxifen treated *VHL*^{f/d}/CreERTM embryos (j, lane 2, 3 and 4) compared with the allele intensities of the *VHL*^{f/d} embryo (j, lane 1).

and blood circulation in the yolk sac was impaired at E14.7 indicating embryo death (Figure 3j and k). Corn oil injection without tamoxifen and low tamoxifen dosage (0.1 mg) did not result in pathological changes in *VHL*^{f/d}/CreERTM embryos dissected at E15.5 (Figure 3i, 3/3) and E18.5 (4/4), respectively, or in littermate controls (Figure 3m, 13/13).

Histological analysis of the *VHL*-inactivated embryos before death (E14.5) showed blood cell leakage from blood vessels, particularly in subcutaneous tissues, dilated blood vessels including blood

vessels in the heart, and focal necrosis in the liver (Figure 4). However, we did not detect significant histological defects in the placenta of the affected embryos. The placental vasculature and labyrinthine layer thickness of the *VHL*^{f/d}/CreERTM embryos were comparable to those of their littermate controls. To look for any changes in cell proliferation or cell death preceding embryonic death, we performed Ki-67 and TUNEL staining of the embryos at E14.5. We did not find significant increase in cell death or decrease in cell prolifera-

tion in the affected embryos (data not shown). Thus, it is likely that vascular leakage induced by *VHL* inactivation is a major cause of embryo death.

We also inactivated *VHL* by tamoxifen injection to pregnant females at E9.0 and E12.5 to see the developmental stage specific effect of *VHL*-inactivation. Injection of 2 mg of tamoxifen was toxic to the embryos at E9.0 since 30% (4/12) of embryos showed lethality irrespective of their genotypes. When *VHL* was inactivated at E12.5 by injecting

2–4 mg of tamoxifen, we saw no pathological defects in dissected E17.5 or E18.5 *VHL^{f/d}/CreERTM* embryos (3/3) or in the placentas. However, 5–6 mg of tamoxifen injection induced embryo lethality in all the *VHL^{f/d}/CreERTM* embryos (4/4, 100%) at E17.5 or E18.5 and some of the control embryos (6/15, 40%). Gross morphological defects were also observed in the control embryos (2/15, 13%). Thus injection of high doses of tamoxifen at E12.5 induced toxicity to embryos.

Figure 3 Time course experiment shows lethality of the *VHL* inactivated embryos between E14.5 and E15.0. Embryos were collected at different time points between E12.5 and E15.5 after tamoxifen (2 mg) injection of pregnant females at E10.5. No significant changes were observed in the *VHL^{f/d}/CreERTM* embryos (a, 4/4 from two litters) before E14.5. Hemorrhage was observed in the *VHL^{f/d}/CreERTM* embryos at E14.5 (b, 2/2 embryos) and became extensive at E14.7 (c, 5/5 embryos) and later (d, 7/7 embryos). All the *VHL^{f/d}/CreERTM* embryos (12/12 embryos from four litters) were dead after E14.5. However, most of their littermate controls showed normal development (22/24 embryos from four litters, 92%). Blood circulation was normal in the *VHL^{f/d}/CreERTM* yolk sac up to E14.5 (j), but was impaired at E14.7 (k) and later (l). The *VHL^{f/+}/CreERTM* controls showed normal development (e–h) and normal blood circulation in the yolk sac (n–p). Higher magnification of the *VHL^{f/d}/CreERTM* embryo at E14.7 showed abnormal vasculature in the body (box in c). Both *VHL^{f/d}/CreERTM* and *VHL^{f/+}/CreERTM* embryos at E15.5 from the tamoxifen untreated (corn oil only) females showed normal development (i and m, respectively).

Figure 4 Histological analysis of the embryos showed hemorrhage, dilated blood vessels and necrotizing liver damage in the *VHL*-inactivated embryos. H&E staining of *VHL^{f/d}/CreERTM* embryos (b, e, and h) and placenta (k) before embryo death (E14.5) and comparison with *VHL^{f/+}/CreERTM* controls (a, d, g and j). Dilated blood vessels (arrow head), vascular lesion (arrow head) and blood cell leakage to surrounding tissues were often observed in the dorso-lateral region of the embryo (b and c). Focal necrotizing liver damage (asterisks in e and g), dilated blood vessels in heart (arrow head in h and i) and hemorrhage in the forebrain (white arrow in l) were observed. No significant developmental defects were observed in the *VHL^{f/d}/CreERTM* placenta (k) compared with the *VHL^{f/+}/CreERTM* placenta (j). *VHL*-inactivated embryo immediately following death (E14.7) displays large distended blood vessels (arrow heads in n) and hemorrhage (n) while the control (*VHL^{f/+}/CreERTM*) shows normal development (m). Higher magnification of the spinal cords in 'n' reveals extensive necrosis (o).

Upregulation of HIF-1 α and VEGF by the Inactivation of the *VHL* Gene

In order to study biochemical changes induced by homozygous *VHL* inactivation, we established and analyzed mouse embryo fibroblast (MEF) cell lines from E13.5 embryos harboring different *VHL* genotypes (*VHL*^{f/+}, *VHL*^{f/+}/*CreER*TM, *VHL*^{f/d} and *VHL*^{f/d}/*CreER*TM). During MEF cell culture, we inactivated the *VHL* gene in an acute manner in the *VHL*^{f/d}/*CreER*TM cells by addition of 4-hydroxy tamoxifen (4OHT) to the culture media. The *VHL* gene was inactivated in more than 90% of the cells by the action of *CreER*TM recombinase after 3 days of culture with tamoxifen (Figure 5a). As a result of *VHL* inactivation, VHL protein and mRNA expression were greatly reduced (Figure 5c left panel). As expected, HIF-1 α accumulated in the *VHL*-inactivated cells (Figure 5b). However, we could not detect HIF-2 α in the MEFs (data not shown). HIF-1 α accumulation resulted in upregulation of its downstream target, *VEGF*, in MEFs (Figure 5c right panel). Thus, we have established primary cells, in which the *VHL* gene could be inactivated in an acute manner by 4OHT providing an *in vitro* system for dissecting biochemical changes resulting from *VHL* inactivation.

Acidification of Culture Media by the Inactivation of the *VHL* Gene in the MEFs

We found that *VHL* inactivation of *VHL*^{f/d}/*CreER*TM MEFs by 4OHT induced acidification of the culture media based on phenol red indicator color change observed 7 days after tamoxifen addition. However, the media pH of the control MEFs (*VHL*^{f/+}/*CreER*TM) was maintained neutral with or without 4OHT treatment (Figure 5d). Upregulation of HIF mimics the hypoxic state, and glycolytic rate and lactate production were shown to be elevated in *VHL*^{-/-} embryonic stem cells.¹⁶ As in the *VHL*^{-/-} embryonic stem cells, lactate production was increased in MEFs with *VHL* inactivation (Figure 5e). Lactate production was not related to increased cell growth of the *VHL*-inactivated MEFs. Cell growth, determined by cell count 7 days after tamoxifen treatment, was actually decreased by *VHL* inactivation (Figure 5f). Decreased cell growth of the *VHL*^{-/-} MEFs has also been reported.²⁴ In addition to lactate production, the expression of *CAIX*, which regulates extracellular pH and is a target gene of HIF, was increased after *VHL* inactivation in the MEFs (Figure 5g).

Discussion

In this study, we inactivated the *VHL* gene at the mid-gestational stage of embryogenesis using a tamoxifen-inducible *CreER*TM system to avoid the early embryo lethality (E10.5–E12.5) seen in conventional *VHL*-KO mouse models. The conventional

VHL-KO mouse reported by Gnarr *et al*¹⁸ died during early embryogenesis due to placental defects. Using the inducible *CreER*TM system, we have overcome early embryonic lethality by inactivating the *VHL* gene at mid-gestation (E10.5), circumventing early placental defects, and have observed the effect of *VHL* inactivation specifically in the embryos at E14.5 or later.

We saw differential effects of tamoxifen-induced *VHL* inactivation in the embryo and in the placenta. Although we observed dramatic pathological changes in the embryos, we were unable to detect gross placental defects at the time of embryo death. One possible explanation is the restricted *Cre* recombinase expression in the placenta. *Cre* recombinase activity was primarily observed in the chorionic plate and umbilical vessels, and was nearly absent from the labyrinthine layer (Figure 1f), where *VHL* expression was high.¹⁸ In support of these observations the overall *VHL*-allele excision rate was much lower in the placenta compared with the embryo (Figure 2i). Thus, the phenotypes in the embryos most likely resulted from inactivation of the *VHL* gene in the embryo rather than from the indirect effect of *VHL* inactivation in the placenta. However, we cannot completely exclude possible involvement of the placenta in the defects of the embryos. Although we could not observe significant pathological changes at the time of embryo death, dilated blood vessels were often observed in the labyrinthine layer in the *VHL*-inactivated placenta at E16.5 (Figure 2g). Thus, it is likely that the limited effect of *VHL*-inactivation on the placenta at the time of embryo death becomes more pronounced later following a longer duration of time.

As in the conventional *VHL*-KO mouse model, *VHL* inactivation at mid-gestation led to embryonic death. However, in contrast to the conventional mouse model, *VHL*-inactivation at mid-gestation delayed embryo death until E14.5 and caused extensive hemorrhage, vascular defects and liver damage in the embryos but did not cause placental defects prior to embryo death. The technical improvement provided by the inducible *CreER*TM system allowed us to evaluate for the first time specific *VHL*-null-associated defects observed in the mid-gestational stage embryos. As with the *VHL* gene, inactivation of a number of genes has been found to cause early embryo lethality. Therefore, it is difficult to study the function of these genes at later stages of embryo development. However, as we report in this study, early embryonic lethality may be overcome by inactivating the gene of interest at mid-gestation through the use of inducible *Cre/lox* technology. To our knowledge, this is the first study showing the application of the *CreER*TM system to the analysis of a gene's function during embryogenesis. We found the *CreER*TM system to be useful for the study of genes, specifically *VHL*, which cause embryonic lethality when inactivated in mice.

Figure 5 *VHL* inactivation in MEFs. (a) The *VHL* gene was inactivated in *VHL^{f/d}/CreERTM* MEFs but not control MEFs by addition of 4OHT (1 μ M) as revealed by Southern blot analysis of the genomic tail DNA with a *VHL*-specific probe. (b) HIF-1 α accumulated in the *VHL* inactivated MEFs. (c) *VHL* protein and mRNA expression were abolished and VEGF mRNA expression was induced by *VHL* inactivation. (d) Culture media was acidified upon inactivation of the *VHL* gene by 5–400 nM 4OHT treatment as indicated by the color change of the phenol red indicator in media. MEFs were plated on six-well plates (7×10^4 cells/well) and 4OHT was added 24 h later. Media color change was monitored 7 days after 4OHT treatment. (e) 24 h lactate accumulation in the culture media was measured 7 days after 4 OHT treatment and normalized to cell number as described in the Materials and methods. Lactate production (per 1×10^6 cells) was increased upon inactivation of *VHL*. (f) However cell growth, based on cell counts performed 7 days after 4OHT addition and normalized to untreated control cultures, was decreased upon *VHL* inactivation. (g) CAIX mRNA expression was induced by the inactivation of *VHL* as revealed by semi-quantitative RT-PCR. *flox*, floxed *VHL* allele; *del*, deleted *VHL* allele; +, wild-type.

In addition, we established MEF cell lines, in which the *VHL* gene could be inactivated in an acute manner. *In vitro* analysis of the *VHL*-null MEFs

revealed HIF-1 α accumulation and VEGF overproduction. We also could observe metabolic and physiological changes in the *VHL*-null

MEFs produced more lactate, indicating increased glycolysis, and overexpressed *CAIX*, which contributed to acidification of the culture media. The biochemical analyses of the *VHL*-inactivated MEFs provide indirect evidence that the *VHL* gene plays an important role in the maintenance of vascular structure and integrity, and in protecting against liver damage during embryogenesis by regulating HIF-1 α and its target genes. However, a more direct way to test whether the highly vascular phenotype in the *VHL*-inactivated embryos is a consequence of HIF α activation would be to perform these experiments in a conditional *VHL* knockout mouse model in which HIF activation was conditionally suppressed during *VHL* inactivation and observe the subsequent effects on the vascular phenotype.

VEGF, one of the most studied targets of HIF, is a key regulator of vasculogenesis, angiogenesis, hematopoiesis and recruitment of leukocytes to inflammation sites.²⁵ The *VEGF* gene family consists of at least five members, including *VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGF-D* and placental growth factor (*PlGF*). These ligands bind distinct receptor tyrosine kinases, including VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), VEGFR-3 (Flt-4) and Neuropilin-1. VEGF receptors are expressed by both hematopoietic stem cells and angioblasts, which are precursors of blood cells and endothelial cells, respectively. VEGF signaling was shown to be critical to both hematopoiesis and vasculogenesis in studies with knockout mouse models of the ligand and its receptors. Embryos heterozygous for the *VEGF* gene die *in utero* between E11 and E12 showing defective vascular development and reduced blood cells within the blood island in the yolk sac.^{26,27} Gene targeting of the VEGFR-2 displayed a phenotype similar to the VEGF mutant with almost complete lack of endothelial cells and hematopoietic cells.²⁸

In this study, we inactivated the *VHL* gene at mid-gestation (E10.5) in an acute manner. Vasculogenesis and hematopoiesis begin as early as E7.5 and blood circulation in the embryo and yolk sac can be seen from E9.0. During mid-gestation the pre-existing vasculature, through which nutrients and oxygen are supplied to the developing embryo, becomes extensive by means of active angiogenesis. *VHL* inactivation at mid-gestation affected vascular stability and vascular structure in the developing embryos. VEGF, known as vascular permeability factor (VPF), increases vascular permeability during pathogenic blood vessel growth.²⁹ Increased permeability leads to leakage of small and large molecules, and often blood cells, into adjacent tissues. Hemorrhage is a consequence of blood cell leakage from blood vessels. Thus, the extensive hemorrhage of the *VHL*-inactivated embryos is most likely a result of increased vascular permeability by overproduced VEGF. The dilated blood vessels and disorganized vasculature are also probable consequences of

uncontrolled growth of endothelial cells by overproduced VEGF.

In addition to the vascular phenotype, we observed focal liver damage in the developing embryos after *VHL* inactivation. Such localized focal damage can be explained by the low Cre activity seen in the liver of the *CreERTM/ROSA26* transgenic embryos. During embryogenesis, hematopoiesis shifts from yolk sac to liver by E11.5. Thus, fetal liver generates blood cells as well as hepatocytes. As VEGF receptors are expressed by hematopoietic stem cells, it is intriguing to consider whether unbalanced production of VEGF induces this phenotype through VEGF signaling. We cannot rule out the possibility that other HIF-regulated cytokines, such as EPO, PDGF and TGF- α , are involved in liver damage. Of note, *VHL* heterozygous null mice develop hemangiomas in the liver spontaneously.^{12,13} Thus, the liver appears to be the organ most susceptible to *VHL* inactivation.

A common characteristic of tumors is their acidic environment. It has been shown that the low pH of the tumor environment is caused by excessive lactate production in tumor cells as well as by hydration of CO₂ to carbonic acid by carbonic anhydrase. As glycolysis is a dominating metabolic pathway in cancer cells, lactate is produced even under aerobic conditions. Generally tumors are in a hypoxic state, in which the glycolytic rate is increased by upregulation of the expression of glycolytic enzymes. *CAIX* and *CAXII* are induced by hypoxia.⁸ HIF is responsible for the expression of glycolytic enzymes and carbonic anhydrases under hypoxic conditions. Thus, acidification of culture media by *VHL* inactivation is regarded as a consequence of HIF stabilization in the MEFs caused by lactate overproduction and increased *CAIX* expression in these *VHL*-inactivated MEFs.

The results of our study support a role for the *VHL* gene in maintenance of vascular integrity and protecting liver function for hematopoiesis during mouse embryogenesis. This report is the first to provide a functional analysis of the *VHL* gene in the embryo during development. As many pathological processes involve increased vascular permeability and angiogenesis, our study suggests a possible involvement of the *VHL* gene in those processes. This inducible *VHL*-inactivation system should prove useful as an *in vivo* model for evaluating drugs that can inhibit vascular permeability and/or target HIF and HIF-regulated genes such as VEGF.

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References

- 1 Maher ER, Bentley E, Yates JR, *et al*. Mapping of the von Hippel-Lindau disease locus to a small region of chromosome 3p by genetic linkage analysis. *Genomics* 1991;10:957–960.
- 2 Latif F, Tory K, Gnarr J, *et al*. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 1993;260:1317–1320.
- 3 Iliopoulos O, Levy AP, Jiang C, *et al*. Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proc Natl Acad Sci USA* 1996; 93:10595–10599.
- 4 Maxwell PH, Wiesener MS, Chang GW, *et al*. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 1999;399:271–275.
- 5 Clifford SC, Cockman ME, Smallwood AC, *et al*. Contrasting effects on HIF-1 α regulation by disease-causing pVHL mutations correlate with patterns of tumorigenesis in von Hippel-Lindau disease. *Hum Mol Genet* 2001;10:1029–1038.
- 6 Flamme I, Krieg M, Plate KH. Up-regulation of vascular endothelial growth factor in stromal cells of hemangioblastomas is correlated with up-regulation of the transcription factor HRF/HIF-2 α . *Am J Pathol* 1998;153:25–29.
- 7 Pioli PA, Rigby WF. The von Hippel-Lindau protein interacts with heteronuclear ribonucleoprotein a2 and regulates its expression. *J Biol Chem* 2001;276: 40346–40352.
- 8 Ivanov S, Liao SY, Ivanova A, *et al*. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. *Am J Pathol* 2001; 158:905–919.
- 9 Hoffman MA, Ohh M, Yang H, *et al*. von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. *Hum Mol Genet* 2001;10:1019–1027.
- 10 Hergovich A, Lisztwan J, Barry R, *et al*. Regulation of microtubule stability by the von Hippel-Lindau tumour suppressor protein pVHL. *Nat Cell Biol* 2003; 5:64–70.
- 11 Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003;3:721–732.
- 12 Haase VH, Glickman JN, Socolovsky M, *et al*. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. *Proc Natl Acad Sci USA* 2001;98:1583–1588.
- 13 Ma W, Tessarollo L, Hong SB, *et al*. Hepatic vascular tumors, angiectasis in multiple organs, and impaired spermatogenesis in mice with conditional inactivation of the VHL gene. *Cancer Res* 2003;63:5320–5328.
- 14 Biju MP, Neumann AK, Bensinger SJ, *et al*. Vhlh gene deletion induces Hif-1-mediated cell death in thymocytes. *Mol Cell Biol* 2004;24:9038–9047.
- 15 Pfander D, Kobayashi T, Knight MC, *et al*. Deletion of Vhlh in chondrocytes reduces cell proliferation and increases matrix deposition during growth plate development. *Development* 2004;131:2497–2508.
- 16 Mack FA, Rathmell WK, Arsham AM, *et al*. Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. *Cancer Cell* 2003;3:75–88.
- 17 Rathmell WK, Hickey MM, Bezman NA, *et al*. *In vitro* and *in vivo* models analyzing von Hippel-Lindau disease-specific mutations. *Cancer Res* 2004;64: 8595–8603.
- 18 Gnarr JR, Ward JM, Porter FD, *et al*. Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. *Proc Natl Acad Sci USA* 1997;94:9102–9107.
- 19 Guo C, Yang W, Lobe CG. A Cre recombinase transgene with mosaic, widespread tamoxifen-inducible action. *Genesis* 2002;32:8–18.
- 20 Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999; 21:70–71.
- 21 Lin CY, Chen SH, Kou GH, *et al*. An enzymatic microassay for lactate concentration in blood and hemolymph. *Acta Zool Taiwan* 1999;10:91–101.
- 22 Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000;132:365–386.
- 23 Boley SE, Wong VA, French JE, *et al*. p53 heterozygosity alters the mRNA expression of p53 target genes in the bone marrow in response to inhaled benzene. *Toxicol Sci* 2002;66:209–215.
- 24 Mack FA, Patel JH, Biju MP, *et al*. Decreased growth of Vhl $^{-/-}$ fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. *Mol Cell Biol* 2005;25:4565–4578.
- 25 Ferrara N. Molecular and biological properties of vascular endothelial growth factor. *J Mol Med* 1999;77: 527–543.
- 26 Carmeliet P, Ferreira V, Breier G, *et al*. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;380:435–439.
- 27 Ferrara N, Carver-Moore K, Chen H, *et al*. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;380: 439–442.
- 28 Shalaby F, Rossant J, Yamaguchi TP, *et al*. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995;376:62–66.
- 29 Bates DO, Harper SJ. Regulation of vascular permeability by vascular endothelial growth factors. *Vasc Pharmacol* 2002;39:225–237.