Acute *Vhl* Gene Inactivation Induces Cardiac HIF-Dependent Erythropoietin Gene Expression

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### Abstract

Von Hippel Lindau (*Vhl*) gene inactivation results in embryonic lethality. The consequences of its inactivation in adult mice, and of the ensuing activation of the hypoxia-inducible factors (HIFs), have been explored mainly in a tissue-specific manner. This mid-gestation lethality can be also circumvented by using a floxed *Vhl* allele in combination with an ubiquitous tamoxifen-inducible recombinase Cre-ER 12. Here, we characterize a widespread reduction in *Vhl* gene expression in *Vhl* floxed UBC-Cre-ER 12 adult mice after dietary tamoxifen administration, a convenient route of administration that has yet to be fully characterized for global gene inactivation. *Vhl* gene inactivation rapidly resulted in a marked splenomegaly and skin erythema, accompanied by renal and hepatic induction of the erythropoietin (*Epo*) gene, indicative of the in vivo activation of the oxygen sensing HIF pathway. We show that acute *Vhl* gene inactivation also induced *Epo* gene expression in the heart, revealing cardiac tissue to be an extra-renal source of EPO. Indeed, primary cardiomyocytes and HL1 cardiac cells both induce *Epo* gene expression when exposed to low O2 tension in a HIF-dependent manner. Thus, as well as demonstrating the potential of dietary tamoxifen administration for gene inactivation studies in UBC-Cre-ER 12 mouse lines, this data provides evidence of a cardiac oxygen-sensing VHL/HIF/EPO pathway in adult mice.

### Introduction

The ability of cells to respond to low O2 supply (hypoxia) is fundamental in numerous pathological scenarios [1]. Hypoxia-inducible transcription factors (HIFs) are oligomeric transcription factors that stabilize under hypoxic conditions. They consist of two subunits: an alpha (α) and a beta (β) isoform. The presence of the β subunit is a discriminant factor in the tissue-specificity of HIFs, whereas the agony of the α subunits is regulated by the presence of oxygen and the hydroxylation of specific prolyl residues by prolyl-hydroxylase domain proteins (PHDs) 1, 2 and 3, and the von Hippel Lindau (*VHL*) protein [2,3]. This prolyl hydroxylation marks the α subunits for degradation by the proteasome [4,5]. As a result, these HIFα isoforms are stabilized and form a heterodimer with the HIFβ subunit, promoting the expression of many genes involved in cellular adaptation to hypoxia [6]. This includes the expression of erythropoietin (*Epo*) in the kidney and liver in order to facilitate oxygen delivery to hypoxic tissues [7,8,9,10]. Global *Vhl* gene inactivation in mice, and the ensuing HIF activation, can be used as a strategy to explore hypoxia signalling *in vivo*. However, conventional global *Vhl* gene inactivation is lethal in embryos [11], although this can be circumvented by only inducing *Vhl* gene inactivation in adult mice.

Widespread and acute gene inactivation in adult mice can be achieved through the ubiquitous expression of an inducible Cre recombinase, which can be used to eliminate the *Vhl* allelic region flanked by two loxP sites (a floxed *Vhl* allele). The nuclear activity of Cre can be induced by fusing it to a mutant form of the human estrogen receptor (ER) 12 that does not recognize its natural ligand (17β-estradiol) at physiological concentrations but rather, it binds the synthetic estrogen receptor ligand 4-hydroxystamoxifen (4-HT) [12]. This Cre-ER 12 is retained in the cytoplasm and only enters the nucleus in the presence of 4-HT, where it binds to loxP sites of the corresponding floxed alleles. Like other ubiquitous promoters, widespread Cre-ER 12 expression can be achieved in mice using the human ubiquitin C (UBC) promoter (UBC-Cre-ER 12 mice) [13]. Several means of administering tamoxifen have been described in rodents, including intraperitoneal injections and gavage [13]. However, the addition of 4-HT to powdered food or the administration of tamoxifen via drinking water is hampered by its poor solubility, its delivery in food has been successfully described in rodents, including intraperitoneal injections and gavage [13]. However, the addition of 4-HT to powdered food or the administration of tamoxifen via drinking water is hampered by its poor solubility, its delivery in food has been successfully achieved in several mouse lines [14,15,16]. While the administration of tamoxifen via drinking water is hampered by its poor solubility, its delivery in food has been successfully achieved in several mouse lines [14,15,16]. However, to date, the full potential of a tamoxifen diet and its efficacy in inducing global Cre-ER 12 activity in different organs of a Cre-ER 12 transgenic mouse line (e.g. UBC-Cre-ER 12 mice) has not been fully explored.

Here we have successfully employed diet-based tamoxifen administration, a timesaving and convenient mean of delivering
tamoxifen in order to induce widespread inactivation of the Vhl gene in a Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mouse line. After validating the efficiency of tamoxifen dietary administration, we characterized VHL/HIF oxygen-sensing dependent events that were readily induced by global Vhl inactivation in <i>vo vo</i> (within just a few days) in contrast to other works that have mainly studied the in <i>vo</i> consequences of Vhl gene inactivation over several weeks [17,18]. This study validates the use of the tamoxifen diet in UBC-Cre-ERT<sup>2</sup> mouse lines for global gene inactivation, and it identifies an oxygen-sensing VHL/HIF pathway controlling extra-renal <i>Epo</i> gene expression in cardiac tissue.

**Results**

**Postnatal tamoxifen diet-mediated Vhl gene inactivation**

Global Vhl gene inactivation results in embryonic lethality, at least in part due to placental dysfunction [11], preventing the study of the global loss of this gene in adult mice. We were interested in the short-term effects of activating the oxygen-sensing HIF pathway in <i>vo vo</i>, as a result of global Vhl gene inactivation in adult Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice through dietary tamoxifen administration. Since the full potential of dietary tamoxifen administration for global gene inactivation has not been explored previously, we first validated the efficacy of the tamoxifen diet in reducing Vhl gene expression in the Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mouse line. Age-matched Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> as well as control mice Vhl<sup>floxed</sup> and Vhl<sup>wt-</sup>-UBC-Cre-ERT<sup>2</sup> were maintained for 10 days on an ad <i>libitum</i> diet of tamoxifen pellets (containing 400 mg/kg tamoxifen), before they were switched to a diet of normal chow for a further 10 days and Vhl gene expression was analyzed by quantitative real-time PCR in the different mouse organs. Hereinafter, the terms Vhl<sup>floxed</sup>, Vhl<sup>wt-</sup>-UBC-Cre-ERT<sup>2</sup> and Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> refer to mice that have been administered a tamoxifen diet as indicated above. The tamoxifen diet significantly reduced Vhl gene expression in the kidney, spleen, liver, skeletal muscle, brown adipose tissue (BAT), heart, lung and brain of Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice, reflecting widespread Vhl gene inactivation (Figure 1A). No differences in tamoxifen intake were observed between Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> and control mice (Figure 1B). Significantly, gene inactivation was not homogeneous and expression of the Vhl gene was more strongly downregulated in the kidney and spleen, and less so in other tissues such as the brain and lung (Figure 1A). To further validate the specificity of Vhl gene inactivation, we also quantified Vhl gene expression in another UBC-Cre-ERT<sup>2</sup> system, the Hif1α<sup>flox</sup>-UBC-Cre-ERT<sup>2</sup> mouse line and their corresponding Hif1α<sup>−/−</sup> and Hif1α<sup>−/−</sup>-UBC-Cre-ERT<sup>2</sup> control mice. While there were no significant differences in tamoxifen intake between the different lines (Figure 1D), Hif1β gene expression was dramatically and globally reduced, while Vhl gene expression was not affected in tamoxifen fed Hif1α<sup>flox</sup>-UBC-Cre-ERT<sup>2</sup> mice (Figure 1C, E).

As mice were transiently exposed to a different diet, we evaluated their body weight before and after tamoxifen treatment. Baseline body weight diminished in a similar way (~10%) in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> and control mice after 10 days on the tamoxifen diet (Figure 2). However, while the body weight of control mice returned to pre-tamoxifen levels just one day after switching back to a normal diet (Figure 2), that was not the case in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice following tamoxifen treatment (Figure 2), suggesting that body weight was rapidly compromised by Vhl gene inactivation.

**Gross appearance of mice shortly after acute Vhl inactivation**

To further evaluate the efficacy of the tamoxifen diet on Vhl gene inactivation, we studied the biological consequences of acute Vhl inactivation soon after the mice returned to a normal diet (10 days). We evaluated spleen size and skin erythema as macroscopic indicators of activation of the HIF oxygen-sensing pathway in <i>vo vo</i> [19]. All tamoxifen-treated Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice analyzed exhibited marked splenomegaly when compared with controls (Figure 3 A, B). Moreover, some mice displayed obvious reddening of their paws and snouts (Figure 3 C, D). These external signs of skin erythema appeared as early as the ninth day of tamoxifen administration (data not shown), suggesting that this phenotype represents an acute manifestation of Vhl gene inactivation. Overall, these data confirm that dietary administration of tamoxifen is an efficient and convenient mean to induce widespread and rapid gene inactivation of floxed alleles in UBC-Cre-ERT<sup>2</sup> mice and in particular, to study the short-term biological consequences of Vhl inactivation.

**Acute Vhl inactivation induces cardiac Epo gene expression**

Splenomegaly and erythema are recognized signs of activation of the oxygen-VHL/HIF/EPO pathway, and they have been reported previously in transgenic mice overexpressing EPO [20,21]. Given that the kidney and liver are the main sites of EPO production in adults [7,22,23], we investigated Epo gene expression in these organs in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice shortly after Vhl gene inactivation. When we analyzed renal and hepatic Epo gene expression in tamoxifen-treated Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice, we found a strong induction of this gene in the kidney (~200 fold, Figure 4A) and an even stronger increase in the liver when compared to control mice (Figure 4B). The marked difference between these two organs is probably due to the very low basal levels of hepatic Epo gene expression, which results in more marked differences when Vhl is inactivated. These differences cannot simply be attributed to differences in Vhl inactivation, as Vhl is inactivated to a greater extent in the kidney than in the liver (Figure 1A). Moreover, serum EPO levels were drastically elevated in tamoxifen-treated Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> when compared with tamoxifen-treated control mice (pg of EPO/ ml: 150.5 ± 22.6 in Vhl<sup>floxed</sup> versus 49835.5 ± 21586 in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup>; n = 4, p < 0.05). These mice showed a remarkable reticulocytosis. Indeed, the number of circulating reticulocytes as well as splenic reticulocytes increases in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice (Circulating reticulocytes x10<sup>6</sup>/ml : 603.92 ± 437 in Vhl<sup>floxed</sup> versus 6391.53 ± 1381 in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup>; n = 3, p = 0.018) (Splenic reticulocytes x10<sup>6</sup>/ml : 32 ± 6.08 in Vhl<sup>floxed</sup> versus 269.08 ± 4.4 in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup>; n = 3, p = 0.01). However, a parallel hemocytometry showed that hemocrit is not significantly elevated in tamoxifen-treated Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> when compared with control mice (hematocrit %: 40.8 ± 2.02 in Vhl<sup>floxed</sup> versus 43 ± 5.3 in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup>; n = 5, p = NS). Furthermore, a follow up of Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice revealed that they started to show anemia after a longer time period upon Vhl gene inactivation (hematocrit %: 42.62 ± 2.22 in Vhl<sup>floxed</sup> versus 33.2 ± 5.8 in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup>; n = 7, p = 0.041). In addition, the proportion of Hoechst 33342 negative CD71 negative cells decrease in the spleens of Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice (% of total number of splenic cells: 27.10 ± 5.3 in Vhl<sup>floxed</sup> versus 3.22 ± 1.5 in Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup>; n = 3, p < 0.01). This possibly reflects a specific VHL-dependent effect on mature red blood cells survival that will be further explored in futures studies.

In line with other studies, baseline Epo gene expression was particularly weak in the heart [8], although we found a remarkable elevation in cardiac Epo gene expression in tamoxifen fed Vhl<sup>floxed</sup>-UBC-Cre-ERT<sup>2</sup> mice (Figure 4D). Given that cardiac Vhl
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expression is not fully ablated in tamoxifen-treated Vhl\textsuperscript{floxed-UBC-Cre-ER\textsuperscript{T2}} mice (Figure 1A), we presumed that cardiac Epo gene expression could be potentially higher if Vhl deletion were more prominent. Expression of glucose transporter-1 (Glut1), a HIF-dependent gene [24], was also elevated in the hearts of tamoxifen treated Vhl\textsuperscript{floxed-UBC-Cre-ER\textsuperscript{T2}} mice (Figure 4D). In addition, Epo gene expression was markedly upregulated in the brain (Figure 4C), possibly reflecting oxygen-sensing VHL/HIF-dependent EPO production in glial cells, as described previously [25]. Induction of Epo gene expression was stronger in cardiac tissue than in the brain, perhaps due to the weak basal expression of the Epo gene in the heart. These data suggest that the oxygen-sensing VHL/HIF/EPO pathway is not restricted to classical EPO-producing tissues, and they demonstrate that the heart can express EPO upon Vhl inactivation. To determine whether cardiomyocytes could be contributing to this VHL-dependent response, Epo gene expression was analyzed in isolated primary rat cardiomyocytes exposed to low oxygen tension. While weak basal expression of the Epo gene was observed in normoxic cardiomyocytes, hypoxia (1% O\textsubscript{2}) augmented markedly its expression (Figure 5A). Likewise, Glut1 expression was also induced, which indicates an effective induction of the HIF pathway in these experimental conditions (Figure 5B). We further evaluated the role of the HIF system in hypoxia-induced Epo gene expression in cardiac cells in the HL-1 cell line, a well-recognized cardiac cell model that retains a differentiated cardiac myocyte phenotype and maintains contractile activity [26].

Discussion

The oxygen-sensing VHL/HIF dependent pathway plays a central role in cellular adaptation to oxygen fluctuations [27,28]. This role has primarily been explored in mouse models in which HIF is chronically overactivated following tissue-specific Vhl inactivation [22,25,29]. Here, we characterize the short-term \textit{in vivo} responses following global inactivation of Vhl in the Vhl\textsuperscript{floxed}.

![](image)

**Figure 1.** Vhl and Hif1\textalpha gene expression in tamoxifen-fed Vhl\textsuperscript{floxed-UBC-Cre-ER\textsuperscript{T2}} and Hif1\textsuperscript{a-floxed-UBC-Cre-ER\textsuperscript{T2}} mice. (A) Vhl\textsuperscript{wt-UBC-Cre-ER\textsuperscript{T2}} (n = 3), Vhl\textsuperscript{floxed} (n = 6) and Vhl\textsuperscript{floxed-UBC-Cre-ER\textsuperscript{T2}} (n = 6) mice were placed on a tamoxifen diet for ten days followed by ten additional days on a normal diet. Gene expression was assessed by RT-PCR in the tissues indicated, the expression of the Vhl gene was normalized to that of Hprt and it was expressed as the change relative to Vhl\textsuperscript{floxed} mice. (B) Tamoxifen intake was measured over the 10 days of tamoxifen administration in Vhl\textsuperscript{wt-UBC-Cre-ER\textsuperscript{T2}} (n = 3), Vhl\textsuperscript{floxed} (n = 6) and Vhl\textsuperscript{floxed-UBC-Cre-ER\textsuperscript{T2}} (n = 6) mice. (C-E) Hif1\textsuperscript{a-floxed-UBC-Cre-ER\textsuperscript{T2}} (n = 4), Hif1\textsuperscript{a-floxed} (n = 3) and Hif1\textsuperscript{a-floxed-UBC-Cre-ER\textsuperscript{T2}} (n = 5) mice were administered tamoxifen as indicated above. Hif1\textsuperscript{a} or Hif1\textsuperscript{a} gene expression was normalized to that of Hprt and expressed as the change relative to Hif1\textsuperscript{a-floxed} mice. (D) Tamoxifen intake in Hif1\textsuperscript{a-floxed-UBC-Cre-ER\textsuperscript{T2}} (n = 4), Hif1\textsuperscript{a-floxed} (n = 3) and Hif1\textsuperscript{a-floxed-UBC-Cre-ER\textsuperscript{T2}} (n = 5) mice was measured as in B. Total intake per day was expressed relative to the body weight at the end of the tamoxifen treatment and the values represent the mean ± SEM. Statistical significance was assessed using a two-tailed Student’s t-test, (*, p < 0.05; **, p < 0.01) when comparing Vhl\textsuperscript{wt-UBC-Cre-ER\textsuperscript{T2}} or Hif1\textsuperscript{a-floxed-UBC-Cre-ER\textsuperscript{T2}} with Vhl\textsuperscript{floxed-UBC-Cre-ER\textsuperscript{T2}} or Hif1\textsuperscript{a-floxed-UBC-Cre-ER\textsuperscript{T2}} respectively; (##, p < 0.01) when comparing Vhl\textsuperscript{floxed} or Hif1\textsuperscript{a-floxed} with Vhl\textsuperscript{floxed-UBC-Cre-ER\textsuperscript{T2}} or Hif1\textsuperscript{a-floxed-UBC-Cre-ER\textsuperscript{T2}} respectively.

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**Figure 2.** Body weight during and after tamoxifen diet administration in Vhl\textsuperscript{floxed-UBC-Cre-ER\textsuperscript{T2}} and control mice. Body weight of Vhl\textsuperscript{wt-UBC-Cre-ER\textsuperscript{T2}} (n = 3), Vhl\textsuperscript{floxed-UBC-Cre-ER\textsuperscript{T2}} (n = 6) control Vhl\textsuperscript{floxed} (n = 6) mice was measured before tamoxifen treatment (TFX 0d), at the end of 10 days on a tamoxifen diet (TFX 10d) and one day after returning to a normal diet (N +1d). Statistical significance was assessed using a two-tailed Student’s t-test, (*, p < 0.05; **, p < 0.01; ns, no significant differences).

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The full potential for global gene inactivation was not previously explored. Indeed, dietary administration of tamoxifen has been characterized in mice with specific Cre-ERT2 expression in the heart, forebrain or in endothelial cells [14,15,16]. However, a comparative analysis of the efficiency of tamoxifen diet in different organs to determine its full potential to induce widespread gene inactivation has not been performed. Moreover, some of these studies have required several weeks on a tamoxifen diet. Here, we describe global gene inactivation in UBC-Cre-ERT2 mouse lines shortly (a few days) after tamoxifen administration.

In this first place, it appears that UBC-Cre-ERT2 is suitable to produce global gene inactivation in animals fed with a tamoxifen diet. However, tamoxifen-mediated Vhl gene inactivation was less pronounced in the Vhlfloxed-UBC-Cre-ERT2 line than Hif1α gene inactivation in Hif1αlox/lox-UBC-Cre-ERT2 line, an effect that could not be attributed to differences in tamoxifen intake. This differential inactivation may reflect the specific efficacy of the Cre-ERT2 recombinase to act on the floxed region of the Vhl and Hif1α alleles. Thus, optimization of the tamoxifen diet may be necessary to achieve comparable effects in distinct UBC-Cre-ERT2 mouse lines. Nevertheless, the extent to which Vhl gene expression was reduced in these mice was sufficient to induce the activation of oxygen-sensing HIF pathways in vivo. Indeed, macroscopic

Figure 3. Gross appearance of tamoxifen-fed Vhlfloxed-ERT2 mice. (A) Vhlwt-UBC-Cre-ERT2 (n = 3), Vhlfloxed (n = 9) and Vhlfloxed-UBC-Cre-ERT2 (n = 10) mice were administered tamoxifen as indicated in Figure 1 and the spleen/body weight ratio was then determined. Statistical significance was assessed using a two-tailed Student’s t-test (*, p<0.05; **, p<0.01). Representative images of spleens (B), snouts (C) and paws (D) of Vhlfloxed-UBC-Cre-ERT2 and control Vhlfloxed mice are shown.

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examination of tamoxifen-treated Vhl\textsuperscript{floxed}-UBC-Cre-ERT\textsuperscript{2} mice revealed marked splenomegaly, an indicator of increased activity of the oxygen-VHL/PHD/HIF sensing pathway, as seen in Phd2 deficient and Phd1:Phd3 double knock-out mice [19]. Tamoxifen-treated Vhl\textsuperscript{floxed}-UBC-Cre-ERT\textsuperscript{2} mice also rapidly show signs of skin erythema (Figure 3). Indeed, reddening of the paws and snouts can be apparent as early as the ninth day of tamoxifen administration (data not shown). This could reflect an increased blood flow to the skin as a consequence of local HIF-induced nitric oxide (NO) release and subsequent local vasodilatation as has been previously shown upon chronic epidermal Vhl deletion [30]. Boutin et al. also show that this increased cutaneous perfusion, as a consequence of epidermal Vhl gene inactivation, subsequently reduces liver/skin blood flow ratio leading to elevated hepatic \textit{Epo} gene expression.

**Figure 4. Erythropoietin gene expression in the kidney, liver, brain and heart of tamoxifen-fed Vhl\textsuperscript{floxed}-UBC-Cre-ERT\textsuperscript{2} mice.** Vhl\textsuperscript{wt}, UBC-Cre-ERT\textsuperscript{2} (n = 3), Vhl\textsuperscript{floxed} (n = 6) and Vhl\textsuperscript{floxed}-UBC-Cre-ERT\textsuperscript{2} (n = 6) mice were administered tamoxifen as indicated in Figure 1. Gene expression was assessed by RT-PCR in the kidney (A), liver (B), brain (C) and heart (D). The expression of \textit{Epo} and \textit{Glut1} was normalized to that of \textit{Hprt} and expressed as the change relative to Vhl\textsuperscript{floxed} mice. Statistical significance was assessed using a two-tailed Student’s t-test (*, p < 0.05; **, p < 0.01).

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gene expression [30]. However, the increased hepatic Epo gene expression observed in tamoxifen-treated Vhl\textsuperscript{floxed}-UBC-Cre-ERT\textsuperscript{2} mice is most likely a consequence of local Vhl gene deletion and HIF2\textalpha activation in liver.

It should be noted that other similar genetic systems have been developed to achieve inactivation of floxed alleles. Indeed, the tetracycline-dependent (Tet) system has been used for renal-specific Cre expression and subsequent inactivation of the tuberous sclerosis complex-1 (Tsc-1) when doxycycline is administered in the drinking water [31]. However, some difficulties in activating this doxycycline-dependent system in certain tissues have been reported [32,33,34]. By contrast, gene expression is significantly reduced in all the tissues analyzed from both Vhl\textsuperscript{floxed}-UBC-Cre-ERT\textsuperscript{2} and Hif1\alpha\textsuperscript{floxed}-UBC-Cre-ERT\textsuperscript{2} mouse lines exposed to tamoxifen diet.

Tamoxifen-treated Vhl\textsuperscript{floxed}-UBC-Cre-ERT\textsuperscript{2} mice have identified the heart as an additional site of EPO production upon Vhl inactivation. Indeed, the baseline expression of the Epo gene in the heart is weak but is elevated dramatically upon inactivation of Vhl gene expression. Experiments on isolated neonatal rat cardiomyocytes

![Figure 5. Erythropoietin and glucose transporter-1 gene expression in isolated primary cardiomyocytes in response to hypoxia.](image-url)

Isolated rat cardiomyocyte cultures were subjected to basal normoxic conditions and/or hypoxia (1% O\textsubscript{2}) for 24 hours. Epo (A) and Glut1 (B) expression was then analyzed by RT-PCR and normalized to that of Hprt. The data from four independent experiments are expressed as the change relative to the normoxic values. Statistical significance was assessed using a two-tailed paired t-test (*, p<0.05).

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![Figure 6. Erythropoietin and glucose transporter-1 gene expression in HL-1 cardiomyocyte cell line in response to activation of the oxygen-sensing HIF pathway.](image-url)

(A,B,C) HL-1 cells were transfected with a siRNA for Hif1\alpha (siHif1\alpha) or a scrambled siRNA control (siSCR) and 24 hours after transfection, the cells were exposed to normoxic or hypoxic (1% O\textsubscript{2}) conditions. The expression of Epo, Glut1 and Hif1\alpha was measured as described above and the data from three independent experiments are expressed as the change relative to the normoxic values. Statistical significance was assessed using a two-tailed Student's t-test (*, p<0.05).

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ocytes revealed that EPO upregulation is an autonomous cardiomyocyte response to hypoxia that is mediated by the oxygen-sensing VHL/HIF pathway. This response is also observed in the HL-1 cardiac cell line, an experimental model suitable to study EPO production in adult cardiac cells. HL-1 is a cardiac cell line derived from the AT-1 adult mouse atrial cardiomyocyte tumor lineage, and these cells retain a differentiated cardiomyocyte phenotype and they maintain contractile activity [26]. Moreover, erythropoietin production has been demonstrated after myocardial infarction [35], which on the basis of our data could be mediated by cardiac HIF activation.

HIF1α gene expression is higher than HIF2α in HL-1 cells, which may explain the predominant contribution of HIF1α to hypoxia-induced Epo gene expression in these cardiac cells. However, the relative contribution of each isoform may differ in vivo and indeed, immunohistological studies have identified both HIF1α and HIF2α in cardiomyocytes of mice subjected to ischemia or atmospheric hypoxia [36,37,38,39,40]. Hif1α floxed mice expressing Cre driven by myosin light chain 2v (MLC2v) cardiac promoter (Hif1α floxed-MLC2v-Cre mice) markedly reduced HIF1α mRNA and protein expression in the heart, providing genetic evidence of Hif1α gene expression in cardiomyocytes [36]. Several studies have demonstrated a critical role for HIF1α in multiple cardiac oxygen-sensing pathways in vivo [29,36,41]. Thus, HIF1α could potentially drive cardiac Epo gene expression upon Vhl gene inactivation. However, HIF2α is the main contributor to HIF-induced Epo gene expression upon Vhl gene inactivation in the kidney, liver and glial cells [22,25,42,43]. Further studies will therefore be required to assess the relative contribution of these isoforms in vivo, and especially that of HIF2α to VHL/HIF-dependent cardiac EPO expression. It should be also noted that HIF1α and HIF2α are also found in cardiac stromal cells. Indeed, cardiac endothelial cells abundantly express both HIF isoforms when oxygen supply to myocardium becomes limited, as do cells in the vessel wall that are presumably smooth muscle cells, [36,37,38,39,40]. Therefore, cardiac Epo gene expression upon Vhl gene inactivation involves HIF activation in cardiomyocytes, although we cannot rule out the involvement of HIF activation in other cardiac cell types.

Elevation of cardiac Epo gene expression is very remarkable, although it occurs to a lesser extent than in the liver and kidney. Therefore, it is conceivable that cardiac EPO production serves a local autocrine or paracrine function when oxygen supply to cardiac tissue becomes limited. Indeed, several studies have shown that EPO protects cardiac tissue during ischemia and the ischemia-reperfusion insult, particularly by activating the serine threonine kinase AKT, as well as through other pathways involving sonic hedgehog [44,45,46]. Indeed, the myocardium of patients undergoing bypass is protected when pyruvate, a previously recognized suppressor of PHD activity, is used [47], which correlates with a remarkable upregulation of Epo gene expression [48]. However, the effect of pyruvate on Epo gene expression was not directly assessed in cardiac cells, nor was the direct contribution of HIF activity, as we have studied in this work. Furthermore, cardiac tolerance to ischemic damage induced by ischemic preconditioning in the heart involves HIF1α mediated upregulation of key cardioprotective molecules, such as ecto-5'-nucleotidase CD73 that generates adenosine, and the A2B adenosine receptor (A2BAR) [49]. Therefore, the cardiac oxygen-sensing VHL/HIF/EPO pathway may represent an endogenous cardioprotective response that works in tandem with other pathways (e.g. adenosine) to locally induce cardiomyocyte tolerance against ischemia or ischemia-reperfusion damage.

**Materials and Methods**

**Ethics Statement**

All the experimental procedures were approved by the Research Ethics Committee at the UAM (Autonomous University of Madrid) and they were carried out under the supervision of the Head of Animal Welfare and Health at the UAM in accordance with Spanish and European guidelines (B.O.E., 18 March 1988, and 86/609/ECC European Council Directives).

**Cell culture and hypoxic conditions**

The murine HL-1 cardiac cell line was cultured in Claycomb medium [26] containing 10% heat-inactivated Fetal Bovine Serum (FBS: Cambrex) and supplemented with 0.1 mM norepinephrine (Sigma) and 2 mM GLUTAMAX-I (Invitrogen). Cells were plated on gelatin (Difco) and fibronectin (Sigma) precoated surfaces, and cultured at 37°C for 16 hours. Neonatal rat cardiomyocytes were isolated from the hearts of 1 day-old Wistar rats using the Neomycin isolation system (Cellutron Life Technologies). To remove contaminating cardiac fibroblasts, dissociated cells were pre-plated for 1 hour on uncoated culture plates. The resulting suspension of cardiomyocytes was plated (2–3 million cells/60 mm plate) and cultured for 24 hours in medium supplemented with 10% FBS and 10 mM 5-bromo-2'-deoxyuridine (Brdu; Sigma, B5002), and then for an additional 24 hours in serum-free conditions. The cells were subjected to hypoxia in DMEM + 10% FBS. All media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 1% HEPES buffer. Normoxic cells (21% O2) were maintained at 37°C in an incubator with 5% CO2. To induce hypoxia, cell culture dishes were placed into an Invivo2 400 humidified hypoxia workstation (Ruskinn Technologies, Bridgend, UK) with 1% O2.

**Mice**

C57BL/6N-Jcl/Hsdmcr8-JsbKsJ mice (Jackson Laboratories, stock no. 4081) were used to generate the Vhl floxed-UBC-Cre-ER2 mice. These mice harbor two loxP sites flanking the promoter and exon 1 of the Vhl locus [50]. C57BL/6N-Jcl/Hsdmcr8-JsbKsJ mice were crossed with B6.Cg-Tg(UBC-Cre/ER2)1Ejb/J mice (Jackson Laboratories, stock no. 008085) which ubiquitously express a tamoxifen-inducible Cre recombinase (Cre-ER2), [13]. Vhl floxed-UBC-Cre-ER2 mice were generated through the appropriate crosses, along with the corresponding controls, Vhl floxed-UBC-Cre-ER2 and Vhl floxed- Hif1α floxed-UBC-Cre-ER2 mice were generated from B6.129-Hif1atm3Rsjo/J mice (Jackson Laboratories, stock no. 007561), which harbor two loxP sites flanking exon 2 of the murine Hif1α locus [51]. These mice were crossed with Tg(UBC-Cre/ER2)1Ejb/J mice as described above to generate Hif1α floxed, UBC-Cre-ER2 mice and their corresponding controls, Hif1α floxed, UBC-Cre-ER2 and Hif1α floxed mice.

The mice were bred and housed in a specific pathogen free (SPF) animal area of the animal facility at the Autonomous University of Madrid (UAM). For gene inactivation, Vhl floxed-UBC-Cre-ER2, Hif1α floxed-UBC-Cre-ER2 and the corresponding control males (10–5 weeks old) were fed ad libitum for ten days with Teckland CRD TAM 400/CreER tamoxifen pellets (Harlan Teklad), which contain 400 mg tamoxifen citrate/kg. Subsequently, they were returned to a diet of standard mouse chow (Safe®, Augy, France) for an additional 10 days.

**Reticulocyte counts and hematocrit measurement**

The number of circulating or splenic reticulocytes was determined by counting total blood or splenic cells respectively followed by a flow cytometry analysis to determine the proportion of reticulocytes identified as CD71 positive cells (using the anti-
Quantitative real-time PCR analysis and primers
Mice were anesthetized by intraperitoneal administration of ketamine (Ketolar® 50 mg/ml) and xylazine (Rompun® 20 mg/ml), and the tissues of interest were then removed and snap-frozen in liquid nitrogen. Subsequently, the tissue was homogenized in Trizol (Invitrogen) with two freeze/thaw cycles and total RNA was isolated using the RNeasy RNA extraction kit (Qiagen). cDNA was prepared by reverse transcription of RNA (1 ng) using Improm-II reverse transcriptase (Promega), and polymerase chain reaction (PCR) amplification was performed using a Power SYBR Green PCR Master Mix Kit (Applied Biosystems). The following primer sets were used: mouse VHL (forward, 5'-TCAGCCGC-TACGCGATCTCCG-3'; reverse, 5'-ATCCGCGAGGCGAAAGATGA-3'); mouse HIF-1α (forward, 5'-CAGGATTTGGCCATGGA-3'; reverse, 5'-AGGGCA-TATCCAAACGAAACTT-3'); rat HIF-1α (forward, 5'-GTCCCCTGGTGTTGACCTTCTCTCTGGAAG-3'; reverse, 5'-TGGAGTGCACATTGGAACATAGGCA-3'); mouse erythropoietin (EPO) (forward, 5'-TCATCTGCGACAGTCGAGTTCT-3'; reverse, 5'-TTTCTCAATCGTCGACAGTTCTCGTA-3'); mouse glu- optic acid transaminase (GOT; forward, 5'-AGGGCA-TATCCAAACGAAACTT-3'; reverse, 5'-AGGGCA-TATCCAAACGAAACTT-3'); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, 5'-GACCAAA-3'; reverse, 5'-GACCAAA-3'); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, 5'-GACCAAA-3'; reverse, 5'-GACCAAA-3').

Statistical analysis
The data are presented as the mean ± SEM. Statistical significance was assessed using a two-tailed Student’s t-test in all figures, except in Figures 6A and B in which a two-tailed paired t-test was used.

Author Contributions
Conceived and designed the experiments: MM-M AE IS-A LA JA. Performed the experiments: MM-M AE IS-A LA-A AV-V SV. Analyzed the data: MM-M AE IS-A MOL JA. Contributed reagents/materials/analysis tools: AO EB AV-V SV EF CF-C. Wrote the paper: MM-M AE IS-A LA-A MOL JA.

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Cardiac HIF-Dependent Erythropoietin Expression

siRNA transfection
HL-1 cells were transfected with a siRNA targeting mouse HIF-1α (50 nM, sc-44225; Santa Cruz) or a scrambled control siRNA (sc-37007), using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 24 hours after transfection the cells were exposed to normoxic or hypoxic conditions for an additional 24 hours.

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Author Contributions
Conceived and designed the experiments: MM-M AE IS-A LA JA. Performed the experiments: MM-M AE IS-A LA-A AV-V SV. Analyzed the data: MM-M AE IS-A MOL JA. Contributed reagents/materials/analysis tools: AO EB AV-V SV EF CF-C. Wrote the paper: MM-M AE IS-A LA-A MOL JA.
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