

## Hypoxia Induces Myocardial Regeneration in Zebrafish

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**Background**—Hypoxia plays an important role in many biological/pathological processes. In particular, hypoxia is associated with cardiac ischemia, which, although initially inducing a protective response, will ultimately lead to the death of cardiomyocytes and loss of tissue, severely affecting cardiac functionality. Although myocardial damage/loss remains an insurmountable problem for adult mammals, the same is not true for adult zebrafish, which are able to completely regenerate their heart after extensive injury. Myocardial regeneration in zebrafish involves the dedifferentiation and proliferation of cardiomyocytes to replace the damaged/missing tissue; at present, however, little is known about what factors regulate this process.

**Methods and Results**—We surmised that ventricular amputation would lead to hypoxia induction in the myocardium of zebrafish and that this may play a role in regulating the regeneration of the missing cardiac tissue. Using a combination of O<sub>2</sub> perturbation, conditional transgenics, in vitro cell culture, and microarray analysis, we found that hypoxia induces cardiomyocytes to dedifferentiate and proliferate during heart regeneration in zebrafish and have identified a number of genes that could play a role in this process.

**Conclusion**—These results indicate that hypoxia plays a positive role during heart regeneration, which should be taken into account in future strategies aimed at inducing heart regeneration in humans. (*Circulation*. 2012;126:3017-3027.)

**Key Words:** cardiac myocyte dedifferentiation ■ cardiac myocyte hypoxia ■ cardiac myocyte proliferation ■ regeneration zebrafish

Hypoxia has been found to regulate a number of cellular processes that are also associated with successful heart regeneration in zebrafish such as cellular dedifferentiation and proliferation.<sup>1–5</sup> The family of transcription factors, hypoxia-inducible factors (HIFs), are the direct effectors of the hypoxic response. HIF-induced transcription involves the formation of a dimer between either HIF1 $\alpha$  or HIF2 $\alpha$  and HIF1 $\beta$  to subsequently target a plethora of downstream genes.<sup>6</sup> HIF1/2 $\alpha$  transcriptional activity is regulated primarily by their level of expression, which, under normoxic conditions, remains relatively low but will be rapidly elevated when hypoxia ensues. The level of HIF1/2 $\alpha$  expression is maintained by the prolyl hydroxylase dioxygenase family of proteins. Under normoxic conditions, prolyl hydroxylase dioxygenase enzymes hydroxylate HIF1/2 $\alpha$ , which subsequently binds to the von Hippel Lindau E3 ligase and is targeted for proteasomal degradation. During periods of hypoxia, the lack of oxygen inhibits prolyl hydroxylase dioxygenase activity; subsequently, the expression levels of HIF1/2 $\alpha$  become elevated.<sup>6</sup>

### Clinical Perspective on p 3027

Cardiac ischemia is typically caused by an occlusion of the coronary vasculature. The resulting reduction in the blood supply to the tissue leads to a drastic decrease in oxygen (hypoxia).<sup>5</sup> Consequently, HIF1 $\alpha$  is no longer targeted for degradation, and its expression levels rise sharply, allowing it to dimerize with the HIF1 $\beta$  and activate an array of downstream targets. Initially, this response serves to preserve the cardiomyocytes by inducing a range of cardioprotective genes that allow the cells to survive in the hypoxic environment that ensues after an ischemic episode.<sup>7</sup> Ultimately, however, prolonged exposure to hypoxic conditions will result in apoptosis and necrosis and the subsequent loss of cardiac tissue. Several studies have examined the consequences of perturbing HIF1 $\alpha$  expression levels in cardiomyocytes. For example, transgenic mice overexpressing HIF1 $\alpha$  in the myocardium show a reduction in the amount of damage caused by cardiac ischemia and show a marked improvement in cardiac function.<sup>8</sup> Similarly, reducing prolyl hydroxylase

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dioxygenase expression levels by siRNA-mediated knock-down results in elevated HIF1 $\alpha$  expression and consequently a similar beneficial response to that seen in HIF1 $\alpha$ -overexpressing mice.<sup>9</sup>

Hypoxia has also been found to play a role in promoting the proliferation of a variety of mammalian cell types such as embryonic stem cells,<sup>2</sup> smooth muscle cells,<sup>10</sup> endothelial cells,<sup>11</sup> and fibroblasts.<sup>12</sup> Furthermore, in adult zebrafish, exposure to chronic hypoxia leads to an increased number of cardiomyocytes in the heart.<sup>13</sup> Because ventricular amputation damages the myocardium and impairs heart function, we hypothesized that it will lead to hypoxia induction and that this may play a role in regulating heart regeneration in adult zebrafish.

## Methods

### Constructs and Zebrafish Lines

All constructs and transgenic lines were generated with the Tol2 Kit as described.<sup>14,15</sup> Dominant-negative (dn) zf HIF1 $\alpha$ b was generated as described.<sup>16</sup> For the Tg(*cmcl2a*:LrL:dnHIF1 $\alpha$ ) construct, the 5' entry clone *cmcl2a* contained a 1-kb polymerase chain reaction fragment of the *cmcl2a* promoter,<sup>17</sup> the middle entry clone contained a floxed red fluorescent protein stop cassette amplified from pBOB-LRL-CBREGFPPa (a kind gift from Geoff Whal), and the 3' entry clone contained zebrafish dnHIF1 $\alpha$ b.

### Zebrafish Oxygen Conditions

For maintaining adult zebrafish in hyperoxic conditions, 3 fish were placed in a transport bag (SERA) with 500 mL aquaria water; 100% O<sub>2</sub> was used to fill the bag before it was tightly sealed with a rubber band. Sealed bags were then placed in an incubator and maintained at 28°C. Each subsequent day for the duration of the experiment, the water was changed; during this time, the fish were also fed (a procedure lasting  $\approx$  1 hour) before the bag was refilled with 100% O<sub>2</sub> and sealed. The average O<sub>2</sub> concentration in the water was 17.21 mg/L (213% saturation; average calculated from 5 consecutive days).

For maintaining adult zebrafish in normoxic conditions (and to ensure that any differences observed between normoxia and hyperoxia were not due to the way the zebrafish were maintained), we followed the exact same procedure as for the hyperoxic conditions, but instead of adding 100% O<sub>2</sub>, we left the bag open and not sealed. The average O<sub>2</sub> concentration in the water was 6.22 mg/L (77% saturation; average calculated from 5 consecutive days).

### Phenylhydrazine Treatment

For induction of anemic hypoxia, 5 adult fish were placed in a crossing box containing 1 mg phenylhydrazine (Sigma) per liter of aquarium water for 1 hour and then returned to the aquarium to recover for 3 days. This procedure was then repeated before amputation.

### Hypoxyprobe Treatment

Hypoxyprobe was dissolved in PBS at a concentration of 5 mg/mL. For in vitro cell culture, 50  $\mu$ L was added to 3 mL culture medium. For in vivo analysis, 20  $\mu$ L was injected intraperitoneally each day for the duration of the experiment.

### Cre/Tamoxifen Induction and Heart Amputation

Transgenes were expressed by inducing Cre-mediated recombination with tamoxifen as described.<sup>15</sup> All amputations were performed as described.<sup>15</sup>

### BrdU Labeling

BrdU treatment was performed essentially as described.<sup>18</sup> Meta-morph software (Molecular Devices) was used to count BrdU-labeled cardiomyocytes.

### Cardiomyocyte Isolation

Fish were euthanized in tricaine. Hearts were collected and put in PBS with penicillin, streptomycin, and 10 U/mL heparin. The outflow tracts were then removed and ventricles and atriums were opened to get rid of the blood. They were then washed 3 times in perfusion buffer (PBS; 10 mmol/L HEPES, 30 mmol/L taurine, 5.5 mmol/L glucose, and 10 mmol/L 2,3-butanedione monoxime [BDM]) and placed in digestion buffer (perfusion buffer plus 12.5  $\mu$ mol/L calcium chloride and collagenase II and IV, 5 mg each [Gibco]) to digest for 2 hours at 32°C in a thermomixer at 800 rpm. Next, an equal volume of stop buffer 1 (perfusion buffer plus 12.5  $\mu$ mol/L calcium chloride and 10% FBS) was added, and cells were mechanically separated. Undigested material was left to sediment, and cells suspended in the supernatant were pelleted by centrifugation at 250g for 5 minutes. The cells were then resuspended in stop buffer 2 (perfusion buffer plus 12.5  $\mu$ mol/L and 5% FBS), and calcium reintroduction was performed by gradually raising the concentration to 62, 112, 212, 500, and 1000  $\mu$ mol/L. Cells were then pelleted again and resuspended in plating medium (minimum essential medium; 5% FBS, 10 mmol/L BDM, 2 mmol/L Glutamax, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin). Cell preparations were plated onto collagen type I (BD Biosciences; reference No. 354236) –treated chamber slides (Nunc; reference No. 177445) and allowed to attach overnight.

### Cardiomyocyte Culture Oxygen Conditions

For maintaining adult zebrafish cardiomyocytes in hyperoxic conditions, cultures were placed in a hypoxia chamber (Stem Cell Technologies) filled with 100% O<sub>2</sub>. Each subsequent day, the chamber was flushed and filled with 100% O<sub>2</sub>.

For maintaining adult zebrafish cardiomyocytes in hypoxic conditions, cultures were placed in a hypoxia chamber (Stem Cell Technologies) filled with 3% O<sub>2</sub>. Each subsequent day, the chamber was flushed and filled with 3% O<sub>2</sub>.

For maintaining adult zebrafish cardiomyocytes in normoxic conditions, cultures were placed in a standard cell culture incubator at 21% O<sub>2</sub> and 5% CO<sub>2</sub>.

### Immunohistochemistry

Immunohistochemistry was performed on 10- $\mu$ m cryosections as previously described.<sup>19</sup> The antibodies used are anti-green fluorescent protein (GFP; GFP-1020, AVES), anti-BrdU (sc-70441, Santa Cruz), anti-phospho-histone H3 (PHH3; 06-570, Upstate), anti- $\alpha$ -sarcomeric actin (ASA; A2172, Sigma), anti-tropomyosin (TPM; T2780, Sigma), anti-MEF2c (ab79436, Abcam), and Hypoxyprobe Mab1 (Hypoxyprobe-1 kit).

### In Situ Hybridization

In situ hybridization was performed as described previously.<sup>18</sup> The dnHIF1 $\alpha$  probe was generated by subcloning dnHIF1 $\alpha$  into the *Pst*I and *Xba*I sites of pBSK-.

### Confocal Microscopy

Confocal microscopy was performed with a Leica SP5. For colocalization analysis, the following formula was used to calculate the axial resolution:

$$Dz = \sqrt{[(1 \times n/NA^2) + (AU \times n \times \sqrt{2} \times 1.22 \times 1/NA^2)^2]},$$

where emission ( $\lambda$ )=500 nm, refractive index=1.518, NA=1.4, and AU (airy units)=1. This results in a section thickness of  $z=0.773 \mu$ m.

### Statistical Analysis

#### BrdU-Positive Cardiomyocyte Assay

For each condition, 5 animals were analyzed. For each animal, BrdU-positive cardiomyocytes were counted on 3 separate sections, and the average was then calculated. Multiple-sample statistical analysis was performed by 1-way ANOVA with the Holm-Sidak

multiple-comparisons test using the average number of BrdU-positive cardiomyocytes per animal.

#### **PHH3 Cardiomyocyte Assay**

For each condition, 3 experiments were performed. For each experiment,  $10^5$  cardiomyocytes were analyzed. The number of PHH3-positive cardiomyocytes was subsequently calculated for each experiment. Multiple-sample statistical analysis was performed by 1-way ANOVA with the Holm-Sidak multiple-comparisons test using the number of PHH3-positive cardiomyocytes per experiment.

#### **Dedifferentiated Cardiomyocyte Assay**

For each condition, 6 experiments were performed. For each experiment, 5 random nonoverlapping images were analyzed. The average percentage of dedifferentiated cardiomyocytes was subsequently calculated for each experiment. Multiple-sample statistical analysis was performed by 1-way ANOVA with the Holm-Sidak multiple-comparisons test using the average number of dedifferentiated cardiomyocytes per experiment.

#### **Microarray and Quantitative Polymerase Chain Reaction Analysis**

A detailed description of the microarray and quantitative polymerase chain reaction analysis is provided in the online-only Data Supplement.

## **Results**

### **Ventricular Amputation Induces Hypoxia in Cardiac Tissue**

To determine whether ventricular amputation induces hypoxia in adult zebrafish, we first established a protocol for detecting hypoxia using the commercially available chemical Hypoxyprobe as an indirect measure of tissue oxygen content (Pimonidazole binds to thiol groups at low oxygen tension, which can subsequently be detected with immunohistochemistry).<sup>20</sup> To establish that this system was functioning in zebrafish, we cultured adult zebrafish cardiomyocytes in either normoxic or hypoxic conditions and treated the culture with Hypoxyprobe for 24 hours. We were unable to detect any Hypoxyprobe signal in cardiomyocytes cultured under normoxic culture (Figure 1A–1C). However, we were able to detect a robust Hypoxyprobe signal in cardiomyocytes cultured under hypoxic conditions (Figure 1D and 1E). This indicates that the Hypoxyprobe system functions reliably in zebrafish cells.

To determine whether hypoxia is a feature of heart regeneration, we treated unamputated adult zebrafish with Hypoxyprobe for a period of 7 days, during which time they were maintained under normoxic conditions. Subsequently, we were unable to detect any Hypoxyprobe signal in the hearts of these animals (Figure 1G–1I). For hypoxia, we tested a number of different techniques (data not shown) and found that the most reliable and reproducible results were obtained by inducing anemia in adult zebrafish with the chemical phenylhydrazine<sup>21</sup> (It is well established that anemia, a lack of red blood cells, leads to hypoxia<sup>22</sup>). Consequently, after anemia induction, adult zebrafish were treated with Hypoxyprobe for a period of 7 days. Immunohistochemical analysis revealed a robust Hypoxyprobe signal in the hearts of all the anemic zebrafish we analyzed ( $n=5$ ), indicating that phenylhydrazine-induced anemia leads to hypoxia induction in the heart (Figure 1J–1L). We next sought to determine whether ventricular amputation also leads to hypoxia induc-

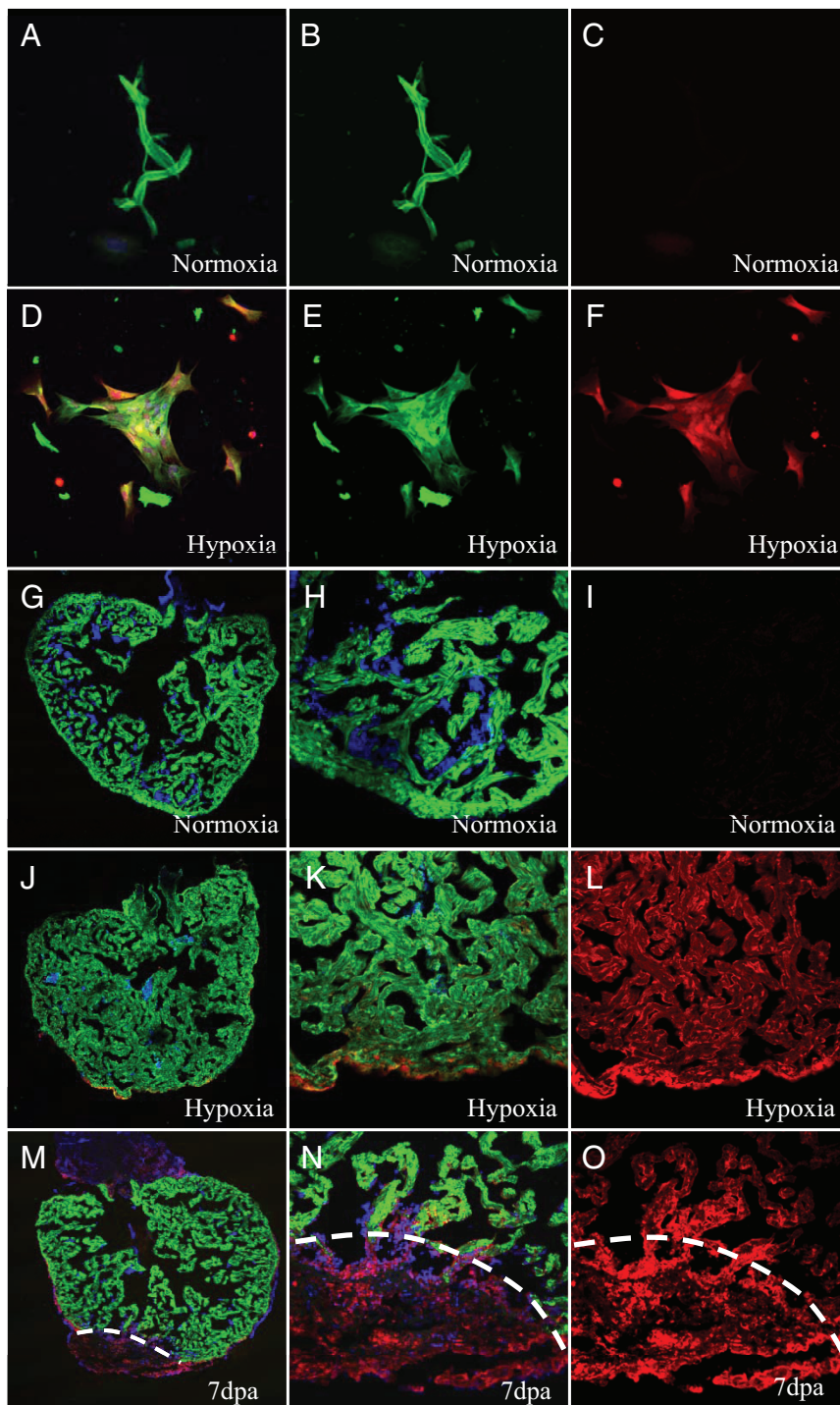
tion. After ventricular amputation, adult zebrafish were treated with Hypoxyprobe throughout the 7-day postamputation period. We were able to detect a strong Hypoxyprobe signal throughout the heart, which was at its most intense in both the fibrin clot, similar to a previous report,<sup>23</sup> and the cardiomyocytes/cells close to the site of amputation ( $n=5$ ; Figure 1M–1O). To ensure the validity of using Hypoxyprobe, we also amputated adult zebrafish and maintained them in hyperoxic conditions throughout the 7-day recovery period. Consequently, we were unable to detect any Hypoxyprobe signal in the hearts of these animals (Figure 1A–1C in the online-only Data Supplement). These results indicate that ventricular amputation of adult zebrafish leads to hypoxia induction in cardiac tissue.

Next, we analyzed what effect perturbation of normoxic conditions has on heart regeneration. To achieve this, we adopted several strategies that involved either modulating the  $O_2$  concentration or using transgenic zebrafish. Because HIF1 $\alpha$  is the direct effector of hypoxia signaling, we generated transgenic lines in which we could specifically and conditionally express dnHIF1 $\alpha$ <sup>16,24,25</sup> in cardiomyocytes using the Cre/tamoxifen system we have described previously.<sup>15</sup> The transgenic zebrafish were generated by crossing the previously described Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LrL:GFP) line<sup>15</sup> with a Tg(cmlc2a:LrL:dnHIF1 $\alpha$ ) line that contains a floxed red fluorescent protein stop cassette (LrL) between the cardiomyocyte-specific promoter (cmlc2a) and dnHIF1 $\alpha$ . Treatment of embryos or adult zebrafish with 4-hydroxytamoxifen results in efficient recombination of the floxed stop cassette and subsequently a rapid, cardiomyocyte-specific induction of dnHIF1 $\alpha$ . To establish that our system was functional, we induced dnHIF1 $\alpha$  expression in embryos by administering 4-hydroxytamoxifen and monitored them throughout early development (up to 5 days after fertilization). In situ hybridization using a dnHIF1 $\alpha$  antisense probe revealed a strong expression of dnHIF1 $\alpha$  in the hearts of 3dpf embryos treated with 4-hydroxytamoxifen (Figure IID and IIE in the online-only Data Supplement). Although untreated embryos naturally express HIF1 $\alpha$  in the brain, we could not detect any endogenous signal in the heart (Figure IIA and IIB in the online-only Data Supplement). Furthermore, we were unable to detect any observable defects in cardiogenesis between wild-type embryos and embryos expressing dnHIF1 $\alpha$  in their cardiomyocytes (Figure IIC and IIF in the online-only Data Supplement).

### **Hypoxia Positively Regulates Heart Regeneration in Adult Zebrafish**

To establish what effect perturbation of the normoxic conditions has on regeneration, we amputated adult zebrafish and maintained them in normoxic conditions, hypoxic conditions (phenylhydrazine-induced anemia), or hyperoxic conditions. In parallel, we also induced cardiomyocyte-specific expression of dnHIF1 $\alpha$  in adult Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LrL:GFP)(cmlc2a:LrL:dnHIF1 $\alpha$ ) transgenic zebrafish before ventricular amputation. To establish a baseline, we first amputated wild-type zebrafish and maintained them under normoxic conditions throughout the postamputation period. Subsequently, we were able to detect a robust regenerative response after 14 days (Figure 2A and 2B). We

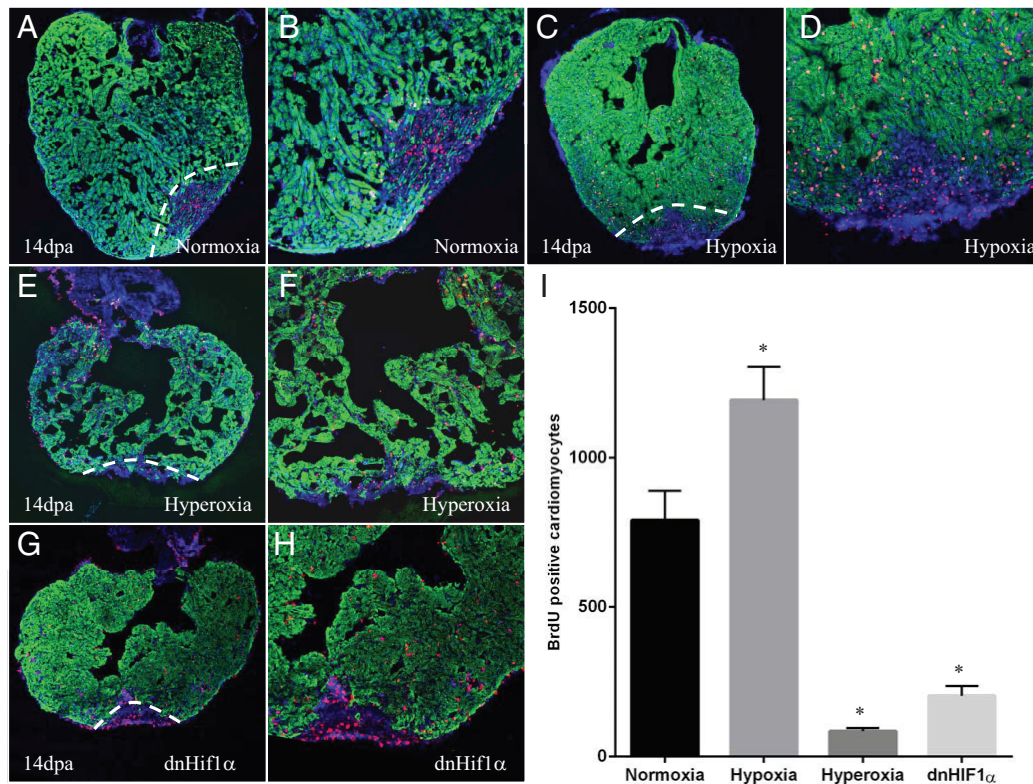




**Figure 1.** Hypoxyprobe analysis of adult zebrafish cardiomyocytes in vitro and in vivo. Adult zebrafish cardiomyocytes cultured under normoxic conditions were immunolabeled for tropomyosin (green; **A**) and Hypoxyprobe (red; **C**). **A**, A merged image with tropomyosin (green) and Hypoxyprobe (red). Adult zebrafish cardiomyocytes cultured under hypoxic conditions were immunolabeled for tropomyosin (green; **E**) and Hypoxyprobe (red; **F**). **D**, A merged image with tropomyosin (green) and Hypoxyprobe (red). A section from a wild-type adult zebrafish heart maintained under normoxic conditions immunolabeled for  $\alpha$ -sarcomeric actin (green), DAPI (blue), and Hypoxyprobe (red; **G**). A higher-magnification image of the same heart with  $\alpha$ -sarcomeric actin (green), DAPI (blue), and Hypoxyprobe (red; **H**). **I**, The same image in **H** with only Hypoxyprobe (red). A section from a wild-type adult zebrafish heart maintained under hypoxic (anemia) conditions immunolabeled for  $\alpha$ -sarcomeric actin (green), DAPI (blue), and Hypoxyprobe (red; **J**). A higher-magnification image of the same heart with  $\alpha$ -sarcomeric actin (green), DAPI (blue), and Hypoxyprobe (red; **K**). **L**, The same image in **K** with only Hypoxyprobe (red). A section from a wild-type adult zebrafish heart 7 days after amputation immunolabeled for  $\alpha$ -sarcomeric actin (green), DAPI (blue), and Hypoxyprobe (red; **M**). A higher-magnification image of the same heart with  $\alpha$ -sarcomeric actin (green), DAPI (blue), and Hypoxyprobe (red; **N**). **O**, The same image in **N** with only Hypoxyprobe (red). DAPI indicates 4'-6'-diamidino-2-phenylindole.

confirmed this by BrdU labeling the regenerating animals and analyzing the number of BrdU-positive cardiomyocytes (Figure 2I and Figure IIIA and IIIB in the online-only Data Supplement). In this manner, we were able to detect numerous BrdU-positive cardiomyocytes in the hearts of regenerating zebrafish. Next, we preconditioned adult zebrafish by treating them with phenylhydrazine to induce anemia/hypoxia 7 days before amputation. These hypoxic zebrafish showed an even more profound regenerative response (Figure 2C and 2D) that was associated with a significantly increased number of BrdU-positive cardiomyocytes (Figure 2I). Conversely,

adult zebrafish maintained under hyperoxic conditions failed to regenerate their hearts substantially after 14 days (Figure 2E and 2F) and had significantly fewer BrdU-positive cardiomyocytes than the normoxic controls (Figure 2I). Similarly, adult Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LnL:GFP) (cmlc2a:LrL:dnHIF1 $\alpha$ ) transgenic zebrafish, in which cardiomyocyte-specific expression of dnHIF1 $\alpha$  had been induced before amputation, also failed to regenerate their hearts (Figure 2G and 2H), resulting in significantly fewer BrdU-positive cardiomyocytes (Figure 2I). As an additional control, we repeated these experiments using an anti-MEF2c antibody



**Figure 2.** Perturbing normoxia effects heart regeneration in adult zebrafish. A section from a 14-day postamputation (dpa) regenerating wild-type adult zebrafish heart maintained under normoxic conditions immunolabeled for  $\alpha$ -sarcomeric actin (green), BrdU (red), and DAPI (blue). The white dashed line indicates the plane of amputation (A). The same heart at higher magnification (B). A section from a 14-day postamputation regenerating wild-type adult zebrafish heart maintained under hyperoxic conditions immunolabeled for  $\alpha$ -sarcomeric actin (green), BrdU (red), and DAPI (blue). The white dashed line indicates the plane of amputation (C). The same heart at higher magnification (D). A section from a 14-day postamputation regenerating wild-type adult zebrafish heart maintained under hypoxic conditions immunolabeled for  $\alpha$ -sarcomeric actin (green), BrdU (red), and DAPI (blue). The white dashed line indicates the plane of amputation (E). The same heart at higher magnification (F). A section from a 14-day postamputation regenerating Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LnL:GFP)(cmlc2a:LnL:dnHIF1 $\alpha$ ) adult zebrafish heart immunolabeled for  $\alpha$ -sarcomeric actin (green), BrdU (red), and DAPI (blue). The white dashed line indicates the plane of amputation (G). The same heart at higher magnification (H). I, A graph representing the average number of BrdU-positive cardiomyocytes per section in each of the 4 conditions  $\pm$  SEM. dnHIF1 $\alpha$  indicates dominant-negative hypoxia-inducible factor-1 $\alpha$ .  $P < 0.0001$ , ANOVA. \* $P < 0.05$ , Holm-Sidak test. DAPI indicates 4',6'-diamidino-2-phenylindole; and BrdU, 5-bromo-2'-deoxyuridine.

(which labels cardiomyocyte nuclei) and obtained similar results (Figure IVA–IVG in the online-only Data Supplement). These findings were further confirmed by repeating the experiments and allowing a 30-day recovery period. At this stage, regeneration is virtually complete; thus, amputated hearts taken from fish maintained under normoxic conditions appeared virtually indistinguishable from unamputated controls (Figure 3A and 3B). However, adult zebrafish, both those maintained in hyperoxic conditions and Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LnL:GFP)(cmlc2a:LnL:dnHIF1 $\alpha$ ) transgenic zebrafish, failed to regenerate normally with obvious areas of missing myocardium (Figure 3C–3F). These results indicate that hypoxia positively regulates heart regeneration in adult zebrafish and furthermore that this is achieved via HIF1 $\alpha$ .

### Hypoxia Positively Regulates Cardiomyocyte Proliferation

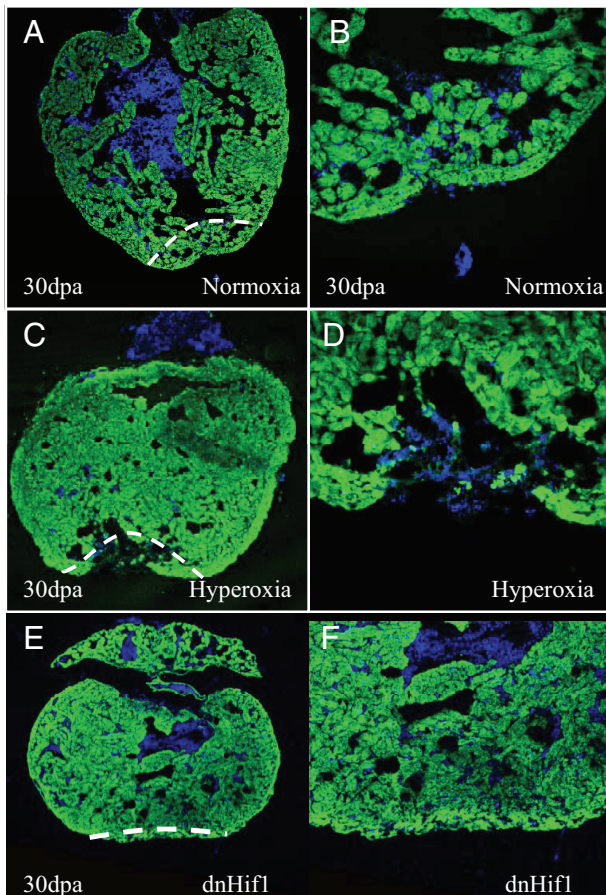
To elucidate the effect that hypoxia has on cardiomyocytes, we examined these cells in vitro to determine how they responded to changes in environmental oxygen. Under normoxic conditions, we established that adult zebrafish cardiomyocytes, unlike adult

mammalian cardiomyocytes, proliferate as evidenced by numerous PHH3-positive cardiomyocytes (46.6 PHH3-positive cardiomyocytes per  $10^5$  cells), which is substantially more than reported for adult mammalian cardiomyocytes<sup>26</sup> (Figure 4A–4C). Having established this basal proliferation rate, we cultured adult zebrafish cardiomyocytes in either hyperoxic or hypoxic conditions. Under hyperoxic conditions, we found that the number of PHH3-positive cardiomyocytes was significantly reduced compared with normoxia (4.1 PHH3-positive cardiomyocytes per  $10^5$  cells; Figure 4A–4C). However, under hypoxic conditions, we observed a significant increase in the number of PHH3-positive cardiomyocytes compared with the control normoxic conditions (59.3 PHH3-positive cardiomyocytes per  $10^5$  cells; Figure 4A–4C). This indicates that hypoxia positively regulates the proliferation of adult zebrafish cardiomyocytes.

### Hypoxia Induces Cardiomyocytes to Dedifferentiate

We have previously reported that zebrafish cardiomyocytes dedifferentiate during heart regeneration, a process involving





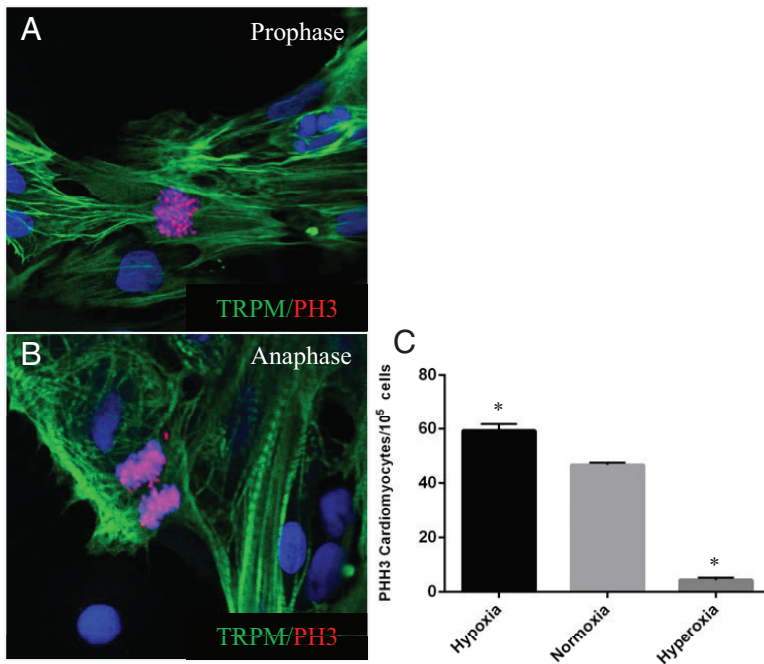
**Figure 3.** Perturbing normoxia affects heart regeneration in adult zebrafish. A section from a 30-day postamputation (dpa) regenerating wild-type adult zebrafish heart maintained under normoxic conditions immunolabeled for  $\alpha$ -sarcomeric actin (green), BrdU (red), and DAPI (blue). The white dashed line indicates the plane of amputation (A). The same heart at higher magnification (B). A section from a 14-day postamputation regenerating wild-type adult zebrafish heart maintained under hyperoxic conditions immunolabeled for  $\alpha$ -sarcomeric actin (green), BrdU (red), and DAPI (blue). The white dashed line indicates the plane of amputation (C). The same heart at higher magnification (D). A section from a 14-day postamputation regenerating Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LnL:GFP)(cmlc2a:LrL:dnHIF1 $\alpha$ ) adult zebrafish heart immunolabeled for  $\alpha$ -sarcomeric actin (green), BrdU (red), and DAPI (blue). The white dashed line indicates the plane of amputation (E). The same heart at higher magnification (F). dnHIF1 $\alpha$  indicates dominant-negative hypoxia-inducible factor-1 $\alpha$ ; DAPI, 4',6'-diamidino-2-phenylindole; and BrdU, 5-bromo-2'-deoxyuridine.

the disassembly of the sarcomere.<sup>15</sup> This phenomenon has also been reported in mammalian cardiomyocytes both in vitro and in vivo; however, there is little or no proliferation/regeneration associated with this process in mammals.<sup>26–29</sup> Although mammalian cardiomyocytes do not proliferate naturally, they can be stimulated to divide in vitro.<sup>30</sup> To proliferate, the adult cells first need to dedifferentiate, which involves disassembly of the sarcomere and re-expression of fetal genes.<sup>26</sup> In vitro and under normoxic conditions, we also readily observed dedifferentiation of zebrafish cardiomyocytes (similar to a previously published report<sup>31</sup>). Immunohistochemical analysis of differentiated zebrafish cardiomyocytes using anti-ASA and anti-TPM antibodies reveals a

typical organized/striated sarcomere (Figure 5A–5C). However, dedifferentiated cardiomyocytes, in which the sarcomere has been disassembled, display reduced/disorganized ASA expression and loss of z-disk striations<sup>26</sup> yet still retain a clear TPM signal (Figure 5D–5F). This difference between ASA and TPM allowed us to quantify the number of dedifferentiated cardiomyocytes (differentiated=ASA<sup>+</sup>/TPM<sup>+</sup>; dedifferentiated=ASA<sup>−</sup>/TPM<sup>+</sup>; Figure 5G). In this manner, we were able to determine that 46% of zebrafish cardiomyocytes were dedifferentiated after 7 days of culture under normoxic conditions (Figure 5H). Surprisingly, when zebrafish cardiomyocytes were cultured under hypoxic conditions, we observed a significant increase in the number of dedifferentiated cardiomyocytes (75%; Figure 5H). Furthermore, if zebrafish cardiomyocytes were cultured under hyperoxic conditions, then the number of dedifferentiated cells dropped significantly (28%; Figure 5H). These results indicate that the environmental oxygen concentration plays a significant role in regulating cardiomyocyte dedifferentiation. This also explains the differences in the amount of proliferating zebrafish cardiomyocytes that we observed under the different oxygen conditions in vitro. Because the cardiomyocytes need to dedifferentiate in order to proliferate, enhancing the amount of dedifferentiation will undoubtedly increase the number of proliferating cells.

### Mechanisms of Hypoxia During Heart Regeneration

To understand more about how hypoxia regulates zebrafish heart regeneration, we performed microarray analysis to identify genes that could play a role in this process. We decided to examine only the differences in gene expression between wild-type and Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LnL:GFP)(cmlc2a:LrL:dnHIF1 $\alpha$ ) transgenic zebrafish because this would provide a cardiomyocyte-enriched list of genes as a result of the restricted expression of dnHIF1 $\alpha$ . To further refine the identification of genes involved in zebrafish heart regeneration, we decided to focus only on 7-day postamputation zebrafish to avoid the initial wound/inflammatory response.<sup>31</sup> To this end, we amputated both wild-type and Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LnL:GFP)(cmlc2a:LrL:dnHIF1 $\alpha$ ) zebrafish, allowed them to regenerate for 7 days, and then compared their transcriptomic profile with those of unamputated wild-type and Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LnL:GFP)(cmlc2a:LrL:dnHIF1 $\alpha$ ) zebrafish, respectively. Our analyses focused on genes that were not significantly different between the wild-type and dnHIF1 $\alpha$  in the unamputated samples ( $P \leq 0.05$  and  $\log FC \leq 1$  or  $\log FC \geq -1$ ) but were subsequently found to be either significantly differentially expressed between unamputated and 7-day postamputation wild-type zebrafish ( $P \leq 0.05$  and  $\log FC \geq 1$  or  $\log FC \leq -1$ ) but showed no significant change in expression between unamputated and 7-day postamputation Tg(cmlc2a:Ert2-Cre-Ert2/cmlc2a:LnL:GFP)(cmlc2a:LrL:dnHIF1 $\alpha$ ) zebrafish ( $\log FC \leq 0.5$  or  $\log FC \geq -0.5$ ) or vice versa because this would identify putative HIF1 $\alpha$ -regulated genes. In this manner, we were able to identify 114 differentially expressed genes in the wild-type versus dnHIF1 $\alpha$  samples (Tables I and II in the online-only



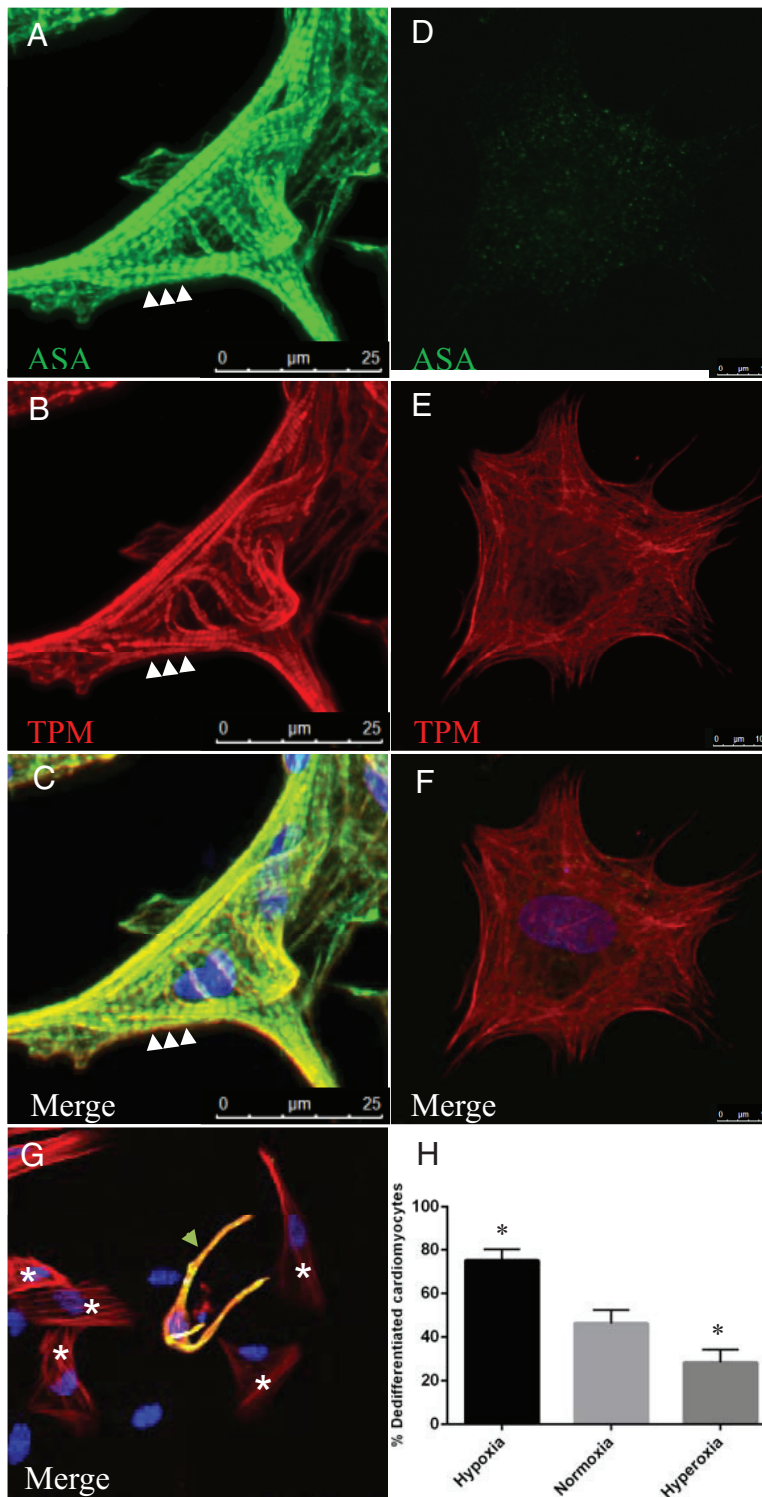
**Figure 4.** Hypoxia increases the number of proliferating adult zebrafish cardiomyocytes in vitro. **A**, A prophase adult zebrafish cardiomyocyte immunolabeled for tropomyosin (TRPM; green), phospho-histone H3 (PH3; red), and DAPI (blue). **B**, An anaphase adult zebrafish cardiomyocyte immunolabeled for tropomyosin (green), phospho-histone H3 (PH3; red), and DAPI (blue). **C**, A graph representing the average number of phospho-histone H3-positive cardiomyocytes per 10<sup>5</sup> cells  $\pm$  SEM.  $P < 0.0001$ , ANOVA.  $^*P < 0.05$ , Holm-Sidak test. DAPI indicates 4',6'-diamidino-2-phenylindole.

Data Supplement). Furthermore, we were also able to detect another 20 genes that were differentially expressed between the dnHIF1 $\alpha$  and wild-type samples (Tables III and IV in the online-only Data Supplement). To confirm the validity of our microarray data, we independently performed real-time quantitative polymerase chain reaction analysis on a subset of differentially expressed genes and confirmed that the changes in gene expression were the same (Figure V in the online-only Data Supplement). Of the 114 genes found to be differentially expressed in the wild-type versus dnHIF1 $\alpha$  sample, 102 of these (55 annotated and 47 unannotated) are upregulated and most likely represent genes that are either directly or indirectly positively regulated by HIF1 $\alpha$  (Table I in the online-only Data Supplement). Within this group, we were able to find genes that are known to be induced by hypoxia (the Table), including several that are directly regulated by HIF1, namely *jak2a*, *mcl1a*, *cdkn1b*, *cxc4b*, and *gata1a*.<sup>6,32–40</sup> Furthermore, we were able to identify genes within the upregulated group that have a heart-associated function (the Table) such as *thbs4b*, *jak2a*, *txnipa*, *pim1*, *slc4a1a*, and *cxc4b*.<sup>39,41–45</sup> A number of proproliferative genes were also shown to be upregulated in the wild-type sample compared with the dnHIF1 $\alpha$  sample (the Table).<sup>46–51</sup> Although the positive aspects of dedifferentiation have not been intensely investigated, we were able to identify a couple of genes that have been linked to this phenomenon, namely *jak2a* and *cxc4b*, albeit these are most commonly associated with cancer cell dedifferentiation.<sup>52,53</sup> Additionally, we were able to identify numerous components of the Jak-STAT signaling pathway (the Table). *Jak2a* expression is significantly increased in the wild-type sample but shows no significant change in the dnHIF1 $\alpha$  sample; *stat2* expression also was increased in the wild-type sample. Although this was just below our stringency ( $\log_{2}FC = 0.99$ ), we were able to confirm the change in expression via quantitative polymerase chain reaction (Figure V in the online-only Data Supplement).

Conversely, we also detected downregulation of a negative regulator of Jak-STAT signaling, *cish* (or *socs*),<sup>54</sup> which, although just below our level of stringency in the dnHIF1 $\alpha$  sample ( $\log_{2}FC = -0.51$ ), was dramatically reduced in the wild-type sample ( $\log_{2}FC = -2.18$ ). Upstream of the Jak-STAT pathway, *cxc4b*<sup>55</sup> was found to be upregulated in the wild-type sample but showed no change in the dnHIF1 $\alpha$  sample (Table I in the online-only Data Supplement), although a putative downstream target of the Jak-STAT pathway, *pim1*,<sup>56</sup> is upregulated in the wild-type sample ( $\log_{2}FC = 1.44$ ) but does not change in the dnHIF1 $\alpha$  sample ( $\log_{2}FC = 0.32$ ; Table I in the online-only Data Supplement). Taken together, these results indicate that numerous genes are regulated by hypoxia to positively influence the regenerative process. Furthermore, our analyses have uncovered various components of the Jak-STAT pathway.

## Discussion

Here, we have shown that ventricular amputation in zebrafish leads to rapid and robust induction of hypoxia in the heart and that, by inhibiting this process either through hyperoxia or by expressing dnHIF1 $\alpha$ , we can effectively block regeneration. Conversely, preconditioning adult zebrafish with hypoxia (via anemia) produces a more profound regenerative response. In mammals, cardiac ischemia also results in hypoxia induction and elevated levels of HIF1 $\alpha$ .<sup>57</sup> However, mammals fail to mount any significant kind of regenerative response, which suggests that whatever factors are missing or are inhibiting this process are downstream of the hypoxic response. Furthermore, our data indicate that hyperoxic conditions inhibit the regenerative process in zebrafish. In humans, current therapeutic strategies aim to reduce the hypoxia associated with cardiac ischemia.<sup>58</sup> Although this undoubtedly has a beneficial effect in the immediate aftermath of a cardiac ischemia/infarction, prolonged treatment may obscure any favorable response associated with hypoxia.



**Figure 5.** Hypoxia increases the number of dedifferentiated adult zebrafish cardiomyocytes in vitro. Differentiated adult zebrafish cardiomyocytes immunolabeled for  $\alpha$ -sarcomeric actin (ASA; green; **A**) and tropomyosin (TPM; red; **B**). **C**, A merged image with  $\alpha$ -sarcomeric actin (green), tropomyosin (red), and DAPI (blue). Dedifferentiated adult zebrafish cardiomyocytes immunolabeled for  $\alpha$ -sarcomeric actin (green; **D**) and tropomyosin (red; **E**). **F**, A merged image with  $\alpha$ -sarcomeric actin (green), tropomyosin (red), and DAPI (blue). **G**, A lower-magnification merged image of adult zebrafish cardiomyocytes in vitro with  $\alpha$ -sarcomeric actin (green), tropomyosin (red), and DAPI (blue). **H**, A graph representing the average percent of dedifferentiated cardiomyocytes in 3 conditions  $\pm$  SEM.  $P < 0.0001$ , ANOVA.  $*P < 0.05$ , Holm-Sidak test. DAPI indicates 4',6'-diamidino-2-phenylindole.

We have also shown here that hypoxia increases the amount of dedifferentiated zebrafish cardiomyocytes in vitro, a process involving disassembly of the sarcomere, which would otherwise physically impede cell division. It is not unusual to find hypoxia playing a role in regulating cellular dedifferentiation. Indeed, hypoxia is well known to be able to induce dedifferentiation in a wide variety of cell types such as adipocytes,<sup>59</sup> renal cells,<sup>1</sup> and astrocytes.<sup>60</sup> Hypoxia is also notorious for inducing tumors to dedifferentiate and subse-

quently to become more aggressive<sup>61</sup> (Hypoxypromote is most commonly used in tumor diagnostics). In mammals, 1 feature often associated with cardiac ischemia (or indeed hypoxia) is cardiomyocyte dedifferentiation (also called hibernating myocardium), which involves the induction of fetal gene expression accompanied by a disassembly of the sarcomeric structure.<sup>29,62</sup> Although this is widely regarded as being part of the cardioprotective program, other reports in which adult mammalian cardiomyocytes have been induced to proliferate



**Table. Functional Groups of Differentially Expressed Genes**

Gene Identification	Gene	LogFC	Reference
<b>Hypoxia induced</b>			
30307	<i>jak2a</i>	1.44	32, 33
497283	<i>hif1a2</i>	2.06	6
58122	<i>mcl1a</i>	1.01	34
368359	<i>txnipa</i>	1.15	35
58054	<i>pim1</i>	1.44	36
368329	<i>cdkn1b</i>	1.06	37, 38
114447	<i>cxcr4b</i>	1.41	39
30481	<i>gata1a</i>	1.08	40
<b>Heart associated</b>			
252850	<i>thbs4b</i>	1.94	41
30307	<i>jak2a</i>	1.44	54
368359	<i>txnipa</i>	1.15	43
58054	<i>pim1</i>	1.44	42
84703	<i>slc4a1a</i>	2.33	44
114447	<i>cxcr4b</i>	1.41	45
<b>Proliferation</b>			
30307	<i>jak2a</i>	1.44	46
58054	<i>pim1</i>	1.44	47
114447	<i>cxcr4b</i>	1.41	48
30766	<i>tal1</i>	1.01	49
321283	<i>tacc3</i>	1	50
30481	<i>gata1a</i>	1.08	51
<b>Dedifferentiation</b>			
30307	<i>jak2a</i>	1.44	52
114447	<i>cxcr4b</i>	1.41	53

indicate that dedifferentiation is a necessary prelude to proliferation.<sup>26</sup> Mechanistically, our microarray analyses has uncovered numerous genes that are potentially involved in hypoxia-mediated heart regeneration. We found that *pim1* expression was increased during heart regeneration in wild-type zebrafish; however, this effect was subdued in the presence of dnHIF1 $\alpha$ . *Pim1* is a serine/threonine kinase; its classification as an oncogene is indicative of its proliferative capacity.<sup>56</sup> Interestingly, *pim1* has previously been shown to be capable of inducing adult mammalian cardiomyocytes to proliferate.<sup>42,63</sup> Furthermore, we have uncovered what appears to be an entire signaling pathway. Numerous components of the Jak-STAT pathway are shown to be differentially expressed in our microarray analyses. Moreover, a recent report has indicated that HIF1 $\alpha$ -induced activation of the Jak-STAT pathway induces pulmonary arterial smooth muscle cells to dedifferentiate and proliferate.<sup>32</sup>

## Conclusions

It appears then that the hypoxia associated with both ischemia in mammals and, as we have shown here, ventricular amputation in zebrafish induces cardiomyocytes to dedifferentiate, the obvious difference being that adult zebrafish cardiomyocytes are able to subsequently proliferate whereas adult mammalian cardiomyocytes cannot. It is likely that whatever

is blocking heart regeneration from occurring in mammals (whether it be a lack of specific factors or an active inhibition of this process) is downstream of the hypoxic response and of the dedifferentiation process. Future strategies aimed at trying to induce myocardial regeneration in mammals should take into account that hypoxia signaling appears to be a key positive component of this process.

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## Disclosures

None.

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### CLINICAL PERSPECTIVE

Cardiac ischemia leads to a reduction in oxygen supply and subsequent induction of hypoxia. At present, the consensus is that the hypoxia associated with cardiac ischemia initially induces a cardioprotective response; however, prolonged exposure ultimately leads to necrosis and loss of myocardial tissue. Treatment of patients who have suffered an ischemic episode aims to reduce hypoxia. Our data now indicate that hypoxia could also play a beneficial role. We have found that hypoxia positively regulates myocardial regeneration in zebrafish. We have also found that some of the processes that are regulated by hypoxia during zebrafish heart regeneration have also been reported in mammals/humans after cardiac ischemia. In particular, we have observed that hypoxia induces zebrafish cardiomyocytes to dedifferentiate, a process that is also associated with cardiomyocytes in ischemic patients. Unfortunately, although they are capable of this initial dedifferentiation, human cardiomyocytes fail to proliferate and subsequently regenerate the heart. Future strategies attempting to regenerate a damaged human heart should take into account that hypoxia can play a beneficial role in inducing a regenerative response.