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Accumulation of hypoxia-inducible factor-1α is limited by transcription-dependent depletion

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In the presence of oxygen and iron, hypoxia-inducible factor (HIF-1α) is rapidly degraded via the prolyl hydroxylases (PHD)/VHL pathways. Given striking similarities between p53 and HIF-1α regulation, we previously suggested that HIF-1 transcriptionally initiates its own degradation and therefore inhibitors of transcription must induce HIF-1α. Under normoxia, while inducing p53, inhibitors of transcription did not induce HIF-1α. Under hypoxia or low iron (DFX), inhibitors of transcription dramatically super-induced HIF-1α. Removal of inhibitors resulted in outburst of the HIF-1-dependent transcription followed by depletion of HIF-1α. Although hypoxia/DFX induced PHD3, we excluded the PHD/VHL pathway in the regulation of HIF-1α under hypoxia/DFX. The transcription-dependent degradation of HIF-1α under hypoxia occurs via the proteasome and is accelerated by protein acetylation. Thus, HIF-1α is regulated by two distinct mechanisms. Under normoxia, HIF-1α is degraded via the classic PHD/VHL pathway, is expressed at low levels and therefore does not activate the feedback loop. But under hypoxia, HIF-1α accumulates and transcriptionally activates its own degradation that is independent from the PHD/VHL pathway.
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Introduction

In normal conditions (in the presence of oxygen and iron), hypoxia-inducible factor 1α (HIF-1α) is hydroxylated by three prolyl hydroxylases (PHD 1, 2, 3) (Semenza, 2001; Giaccia *et al*., 2004; Poellinger and Johnson, 2004). Then, HIF-1α binds VHL, which targets HIF-1α for ubiqitination and degradation (Maxwell *et al*., 1999; Ivan *et al*., 2001; Jaakkola *et al*., 2001; Yu *et al*., 2001). When levels of either oxygen or iron are low (e.g., hypoxia and treatment with the iron chelator – DFX, respectively), PHDs cannot hydroxylate HIF-1α (Ivan *et al*., 2001; Jaakkola *et al*., 2001). Then, HIF-1α does not bind VHL and is rapidly accumulated.

Thus, HIF-1 and p53 transcription factors share a unique mode of regulation by VHL and Mdm-2, respectively. Mdm-2 binds p53 and causes its ubiquitination and degradation (Haupt *et al*., 1997; Kubbutat *et al*., 1997). Similarly, VHL binds HIF-1α, causes its ubiquitination and degradation. In normal conditions, both p53 and HIF-1α are expressed at low levels. In response to DNA damage and hypoxia, respectively, p53 and HIF-1α accumulate. DNA damage and hypoxia abolish binding of p53 to Mdm-2 and binding of VHL to HIF-1α, respectively. It is important that p53 transcriptionally induces Mdm-2, thus completing a feedback loop: p53 transactivates *Mdm-2* and Mdm-2 initiates degradation of p53 (Haupt, 2004). By disrupting a transcription-dependent feedback loop, inhibitors of transcription (actinomycin D (ActD), DRB, flavopiridol (FL) induce p53 (Blagosklonny *et al*., 2002; Demidenko and Blagosklonny, 2004; Ljungman and DP, 2004). In analogy, a feedback loop involving HIF-1α and VHL has been suggested (Blagosklonny, 2001). If HIF-1 is regulated by a feedback loop, then inhibitors of transcription would induce HIF-1α. For example, by inhibiting Mdm-2, inhibitors of transcription induce p53 (Blagosklonny *et al*., 2002). This is the ultimate hallmark of transcription-mediated feedback loop. However, we found that inhibitors of transcription alone did not induce HIF-1 but caused its super-induction, when HIF-1α was induced by DFX or hypoxia. This indicates that HIF-1α is regulated by two mechanisms: (i) at low HIF-1 levels, it is regulated by the availability of oxygen and iron, whereas (ii) at high HIF-1 levels, it is kept under control by transcription-dependent degradation.

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Results

Hypoxia and DFX do not induce the VHL mRNA

Our initial hypothesis was that HIF-1 induces VHL, which in turn decreases HIF-1α. It has been shown that hypoxia can either induce (Turcotte *et al*., 2004) or decrease VHL in different cells (Kim *et al*., 2001). This prompted us to investigate the effects of hypoxia, DFX, and cobalt on the VHL mRNA levels at multiple time points. Here we found that neither hypoxia, nor DFX induced VHL (Figure 1). Furthermore, hypoxia slightly downregulated the VHL mRNA (Figure 1). The same results were obtained in several cell lines including MCF-7, A549, H157, UP293 (Figure 1 and data not shown).

Super-induction of HIF-1

As we discussed, it has been suggested that inhibitors of transcription may induce HIF-1α. Inhibitors of transcription such as actinomycin D (ActD) induce p53, because they disrupt transcription-dependent feedback loop (Blagosklonny *et al*., 2002). Therefore, if HIF-1α degradation is transcription-dependent, then inhibitors of transcription should induce HIF-1α. First, we compared effects of ActD (an inhibitor of transcription) on HIF-1α and p53. As expected, ActD induced p53 (Figure 2). In contrast, ActD did not induce HIF-1α (Figure 2). As expected, DFX induced HIF-1α (Figure 2). Most importantly, in the presence of DFX, ActD dramatically induced HIF-1α (Figure 2). Similar results were obtained in MCF-7 and A549 cells (Figure 2). Thus, once induced by DFX, HIF-1α was further induced by ActD.

Following removal of DFX, HIF-1α disappeared (Figure 2), consistent with its degradation under normal conditions. In contrast, when cells were incubated with DFX plus ActD, removal of DFX (change of the medium) did not result in degradation of HIF-1α (Figure 2b, washed). (It is important to emphasize that ActD, which binds DNA, cannot be washed out from cells. Therefore, only DFX was washed out in this experiment.) We conclude that the iron-initiated degradation of HIF-1α requires transcription.

Next, we excluded that under hypoxia ActD somehow induced HIF-1α mRNA (Figure 3). As expected, ActD slightly decreased HIF-1α mRNA (Figure 3), while HIF-1α protein was dramatically super-induced. ActD is a

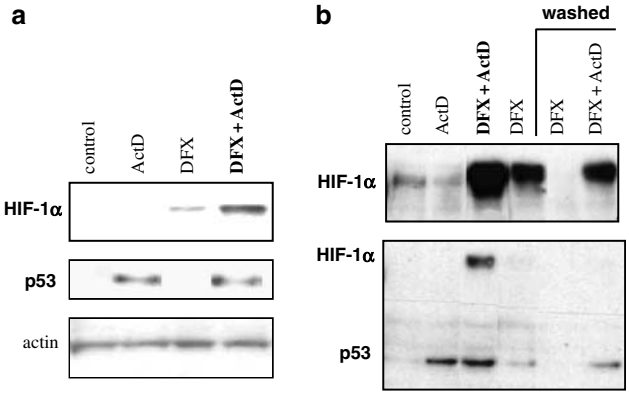

Figure 2 Super-induction of HIF-1. (**a**) MCF-7 cells were treated for 16 h with 260 μM DFX and 5 μg/ml ActD, as indicated. Immunoblot for HIF-1α, p53, and actin was performed. (**b**) A549 cells were treated for 24 h with 260 μM DFX and ActD. When indicated 'washed', the medium was changed for the last 8 h. Immunoblot for HIF-1α and p53 was performed. Upper panel, the film is overexposed to demonstrate induction of HIF-1α by DFX alone

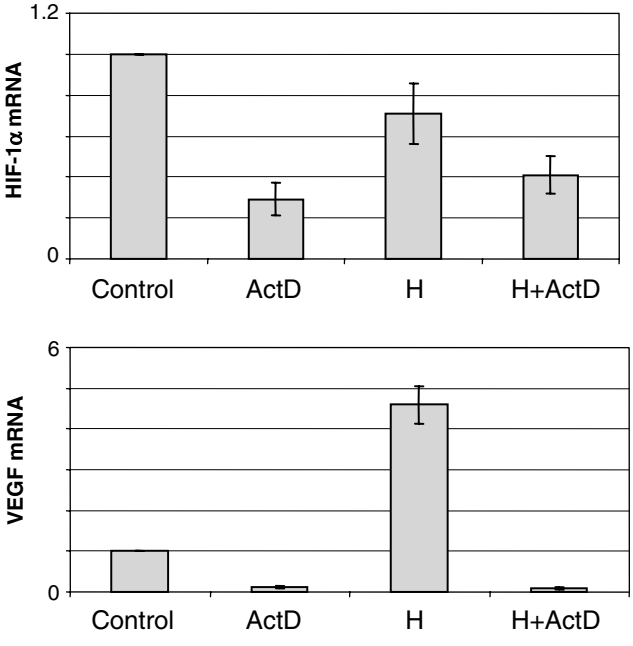

Figure 3 ActD does not induce HIF-1α mRNA. A549 cells were treated with ActD 5 μM, hypoxia 1%, ActD + hypoxia, or left untreated (control). (The same conditions as Figure 2b). After 24 h, mRNA for HIF-1α and VEGF (a HIF-1-inducible gene) were measured by real-time PCR as described in Materials and methods

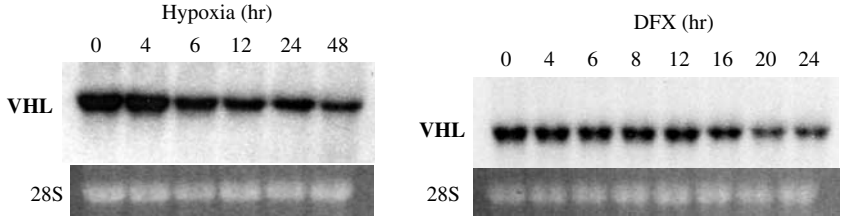

Figure 1 Effects of hypoxia and hypoxia mimicking conditions on VHL mRNA expression. MCF-7 cells were incubated under indicated conditions and Northern blot for VHL was performed as described in Materials and methods

DNA-damaging agent, which may have effects independent from inhibition of transcription. Therefore, it was necessary to confirm super-induction of HIF-1α using inhibitors of transcription with a different mechanism of action. We compared ActD and FL, which blocks transcription by inhibiting CDK9, which is required for transcriptional elongation (Chao and Price, 2001; de Azevedo *et al*., 2002; Demidenko and Blagosklonny, 2004). In fact, FL prevented hypoxia-mediated induction of VEGF and Cap43, HIF-1-inducible genes (Figure 4a). In agreement, FL inhibited HIF-1-dependent transcription of HIF-response element luciferase (HRE-Luc). As shown in Figure 4b, whereas DFX induced HRE-Luc, 400 nM FL completely blocked this induction. We next compared effects of ActD and FL on induction of HIF-1α by DFX (Figure 4c). DFX alone induced HIF-1α, which in turn induced Cap43 (a HIF-1-inducible protein). So DFX induced both HIF-1α and Cap43 (Figure 4c). Neither ActD nor FL alone induced HIF-1α. However, both ActD and FL dramatically super-induced HIF-1α in the presence of DFX. This super-induction of HIF-1α was not accompanied by Cap43 (Figure 4c). Similarly, DRB, another inhibitor of transcription, super-induced HIF-1α while inhibiting Cap43 (data not shown).

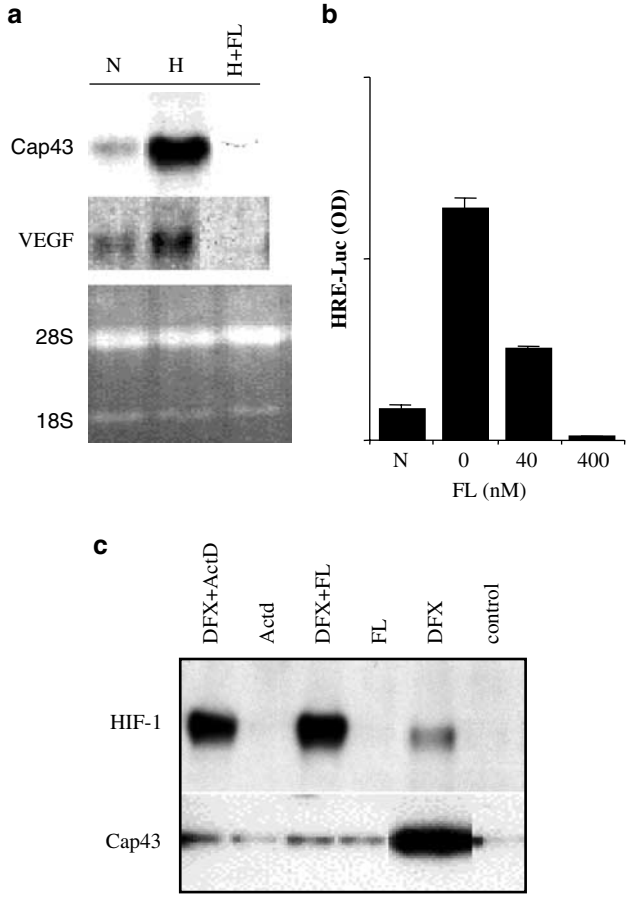

Figure 4 FL inhibits HIF-1-dependent transcription and super-induces HIF-1α. (**a**) A549 cells were incubated overnight under normoxia (N) and hypoxia with or without FL (400 nM flavopiridol) and then Northern blot was performed. (**b**) A549 cells were transfected with HRE-Luc and then either left untreated (N) or treated with DFX. DFX-treated cells were incubated with FL at indicated concentrations: 0, 40, 400 nM. After 16 h, luciferase activity was measured. (**c**) A549 cells were treated with ActD and FL with or without DFX. Immunoblot for HIF-1α and Cap43 (a HIF-1-inducible protein) was performed after 16 h

Super-induction of HIF-1-dependent transcription and HIF-1 degradation

Thus, inhibition of transcription during treatment with DFX caused dramatic accumulation of HIF-1α. FL can be washed out, thus restoring transcription (Demidenko and Blagosklonny, 2004). We predicted that this must result in rapid depletion of HIF-1α. We have transfected cells with HRE-Luc (a marker of HIF-1 transcriptional activity) and then treated cells with DFX either in the absence (Figure 5: lanes 1–3) or in the presence of FL (Figure 5: lanes 4–6). Without FL, DFX induced HIF-1α, leading to HRE-Luc induction (lane 2). When DFX was washed out (Figure 5: DFX (w)), HIF-1α was decreased, as expected, followed by decreased HRE-Luc expression (lane 3). FL downregulated HRE-Luc expression below basal levels (lane 4) and prevented its induction by DFX (lane 5). In the presence of FL, DFX did not induce HRE-Luc and, therefore without feedback, HIF-1α was dramatically induced (lane 5). In FL-treated cells, removal of FL resulted in induction of HIF-1-dependent transcription (Figure 5: lane 6), because the overexpressed HIF-1 started to transactivate. This in turn was accompanied by a decrease

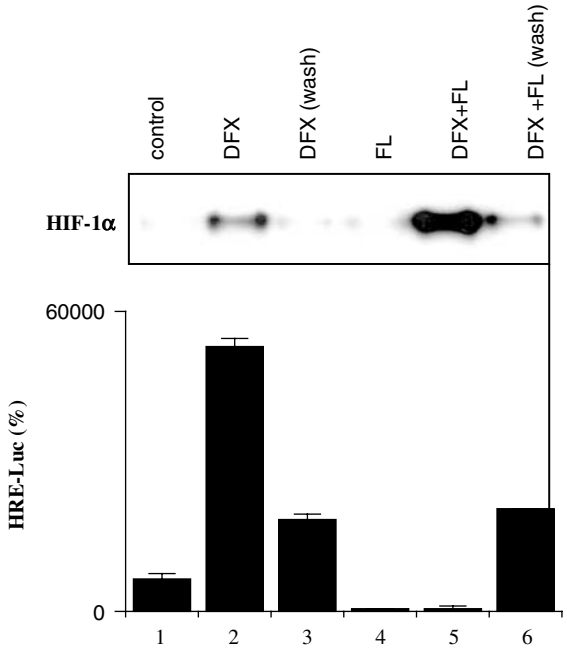

Figure 5 Reciprocal changes of HIF-1-dependent transcription and HIF-1α expression. A549 cells were cultured without or with 400 nM FL. DFX was added to lanes 2,3 and 5,6 for 24 h. In lanes 3 and 6, the medium was changed for the last 8 h. Luciferase activity (lower panel) and HIF-1α protein (upper panel) were measured

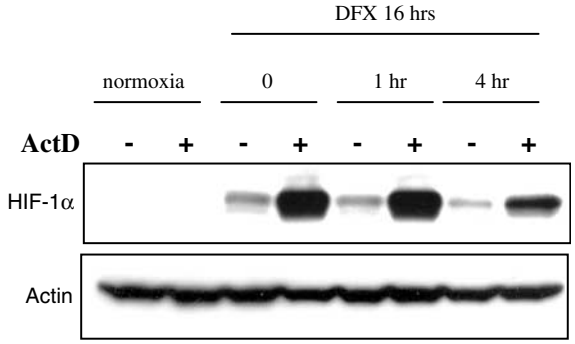

Figure 6 Time-dependent super-induction of HIF-1α. A549 cells were treated with DFX, and 5 μM ActD was added simultaneously (0 h), 1 and 4 h after. Cells were incubated for 16 h and immunoblot was performed as described in Materials and methods

of HIF-1α (Figure 5). Thus, restoration of transcription caused HIF-1α depletion.

HIF-1 super-induction is weakened, when ActD is delayed

If prevention of HIF-1-transcription is responsible for super-induction of HIF-1α by ActD, then super-induction of HIF-1α would be abated when ActD is added several hours *after* hypoxia/DFX. We treated A549 cells with DFX and simultaneously with ActD (0 h), ActD after 1 h, and ActD after 4 h (Figure 6). When ActD was added 4 h after DFX, super-induction of HIF-1 was relatively low (Figure 6). This is consistent with the data that HIF-1-inducible genes are already expressed by 4 h (Figure 7).

Transcription-dependent induction of PHD3 by hypoxia

We next investigated induction of PHDs, which (by hydroxylating HIF-1α) initiate degradation of HIF-1α. Therefore, PHDs could be implicated in the feedback control. ActD inhibited PHDs mRNA expression below basal levels (Figure 7). Importantly, hypoxia and DFX (Figure 7 and data not shown) highly induced PHD3 but did not affect expression of PHD1. PHD2 was slightly induced (twofold, Figure 7b). As expected, ActD downregulated PHD3 below basal levels, even under hypoxia (Figure 7a). We next investigated time-course of PHD3 induction. After 8 h, both hypoxia and DFX comparably induced PHD3 (Figure 7b). Interestingly, DFX was more proficient than hypoxia in induction of PHD3. For example, after 4 h, DFX induced PHD3, whereas hypoxia caused only marginal effect. We also confirmed that induction of PHD2 was marginal (Figure 7b).

HIF-1 super-induction is PHD/VHL independent

It was tempting to propose that PHD2–3 and HIF-1α form a feedback loop, because PHD2–3 are induced by HIF-1α. Yet, HIF-1α is accumulated precisely because PHDs are not working under hypoxia/DFX. To exclude a residual activity of PHDs, we investigated combina-

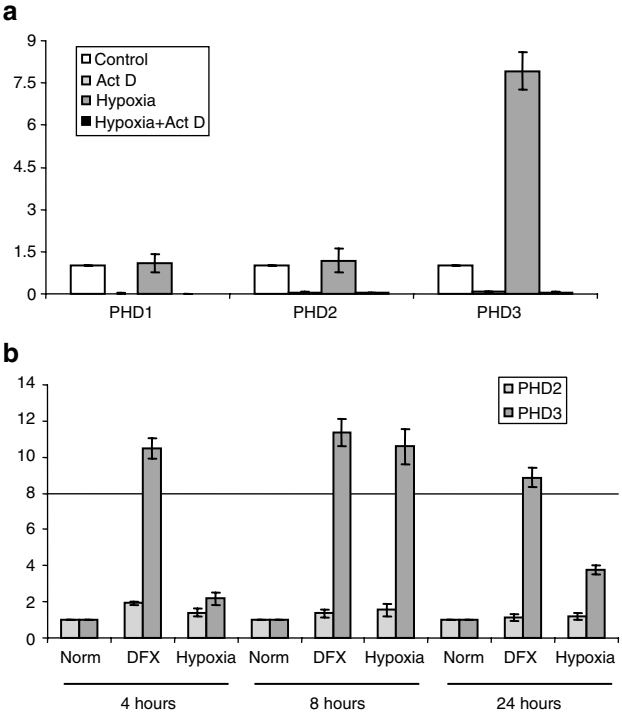

Figure 7 Effects of hypoxia and ActD on expression of PHDs mRNA. (**a**) A549 cells were treated with ActD 5 μM, hypoxia 1%, ActD + hypoxia, or left untreated (medium, med). After 24 h, cells were lysed and mRNA for PHD1, 2, 3 were measured by real-time PCR as described in Materials and methods. (**b**) Time course of induction of PHD2 and PHD3 by hypoxia and DFX. A549 cells were incubated under hypoxia (1% oxygen), normoxia (norm), or DFX hypoxia for 4, 8, 24 h as indicated. mRNA for PHD2 and PHD3 were measured by real-time PCR as described in Materials and methods

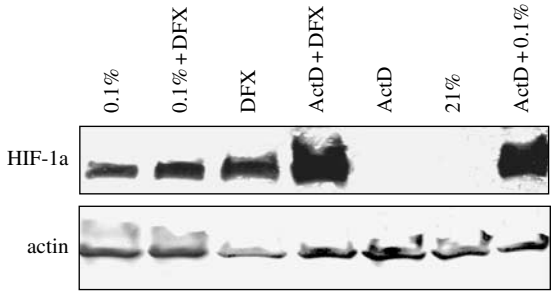

Figure 8 DFX plus anoxia do not super-induce HIF-1. A549 cells were cultured under indicated conditions for 16 h. Hypoxia is anoxia with levels of oxygen below 0.1%. Concentrations of DFX is 260 μM and ActD 5 μg/ml. Immunoblot was performed as described in Materials and methods

tions of both hypoxia (below 0.1% oxygen) and DFX. DFX plus hypoxia did not produce higher levels of HIF-1α, compared with each condition alone (Figure 8). ActD super-induced HIF-1 under hypoxia and DFX (Figure 8). We also showed that an increase in DFX from 60 to 1000 μM did not further increase HIF-1α (data not shown), indicating that PHDs are fully inhibited by 60 μM DFX. Thus, we found no evidence

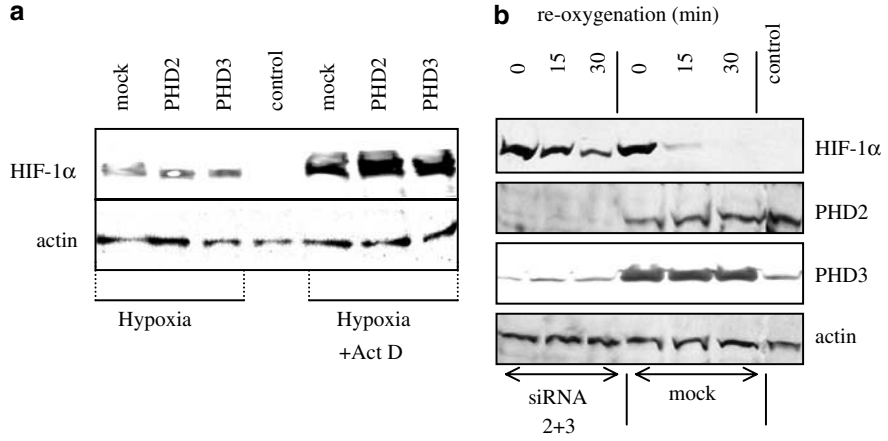

Figure 9 Role of PHD2 and PHD3 in the feedback loop. (**a**) PHD2 and PHD3 siRNA do not super-induce HIF-1. A549 were transfected with siRNAs for 16 h. Then, cells were cultured under hypoxia (<0.1% oxygen) for 16 h. If indicated + ActD (5 μM ActD). Act immunoblot was performed as described in Materials and methods. (**b**) PHD2 plus PHD3 siRNA prevent depletion of HIF-1 following reoxygenation. A549 were transfected with siRNAs for 12 h. Then cells were cultured under hypoxia (<0.1% oxygen) for 16 h. Re-oxygenation for 15 and 30 min. Immunoblot was performed as described in Materials and methods

that PHDs are involved in the HIF-1 degradation under DFX or hypoxia. Using siRNAs, we performed experiments to exclude PHD2 and 3. Under hypoxia, neither siRNA PHD2 nor siRNA PHD3 induced HIF-1α (Figure 9a). In contrast, Act D dramatically super-induced HIF-1α. Furthermore, ActD still super-induced HIF-1α in the presence of siRNA PHD2 and PHD3 (Figure 9a). This indicates that neither PHD2 nor PHD3 downregulates HIF-1α under hypoxia.

Next, it was necessary to demonstrate that siRNA actually inhibited PHDs. We reasoned that depletion of PHDs should prevent depletion of HIF-1α following reoxygenation. PHD2 and PHD3 have redundant functions. Cells were transfected with both siRNA for PHD2 and PHD3. Untransfected cells and PHD2 + 3 transfected cells were then placed at oxygen below 0.1%. Following 16 h, cells were lysed either immediately (0 min) or were reoxygenated for 15 and 30 min (Figure 9b). First, we confirmed that hypoxia induced the PHD3 protein (Figure 9b) and siRNA prevented this induction (Figure 9b). Second, PHD2 was also downregulated by siRNA. Most importantly, depletion of PHD2 + 3 did not increase HIF-1α under hypoxia. Finally, upon reoxygenation HIF-1α was rapidly depleted in control (mock), but this depletion was delayed after pretreatment with siRNA PHD2 + 3 (Figure 9b).

This indicates that PHDs, which are induced during hypoxia, participate in rapid degradation of HIF-1 upon re-oxygenation (conditions when PHD are active) but not in anoxia. These results exclude PHDs as transcription-dependent feedback regulators of HIF-1. This suggests that VHL may be also dispensable for feedback control because it binds HIF-1α, only when PHDs are active.

In VHL−/− cells (UMRC2 cells), the PHD-dependent pathway of HIF-1α degradation is blocked downstream from PHDs. In such conditions, when

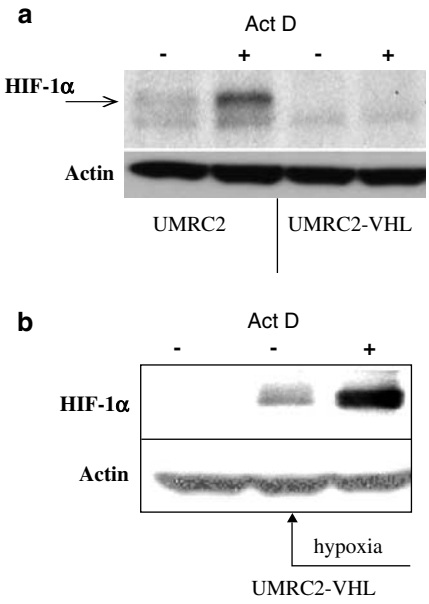

Figure 10 ActD induces HIF-1 in UMRC2 cells lacking VHL. (**a**) Cells were incubated with 5 μg/ml ActD for 8 h as indicated. (**b**) 1% hypoxia started 30 min after ActD treatment. Immunoblot was performed after 8 h as described in Materials and methods

HIF-1α is constantly increased, we expected to find the evidence for the feedback pathway of HIF-1α degradation. We expected that that ActD will induce HIF-1α in VHL−/− cells in normoxic conditions. This prediction was confirmed in UMRC2 cells. Reintroduction of VHL in UMRC2 cells (UMRC2-VHL cells) activated the VHL-mediated degradation of HIF-1α in normoxia. Following restoration of VHL, ActD did not induce HIF-1α (Figure 10a). In agreement with results in cell lines with wt VHL, ActD super-induced HIF-1α in UMRC2-VHL cells under hypoxia (Figure 10b).

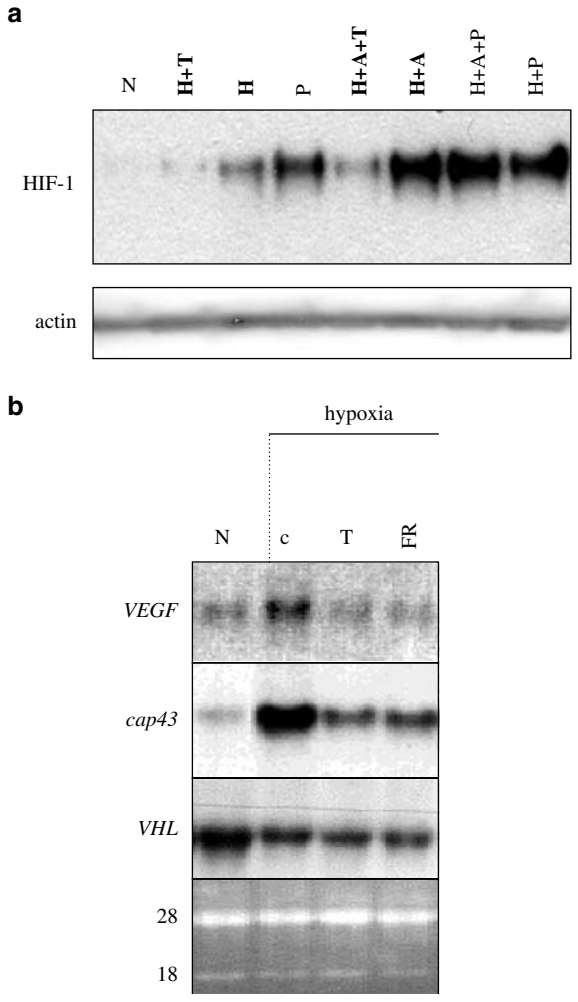

Figure 11 Regulation of HIF-1α degradation under hypoxia. (**a**) A549 cells were incubated under normoxia (N) or hypoxia (H) with 200 ng/ml TSA (T), 5 μM PSA-341 (P), 5 μM ActD (A), as indicated. After 16 h, HIF-1a was measured by immunoblot as described in Materials and methods. (**b**) A549 cells were incubated under normoxia (N) and hypoxia (h) with 200 ng/ml TSA (T) or 10 ng/ml FR901228 (FR) for 16 h and then the Northern blot for Cap43, VHL, and VEGF mRNAs was performed as described in Materials and methods

Characterization of transcription-dependent pathway

First, we wished to determine whether degradation of HIF-1α under hypoxia occurs via the proteasome. As expected, hypoxia induced HIF-1α and ActD super-induced HIF-1α under hypoxia (Figure 11: H vs H + A). PS341, a proteasome inhibitor, also induced HIF-1 by itself, consistent with a proteasomal-dependent degradation of HIF-1α in normal conditions. Noticeably, this induction was higher than those caused by hypoxia. Under hypoxia, PS super-induced HIF-1 (H + PS) to the levels that are achieved by hypoxia plus ActD (H + A), supporting the notion that all transcription-dependent degradation occurs via the proteasome. In agreement, PS341 did not affect HIF-1α in the presence of ActD (Figure 11, H + A = H + A + PS).

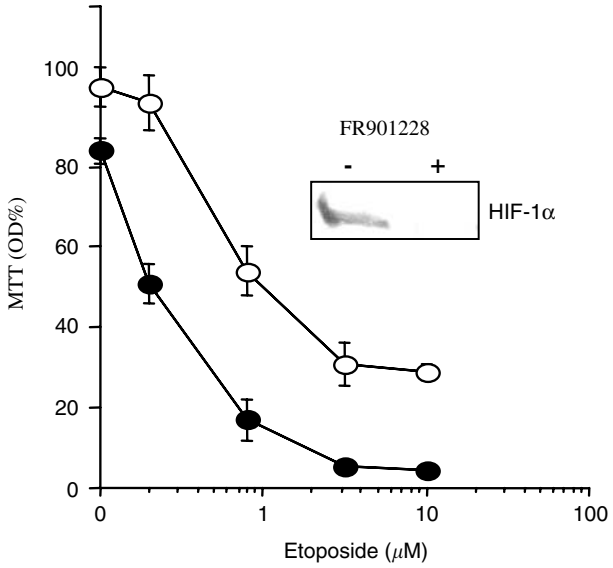

Figure 12 FR901228 sensitizes UMRC2 cells to etoposide. UMRC2 cells were incubated with (closed circles) or without (open circles) 1 ng/ml FR901228, and were treated with indicated concentrations of etoposide. After 2 days, MTT assay was performed as described in Materials and methods. Inset: HIF-1α was measured by immunoblot after 16 h

To further characterize the feedback pathway of HIF-1 degradation, we investigated inhibition of deacetylases. It has been previously shown that acetylation of HIF-1 may be involved in its destabilization. We found that TSA decreased HIF-1 under hypoxia. Most importantly, TSA partially prevented the super-induction of Act. This allows us to suggest that ActD blocks induction of acetylases, thus preventing HIF-1α degradation. TSA, by blocking deacetylases, shifted the balance towards HIF-1 degradation. Downregulation of HIF-1 was accompanied by downregulation of its target genes (Figure 11b).

Applications for cancer therapy

It is known that the renal cancer cells, which lack VHL, are notoriously resistant to chemotherapy. One reason is that HIF-1 can act as a survival factor. We have suggested that HIF-1 degradation via feedback (VHL-independent) pathway can be accelerated by inhibitors of deacetylases. FR902281, an inhibitor that is currently undergoing clinical trials, decreased HIF-1 levels in RCC (Figure 12). Importantly, FR902228 increased the sensitivity of RCC to etoposide, a DNA-damaging anticancer drug (Figure 12). This result indicates clinical potentials for activation of VHL-independent degradation of HIF-1α.

Discussion

In normal conditions, VHL binds HIF-1α, leading to ubiquitination and degradation of HIF-1α. Hypoxia abrogates binding of HIF-1α and VHL, causing HIF-1α

accumulation. This mirrors the mode of regulation of p53 by Mdm-2. In fact, Mdm-2 binds p53, causing its degradation, whereas DNA damage abrogates the binding, causing p53 accumulation. Finally, p53 transactivates Mdm-2, thus completing a feedback loop. Both HIF-1 and p53 are transcription factors. In analogy with the p53 feedback loop, it was tempting to suggest that HIF-1 trans-activates VHL. Here, we demonstrated that neither hypoxia nor DFX induced VHL. On the other hand, it was recently shown that hypoxia induces either PHD3 (Cioffi *et al*., 2003; Aprelikova *et al*., 2004) or PHD2 (Berra *et al*., 2003; D'Angelo *et al*., 2003) or both (Del Peso *et al*., 2003; Metzen *et al*., 2003) in different cell types (Appelhoff *et al*., 2004). We confirmed here that hypoxia and DFX preferentially induced PHD3. Therefore, it may seem plausible that the feedback loop involves PHD3 (Cioffi *et al*., 2003; Marxsen *et al*., 2004). In agreement with previous reports (Berra *et al*., 2003; Marxsen *et al*., 2004), we found that inhibitors of transcription blocked accumulation of PHD during hypoxia and thus prevented depletion of HIF-1α following reoxygenation. Using PHD2 and PHD3 siRNA, we demonstrated that accumulation of PHDs during hypoxia is responsible for a rapid decrease of HIF-1α upon reoxygenation.

We demonstrated that inhibitors of transcription did not, however, induce HIF-1α under normal conditions. When HIF-1α was induced by hypoxia, inhibitors of transcription induced HIF-1α further (super-induction). Super-induction of HIF-1α by inhibitors of transcription was observed under different conditions that induce HIF-1α as hypoxia, DFX, and cobalt. This indicates that, when HIF-1 is induced, it trans-activates a feedback loop. This feedback loop may explain why induction of HIF-1α by hypoxia is limited. For example, HIF-1α reaches a peak at 4 h and then it decreases almost to basal line by 12–16 h (Mottet *et al*., 2003). In the absence of feedback control, HIF-1α would continuously accumulate under hypoxia. Thus, transcriptional control limits HIF-1 expression under hypoxia. Removal of inhibitors of transcription resulted in dramatic induction of HIF-1-dependent transcription with a subsequent depletion of HIF-1α (Figure 5).

Seemingly, a feedback loop may involve HIF-1 and PHDs. The paradox is that PHDs are induced by HIF-1 under unfavorable (for PHDs) conditions (low oxygen or iron). Although certain activity of PHDs is expected under *moderate* hypoxia, there is no further induction of HIF-1α by decreasing oxygen levels below 0.5% (Jiang *et al*., 1996). This indicates that HIF-1α is not affected by PHDs at so low oxygen.

We provide the evidence that PHDs are not involved in the feedback downregulation of HIF-1α under DFX/hypoxia. First, in the presence of DFX or hypoxia, neither combinations of these conditions, nor increase in concentration of DFX caused any additional induction of HIF-1α. This actually excluded a residual activity under anoxia or DFX that can detectably affect levels of HIF-1α. In contrast, inhibitors of transcription caused dramatic super-induction of HIF-1α under anoxia or DFX. Second, while depletion of PHD2 and PHD3 by siRNAs prolonged HIF-1α expression upon reoxygenation, this depletion did not super-induce HIF-1α under anoxia or DFX.

Thus, the role of the PHD3/HIF-1 feedback loop is limited to rapid degradation of HIF-1α upon reoxygenation. Finally, inhibition of transcription further increased levels of HIF-1α in VHL−/− cells under normoxia. After reintroduction of VHL, ActD induced HIF-1α under hypoxia but not under normoxia. It is noteworthy that normoxic induction of HIF-1α by ActD was modest in VHL−/− UMRC2 cells. Since HIF-1-inducible gene products pre-exist in VHL−/− cells, this situation parallels a weaker super-induction of HIF-1α in A549 cells, when ActD was added 4 h after hypoxia (Figure 6).

Our data suggest that transcription-dependent degradation (under hypoxia/DFX) is independent from PHDs and VHL. In agreement, degradation of HIF-1 can be VHL-independent (Isaacs *et al*., 2002; Tang and Lasky, 2003). We demonstrated that ActD induced HIF-1α in VHL−/− cells, indicating that it is still degraded in transcription-dependent manner in the absence of VHL. We also show that, under hypoxia, degrdation of HIF-1 occurs via the proteasome and can be accelerated by the inhibition of deacetylation.

Based on all results, we suggest the following model. When HIF-1α is low, it does not trans-activate and is not regulated via a transcription-dependent loop. When oxygen and iron are decreased, HIF-1α accumulates thus establishing a feedback loop. Whereas PHDs participate in a rapid degradation after reoxygenation or after removal of DFX, they are inactive under hypoxia/DFX. Thus, HIF-1α is regulated by the alternative pathway, when the classic pathway of HIF-1α degradation is blocked. We describe a transcription-dependent regulation of HIF-1α, which is PHD/VHL independent and occurs only when HIF-1 is accumulated. Proteasomal inhibitors further increase HIF-1α under hypoxia/DFX, indicating that it is still degraded via the proteasome under hypoxia. This pathway does not depend on hydroxylation and may involve other modifications such as acetylation, which accelerates HIF-1α degradation. In fact, acetylation reaction is independent from oxygen and iron and may occur under anoxia/DFX (Jeong *et al*., 2002; Lin *et al*., 2004). This alternative pathway may be dysregulated in cancer, given that HIF-1-inducible transcription is very transient in normal prostate epithelial cells compared with cancer cell lines (Salnikow *et al*., 2000). It is also noteworthy, that topoisomerase I inhibitors blunted induction of HIF-1α by hypoxia, whereas inhibitors of transcription prevented this downregulation (Rapisarda *et al*., 2002, 2004b). Thus, VHL-independent pathway may provide a means for pharmacological inhibition of HIF-1. Here we demonstrated that FR90228 (an inhibitor of deacetylation) downregulated HIF-1α and sensitized renal carcinoma cells to etoposide, a DNA-damaging anticancer drug. In theory, pharmacological manipulation of HIF-1α may find clinical applications in numerous diseases, including cancer (Semenza, 2003; Giaccia *et al*., 2003; Rapisarda *et al*., 2004a).

Materials and methods

Cell lines and chemotherapeutic agents

Human cancer cell lines MCF-7, A 549, H157 and DU 145 were obtained from American Type Culture Collection (Manassas, VA, USA). VHL-deficient UMRC2 cells and these cells transfected with VHL (UMRC2-VHL) were obtained from Dr Len Neckers (NCI, NIH). Other renal cell lines were obtained from Dr El-Deiry (University of Pennsylvania) (Corn *et al*., 2003). Cell lines were maintained in RPMI-1640 medium and 10% FBS. Adriamycin, ActD, α-amanitin, 5,6-dichloro-1-D-ribofuranosylbenzimidazole (DRB), desferoxamine (DFX) were purchased from Sigma (St Louis, MO, USA). Adriamycin was dissolved in DMSO as a 2 mg/ml stock solution. ActD was dissolved in water as 2 mg/ml solutions. DRB was prepared as 100 mM stock solution. FL was obtained from the Development Therapeutics Program, NCI, and was prepared as 10 mM stock solution in DMSO.

Cells were cultured at 37°C in 20% O_2, 5% CO_2, 75% N_2 for normoxic conditions. The hypoxia induction was achieved either by hypoxia mimetics: 100 μM $CoCl_2$, 260 μM and 100 μM DFX mesylate (both from Sigma, St Louis, MO, USA), or by culturing cells in a hypoxia chamber at 37°C with 1% O_2, 5% CO_2, 94% N_2 atmosphere.

Transient transfections

PG13-Luc, containing a generic p53 response element, were obtained from Dr El-Deiry. The control luciferase plasmid, pGL2-control, driven by SV40 promoter and enhancer sequences, were purchased from Promega (Madison, WI, USA). HRE-Luc was obtained by subcloning of three copies of the double-stranded 21 bp oligonucleotide (5′-AGT-GAC-TAC-GTG-CTG-CCT-3′) in the pGL3 promoter vector (Promega) digested with *Kpn*I and *Mlu*I (Rapisarda *et al*., 2004b). This oligonucleotide used encompasses the hypoxia-responsive element of the iNOS promoter. A total of 50,000 cells were plated in 24-well plates (Costar, Acton, MA, USA). The next day, cells were transfected with plasmids as described previously (Blagosklonny *et al*., 2001, 2002). After 6–16 h, the medium was changed and cells were treated with FL and or DFX for an additional 16 h. Cells were lysed and analysed for luciferase activity (Blagosklonny *et al*., 2002).

Northern blot analysis

Immediately after treatment for the indicated times, cells were washed once in PBS, and total RNA was extracted using the guanidine isothiocyanate and cesium chloride method. In all, 15 μg of RNA was loaded per lane, run in 1% agarose gels containing 2.2 M formaldehyde, blotted by capillarity onto nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH, USA), and baked for 2 h at 80°C. Equal loading and integrity of RNA were monitored by ethidium bromide staining of the 28S subunit of rRNA. The human HIF-1 cDNA probe was also produced by RT–PCR (1308-bp product) using oligonucleotide primers: 5′-CGGCGCGAAC GACAAGAAAAAGAT-3′ (sense, bp 43–66) and 5′-TCGTT GGGTGAGGGGAGCATTACA-3′ (antisense, bp 1327–1350). Numbering of the nucleotide base positioning was taken from the GenBank profile Accession no. U22431 (human HIF-1 mRNA). All RT–PCR products were sequenced to validate base integrity of the probes (Garayoa *et al*., 2000). NDRG-1/Cap43 gene was described previously (Salnikow *et al*., 2002). The VEGF probe was a kind gift from K Claffey (University of Connecticut) (Salnikow *et al*., 2002). VHL containing pCEP4VHL vector was provided by Dr Kuzmin (NCI-Frederick, NIH).

Probes were labeled with [−32P]dCTP (3000 Ci/mmol; NEN Life Science Products, Boston, MA, USA) by random priming, and unincorporated nucleotides were removed by ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization was carried out overnight at 42°C in a hybridization buffer containing 40% formamide (Garayoa *et al*., 2000). After stringency washes, blots were exposed to XAR film (Eastman Kodak Co., Rochester, NY, USA) at −80°C for varying times. Densitometry of the autoradiograms was performed using a ChemiImager 4000 (Alpha Innotech Corp., San Leandro, CA, USA).

siRNA transfections

siRNA oligonucleotides were purchased from Qiagen. The siRNA targeting PHD2 mRNA (GenBank accession number NM_022051) corresponds to nt 891–909 (5′-AGCCAUGG UUGCUUGUUAU-3′); the siRNA to PHD3 (GenBank accession number NM_022073) corresponds to nt 357–375 (5′-GGCAAUGGUGGCUUGCUAU-3′). Cells were seeded at 30–50% confluence in antibiotic-free medium 24 h prior to transfection. Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect cells with 20 μM siRNA duplex according to the transfection procedure protocol (Invitrogen). The efficacy of the siRNA transfection in each experiment was ascertained by immunoblotting for PHD2 and PHD3.

Immunoblot analysis

Cells were lysed and soluble proteins were harvested in TNES buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 1% (v/v) NP40) containing protease inhibitors (20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM PMSF). Proteins were resolved with 7.5% SDS–PAGE for detection of mdm-2, PARP, and p53 or with 12.5% SDS–PAGE for detection of p21, cyclin D1, and p53 as previously described (Blagosklonny *et al*., 2002). Alternatively, proteins were resolved on NuPAGE 4–12% Bis-Tris gel with MOPS running buffer (NOVEX, San Diego, CA, USA) according to the manufacturer's instructions. Whole-cell lysate was separated on a 4–20% Tris-Glycine gel (Invitrogen) electroblotted on a polyvinylidene difluoride membrane (Invitrogen) and subjected to immunoblot analysis. Monoclonal anti-HIF-1 antibody was purchased from BD-Transduction Laboratories (Lexington, KY, USA) and used at a 1:300 dilution. Monoclonal anti-HIF-1β antibody was purchased from Novus Biologicals (Littleton, CO, USA) and used at a 1:1200 dilution; monoclonal mouse anti-human p21 (EA10; Oncogene, Calbiochem, San Diego, CA, USA), monoclonal mouse anti-human p53 (Ab 2 and Ab 6; Oncogene, Calbiochem). Polyclonal anti-human PHD2 and PHD3 were purchased from Novus (Littleton). Anti-human actin and tubulin were used for loading controls. Anti-human polyclonal Cap43 were described previously (Salnikow *et al*., 2002). Horseradish peroxidase-conjugated mouse and rabbit IgG (1:10 000 dilution) and enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech. Immunoblots were developed using an HRP-conjugated secondary antibody (Bio Rad) and a chemiluminescence detection kit (Dupont NEN).

MTT assay

A total of 5000 cells were plated in 96-well flat bottom plates and then exposed to tested agents. After 48 h, 20 μl of 5 mg/ml MTT (3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-

zolium bromide (Sigma) was added to each well for 4 h. After removal of the medium, 170 μl of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a Biokinetics plate reader as previously described (Demidenko and Blagosklonny, 2004). Standard deviations were determined in triplicates.

Real-time PCR

Total RNA was obtained using RNA Mini Kit (Qiagen, Inc.). Reverse transcription-PCR was performed using a reverse transcription-PCR kit (PE Biosystems, Foster City, CA, USA) as described previously (Rapisarda *et al*., 2004b). To measure human VEGF and HIF-1 expression, real-time PCR was performed using an ABI-Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) as described previously (Rapisarda *et al*., 2004b). Primers and specific probes were obtained from Applied Biosystems. The following primers and probes were used:

HIF-1 forward 5′-CCAGTTACGTTCCTTCGATCAGT-3′; and reverse 5′-TTTGAGGACTTGCGCTTTCA-3′.

Human VEGF primers and probes were described previously (Rapisarda *et al*., 2004b).

VEGF forward, 5′-TACCTCCACCATGCCAAGTG-3′;
VEGF reverse, 5′-ATGATTCTGCCCTCCTCCTTC-3′;
PHD1 forward 5′-ACGGGCTCGGGTACGTAAG-3′;
PHD1 reverse 5′-CCCAGTTCTGATTCAGGTAATAGATA CA-3′;
PHD2 forward 5′-GACCTGATACGCCACTGTAACG-3′;
PHD2 reverse 5′-CCCGGATAACAAGCAACCAT-3′;
PHD3 forward 5′-AACTGAATCTGCCCTCACTGAAG-3′;
PHD3 reverse 5′-ATAATTCAGGAACCGTTACTAAAAT GA-3′.

Used as an internal control, 18S rRNA was assessed using premixed reagents from Applied Biosystems. Detection of VEGF and 18S rRNA was performed using TaqMan Universal PCR Master Mix (Applied Biosystems), and HIF-1 detection was performed using Sybr Green PCR Master Mix (Applied Biosystems).

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