Published in final edited form as:

Trends Mol Med. 2017 November ; 23(11): 1024–1036. doi:10.1016/j.molmed.2017.08.008.

Oxygen, Metabolism, and Regeneration – Lessons from Mice

Ellen Heber-Katz, PhD

Professor, Laboratory of Regenerative Medicine, Lankenau Institute for Medical Research,
Wynnewood, PA 19096

Abstract

The discovery that the Murphy Roths Large (MRL) mouse strain is a fully competent, epimorphic tissue regenerator, proved that the machinery of regeneration was preserved through evolution from hydra, to salamanders, to mammals. Such concepts have allowed translation of the biology of amphibians -- and their ability to regenerate -- to a mammalian context. In particular, the ancient hypoxia inducible factor (HIF-1 α) pathway, operating through prolyl hydroxylase domain proteins (PHDs), was identified as a central player in mouse regeneration. Thus, the possibility of targeting PHDs or other HIF-1 α modifiers to effectively recreate the amphibian regenerative state has emerged. We posit that these regenerative pathways are critical in mammals. Moreover, the current approved use of PHD inhibitors in the clinic should allow fast-track translation from mouse studies to drug-based regenerative therapy in humans.

PROLYL HYDROXYLASES AND TISSUE REGENERATION

Prolyl hydroxylase domain proteins (PHD) are ubiquitous in biology but most recently, and unexpectedly, have been seen to play a central role in mammalian regeneration. Attempting to present a unified biological picture of regeneration and PHDs is at this time impossible, given our evolving understanding of their function. This is further confounded by the tortuous nomenclature of PHDs, which varies from P4H, to HIF-P4H, to EGLNs, to PHDs, along with their respective isoforms. Indeed, PHD pathways are complex, serving a wide range of functions, including, protecting cells against the deleterious effects of oxygen, stabilizing collagen structures, inducing the severe scarring of tissues, orchestrating the effects HIF-1 α in cells, and influencing tissue regeneration. Nonetheless, emerging PHD inhibitors are now being considered in humans for the treatment of scarring, anemia, chronic kidney disease (CKD), as well as myeloid dysplasia syndrome (MDS). Consequently, a greater understanding of PHD pathways in tissue regeneration will surely come from this clinical direction. The possible extension of using such FDA-approved drugs to achieve mammalian tissue regeneration-- and potentially for all tissue types, creates an exciting time for drug-based regenerative medicine, with new clinical possibilities and solutions.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

In this Opinion piece, we attempt to show how clues derived from salamander regeneration and unique aspects of the Murphy Roths Large (MRL) mouse strain, including the use of aerobic glycolysis, enhanced inflammation, cell cycle differences, and genetics, have led to the hypothesis that PHDs and HIF-1 α play a central role in mammalian tissue regeneration (Figure 1).

CHEMISTRY OF PROLINE HYDROXYLASES

One molecule that appeared early in evolution was the oxygen sensor prolyl hydroxylase (P4H), a non-heme dioxygenase that interacts with Fe⁺² (46). Fe⁺² is an important binder of molecular oxygen which can become inactive Fe³⁺ but is reactivated through its reduction by ascorbic acid. P4H can also interact with 2-oxoglutarate or α -ketoglutarate which also binds oxygen. P4H modifies collagen, the most abundant protein in the body that is found in all connective tissue. In fact, one third of all bodily protein is collagen, and 38% of the prolines within collagen are hydroxylated as hydroxyproline (Hyp) by P4H (46,47). Nascent collagen single strands are modified by hydroxylation and this pre-organizes collagen so that it can form trimers and produce a structurally stable triple helix (46). This is a major function of P4Hs, first shown to be responsible for this chemical modification in the mid 1960's (48,49) (see Box 2).

Box 2

The structure and function of prolyl 4 hydroxylases (P4H)

The structure of the P4H molecule in mammals is an $\alpha\beta$ tetramer in which the alpha subunit contains the binding domain for substrate and the enzymatic active site (50). It was shown that there is a two stage catalytic process, the first is proline-independent and involves a highly reactive Fe-oxygen species and a second stage in which proline is decarboxylated by this species (46). The motif that is recognized in collagen by the P4H is XaaYaaGly where X and Y are most commonly Pro (46). This motif is found in many molecules including acetyl choline esterase, **C1q complement, collectins and hibernation proteins** which all form trimers and are stabilized by proline hydroxylation (46). Many of these are involved in innate and adaptive immune responses, as well as in the recognition/presentation of multivalent immune epitopes (46). Elastin (51) and argonaute2, which is involved in **RISC** complexes (52), are also substrates of P4H.

A related small group (3 isoforms) of prolyl hydroxylases, show a greater affinity for molecular oxygen and are only partially homologous to the P4H amino acid structure; in fact, there is very little sequence identity (53). These molecules have been more properly identified as intracellular oxygen concentration sensors relative to P4H and have a greater affinity for O₂(53). Prolyl hydroxylases are identified as prolyl hydroxylase domain proteins or PHDs, for isoforms 1–3; EGLNs, for isoforms 2,1,3; or HIF-P4Hs for isoforms 3-1 (54–59). Like P4Hs, PHDs require molecular oxygen, α -ketoglutarate and Fe⁺² and use a similar mechanism for hydroxylation(53).

However, there are many differences between P4Hs and PHDs. P4Hs are found in the endoplasmic reticulum (ER) and Golgi, and PHDs are found in the soluble cytoplasm and

the nucleus”(46). Although they share 3-dimensional structural similarity in the active site, outside of this region their differences may be seen “to accommodate disparate substrates”(46). Thus, their molecular targets are different. PHDs recognize and hydroxylate two prolines in **HIF-1 α** , a key molecule that regulates hundreds of genes involved in oxygen homeostasis, metabolism, angiogenesis, and blood cell formation; P4Hs cannot hydroxylate those same prolines. This is because PHDs are more flexible in their recognition of the LXXLAP motif (alanine being the strictest requirement) (60).

From evolutionary studies, PHDs and specifically PHD2 or EGLN1 arose before the metazoan stage and before HIF-1 α , suggesting that PHDs may have originally had different substrates (61). In fact, there are other known substrates for PHDs, such as the RNA polymerase II (Pol II) subunit RPB and ATF-4, both involved in transcriptional regulation, as well as I κ B, known to regulate nuclear factor kappa light chain enhancer in B cells (NF κ B), and lastly, the beta adrenergic receptor (61).

Though there are many that believe that there are no functional differences between PHD isoforms, except perhaps the difference in localization or levels of expression in different tissues, there have been, in fact, differences found. In the case of RPB1 -- which shares sequence homology with HIF-1 α , including its hydroxylation motif--PHD1 can catalyze hydroxylation, and PHD2 can inhibit hydroxylation (62), showing that there may be distinct functional differences among PHD isoforms.

THE REGULATION OF OXYGEN

As elegantly summarized by Gregg Semenza in his essay “Life with Oxygen” (1), the advent of an oxygen-rich atmosphere following the evolution of photosynthetic plants confronted life with new opportunities and challenges (1). On the one hand, oxygen permits the highly efficient energy conversion mechanism of oxidative phosphorylation (OXPHOS) relative to the evolutionarily earlier glycolytic energy pathway. On the other hand, the highly corrosive property of electro-negative oxygen requires a finely tuned oxygen sensing mechanism to protect the cellular milieu (1). The co-opting of oxygen sensing and regulation for regenerative processes was a marvelous solution for vulnerable organisms at the land-water interface.

AMPHIBIAN AND MAMMALIAN REGENERATION: THE IMPORTANCE OF OXYGEN

The ability to regenerate injured or lost tissues, limbs and organs is a property of organisms such as hydra, sea cucumbers, starfish, and salamanders and has been known for hundreds of years (2). Most organisms that are **super regenerators** exist in a shallow watery environment where changes in oxygen concentrations are most dramatic and where injuries from turbulence or predators are acute and can be repaired by regeneration of missing parts. By contrast, land mammals exposed to a constant level of atmospheric oxygen are overwhelmingly found to be non-regenerating animals, using fibrosis or scar tissue formation as a means to control injuries that can be life threatening (2).

Nevertheless, various cells in mammals can replace themselves. For example, this is certainly known for epithelial cells on the skin surface and the intestinal tract of mice and humans; it is also true for cells of the hematopoietic system, beginning with the yolk sac and fetal liver, and extending to the adult bone marrow. In addition, it is well known that mammalian striated muscle can regenerate through the **satellite cell** system. Finally, the adult liver also harbors regenerative capacity; in mammals, pre-existing multipotent stem cells are found at various anatomical reservoirs such as adipose tissue and bone marrow. Tissue engineering studies of **bioscaffolds** loaded with immature cell populations have revealed that precursor and stem cells bear the potential of repopulating whole organs (3,4). Moreover, emerging methodologies such as the advent of induced pluripotent cells (iPSC) where a cocktail of transcription factors (**Yamanaka factors**) can be used to reprogram adult cell populations into distinct cell lineages are being intensively pursued. For instance, iPSC reprogramming protocols can induce mature fibroblasts to de-differentiate and then re-differentiate into different cell lineages in culture, allowing donor and recipient cells to be identical. However, there are also many known problems with this type of approach, including the potential lack of stability (and teratogenesis) of induced cells.

There is still the issue of how to fully regrow a complete organ in-situ without adding cells, as seen in the regenerating world of non-mammals. This issue is embodied by the concept of **epimorphic regeneration** or epimorphic tissue reprogramming: the growth of new mammalian tissue through the generation of a **blastema**, consisting of de-differentiated cells which can migrate and proliferate, but are able to stop these processes at an appropriate time (5,6). At this point, re-differentiation can occur to produce mature cell types needed to carry out specific functions, identical to those observed in parental cells. The highlight of this type of regeneration is that there appears to be no scarring, multiple tissues can reform in the appropriate location, and complete function is restored (5,6).

The reason that mammals do not have the ability to regenerate as amphibians do has long been debated. One obvious possibility is that the genes and pathways for this trait have been lost with evolution, and mammals are left with the ability to heal via scar formation. However, in 1998, a particular mouse strain, the **MRL mouse** and its parent, the **LG/J mouse** (7–11), were reported to experimentally regenerate injured appendages and organs nearly to completion. Since then, the ability of the MRL mouse to regenerate has been confirmed in many laboratories and include such diverse tissues as articular cartilage, tendon, myocardium, cornea, central nervous system, peripheral nervous system, myometrium, muscle, and digits (11–14). Furthermore, other regenerator mouse strains have been identified. Of course, skepticism and controversy still exist, but nonetheless, a point has been reached where determining the mechanisms of such regenerative responses is becoming an important focus in biology.

A MAMMALIAN MODEL OF REGENERATION: THE MRL MOUSE

Perhaps the easiest epimorphic regenerative amphibian-like mammalian system has been created in mice. In this model, a hole is made in the mouse **ear pinna**; a wild type mouse cannot close the hole during its lifetime. Indeed, because of this, ear punching is commonly used in research to identify mice by using particular punch codes (15). However, MRL mice,

-- widely used as models for autoimmunity and specifically, systemic lupus erythematosus—display an unusual response, by closing ear holes completely, and are able to regrow cartilage as well as hair follicles (16,17). Of note, the ear hole model was first identified in some rabbits as tissue that could regenerate and close completely. Moreover, both rabbits and MRL mice are animals that generate a circular blastema at the lesion site, and thus, can close the hole without scarring (16,17). However the mouse appears to be a more practical and insightful model for studying mammalian regeneration (including for genetic characterization and analysis purposes). Various laboratories, including ours, have investigated the metabolism, genetics, and minimal requirements needed to achieve a full regenerative response in mice.

THE ROLE OF METABOLISM IN REGENERATION

An important piece of the regeneration puzzle was solved in the MRL mouse model of ear regeneration. Specifically, these mice were shown to use aerobic glycolysis as their basal metabolic state, with increased levels of lactate in cells and serum, derived from the conversion to pyruvate from glucose metabolism through increased LDH levels (18,19). This was accompanied by a process where mitochondria operated at highly reduced levels with low mitochondrial membrane potentials, reduced ROS production and reduced glutathione pools, indicating that cytoplasmic glycolysis and not OXPHOS was being used for ATP synthesis (18,19). Furthermore, this metabolic state, characterized by increased glutamate oxidation and reduced fatty acid oxidation, was confirmed, as evidenced from increased levels of carnitine species in plasma, further indicating the occurrence of increased glycolysis and reduced OXPHOS in cells from these mice. (18,19).

Enhanced OXPHOS in this mouse model was induced using the small molecule dichloroacetate (DCA) to enhance pyruvate dehydrogenase (PDH) through the inhibition of pyruvate dehydrogenase kinase (PDK), which in turn, reduced lactate and increased ROS and ATP levels (20–22). This led to inhibition of ear closure (Key Figure, Figure 2), indicating that, indeed, increased OX PHOS was not supportive of a regenerative response in these mice (20–22).

Aerobic glycolysis is known to be important during embryonic development and in tumorigenesis. Indeed, aerobic glycolysis is required in the **pentose phosphate pathway** and in the production of nucleic acids and fatty acids, all necessary components of new cells. In addition, this metabolic state appears to be important for cell proliferation, and since aerobic glycolysis has been implicated in developmental processes, it would not be surprising if this were an ideal pathway to achieve regeneration (for alternatives, see Box 1). A recent report suggested that the mole rat and other hibernating animals can use aerobic glycolysis as a mechanism to resist low oxygen states (26). Thus, a search for a means to reverse OXPHOS has been pursued.

Box 1**Pseudohypoxia and the role of ROS in PHD inhibition**

The reduction in the level of oxygen, which often occurs in wounds when blood vessels become occluded, can be detected by cells using multiple means. They include prolyl hydroxylases (P4H and PHDs), NADH/NADPH oxidases, reactive oxygen species (ROS), and oxygen-sensitive ion channels, and the electron transport system (ETC)(23). Focusing on PHDs during physiological hypoxia, ROS is made by mitochondria from complex III. PHDs are then regulated not only by the level of oxygen but by ROS which can regulate PHD function, through possible oxidation of Fe^{2+} to nonfunctional Fe^{3+} .

Even in the case of normoxia which can occur with infections and toxin exposure, where ROS levels are high due to cell stress, PHDs can be regulated by ROS levels which again can potentially act through the Fe oxidation state. Also, PHD function can be affected by levels of ETC components 2-oxoglutarate and succinate (structurally similar to 2-oxoglutarate). The use of specific PHD inhibitors can also function to block PHD hydroxylation activity. Aside from ROS increases, it has been reported that viral molecules might also act as PHD inhibitors (24).. The regulation of PHDs in the presence of normal oxygen levels is known as **pseudo-hypoxia** (24).

The MRL mouse displays increased levels of HIF-1 α under normoxic conditions. One explanation for this may be that the E3 ligase component, RNF7 -- necessary for the ubiquitination and proteosomal degradation of HIF-1 α , along with pVHL -- is reduced or not expressed in the MRL mouse. If RNF7 were not to be expressed, this would presumably lead to HIF-1 α stabilization. Alternatively, PHDs may be inhibited as well. However, regenerating MRL cells have been reported to have low levels of ROS, suggesting that the alternative pseudohypoxia mechanisms discussed above might not be involved.

One potential mechanism responsible for increased aerobic glycolysis is cellular stress, in which mitochondrial ATP synthesis falls. Mitochondrial function is reduced (as seen in the MRL mouse model) and there is an associated vast increase in aerobic glycolysis and net ATP synthesis (25).

THE ROLE OF THE CELL CYCLE IN REGENERATION

A second clue for the basis of regeneration in the MLR mouse model came from the observation that regenerating MRL-derived fibroblasts -- but not non-regenerating mouse-derived fibroblasts ---were highly proliferative in culture(27,28). An analysis of the cell cycle status in these fibroblasts indicated that regenerative MRL cells showed a pause at G2, with non-regenerative cells showing a G1 peak(27,28). Moreover, the defective G1 arrest in MRL was also accompanied by a lack of Cdkn1a or p21^{cip/waf1} protein expression, leading to an intense DNA damage response(27,28). In order to confirm that G2 arrest was functional in regeneration, p21 knockout mice (in an MLR background) were tested, and demonstrated a positive ear closure response (27,28). Furthermore, a comparative analysis between salamander and MRL mice revealed similar molecular changes expressed during

G2, suggestive of an evolutionary conservation of the regeneration machinery -- and presumably function-- from salamander to mammals. (29).

Another murine study using the ear hole closure model confirmed previous observations that p21 KO mice could regenerate ear tissue, and in addition, established that the protein interaction between stromal cell-derived factor 1 (SDF1, also known as CXCL12) and C-X-C chemokine receptor 4 (CXCR4), -- normally seen in wild type mice --- was reduced in p21 KO mice(30). Indeed, SDF1 was down-regulated after injury, and reduced numbers of CXCR4-expressing cells were found at the injury site, suggesting that their recruitment to the wound repair site was decreased in the absence of p21(30). Furthermore, the drug AM3100 (Plerixafor, 31), delivered through an osmotic pump subcutaneously in wild type mice, was used to block the SDF-1/CXCR4 interaction, which significantly enhanced the degree of ear hole closure and regeneration of cartilage(30).

Of note, the human regenerating liver also displays reduced levels of mRNA transcript coding for p21^{cip/waf} (32), consistent with the fact that liver is one of the few organs that can spontaneously regenerate in mammals. Furthermore, p21 KO mice have been reported to exhibit enhanced liver regeneration in both partial and extreme hepatectomies (33–35). Consequently, it is possible that direct blockade of p21 could be considered as an alternative approach to induce a regenerative liver response, but further research into this pathway would be required.

THE ROLE OF INFLAMMATION AND THE INNATE IMMUNE RESPONSE

Other aspects of regeneration may involve inflammation and innate immunity; these processes, which are driven by aerobic glycolysis (36–38), are necessary to induce a regenerative response, at least in mice. Specifically, in the ear punch MRL mouse model, macrophages, mast cells, and neutrophils have been shown to be present in increased numbers (39–42) and with increased expression of myeloperoxidase (MPO), and mast cell protease 1 (MCP-1), indicative of inflammatory infiltration at the lesion site (40). Moreover, administration of the cyclooxygenase 2(COX2) inhibitor Meloxicam, an anti-inflammatory agent, was shown to block ear hole closure, confirming that inflammation was required to achieve regeneration (40). A compelling study of mice selectively bred for a highly inflammatory phenotype (**AIRmax mouse model**) displayed full ear hole closure and regeneration by contrast to a low inflammation model (**AIRmin**) (42). Here, macrophages were identified and genetic mapping studies revealed that specific alleles of the natural resistance-macrophage protein 1(**NRAMP 1**), a divalent metal transporter in macrophages, was shown to interact with inflammatory alleles to affect healing (42). In a recent study using the related rodent strain **African spiny mouse**, depletion of macrophages led to reduced ear hole closure (43,44) supporting a positive macrophage role in the regenerative response.

The importance of COX-2 in regenerative responses (40) was further demonstrated in a recent study in mice showing that prostaglandin E2 (PGE2)-- the biosynthetic target of COX enzymes, negatively regulated by 15-prostaglandin dehydrogenase (PGDH)-- could be relieved of this inhibition via a small molecule drug, SWO33291 (15-PGDH inhibitor))

injected intraperitoneally (45). When the drug was given to normal mice, enhanced hematopoietic recovery was noted in recipient mice receiving a bone marrow transplant(45). In addition, using models of colon and liver injury, 15-PGDH blockade reduced the severity of dextran sodium sulfate-induced colitis and enhanced liver regeneration following partial hepatectomy, respectively (45). Consequently, 15-PGDH inhibition may prove to be a potential therapeutic strategy for tissue regeneration.

HIF-1 α IN REGENERATION

Since inflammation is regulated in part by two major genes, NF κ B and HIF-1 α (63), their involvement in regulating the MRL regenerative response in mice provided another important mechanistic clue. HIF-1 α protein expression levels are regulated by PHD hydroxylation of HIF-1 α leading to its recognition by the E3 ligase component pVHL, followed by ubiquitination, and proteosomal destruction in the presence of a sufficient amount of molecular oxygen (65). When oxygen is low, PHDs are unable to function, and consequently, HIF-1 α survives and is able to join HIF-1 β in the nucleus, functioning as a DNA-binding transcription factor at specific promoter or enhancer sites known as **hypoxia response element sites (HREs)**. At HREs, there is subsequent activation of genes important for glycolytic metabolism, angiogenesis, growth/survival, cell migration and invasion.(see Figure 2). Another piece of evidence came from genetic mapping studies of MRL/LG vs non-regenerating mice identifying the candidate gene *Rnf7* (64), part of an E3-ligase complex, as necessary for HIF-1 α degradation (65), and which exhibited very low expression levels in regenerating MRL murine tissue (64).

Testing MRL tissue for HIF-1 α protein levels resulted in a biphasic expression profile similar to the kinetics of DCA action (Figure 3). Considering the regenerative phenotypic picture emerging in the MRL mouse, including inflammation, tissue remodeling, increased angiogenesis, and proliferation, it became clear that HIF-1 α could act as an important mediator of all of these processes. Even the process of de-differentiation, important for the conversion of mature cells into immature cells, shown to be functioning in amphibian regeneration (66,67), has been shown to be induced by HIF-1 α (68,69). Indeed, RNA silencing of *Hif1a* in these mice (via si*Hif1a* administration in-vivo) fully eliminated ear hole closure as well as all of the phenotypes examined (70).

Furthermore, a study using PHD knockout mice demonstrated that PHD1 was required for liver regeneration, (PHD3 was not, and PHD2 was inconclusive) (71). In addition, HIF-1 α KO mice showed early reduction (during the first 3 days post-hepatectomy) of a regenerative liver response in the absence of HIF-1 α , but later on, a full replacement of liver mass and function, demonstrating that a HIF-1 α - independent mechanism was functioning later in the regenerative liver response (72).

PHD INHIBITORS AND REGENERATION

It took approximately twenty years since the discovery of PHDs to determine that their inhibition was of biological significance. (73,74). However, PHD involvement in HIF-1 α regulation came from another direction. As early as 1993, HIF-1 α was identified as a key

molecule mediating hypoxia and acting a transcription factor (75,76). In 2001, specific HIF-prolyl hydroxylases or PHDs/EGLNs were characterized as a novel class of oxygen-binding enzymes. As discussed, this group was shown to be similar but not identical to collagen prolyl hydroxylases (53–59). At this point, inhibitory molecules specific for PHDs were generated and shown to block the HIF α pathway (67) (Box 3).

Box 3

Small molecule PHD inhibitors in the Clinic

One major focus of PHD blockade has been to treat anemia. Erythropoietin or EPO, produced by the kidney and liver, is a molecule which regulates red cell production in the bone marrow and is a target of HIF1- α . EPO has been shown to be produced in great quantities upon PHD blockade.

The company FibroGen developed two small molecule drugs, FG-2216 and FG-4592, both PHD inhibitors, which when given orally, could induce high levels of EPO to treat anemia in humans. They were both FDA approved, but after a patient receiving FG2216 died, these agents were temporarily withdrawn, but are now back on the market (78,79). Currently, FG-4592 (roxadustat) is being given orally to treat anemia associated with chronic kidney disease (CKD) and is in Phase 3 clinical trials. It is also being tested for anemia associated with myelodysplastic syndromes (MDS) and is beginning a Phase 2/3 clinical trial for MDS-associated anemia in China. It is also being considered as a potential treatment for mitochondrial disease (80). Another PHD inhibitor, the small molecule drug vadadustat (AKB-6548 from Akebia), is also being used to treat anemia for Stage 3/4 CKD, and is currently in Phase 3 clinical trials (81–83).

PHD inhibitors have been tested in multiple systems, such as vascular and heart disease (84), and to enhance angiogenesis (85). They have also been examined for enhancement of EPO expression in the brain to increase hippocampal memory (86) and to promote neural protection in spinal cord injury (87).

Other studies are exploring the process by which many of the small molecule HIF-PHD inhibitors function as iron chelators; small molecules such as M10 and M30, can get through the blood brain barrier, and have been shown to block iron accumulation in the brain of aged patients at sites of neural degeneration, and --- in addition to being PHD inhibitors---might be considered as potential anti-Alzheimer's disease drugs (88,89). This remains to be validated.

For instance, the compound 1,4-dihydrophenanthroline-4-one-3-carboxylic acid (1,4-DPCA) was identified as a blocker of P4H collagen prolyl hydroxylation in rats, with the potential of reducing collagen crosslinking and scar formation, both inhibiting regeneration (90) and inhibiting human MDA-MB-231 breast cancer metastasis in in vitro 3D cultures (91). However, it was also shown to act on PHDs and to modulate HIF-1 α levels (6). Given the results that HIF-1 α protein expression appeared to be key in the regenerative response of MRL mice (6), 1,4-DPCA was used in combination with a hydrogel delivery system to slowly release low concentrations of the drug over a 5 day period in mice (92). The amount of drug used was 75-fold less than the dose for soluble drug given orally in rats for prolyl

hydroxylation studies in vivo (6,90). Mimicking the kinetics of the MRL- HIF-1 α expression response, 1,4-DPCA/hydrogel was injected subcutaneously at the base of the neck into non-regenerative mice and regenerative ear hole responses assessed (92). Non-regenerative (Swiss Webster) mice that received the drug regenerated ear lesions in the same amount of time as regenerative (MRL) mice (92). The observed phenotypic changes, including remodeling, inflammation, de-differentiation, cell division and migration were replicated in otherwise non-regenerative mice, compared to regenerative mice. Moreover, these changes were blocked when *Hif-1 α* was knocked down (si*Hif1 α*), supporting the notion that these effects were HIF-1 α -related (92). Of note, these regenerative responses have been replicated in the liver (93). Given the promising potential of PHD inhibitors in regeneration, other PHD inhibitors are currently being tested for liver regeneration post hepatectomy using mice receiving dimethyloxalylglycine (DMOG) (94,95) and ethyl-3,4-dihydroxybenzoate (EDHB) (96). Finally, it should be noted that PHD inhibitors may act on other targets besides HIF-1 α as well as being able to inhibit P4Hs and thus, may affect the targets of the latter also.

CONCLUDING REMARKS

Prolyl hydroxylase inhibitors are currently being used in humans in the clinic for amelioration of hypertrophic scars and of anemia associated with kidney disease and MDS, by enhancing EPO production (see Box 3), as well as in Alzheimer's disease.

Recently, a very exciting new direction arose, as discussed, from the findings that such inhibitors could elicit potent, and surprisingly rich regenerative responses, accompanied by varied regenerative effects, including metabolic, pro-inflammatory, remodeling, migratory, proliferative, and de-differentiation responses. This can be followed by re-differentiation of cells and full replacement of normal tissue with normal architecture. This can occur in a very short amount of time, with a treatment of approximately 2 weeks. Clearly, researching the potential applicability to non-human primates and then to humans, would be among the next steps to take.

With these findings, it appears that HIF-1 α is a central regulator of mammalian regeneration, at least in mice. Although many questions remain and there is a long research road ahead of us (see Outstanding Questions and Box 4), it may be possible to bring cells and tissues in need of regeneration back to an embryonic-like, more primitive state, where regeneration might start anew. With lessons learned from mice, and the current human preclinical and clinical trials being undertaken, hopefully, we may see more powerful possibilities for using these PHD inhibitory drugs in human tissue regeneration. It will be quite exciting to follow how this journey unfolds.

Box 4

Clinician's Corner

What was not too long ago considered to be purely in the realm of science fiction can now be envisioned as a clinical possibility. Regeneration through the use of small molecule pharmaceuticals as pills or injectables may become part of the normal clinical

armamentarium. Physicians now in training, during their careers, may reach for these drugs in this same way that they currently prescribe statins, beta-blockers, TNF inhibitors, or antibiotics.

But even more than just the replacement of cells in tissues, epimorphic regeneration, the ability to replace not just some tissues, but multiple tissues in a complete organ or appendage structure with the proper architecture and function and with a functional “reset” to normal, may be a possibility as well. Experiments in mice described in this Opinion piece indicate that these approaches are indeed promising.

Epimorphic regeneration appears to be a dependent property of oxygen regulation which controls the most basic of physiological processes. The promotion of a glycolytic metabolic environment can lead to a pro-inflammatory innate immune response, altered adaptive immunity, and tissue remodeling (6). Such changes may lead to the expression of immature cell markers and cell de-differentiation, as well as migratory effects and enhanced proliferation, all of which may be associated with the status of a tissue regenerative response (6).

The use of HIF prolyl hydroxylase inhibitors which induce a pseudohypoxic condition in cells, and which regulate molecules such as HIF-1 α and NF κ B, inflammation and glycolysis have already been used in humans to upregulate EPO, a factor necessary for red cell growth and treatment of anemia. Those same inhibitors can lead to de-differentiation of cells required for reinduction of developmental processes necessary for regenerative healing without scar formation and presumably, for perfect function and anatomy. Differences in degrees of tissue healing achieved may be related to changes in kinetics, concentrations, and delivery modes of these compounds, but the use of such small molecule inhibitors holds great potential.

Outstanding Questions Box

Can there be too much PHD inhibition? i.e. can the HIF1 levels be too high and for too long?

In the MRL mouse after wounding, systemic HIF-1 α levels immediately rise and peak between days 10 and 14 and then subside for a total regeneration period of one month. This biphasic response correlates well with inflammation and remodeling and the pattern of de-differentiation, followed by re-differentiation of responding tissue. However, we do not know if the MRL response is actually optimal to achieve maximal regeneration in various tissues and different injury types. Tailoring drugs and kinetics may achieve super-regeneration. Obviously, this needs an analysis in larger mammals.

Are there “off target” effects of PHD inhibition?

The drug 1,4-DPCA, used to achieve full epimorphic regeneration in mice, was originally developed to be a P4H inhibitor to reduce collagen crosslinking and accumulation to treat hypertrophic scarring. The fact that HIF-PHD is also inhibited can be considered to be an “off-target” effect, but perhaps a most desirable one. Are there other off target effects at work?

What other target genes besides HIF1a or HIF2a are modified or affected?

Besides PHD inhibition affecting HIF-a's, there are other proteins that are affected as well.

These include NFkB, regulators of transcription like pol II, and beta adrenergic receptors. Drugs that more specifically target these molecules should be examined to see their effect on aspects of the regenerative response. Also, PHD inhibitors are also in many cases P4H inhibitors and this adds other targets including argonaute involved with small RNA modification such as microRNAs and siRNAs and regulation of many pathway genes.

What type of drug delivery is ideal? Is systemic or local inhibition of prolyl hydroxylase necessary to achieve local regeneration?

Previous studies have opted for a systemic approach for the practical reason that the small size of mice makes accurate and reproducible local administration problematic. However, from a medical standpoint, effective local administration is desirable, but perhaps systemic effects are necessary. Experiments in large animals should resolve this question.

Acknowledgments

The author was supported by grants from the National Institutes of Health Grants DE021104, DE021215, and CA180070

Glossary**AIRmax/AIRmin mouse model**

Mouse lines generated by intercrossing eight isogenic strains. Mice selected for their acute inflammatory reactivity (AIR) including cell infiltrates 24 hours post subcutaneous injection of polyacrylamide particles. The AIRmax line has inflammatory infiltrates 25 fold greater than the AIRmin mice and genetic analysis has shown 6 inflammatory quantitative trait loci (QTL). AIRmax mice exhibit MRL-like ear hole closure, but the AIRmin do not. One of the loci, *Slc11a1* in chromosome 1 or *Nramp*, is associated with ear hole closure.

Blastema

In development, aggregation of cells in the early embryo, capable of differentiation into specialized tissue and organs. It has a similar meaning in regenerating tissue where a mass of cells, un-differentiated and pluripotent, can develop at an injury site and grow into a normal organ or appendage.

Bioscaffolds

tissue-engineered biomaterials harboring different cell populations and can be implanted into the body to attempt growing new organs or tissues.

C1q complement

component of the C1-complex pathway involved in complement activation as part of the innate immune response. C1q can bind to antibodies and is formed from 18 peptides formed into 3 subunits.

Collectins

collagen-containing C-type lectins; constitute a group of defense lectins known as soluble pattern recognition receptors (PRRs) involved in the innate immune response.

Ear pinna

part of the ear that resides outside of the head and is composed of skin and cartilage along with muscle and hair follicles. This is also called the auricle and focuses sound waves into the ear canal to be transmitted to the eardrum.

Epimorphic regeneration

type of regeneration defined in amphibians involving de-differentiation of mature structures to form a mass of pluripotent cells known as the blastema just under a rapidly migrating population of epidermal cells. The regenerating blastema grows and forms a new limb or appendage with normal architecture and function. This occurs after an amputation injury.

Hibernation proteins

made by the liver, form a complex consisting of 3 related proteins + a proteinase inhibitor, and circulate in the blood. Currently, it is not clear what the function of these proteins is since they are present in some species of hibernating mammals, but also in animals that do not hibernate.

Hypoxia inhibitory factor-1 α (HIF-1 α)

The heterodimeric transcription factor HIF1 is comprised of two subunits, HIF-1 α and HIF1 β . HIF1 α is a basic helix-loop-helix PAS domain containing protein and is regulated through its interaction with prolyl-hydroxylating PHDs. As a transcription factor, HIF1 is a master regulator of hypoxia-related genes including angiogenesis and vasculogenesis, energy metabolism, migration, and cell survival.

HREs

HIF1 α / β functions as a transcription factor by binding to HIF-responsive elements (HREs) in promoters with the sequence NCGTG where N=A or G.

LG/J mouse

created from a pool of albino mice and then bred and selected for increased body size for over 50 generations. Extensive genetic studies using this mouse strain have been carried out to examine bone length, obesity, and regeneration.

MRL mouse

originally generated to preserve the *cn* gene found in AKR mice. It was further bred to the C57BL/6 and C3H mouse strains and then to LG/J, with 75% of the genome being LG-derived. It has been used to study systemic lupus erythematosus and Sjogren syndrome; found to have an unusual ability to regenerate a broad range of tissues.

NRAMP

membrane-bound protein also known as SLC11A1 is a divalent metal transporter within the endosomal and phagolysosomal membranes of iron-recycling macrophages and monocytes. It has been shown to be involved in ear hole regenerative responses in mice.

Pentose Phosphate Pathway

Branching off the glycolytic pathway at glucose-6-phosphate is the pentose phosphate pathway which produces sugars that make up RNA and DNA but which neither produces nor uses up ATP but generates NADPH.

RISC

complex. RNA-inducing silencing complex, is a 500 kDa ribonucleoprotein complex that incorporates one strand of a short (approx. 22 nt) single stranded microRNA or a double stranded siRNA. This single strand acts as a RISC template to recognize complementary RNA. The protein Argonaute (Ago2), part of the complex, activates and cleaves the mRNA. This is a key process in gene silencing from drosophila to humans.

Satellite cell

multipotent cell found in mature skeletal muscle in mammals. After injury, this cell type is able to differentiate into mature skeletal muscle cells and allows muscle regeneration to occur.

African spiny mouse

rodent mammal with 3 different species, *Acomys kemp*i, *A. percivali*, and *A. cahirinus*; harbor unusual properties in skin shedding and rapid replacement of skin and, similar to the MRL mouse, close ear holes and regenerate cartilage, adipose tissue and hair follicles. This rodent is more related to gerbils than to mice.

Super regenerators

organisms which are able to regenerate lost limbs, appendages, and multiple organs such as planaria, starfish, hydra, sea cucumbers, amphibians, lizards (tails), deer (antlers), and MRL mice (ear holes, digit tissue, heart, etc).

Yamanaka factors

Four transcription factors factors (Oct3/4, Sox2, Klf4, and c-Myc) that are highly expressed in embryonic stem cells and are involved in regulating the developmental signaling network required for ES cell pluripotency. overexpression of these factors in both mouse and human somatic cells leads to a state of de-differentiation and pluripotency and the generation of cell populations known as induced-pluripotent stem (iPSC) cells used for regenerative replacement of tissue.

References

1. Semenza GL. Life with oxygen. *Science*. 2007; 318:62–64. [PubMed: 17916722]
2. Dinsmore, CE., editor. *A History of Regeneration Research: Milestones in the Evolution of a Science*. Cambridge University Press; New York: 1991.
3. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007; 318:1917–20. [PubMed: 18029452]
4. Mao AS, Mooney DJ. Regenerative medicine: Current therapies and future directions. *PNAS*. 2015; 112:14452–14459. [PubMed: 26598661]
5. Marrero L, Simkin J, Sammarco M, Muneoka K. Fibroblast reticular cells engineer a blastema extracellular network during digit tip regeneration in mice. *Regeneration (Oxf)*. 2017; 4:69–84. [PubMed: 28616246]

6. Zhang Y, Strehin I, Bedelbaeva K, Gourevitch D, Clark L, Leferovich J, Messersmith PB, Heber-Katz E. Drug-induced regeneration in adult mice. *Sci Transl Med.* 2015; 7
7. Clark LD, Clark RK, Heber-Katz E. A New Murine Model for Mammalian Wound Repair and Regeneration. *Clinical Immunology and Immunopathology.* 1998; 88:35–45. [PubMed: 9683548]
8. McBrearty BA, Clark LD, Zhang XM, Blankenhorn EP, Heber-Katz E. Genetic analysis of a mammalian wound-healing trait. *Proc Natl Acad Sci USA.* 1998; 95:11792–11797. [PubMed: 9751744]
9. Kench JA, Russell DM, Fadok VA, Young SK, Worthen GS, Jones-Carson J, Henson JE, Nemazee D. Aberrant wound healing and TGF-beta production in the autoimmune-prone MRL/+ mouse. *Clinical Immunology.* 1999; 92:300–310. [PubMed: 10479535]
10. Blankenhorn E, Bryan G, Kossenkova A, Clark L, Zhang XM, Chang C, Horng W, Pletscher L, Cheverud J, Showe L, Heber-Katz E. Genetic loci that regulate healing and regeneration in LG/J and SM/J mice. *Mammalian Genome.* 2009; 20:720–733. [PubMed: 19760323]
11. Heydemann A. The super super-healing MRL mouse strain. *Frontiers in Biology.* 2012; 7:522–538. [PubMed: 24163690]
12. Rai MF, Hashimoto S, Johnson EE, Janiszak KL, Fitzgerald J, Heber-Katz E, Cheverud JM, Sandell LJ. Heritability of articular cartilage regeneration and its association with ear wound healing in mice. *Arthritis Rheum.* 2012; 64:2300–2310. [PubMed: 22275233]
13. Sereysky JB, Flatow EL, Andarawis-Puri N. Musculoskeletal regeneration and its implications for the treatment of tendinopathy. *International Journal of Experimental Pathology.* 2013; 94:293–303. [PubMed: 23772908]
14. Lalley AL, Dymant NA, Kazemi N, et al. Improved Biomechanical and Biological Outcomes in the MRL/MpJ Murine Strain Following a Full-Length Patellar Tendon Injury. *Journal of orthopaedic research.* 2015; 33:1693–1703. [PubMed: 25982892]
15. Dickie, MM. Keeping Records. In: Green, EL., editor. *Biology of the Laboratory Mouse.* New York, New York: Dover Publications; 1975. p. 706
16. Goss RJ, Grimes LN. Epidermal downgrowths in regenerating rabbit ear holes. *J Morph.* 1975; 146:533–54. [PubMed: 1171254]
17. Clark LD, Clark RK, Heber-Katz E. A New Murine Model for Mammalian Wound Repair and Regeneration. *Clinical Immunology and Immunopathology.* 1998; 88:35–45. [PubMed: 9683548]
18. Naviaux RK, Le TP, Bedelbaeva K, Leferovich J, Gourevitch D, Sachadyn P, Zhang XM, Clark L, Heber-Katz E. Retained features of embryonic metabolism in the adult MRL mouse. *Mol Genet Metab.* 2009; 96:133–144. [PubMed: 19131261]
19. Heber-Katz, E., Naviaux, R. Chapter 3. The MRL mouse, a model of regeneration and cancer. In: Berger, NA., editor. *Murine Models of Energy, Balance, and Cancer in Energy and Cancer.* Vol. 3. 2015. p. 319
20. Stacpoole PW. The pharmacology of dichloroacetate. *Metabolism.* 1989; 38:1124–44. [PubMed: 2554095]
21. Baker JC, Yan X, Peng T, Kasten S, Roche TE. Marked differences between two isoforms of human pyruvate dehydrogenase kinase. *J Biol Chem.* 2000; 275:15773–81. [PubMed: 10748134]
22. Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, Lee CT, Lopaschuk GD, Puttagunta L, Bonnet S, Harry G, Hashimoto K, Porter CJ, Andrade MA, Thebaud B, Michelakis ED. A mitochondria-K⁺ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell.* 2007; 11:37–51. [PubMed: 17222789]
23. Giaccia AJ, Simon MC, Johnson R. The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. *Genes and Development.* 2004; 18:2183–2194. [PubMed: 15371333]
24. Sabharwal SS, Schumacker PT. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nature reviews Cancer.* 2014; 14:709–721. [PubMed: 25342630]
25. Zhou Y, Tozzi F, Chen J, Fan F, Xia L, Wang J, Gao G, Zhang A, Xia X, Brasher H, Widger W, Ellis LM, Weihua Z. Intracellular ATP levels are a pivotal determinant of chemoresistance in colon cancer cells. *Cancer Res.* 2012; 72:304–14. [PubMed: 22084398]

26. Park TJ, Reznick J, Peterson BL, Blass G, Omerbaşı D, Bennett NC, Kuich PHJL, Zasada C, Browe BM, Hamann W, Applegate DT, Radke MH, Kosten T, Lutermann H, Gavaghan V, Eigenbrod O, Bégay V, Amoroso VG, Govind V, Minshall RD, Smith ESJ, Larson J, Gotthardt M, Kempa S, Lewin GR. Fructose-driven glycolysis supports anoxia resistance in the naked mole-rat. *Science*. 2017; 356:307–311. [PubMed: 28428423]
27. Bedelbaeva K, Snyder A, Gourevitch D, Clark L, Zhang XM, Leferovich J, Cheverud JM, Lieberman P, Heber-Katz E. Lack of p21 expression links cell cycle control and appendage regeneration in mice. *Proc Natl Acad Sci U S A*. 2010; 107:5845–50. [PubMed: 20231440]
28. Arthur LM, Demarest RM, Clark L, Gourevitch D, Bedelbaeva K, Anderson R, Snyder A, Capobianco AJ, Lieberman P, Feigenbaum L, Heber-Katz E. Epimorphic Regeneration in Mice is p53-independent. *Cell Cycle*. 2010; 9:3667–3673. [PubMed: 20855943]
29. Heber-Katz E, Zhang Y, Bedelbaeva K, Song F, Chen X, Stocum DL. Cell Cycle Regulation and Regeneration. *Curr Top Microbiol Immunol*. 2013; 367:253–276. [PubMed: 23263201]
30. Leung TH, Snyder ER, Liu Y, Wang J, Kim SK. A cellular, molecular, and pharmacological basis for appendage regeneration in mice. *Genes Dev*. 2015; 29:2097–107. [PubMed: 26494786]
31. Hendrix CW, Collier AC, Lederman MM, Schols D, Pollard RB, Brown S, Jackson JB, Coombs RW, Glesby MJ, Flexner CW, et al. Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV- 1 infection. *J Acquir Immune Defic Syndr*. 2004; 37:1253–1262. [PubMed: 15385732]
32. Albrecht JH, Meyer AH, Hu MY. Regulation of cyclin-dependent kinase inhibitor p21 (WAF1/Cip1/Sdi1) gene expression in hepatic regeneration. *Hepatology*. 1997; 25:557–563. [PubMed: 9049198]
33. Weymann A, Hartman E, Gazit V, Wang C, Glauber M, Turmelle Y, Rudnick DA. p21 Is Required for Dextrose-Mediated Inhibition of Mouse Liver Regeneration. *Hepatology*. 2009; 50:207–215. [PubMed: 19441104]
34. Lehmann K, Tschuor C, Rickenbacher A, Jang JH, Oberkofler CE, Tschopp O, Schultze SM, Raptis DA, Weber A, Graf R, Humar B, Clavien PA. Liver Failure After Extended Hepatectomy in Mice Is Mediated by a p21-Dependent Barrier to Liver Regeneration. *Gastroenterology*. 2012; 143:1609–1619. [PubMed: 22960658]
35. Jackson DP, Li H, Mitchell KA, Joshi AD, Elferink CJ. Ah Receptor–Mediated Suppression of Liver Regeneration through NC-XRE–Driven p21^{cip} Expression. *Mol Pharmacol*. 2014; 85:533–541. [PubMed: 24431146]
36. Nathan C. Immunology: Oxygen and the inflammatory cell. *Nature*. 2003; 422:675–676. [PubMed: 12700748]
37. O'Neill LA, Hardie DG. Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature*. 2013; 493:346–355. [PubMed: 23325217]
38. Mills EL, Kelly B, Logan A, Costa AS, Varma M, Bryant CE, Tourlomousis P, Däbritz JH, Gottlieb E, Latorre I, Corr SC, McManus G, Ryan D, Jacobs HT, Szibor M, Xavier RJ, Braun T, Frezza C, Murphy MP, O'Neill LA. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell*. 2016; 167:457–470. [PubMed: 27667687]
39. Heber-Katz E, Gourevitch D. The relationship between inflammation and regeneration in the MRL mouse: potential relevance for putative human regenerative (scarless wound healing) capacities? *Ann N Y Acad Sci*. 2009; 1172:110–114. [PubMed: 19735244]
40. Gourevitch D, Kossenkova AV, Zhang Y, Clark L, Chang C, Showe LC, Heber-Katz E. Inflammation and its Correlates in Regenerative Wound Healing: An Alternate Perspective. *Advances in Wound Care*. 2014; 3:592–603. [PubMed: 25207202]
41. Davis TA, Lennon G. Mice with a regenerative wound healing capacity and an SLE autoimmune phenotype contain elevated numbers of circulating and marrow-derived macrophage progenitor cells. *Blood Cells Mol Dis*. 2005; 34:17–25. [PubMed: 15607695]
42. Canhamero T, Garcia LV, De Franco M. Acute Inflammation Loci Are Involved in Wound Healing in the Mouse Ear Punch Model. *Adv Wound Care (New Rochelle)*. 2014; 3:582–591. [PubMed: 25207201]

43. Simkin J, Gawriluk T, Gensel JC, Seifert AW. Macrophages are necessary for epimorphic regeneration in African spiny mice. *eLife*. 2017; 6
44. Matias Santos D, Rita AM, Casanellas I, Brito Ova A, Araújo IM, Power D, Tiscornia G. Ear wound regeneration in the African spiny mouse *Acomys cahirinus*. *Regeneration (Oxf)*. 2016; 3:52–61. [PubMed: 27499879]
45. Zhang Y, Desai A, Yang SY, Bae KB, Antczak MI, Fink SP, Tiwari S, Willis JE, Williams NS, Dawson DM, Wald D, Chen WD, Wang Z, Kasturi L, Larusch GA, He L, Cominelli F, Di Martino L, Djuric Z, Milne GL, Chance M, Sanabria J, Dealwis C, Mikkola D, Naidoo J, Wei S, Tai HH, Gerson SL, Ready JM, Posner B, Willson JK, Markowitz SD. Inhibition of the prostaglandin-degrading enzyme 15-PGDH potentiates tissue regeneration. *Science*. 2015; 348
46. Gorres KL, Raines RT. Prolyl-4-hydroxylase. *Crit Rev Biochem Mol Biol*. 2010; 45:106–124. [PubMed: 20199358]
47. Ramshaw JAM, Shah NK, Brodsky B. Gly–X–Y tripeptide frequencies in collagen: A context for host– guest triple-helical peptides. *J Struct Biol*. 1998; 122:86–91. [PubMed: 9724608]
48. Peterkofsky B, Udenfriend S. Enzymatic hydroxylation of proline in microsomal polypeptide leading to formation of collagen. *Proc Natl Acad Sci USA*. 1965; 53:335–342. [PubMed: 14294066]
49. Kivirikko KI, Prockop DJ. Enzymatic hydroxylation of proline and lysine in procollagen. *Proc Natl Acad Sci USA*. 1967; 57:782–789. [PubMed: 16591531]
50. Berg RA, Prockop DJ. Purification of [¹⁴C]procollagen and its hydroxylation by prolyl-hydroxylase. *Biochemistry*. 1973; 12:3395–3401. [PubMed: 4354367]
51. Rosenbloom J, Cywinski A. Biosynthesis and secretion of tropoelastin by chick aorta cells. *Biochem Biophys Res Commun*. 1976; 69:613–620. [PubMed: 944578]
52. Qi HH, Ongusaha PP, Myllyharju J, Cheng D, Pakkanen O, Shi Y, Lee SW, Peng J, Shi Y. Prolyl 4-hydroxylation regulates Argonaute 2 stability. *Nature*. 2008; 455:421–424. [PubMed: 18690212]
53. Koivunen P, Tiainen P, Hyvarinen J, Williams KE, Sormunen R, Klaus SJ, Kivirikko KI, Myllyharju J. An endoplasmic reticulum transmembrane prolyl 4-hydroxylase is induced by hypoxia and acts on hypoxia-inducible factor alpha. *J Biol Chem*. 2007; 282:30544–30552. [PubMed: 17726031]
54. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science*. 2001; 294:1337–1340. [PubMed: 11598268]
55. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*. 2001; 292:464–468. [PubMed: 11292862]
56. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim Av, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*. 2001; 292:468–472. [PubMed: 11292861]
57. Semenza GL. HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell*. 2001; 107:11–3.
58. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell*. 2001; 107:43–55. [PubMed: 11595184]
59. Ivan M, Haberberger T, Gervasi DC, Michelson KS, Gunzler V, Kondo K, Yang H, Sorokina I, Conaway RC, Conaway JW, Kaelin WG Jr. Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. *Proc Natl Acad Sci U S A*. 2002; 99:13459–13464. [PubMed: 12351678]
60. Li D, Hirsilä M, Koivunen P, Brenner MC, Xu L, Yang C, Kivirikko KI, Myllyharju J. Many amino acid substitutions in a hypoxia-inducible transcription factor (HIF)-1α-like peptide cause only minor changes in its hydroxylation by the HIF prolyl 4-hydroxylases: Substitution of 3,4-dehydropyrroline or azetidine-2-carboxylic acid for the proline leads to a high rate of uncoupled 2-oxoglutarate decarboxylation. *J Biol Chem*. 2004; 279:55051–55059. [PubMed: 15485863]

61. Rytönen K, Williams TA, Renshaw GM, Primmer CR, Nikinmaa M. Molecular Evolution of the Metazoan PHD-HIF Oxygen- Sensing System. *Mol Biol Evol.* 2011; 28:1913–1926. [PubMed: 21228399]
62. Mikhaylova O, Ignacak ML, Barankiewicz TJ, Harbaugh SV, Yi Y, Maxwell PH, Schneider M, Van Geyte K, Carmeliet P, Revelo MP, Wyder M, Greis KD, Meller J, Czyzyk-Krzeska MF. The von Hippel-Lindau tumor suppressor protein and Egl-9-type proline hydroxylases regulate the large subunit of RNA polymerase II in response to oxidative stress. *Mol Cell Biol.* 2008; 28:2701–2717. [PubMed: 18285459]
63. Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, Johnson RS, Haddad GG, Karin M. NF- κ B links innate immunity to the hypoxic response through transcriptional regulation of HIF-1 α . *Nature.* 2008; 453:807–11. [PubMed: 18432192]
64. Cheverud, Cheverud JM., Lawson, HA., Bouckaert, K., Kossenkova, A., Showe, L., Cort, L., Blankenhorn, EP., Bedelbaeva, K., Gourevitch, D., Zhang, Y., Heber-Katz, E. Fine-mapping quantitative trait loci affecting murine external ear tissue regeneration in the LG/J by SM/J advanced intercross line. *Heredity.* 2014; 112:508–518. [PubMed: 24569637]
65. Tan M, Gu Q, He H, Pamarthy D, Semenza GL, Sun Y. SAG/ROC2/RBX2 is a HIF-1 target gene that promotes HIF-1 α ubiquitination and degradation. *Oncogene.* 2008; 27:1404–11. [PubMed: 17828303]
66. Brockes JP, Kumar A. Comparative aspects of animal regeneration. *Annu Rev Cell Dev Biol.* 2008; 24:525–549. [PubMed: 18598212]
67. Krag M, Knapp D, Nacu E, Khattak S, Maden M, Epperlein H, Henning H, Tanaka EM. Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature.* 2009; 460:60–65. [PubMed: 19571878]
68. Mathieu J, Zhang Z, Zhou W, Wang AJ, Heddleston JM, Pinna CM, Hubaud A, Stadler B, Choi M, Bar M, Tewari M, Liu A, Vessella R, Rostomily R, Born D, Horwitz M, Ware C, Blau CA, Cleary MA, Rich JN, Ruohola-Baker H. HIF induces human embryonic stem cell markers in cancer cells. *Cancer Res.* 2011; 71:4640–4652. [PubMed: 21712410]
69. Mathieu J, Zhou W, Xing Y, Sperber H, Ferreccio A, Agoston Z, Kuppusamy KT, Moon RT, Ruohola-Baker H. Hypoxia-inducible factors have distinct and stage-specific roles during reprogramming of human cells to pluripotency. *Cell Stem Cell.* 2014; 14:592–605. [PubMed: 24656769]
70. Zhang Y, Strehin I, Bedelbaeva K, Gourevitch D, Clark L, Leferovich J, Messersmith PB, Heber-Katz E. Drug-induced regeneration in adult mice. *Sci Transl Med.* 2015; 7
71. Mollenhauer M, Kiss J, Dudda J, Kirchberg J, Rahbari N, Radhakrishnan P, Niemietz T, Rausch V, Weitz J, Schneider M. Deficiency of the oxygen sensor PHD1 augments liver regeneration after partial hepatectomy. *Langenbecks Arch Surg.* 2012; 397:1313–22. [PubMed: 22961008]
72. Tajima T, Goda N, Fujiki N, Hishiki T, Nishiyama Y, Senoo-Matsuda N, Shimazu M, Soga T, Yoshimura Y, Johnson RS, Suematsu M. HIF-1 α is necessary to support gluconeogenesis during liver regeneration. *Biochem Biophys Res Commun.* 2009; 387:789–94. [PubMed: 19643083]
73. Myllyharju J. Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and regulation of the response to hypoxia, and their roles as treatment targets. *Ann Med.* 2008; 40:402–17. [PubMed: 19160570]
74. Hanauske-Abel LHM. Prolyl 4-hydroxylase, a target enzyme for drug development. Design of suppressive agents and the in vitro effects of inhibitors and proinhibitors. *J Hepatol.* 1991; 13(Suppl 3):S8–S15.
75. Wang GL, Semenza GL. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A.* 1993; 90:4304–8. [PubMed: 8387214]
76. Wang GL, Semenza GL. Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J Biol Chem.* 1993; 268:21513–8. [PubMed: 8408001]
77. Ivan Mircea N. HIF-PROLYL hydroxylase inhibitors: From basic science to clinical trials. *MÆDICA - a Journal of Clinical Medicine.* 2006; 1:67–69.

78. FibroGen Reports New Research on Development of HIF Prolyl Hydroxylase Inhibitors Related to Endothelial Progenitor Cells, Anti-Inflammation, Cytoprotection and Erythropoiesis. drugs.com. 2008
79. Hsieh MM, Linde NS, Wynter A, Metzger M, Wong C, Langsetmo I, Lin A, Smith R, Rodgers GP, Donahue RE, Klaus SJ, Tisdale JF. HIF prolyl hydroxylase inhibition results in endogenous erythropoietin induction, erythrocytosis, and modest fetal hemoglobin expression in rhesus macaques. *Blood*. 2007; 110:2140–7. [PubMed: 17557894]
80. Jain IH, Zazzeron L, Goli R, Alexa K, Schatzman-Bone S, Dhillon H, Goldberger O, Peng J, Shalem O, Sanjana NE, Zhang F, Goessling W, Zapol WM, Mootha VK. Hypoxia as a therapy for mitochondrial disease. *Science*. 2016; 352:54–61. [PubMed: 26917594]
81. Pergola PE, Spinowitz BS, Hartman CS, Maroni BJ, Haase VH. Vadadustat, a novel oral HIF stabilizer, provides effective anemia treatment in nondialysis-dependent chronic kidney disease. *Kidney Int*. 2016; 90:1115–1122. [PubMed: 27650732]
82. Pergola PE, Spinowitz BS, Hartman CS, Maroni BJ, Haase VH. Vadadustat, a novel oral HIF stabilizer, provides effective anemia treatment in nondialysis-dependent chronic kidney disease. *Kidney Int*. 2016; 90:1115–1122. [PubMed: 27650732]
83. Martin ER, Smith MT, Maroni BJ, Zuraw QC, deGoma EM. Clinical Trial of Vadadustat in Patients with Anemia Secondary to Stage 3 or 4 Chronic Kidney Disease. *Am J Nephrol*. 2017; 45:380–388. [PubMed: 28343225]
84. Warnecke C, Griethe W, Weidemann A, Jurgensen JS, Willam C, Bachmann S, Ivashchenko Y, Wagner I, Frei U, Wiesener M, Eckardt K-U. Activation of the hypoxia-inducible factor pathway and stimulation of angiogenesis by application of prolyl hydroxylase inhibitors. *The FASEB Journal*. 2003; 17:1186–8. [PubMed: 12709400]
85. Nangaku M, Izuhara Y, Takizawa S, Yamashita T, Fujii-Kuriyama Y, Ohneda O, Yamamoto M, Van Ypersele De Strihou C, et al. A Novel Class of Prolyl Hydroxylase Inhibitors Induces Angiogenesis and Exerts Organ Protection Against Ischemia. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2007; 27:2548–2554.
86. Adamcio B, Sperling S, Hagemeyer N, Walkinshaw G, Ehrenreich H. Hypoxia inducible factor stabilization leads to lasting improvement of hippocampal memory in healthy mice. *Behavioural Brain Research*. 2010; 208:80–84.4. [PubMed: 19900484]
87. Wu K, Kailiang K, Wang Y, Zhou Y, Tian N, Wu Y, Chen D, Zhang D, Wang X, Xu H, Zhang X. Stabilization of HIF-1 α by FG-4592 promotes functional recovery and neural protection in experimental spinal cord injury. *Brain Research*. 2016; 1632:19–26. [PubMed: 26707978]
88. Zheng H, Youdim M, Weiner LM, Fidkin M. Novel potential neuroprotective agents with both iron chelating and amino acid-based derivatives targeting central nervous system neurons. *Biochemical Pharmacology*. 2005; 70:1642–1652. [PubMed: 16226724]
89. Avramovich-Tirosh Y, Bar-Am O, Amit T, Youdim MBH, Weinreb O. Up-Regulation of Hypoxia-Inducible Factor (HIF)-1 α and HIF-Target Genes in Cortical Neurons by the Novel Multifunctional Iron Chelator Anti-Alzheimer Drug, M30. *Current Alzheimer Research*. 2010
90. Franklin MTJ, Morris WP, Edwards PN, Large MS, Stephenson R. Inhibition of prolyl 4-hydroxylase in vitro and in vivo by members of a novel series of phenanthrolinones. *Biochem J*. 2001; 353:333–338. [PubMed: 11139398]
91. Gaofeng X, Deng L, Zhu J, Piotr GR, Xu R. Prolyl-4-hydroxylase α subunit 2 promotes breast cancer progression and metastasis by regulating collagen deposition. *MC Cancer*. 2014; 14:1.
92. Strehin, Gourevitch D, Zhang Y, Heber-Katz E, Messersmith PB. Hydrogels formed by oxo-ester mediated native chemical ligation. *Biomater Sci*. 2013; 1:603–613. [PubMed: 23894696]
93. Latona J, Shah A, Cheng J, Messersmith P, Heber-Katz E. Enhanced Liver Regeneration After Partial Hepatectomy in Mice Treated with a Prolyl Hydroxylase Inhibitor. *Am J Transplant*. 2017; 17(suppl 3)
94. Moris D, Pawlik TM. Liver hypoxia as a trigger to liver regeneration: No more than another piece of the puzzle. *Surgery*. 2017; 161:1176–1177. [PubMed: 27712878]
95. Schadde E, Breitenstein S, Hertl M, Spahn DR, Schlpfer M, Beck-Schimmer B. Reply to: Liver hypoxia as a trigger to liver regeneration: No more than another piece of the puzzle. *Surgery*. 2017; 161:1177–1178. [PubMed: 28109617]

96. Harnoss JM, Platzer LK, Burhenne J, Radhakrishnan P, Cai J, Strowitzki MJ, Weiss J, Ritter AS, Mollenhauer M, Schmidt T, Ulrich A, Haefeli WE, Schneider M. Prolyl Hydroxylase Inhibition Enhances Liver Regeneration Without Induction of Tumor Growth. *Annals of Surgery*. 2017; 265:782–791. [PubMed: 28266966]

Trends Box

The metabolic approach to regeneration can be viewed as being very broad and very deep.

Part of our appreciation for the role of metabolism in regeneration has come from a rodent model of epimorphic regeneration, the MRL mouse. MRL mice exhibit a basal metabolic state of aerobic glycolysis, with increased levels of HIF-1 α and NF κ b, increased levels of lactate, and reduced mitochondrial function. These processes have been implicated in inflammation and active chromatin remodeling that can lead cells to states of immature differentiation.

The modulation of aerobic glycolysis and OXPHOS by drugs can lead to first a de-differentiation state followed by a re-differentiation state returning new regenerated tissue back to normal architecture and normal function.

We expect that the exploitation of metabolic plasticity will be a major medical theme in the future.

Figure 1. Overview of the epimorphic regenerative response in mice

The epimorphic regenerative response to injury in mice involves multiple factors discussed in this opinion piece and displayed in this figure.

Key Figure. Figure 2. Metabolic changes associated with increased levels of mammalian HIF-1α

The left side of this figure displays the activation of aerobic glycolysis whereas the right side displays an OX PHOS metabolic state with the involvement of mitochondrial function.

HIF $\alpha\beta$ or HIF-1 activates a significant number of genes involved in glycolysis (green lines) including hexokinase (HK), glucose transporter 1 (GLUT1), phosphofructokinase in liver (PFKL), aldolase C or fructose-biphosphate (ALDOC), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 2 (PGK2), enolase 1 (ENO1), as well as lactate dehydrogenase (LDH) which converts pyruvate to lactate. Furthermore, it activates pyruvate dehydrogenase kinase (PDK1) which in turn inhibits pyruvate dehydrogenase (PDH), necessary for conversion of pyruvate to acetyl CoA, feeding the TCA cycle and mitochondrial OX PHOS energy production as well as chromatin acetylation.

The drug dichloroacetate (DCA) blocks PDK1 and relieves PDH inhibition, allowing pyruvate conversion to acetyl CoA and OX PHOS to proceed. DCA was used to block the regenerative response and showed its effect during phase I (Fig 1).

The molecules that regulate HIF-1 α degradation/ubiquination/stabilization are prolyl hydroxylase domain proteins (PHDs; blue dots) and are found in the cytoplasm and the nucleus. Inhibition of PHDs by PHD inhibitors (PHDi) including ROS allows HIF-1 α to survive, move into the nucleus, complex with HIF-1 β to form a transcription factor that binds to HRE's and promotes transcription of the genes described above along with many others.

PHDi can also regulate prolyl 4 hydroxylases (P4H) that don't recognize HIF-1 α but other potentially important molecules involved in regeneration such as collagen. Here P4Hs are necessary for collagen crosslinking and scarring, a barrier for regeneration which PHDi's can potentially block. This would occur in the ER and Golgi where the P4H's are found (yellow dots).

Figure 3. Biphasic HIF-1a Expression Levels

A full regenerative response involves phase I with increasing HIF-1a levels followed by phase II with falling HIF-1a responses. In phase I, de-differentiation occurs accompanied by a shift to glycolytic metabolism leading to an M-1 macrophage type inflammatory response and tissue remodeling. Also, there is an increase in cell migration, chromatin changes, the expression of multiple immature pluripotent cell markers, and an increase in biomass along with the activation of the pentose phosphate pathway (PPP) with increased levels of amino acids, nucleotides, and lipids and finally cellular proliferation. In phase II, cells begin to re-differentiate into mature cell phenotypes with the switch to oxidative phosphorylation (OX PHOS) and a qualitative change in innate immunity to an M-2 type response. Non-regenerators show the same increases and decreases in HIF-1a but never reach a level necessary for regenerative processes.