Intrinsic Heart Regeneration in Adult Vertebrates May be Strictly Limited to Low-Metabolic Ectotherms

Anita Dittrich, Kasper Hansen, Mette Irene Theilgaard Simonsen, Morten Busk, Aage Kristian Olsen Alstrup, and Henrik Lauridsen*

The heart has a high-metabolic rate, and its “around-the-clock” vital role to sustain life sets it apart in a regenerative setting from other organs and appendages. The landscape of vertebrate species known to perform intrinsic heart regeneration is strongly biased toward ectotherms—for example, fish, salamanders, and embryonic/neonatal ectothermic mammals. It is hypothesized that intrinsic heart regeneration is exclusively limited to the low-metabolic hearts of ectotherms. The biomedical field of regenerative medicine seeks to devise biologically inspired regenerative therapies to diseased human hearts. Falsification of the ectothermy dependency for heart regeneration hypothesis may be a crucial prerequisite to meaningfully seek inspiration in established ectothermic regenerative animal models. Otherwise, engineering approaches to construct artificial heart components may constitute a more viable path toward regenerative therapies. A more strict definition of regenerative phenomena is generated and several testable sub-hypotheses and experimental avenues are put forward to elucidate the link between heart regeneration and metabolism. Also see the video abstract here https://youtu.be/fZcanaOT5z8.

1. Introduction

The ability to regenerate lost or damaged tissue is a natural phenomenon that has fascinated ancient philosophers and polymaths like Aristotle[1] and the arising Enlightenment scientist since the time of Spallanzani’s Prodromo in the 18th century.[2] While appendage regeneration in fish, amphibians, and reptiles has thus been known in the scientific community for centuries, the ability to regenerate damaged myocardium of the heart is a much more recent discovery first reported in newts in the 1970s.[3] Contrary to appendage regeneration, which can be documented as far back as the Carboniferous in the fossil record,[4] our knowledge of the ancestry of the capacity to restore soft tissues like the heart is very limited, since soft tissue rarely leaves fossil traces. Today, we know of no adult mammal nor bird that is capable of intrinsic heart regeneration, and there are only a few indicative but inconclusive reports of cardiac regenerative capacity in reptiles[5,6] (Figure 1). On the other hand, heart regenerative ability has been documented in a few species of adult fish and amphibians: zebrafish,[7] giant danio,[8] goldfish,[9] Mexican tetra,[10] Eastern newt,[11,12] and axolotl[12] as well as some embryonic and/or neonatal birds and mammals: embryonic chicken,[13] neonatal mouse,[14] neonatal rat,[15] embryonic rabbit,[16,17] embryonic sheep,[18] and possibly embryonic human[19] (Figure 1). There are several characteristics that set the known regenerative vertebrates apart from non-regenerative species, for example, low-pressure cardiac systems, low oxygen availability, less reliance on the adaptive immune systems, mononucleated cardiomyocytes, and a looser structural organization of cardiomyocytes (reviewed in ref. [20]). Another striking characteristic is in temperature regulation and metabolism. All vertebrate animals known to this day that are capable of cardiac regeneration are low-metabolic ectothermic animals (Figure 1). Because metabolism is essential to all biological functions and it is linked to several of
the other characteristics of regenerative hearts, it is tempting to speculate that there is a tight link between cardiac metabolism and regenerative capability.\textsuperscript{[21]} This leads us to the following fundamental hypothesis:

\textit{Fundamental hypothesis: Intrinsic heart regeneration in vertebrates is strictly limited to low-metabolic ectotherms.}

Metabolic pathways are largely shared between endo- and ectotherms (with the exception of the proton-motive force and oxidative phosphorylation uncoupling function of thermogenin found in brown adipose tissue in endothermic eutherians to release energy as heat). Therefore, it is difficult to test this hypothesis in a single stroke. Unambiguous falsification would require the involvement of more than 15 000 endothermic species of birds and mammals and an additional number of partially endothermic reptiles. Therefore, it is necessary to break down this fundamental hypothesis into testable sub-hypotheses that may not provide a final answer, but
Figure 2. Therapeutic strategies for replacement or repair of damaged cardiac tissue. There are two overall approaches for proposed regenerative therapies to the heart: The engineering approach of constructing an artificial (mechanical or biological) heart or heart patches for later transplantation, or the biological approach of repairing the heart in vivo using stem cells or pharmacological/gene therapy stimulation of endogenous repair mechanisms. Some forms of stem cell therapy utilizes techniques affiliated with both biological and engineering approaches, when biological stem cell therapy is supported by engineered hydrogels or other scaffolding/delivery materials.

potentially provide strong indications to unravel the fascinating and potentially clinically important phenomenon of heart regeneration. In this review, several testable hypotheses are presented and discussed in light of the present knowledge in the field.

2. Consequences of Lacking Heart Regenerative Capacity and Strategies to Alleviate It

The presence or absence of heart regeneration in different organisms is an interesting academic question, but it also has obvious implications for human medicine since the adult human heart has very limited regenerative potential. Heart disease and especially the occurrence of ischemic heart disease resulting in myocardial infarction is, next to cancer, the major cause of death in western societies.[25] Due to optimized care, an ever-increasing number of patients survive ischemic heart disease and go on to live with symptoms of impaired cardiac function throughout life as reparative therapies are sorely lacking. These patients often experience reduced quality of life and suffer complications like arrhythmias, pain, and increased risk of a secondary events,[26] even though secondary prophylaxis has improved in recent years.[27]

On the overall, there are two main strategies to follow in the development of regenerative therapies for the human heart (Figure 2). First, the biological approach, aiming to stimulate latent endogenous regenerative capacity of the heart—inspired by animals with impressive cardiac regenerative capabilities.[20,28] This entails repairing the existing tissue or introducing biological elements like stem cells. This approach remains to mature into real therapies, and follow the underlying assumption that heart regeneration can take place in situ while the heart is fulfilling its vital task of supplying the entire body with oxygenated and nutrient rich blood. The engineering approach is fundamentally different in that it aims at replacing damaged tissues with mechanical or artificial elements, producing functional artificial components outside the body to be transplanted into patients. Examples of this tactic include endeavors like mechanical hearts,[29–31] repopulating decellularized hearts with stem cells[32,33] and 3D bioprinting of entire organs.[34] These strategies have, however, not yet been successful in producing a fully functional and/or maintenance free heart.

It is important to realize that the biological and the engineering approaches toward cardiac regeneration fundamentally differ in the demands they place on the infarcted heart. In the biological approach, the diseased heart must allocate resources to perform endogenous myocardial regeneration while continuously executing its vital and labor-intensive primary function. In the engineering approach, artificial tissue is constructed in vitro and can later be implanted in the patient, thus the diseased heart does not need to struggle with both pumping and regenerative processes simultaneously. The interplay between metabolism and regeneration is important. If highly metabolically active hearts are unable to perform intrinsic regeneration as hypothesized, then regenerative therapies may depend on adjustments to cardiac metabolism. These adjustments could potentially be obtained by pharmacological control of cardiac metabolism or a general reduction in metabolic rate by temperature adjustments during regenerative therapy. However, answering the fundamental hypothesis precedes the development of therapeutic methods to put this knowledge into action.
3. Large-Scale Comparative Analysis of Heart Regenerative Capacity

Although, the vertebrate subphylum comprises an insurmountable number of species to test individually for heart regenerative capacity, the phylogenetic approach to the fundamental hypothesis posed above may arguably be the most straightforward and informative. Evolution does not move along a predefined path, and adaptations in one clade is not rock-solid evidence of similar adaptations in sister clades. However, by using a phylogenetically informed approach of testing adaptations in key species of a phylogeny with redundancy, it is possible to draw out information of the evolution of a trait from a much smaller sample size of species than the entire phylogeny.[35–37] The capacity to regenerate myocardium has only been reported for a low number of adult vertebrates (Figure 1), and importantly there is a lack of reported non-regenerative species (lack of cardiac regeneration has been reported in medaka[22] and Pachón et al. of Mexican tetra[18]), thus we have very little information on how widespread this phenomenon is within the vertebrates (Figure 1). Instead of directly approaching the fundamental hypothesis from the viewpoint of endotherms, it may be informative to search the vast majority ectotherms that have highly variable general and cardiac metabolic rate. It is not unlikely that a focused comparative effort of testing regenerative capacity across ≈100 species selected for coverage of the ectothermic vertebrate phylogeny as well as accessibility would provide information of the ancestry and limits of the heart regenerative trait. Additionally, linking the physiological reality of the selected species, for example, high-metabolic tropical versus low-metabolic polar ectotherms, with regenerative capacity would allow for an indirect approach to test the fundamental hypothesis. Additionally, mammals that naturally have the ability to enter a state of low-metabolic dormancy, that is, switch between endothermic and ectothermic lifestyles such as some rodents and bats may provide important cues to understand the link between metabolism and regeneration at different levels of metabolic requirements on the heart.

A key to success in a large-scale comparative study is a standardized injury method. Currently, several injury mechanisms are being used to induce cardiac damage in regenerative experiments. These include mechanical injury (resection, crushing), physical injury (applying freezing or burning), chemical injury (induced ischemia, cardiomyocyte poisoning), and genetic ablation (injury methods reviewed in ref. [20]). In a comparative study it is crucial to standardize the injury type to allow for direct comparisons. Since the importance of coronary vasculature for heart function varies tremendously within vertebrates and likewise the response to cardiac toxins, chemical approaches, for example, by ablating portions of the heart via limiting myocardial oxygenation by coronary ligation, may not be feasible in a large comparative setting. Genetic ablation of cardiomyocytes is an elegant method to induce cell specific injury to the functional components of the heart, but this technique must be tailored to individual species, and is thus unrealistic in a large-scale experiment. Resection of a part of the ventricle is a straightforward surgical procedure that could be applied to a large number of species with a high throughput. However, mechanically removing a portion of the ventricle does not reflect the injury environment of a cardiac infarction where necrotic cardiac tissue must first be cleared before regeneration can take place. Additionally, transmural resection injury may only be tolerated by some number of species that are able to muster rapid clotting of the wound surface, whereas others may bleed out. Remaining are the mechanical and physical injury mechanisms of crushing, cryo-, and cauterezation injuries. These all represent injury techniques that can be induced rapidly on a large number of different animal species, they are heart destructive across all vertebrates, and the injury zone is well defined making it easier to monitor and compare a potential regenerative response.

Another key task is establishing a clear definition of what actually constitutes an intrinsic heart regenerative event. This definition is currently lacking, and reports of heart regeneration have been based on a large variety of methods (systematically reviewed for apical resection injury in zebrafish in ref. [38]). Demonstration of DNA replication and cardiomyocyte mitosis must be used with caution to conclude regeneration, since DNA replication is not necessarily followed by cell proliferation,[39] and even demonstration of mitosis only shows the generation of new cells but not necessarily regeneration, since cardiomyocytes could still take part in the cell cycle in species we know little of. Besides, uncontrolled generation of new tissue is not necessarily similar to a well-controlled regenerative event. Quantitative histology can be used to evaluate anatomical regeneration of a heart by measuring, for example, the infarct fraction at different stages in a regenerative process. Strict unbiased stereological methods must be applied in this context.[40] An often neglected measure of cardiac regeneration is cardiac function prior to injury, during a putative regenerative process and at full recovery. This is surprising since the importance of the heart is in its function and not in its appearance. Thus, a crucial aspect in the demonstration of a heart regenerative event is a functional analysis. Figure 3 represents an updated graphic expansion of the systematic review by Juul Belling et al. (2020) here including also cryo-injury and genetic ablation injury[38] and demonstrating the current variety of methods to demonstrate heart regeneration in studies based on the zebrafish, currently the most popular model in the heart regenerative field. Although there is a relatively high prevalence of papers using methods to demonstrate cardiomyocyte proliferation and anatomical regeneration by qualitative and/or quantitative histology, functional analyses, especially those taking the trabeculated nature of the zebrafish heart into account, are underrepresented (Figure 3).

The above considerations can be summarized into the following proposed definition of a heart regenerative event:

Definition: Intrinsic heart regeneration is a self-initiated response to damage to the heart muscle in which an organism is capable of fully restoring cardiac function by rebuilding damaged cardiac structures, and not by compensatory mechanisms such as hypertrophy of undamaged portions of the heart. Intrinsic heart regeneration can be separated into transient regenerative ability in which regenerative capacity is only present at certain life stages of the organism, i.e., in the embryonic or neonatal stages, and constant regenerative ability in which heart regeneration is possible at all life stages of the organism, also after sexual maturity.

The adoption of this definition and the development of standardized injury methods would allow for a more precise
comparison between heart regenerative phenomena in different vertebrates.

4. Energy Turnover in the Healthy Heart

In the context of regenerative biology, the heart holds a unique position as its continuous physiological function must be upheld to support life. Unlike a broken appendage, which can be immobilized while the injury heals, the heart must operate at a high level of function every second while performing repair and regeneration. The connection between metabolism and response to injury is therefore even more crucial in the heart compared to most other organs and structures. While appendage regeneration does have an ectothermic bias and is most widely found in amphibians and reptiles, it is also found in endothermic mammals in the shape of digit tip regeneration in, for example, mouse and human and in bone (anterior) regeneration in cervids.[41–44] Likewise, regeneration of other organs with a high-metabolic rate like the liver and the epidermis is also present in endotherms,[45] however vital as they are, these organs may be better adapted to function in a somewhat compromised state than the heart.

Overall, metabolic pathways are shared between endo- and ectotherms, but the environment experienced by internal organs can vary considerably between the two. Temperature level and stability is often, but not always, higher and more stable in endotherms than ectotherms. Since the ability to maintain elevated body temperature above ambient temperature in the absence of skeletal muscle contractions, which is only found in birds and mammals[46] is correlated with the evolution of a separate respiratory circulation in these groups, the heart in endothermic vertebrates as a general rule of thumb receives more oxygen than in many ectothermic vertebrates. Cardiac metabolism is most well described in mammals. Since this group comprises an important fraction, at least from an anthropocentric point of view, of vertebrates that under the assumption of the fundamental hypothesis has a mismatch between metabolic and regenerative processes, the following description of cardiac metabolism in healthy and diseased hearts is based on what we currently know of cardiac metabolism in mammals.

Under physiological resting conditions, the adult mammalian heart roughly takes up about 10% of the total amount of energy consumed by the whole body, with oxidative phosphorylation accounting for about 70%, while glycolysis and oxidation of carbohydrates makes up the remaining 30%. The human heart provides an illustrative example of the impressive work that the heart of a medium-sized mammal must carry out on a daily basis and the energy this requires: The human heart typically beats 100,000 times every day, pumping 7200 L of blood through the body, an impressive accomplishment requiring 35 L of oxygen and 6 kg of ATP, a staggering amount of energy consumed to keep the heart operating at a basal level while maintaining ion homeostasis.[48,49] The need for relentless energy producing metabolism is further underlined by the fact that adult human cardiomyocytes hold very limited amounts of stored energy.[50,51] In this light, it makes sense that the heart is omnivorous and able to utilize almost any substrate for energy production.[52] However, it intuitively follows that the heart is exceptionally vulnerable to malfunctions in the supply of fuel and oxygen.

An overview of metabolic pathways most critical to cardiac metabolism can be found in Figure 4. Fatty acids, the heart’s most important fuel source, are converted into acetyl-CoA via β-oxidation. Acetyl-CoA is then shuttled into the mitochondria for further aerobic metabolism in the citric acid cycle, producing NADH and FADH₂ that are utilized in the electron transport chain to yield ATP. In glycolysis, glucose is converted into pyruvate and ATP. Pyruvate can either enter the citric acid cycle or be
metabolized anaerobically to lactate. Lactate can in turn be oxidized to pyruvate under aerobic conditions. The intermediates of the citric acid cycle can alternatively be utilized for biosynthesis (Figure 4), however, they can importantly not do both. The pentose phosphate pathway is also utilized in cardiomyocytes and can be viewed as a parallel pathway to glycolysis in which glucose is converted into pentoses and NADPH. Pentoses are used for the biosynthesis of aromatic amino acids as well as nucleotides. NADPH in turn is an important reducing agent that has been shown to have a regulatory effect on reactive oxygen species in the heart. 

While utilization of fatty acids is more effective than glucose in terms of the number of ATPs produced per carbon or per gram, it also requires more oxygen per ATP due to the reduced state of fatty acids. Biosynthesis, which is required for any anabolic process such as cell proliferation and formation of new tissues, requires metabolic intermediates to be shuttled toward biosynthesis rather than ATP production. Especially in a high-metabolic tissue like mammalian cardiac muscle, the need for energy versus biosynthesis must constantly be carefully balanced.

5. Energy Turnover in the Diseased Heart

During an ischemic incident, oxygen and nutrient supply is limited to the affected area with important implications on cardiac metabolism. Ischemia activates hypoxia inducible factor HIF1α, which suppresses oxidative phosphorylation for instance by inactivating pyruvate dehydrogenase, in effect blocking the conversion of pyruvate into acetyl-CoA. Simultaneously, HIF1α activates a number of genes favoring glycolysis and anaerobic production of lactate, which as a byproduct also increases the production of protons. Increased levels of lactate and protons lowers intracellular pH. As the lack of oxygen reaches critical levels, the electron transport chain screeches to a halt as oxygen normally serves as the final electron acceptor in the chain and glycolysis becomes uncoupled from glucose oxidation. The abundance of protons and reduced ATP-synthase activity triggers reverse electron transfer in the electron transport chain, resulting in accumulation of succinate, which acts as a ticking "ROS-time bomb" ready to go off once oxygen flow is reestablished. At this point, the concentration of Ca²⁺ becomes dysregulated and can result in arrhythmias and cardiac arrest. Intracellular Ca²⁺ can trigger apoptosis by stimulating the opening of mitochondrial permeability transition pores in the inner mitochondrial membrane, however, the low pH somewhat ironically acts in a protective fashion at this point by keeping mitochondrial permeability transition pores shut. The fact remains that if oxygen supply is not restored, the hypoxic state and lack of nutrients results in widespread cell death. Thus, while reperfusion is absolutely critical at this point, it contrarily
often results in additional damage. The abrupt return of oxygen prompts oxidative phosphorylation to start up again, but there is a lag phase in which cardiomyocytes also continue to shuffle glucose toward anaerobic glycolysis.\[61\] This keeps intracellular Ca\(^{2+}\) high while pH normalizes allowing mitochondrial permeability transition pores now to open resulting in apoptosis.\[61\] In addition, superabundant amounts of reactive oxygen species are produced, which are cytotoxic and cause apoptosis of cells that otherwise survived the ischemic incident.\[66\] With continued supply of oxygen and substrates for oxidative phosphorylation Ca\(^{2+}\), pH and ATP production eventually returns to normal. Importantly, because the resulting injury never heals, cardiac function will remain affected with implications for cardiac metabolism. A curious example of tolerance to hypoxia and even anoxia as well as reperfusion is seen in fresh water turtles like pond sliders that can survive weeks of anoxia followed by reoxygenation without cardiac injury. This appears to be thanks to a rewiring of cardiac metabolism that maintains a new steady state of ATP by continuous re-synthesis from ADP, and shuttling of succinate out of the mitochondria by a yet unknown mechanism.\[67\]

### 6. Manipulation of Ambient Oxygen Level and Metabolic Mechanisms

Cardiac metabolism undergoes substantial changes after birth in mammals, coinciding with a loss of regenerative ability within the first few weeks of the neonate life outside the uterus.\[36-68\] During fetal life, mammals are exposed to a hypoxic environment compared to after birth,\[69\] with limited availability of fatty acids compared to carbohydrates.\[70\] Mammalian fetuses and early neonates as a result rely mainly on glycolysis for ATP production and have only limited ability for oxidative phosphorylation.\[50,71,72\] After birth, the mammalian neonate is immediately exposed to higher levels of atmospheric oxygen and cardiac work load increases, prompting a transition toward increased oxygen consumption.\[50,73,74\] oxidative phosphorylation, and increased uptake of fatty acids as these become readily available from ingestion of breast milk.\[75\] At the same time, cardiomyocytes become multinucleated and enter a state of cell cycle arrest.\[39\] They also diminish their store of glycogen in favor of an increase in the number and size of mitochondria.\[50,76\]

It has been suggested that these metabolic adaptations after birth are responsible for the loss of heart regenerative ability in mammals as an increased level of oxidative phosphorylation leads to increased levels of reactive oxygen species resulting in DNA damage and reduced cardiomyocyte proliferation.\[77\] Thus, abandoning the ability to repair damaged cardiac muscle during development may represent an evolutionary tradeoff allowing a high-metabolic heart to function without an uncontrolled accumulation of DNA damage.\[78\] It has been demonstrated in the neonatal mouse that reactive oxygen species, oxidative DNA damage, and markers of DNA damage response were significantly increased during the first week after birth and that the neonatal regenerative period could be extended by treatment with hypoxia.\[79\] Later it was demonstrated that hypoxia could also induce cardiac regeneration via dedifferentiation of existing cardiomyocytes in adult mice.\[79\] This interesting finding however remains to be replicated by others and scrutiny of the quantitative histology performed in the study cannot rule out that the response reported as intrinsic regeneration was instead a non-regenerative hypertrophic response to a life-threatening low oxygen environment (ambient PO\(_2\) = 7 kPa resembling PO\(_2\) just 400 m below the summit of Mount Everest) rather than a truly regenerative response.

The most informative experiments of the role of aerobic metabolism on heart regeneration may in fact not be conducted by exposing highly oxygen-dependent non-regenerative animal models to extreme hypoxia, but rather by investigating the response of organisms with regenerative capacity subjected to abundant availability of oxygen (hyperoxia). This can be formulated into the following hypothesis:

**Sub-hypothesis 1:** Increased oxygen availability is disruptive to heart regeneration in organisms capable of intrinsic regeneration due to increased levels of reactive oxygen species from oxidative phosphorylation resulting in DNA damage.

This hypothesis has been put to the test in zebrafish.\[80\] It was found that a regeneration disruptive effect of hyperoxia occurred by exposing zebrafish to hyperoxic water at 45 kPa (relative to 21 kPa in normoxic water). However, based on the experimental procedure describing a setup where the zebrafish was placed in an oxygen penetrable plastic bag without standardized mixing of gas and a water phases, and no published record of available oxygen tension over a daily cycle, these results should be interpreted with caution. It seems that solid experiments need to be conducted to test sub-hypothesis 1 across a range of species to reveal the link between available oxygen and heart regeneration. In doing so, it could be worth to consider investigating how regeneration competent amphibians respond to varying oxygen availability, since many members of this group are characterized by both water and air breathing, that is, they are exposed to both low and high oxygen concentrations.

It is poignant that the metabolic response to myocardial infarction appears to return the heart to a metabolic profile similar to that of the fetal stage.\[81\] at which some mammals are able to regenerate after injury. A way to falsify the fundamental hypothesis would be to extend the embryonic/neonatal regenerative capacity of endotherms until later life stages. A counter hypothesis can be formulated:

**Sub-hypothesis 2:** Metabolic manipulation of the developing endotherm allows for cardiac regeneration even at life stages of full endothermy.

This hypothesis could be tested in a mammalian setting, for example, in the embryonic or neonatal mouse, but other model systems may be worth considering. The embryonic chicken has been described to possess heart regenerative capacity early in development (Days 3 and 5), but lose this capacity at later embryonic stages (Day 18 and after hatching).\[11\] The chicken egg represents a much cheaper, legislative less restrictive, embryonically easy accessible, and more manipulable model system than any embryonic and neonatal mammal. By regulating gas composition, substrate availability in the yolk and incubation temperature, an avian in ovo model system may be desirable to test sub-hypothesis 2.
7. The Interplay between Metabolism and Regenerative Capacity

The implication of metabolic rate on heart regeneration remains to be investigated. Unless a high-metabolic species capable of heart regeneration is discovered in a comparative study as described above, it is necessary to rely on regeneration competent ectothermic model organisms and design experiments in such a way that the validity of the fundamental hypothesis can be directly tested.

There are two possible mechanisms to consider on how a high-metabolic heart might inhibit a regenerative response (Figure 5): 1) the metabolic cost of regeneration may simply be too high for high-metabolic hearts. The simultaneous processes of maintaining cardiac function and energy demanding formation of new cardiac tissue may not be energetically feasible simultaneously, especially in an injury compromised heart; 2) a direct inhibitory effect of metabolites on regeneration, for instance the higher levels of reactive oxygen species produced.

In zebrafish, a controlled shift from oxidative phosphorylation toward glycolysis appears to be required for cardiac regeneration. This process was found to be driven by Nrg1/ErbB2 signaling and a crucial step in cardiomyocyte cell cycle reentry. When inhibiting glycolysis by administering a glucose analog, the zebrafish could no longer produce new cardiomyocytes. This indicates that the reconstruction of an injured heart in a regeneration competent model which requires cardiomyocyte proliferation is tightly linked to a glycolysis favoring environment. The importance of an ErbB2 driven shift toward glycolysis rather than oxidative phosphorylation has also been studied in neonatal mice. ErbB2 signaling gradually decreases after birth in mice. ErbB2 knockout mice displayed abnormal cardiac development, with reduced levels of cardiomyocyte proliferation resulting in compensatory hypertrophy and reduced ability for cardiac regeneration at neonatal stages where this is normally possible. Conversely, a transgenic mouse model in which ErbB2 was constitutively active was shown to display increased levels of cardiomyocyte dedifferentiation and proliferation. Importantly, these mice suffered from cardiomegaly and reduced cardiac output, likely due to compromised adhesion between cardiomyocytes and the extracellular matrix. During dedifferentiation cardiomyocytes lose attachment to the extracellular matrix. Importantly, transient expression of ErbB2 in adult mice after myocardial infarction resulted in markedly improved outcomes due to cardiomyocyte dedifferentiation as well as improved angiogenesis. The cardiomyocytes of this mouse strain has interestingly been found to display a metabolic profile consistent with an early neonatal phenotype, with increased expression of glycolytic enzymes. It has further been demonstrated that stimulating glucose oxidation directly by activation of pyruvate dehydrogenase or inhibiting oxidative phosphorylation by inhibiting malonyl CoA decarboxylase reduced the size of infarction following myocardial infarction in adult mice. Taken together with the results in zebrafish, this suggests that glycolysis rather than oxidative phosphorylation favors a regeneration permissive environment in both a regeneration competent (zebrafish) and what is generally perceived as a regeneration incompetent model (mouse), and Nrg1/ErbB2 signaling is linked to cardiomyocyte cell cycle reentry in both models.

Another interesting example of the potential interplay between metabolism and regenerative capacity stems from the Mexican tetra (Astyanax mexicanus) that exists both in a surface ecotype capable of heart regeneration as well as several cave ecotypes of which at least the Pachón ecotype is not capable of heart regeneration. Troglobiont species often respond to a radically altered selection pressure in caves such as the lack of light and reduced nutrient access by specific adaptations, for example, the Pachón tetra is colorless, only has rudimentary eyes and altered blood chemistry resulting in lower-metabolic cost of eyes and neural tissue compared to the surface ecotype. Interestingly, general metabolic rate is also lower in the Pachón ecotype than in the surface Mexican tetra, seemingly contradicting the fundamental hypothesis put forward here. However, in a nutrient poor environment like caves, there is also the option that the metabolic cost of heart regeneration is simply too high and/or that the different ecotypes of Mexican tetras constitute a highly useful natural experiment that potentially holds a lot of information of the presumably fine-tuned interplay between metabolism, nutrient availability, and regenerative capacity.

8. Models and Methods to Approach the Metabolic Cost of Regeneration

A large comparative analysis of the distribution of regenerative capacity within vertebrates as described above may yield new candidate species that are particularly adapted to study the mechanisms of regeneration and the interplay with cardiac metabolism. Until then, we have to rely on already established laboratory models, essentially the zebrafish and the axolotl that are currently the two most widely used model animals in the heart regenerative field.

Heart metabolic rate can be manipulated by artificially pacing the heart or pharmacological/genetic blocking of metabolic pathways, but a benefit of using ectothermic animal models like the zebrafish and the axolotl is that regulating overall metabolic rate is easily accomplished simply by regulating ambient temperature and/or the amount and composition of food. A relatively straightforward approach to test the metabolic limits of heart regeneration would be to establish if there exists a level of routine metabolic rate at a temperature above and below the critical thermal minimum and maximum (CT_min and CT_max, i.e., the temperature range within which the animal generally functions) at which heart regeneration is inhibited. This can be formulated in the hypothesis:

Sub-hypothesis 3: In an organism with heart regenerative capacity, a regenerative window exists at a more narrow temperature range than CT_min – CT_max.

Interestingly, it has been demonstrated that newts presented with a gradient of temperatures actively seek a temperature above the normally preferred temperature when they perform limb regeneration. We do not know if this behavior is directly linked to an optimal temperature for the anabolic processes during tissue regeneration or indirect components relevant for regeneration, for example, reduced bacterial growth similar to...
Figure 5. Possible mechanisms inhibiting cardiac regeneration in high-metabolic adult mammals. The hypothesis that metabolic cost inhibits cardiac regeneration is illustrated in animal models (adult mouse, fetal mouse, and axolotl). Green bars show a representation of total energy available to the animal, red bars the energy used to maintain normal cardiac function, and yellow bar energy required for growth. The dashed line represents the hypothetical maximum amount of energy that can be used for cardiac function and growth while still allowing energy allocation for regeneration, represented by the blue bar. The hypothesis that high levels of oxidative phosphorylation inhibit cardiac regeneration is illustrated below animal models.
a fever response in mammals. A similar experiment on heart regeneration would provide useful information, especially in the combination with forced heating and cooling during heart regeneration to directly test sub-hypothesis 3. Classic respirometry measurements of oxygen consumption and CO₂ excretion at different stages of the regenerative process could provide important information of the metabolic cost of heart regeneration.

The study of metabolic processes during regeneration in intact and conscious animals is complicated. Animal behavior profoundly impacts general metabolic rate and many of the chemical substrates and intermediates involved are unstable or quickly metabolized and shuttled into alternative pathways. Because cardiac function and metabolism are reciprocal processes, it can be a challenge to deduce if metabolism is affecting cardiac function or vice versa. In the complicated in vivo system of an intact organism, it can often be a challenge to separate effects of cardiac metabolism from systemic metabolism and filter out the noise posed by animal behavior. One way to largely overcome the behavioral component in vivo would be to develop a regenerative animal model under permanent anesthesia. Assuming that heart regeneration is unchanged under extended periods of anesthesia, this would allow for measurements of metabolic rate unaffected by behavior. Even a potentially small extra cost of cardiac regeneration could in this way be measured by whole animal respirometry.

It is possible in some species to uncouple heart metabolism with systemic metabolism using an ex situ model of heart regeneration. Salamanders are generally receptive of allotransplantation of hearts[88] and in the case of the axolotl, we have previously developed an ex situ heterotopic heart model in which a donor heart is transplanted into the abdomen of a host and is joined to the blood system.[89] In this model, cardiac function of the heterotopic heart is uncoupled with cardiac output of the host animal and manipulations to cardiac function of the ex situ heart does not affect the animal's general capabilities or metabolism.

An even more sophisticated approach would be the development of an ex vivo model of heart regeneration in which an excised heart from a regeneration competent animal model like the zebrafish or axolotl is kept alive in similar fashion as the Langendorff isolated perfused heart model.[90] An explant culture system has been developed for the unloaded zebrafish heart,[91] but it remains to be tested if a working heart can survive in a perfused ex vivo state for a time span relevant for regenerative experiments, that is, for months. If this challenge can be overcome, the ex vivo regenerative heart model has the potential to be used to precisely describe the link between heart regeneration and metabolism as well as the potential factors outside the heart that may play crucial roles for regeneration to take place. One of these factors could be immune cells that have previously been described to play crucial roles for regeneration to take place. One of these factors could be immune cells that have previously been described to play crucial roles for regeneration to take place. If this challenge can be overcome, the ex vivo regenerative heart model has the potential to be used to precisely describe the link between heart regeneration and metabolism as well as the potential factors outside the heart that may play crucial roles for regeneration to take place. One of these factors could be immune cells that have previously been described to be crucially involved in heart regeneration in the axolotl.[92] In ectothermic animals with a generally lower body temperature than endotherms, the mere effect of a less optimal infectious environment at lower temperatures could result in a less severe and less regeneration inhibitory inflammatory response. Uncoupling the heart from the rest of the organism in an ex vivo model would allow for the experimental manipulation of both metabolism and other strong factors like the immune response.

In vitro experimentation on primary cell cultures of heart regenerative species to explore substrate utilization and cellular metabolism is possible. Under the definition of heart regeneration proposed above, an in vitro model is insufficient to claim heart regeneration since it does not resolve anatomical and functional components of the process. However, heart regeneration is a multilevel process and cardiomyocyte proliferation and metabolism even on the primary cell culture level could provide highly useful information of cellular processes, in particular at different stages of heart regeneration.

Metabolic adaptations occur quickly and are in many cases controlled at the posttranscriptional level, which likely explains the inconsistency when comparing gene expression and protein levels of different metabolic enzymes and cofactors.[90,91] It is therefore crucial to design experiments that evaluate cardiac metabolism at the most relevant level. Whole animal metabolic imaging has the benefit of constituting an intact self-supporting system in which metabolic processes can be studied as they happen in vivo. Modern non-invasive medical imaging technologies such as micro positron emission tomography (micro-PET) and hyperpolarized magnetic resonance imaging (micro-MRI) provide some options to follow metabolic processes in regeneration competent animal models. Since these technologies have a rather low resolution (in the mm range) there is a size limit on useful regenerative model species for these technologies. Whereas the zebrafish is too small to constitute a meaningful model using these technologies, the axolotl ranging in body weight from 10 to 100 g in adulthood is well suited. We have conducted pilot experiments in the axolotls to validate the functionality of PET/MRI imaging using [18F]-fluorodeoxyglucose (a non-metabolized glucose analog) and [14C]-acetate tracers (Figure 6). In these experiments, control and animals subjected to cardiac cryo-injury were PET imaged and subsequently hearts were sampled, sectioned, and imaged with autoradiography. An increased glucose and acetate uptake in the border zone of the infarction was observed (Figure 6) both at 10 and at 30 days after cryo-injury, pointing toward a general upregulation of both glycolysis and TCA cycle pathways during early phases of cardiac regeneration. While this data only constitutes early observations and has yet to be coupled with, for example, measurements of cardiomyocyte proliferation in the border zone, it points to the applicability of these methods to measure metabolic processes in regeneration competent animals, which can potentially be combined with other techniques to monitor regenerative mechanisms inspired from the medical field.[94]

9. Conclusions
The development of regenerative therapies to the heart to alleviate or even cure the detrimental effects of ischemic heart disease has the potential to save and improve millions of lives. In this light it is easy to justify the interest in heart regenerative model organisms. However, at this time we know very little of the actual distribution of heart regenerative capacity across vertebrates, but what we do know is that there seems to be a strong bias toward low-metabolic ectotherms. Since the biologically inspired approach toward developing regenerative therapies has yet to demonstrate its validity, and while engineering inspired approaches are rapidly being developed, it may be important to consider that there could be underlying constraints such as lack of metabolic capacity that does not allow high-metabolic hearts like
Figure 6. Preliminary a) whole animal PET imaging and b) heart section autoradiography of [18F]-FDG (glucose analog) and [14C]-acetate (oxidative phosphorylation marker) uptake in regenerating axolotls indicate increased general cardiac metabolism 10 days post myocardial infarction (dpi) and increased glycolysis and oxidative phosphorylation in the regeneration active infarct border zone 30 dpi. At the time of intravenous PET tracer ([18F]-FDG) injection (left image in (a)) a high [18F]-FDG signal originating from blood borne [18F]-FDG can be found in all well-perfused organs, for example, brain, heart, liver, and gills. Three hours post injection [18F]-FDG is much lower in blood and the glucose analog is concentrated in organs with a high glycolytic activity such as the brain, and in the case of a regenerating heart (image to the right) in the myocardium. Autoradiography of representative heart sections in (b) are presented with the same rainbow color map in which red indicates a high degree of radioactive decay, that is, a high concentration of the radiolabeled metabolite (top row: [18F]-FDG; bottom row: [14C] acetate) in a healthy control (left column) and cryo-injured hearts at 10 dpi (middle column), and 30 dpi (left column).
that of the human to perform regeneration while simultaneously providing a high cardiac output. Large-scale comparative endeavors in combination with a strict definition of what constitutes a regenerative event could potentially provide crucial information on the physiological restraints of heart regeneration. At the same time, a judicious use of established model species with transient or constant regenerative capacity in experiments manipulating, for example, metabolic mechanisms and rate, availability of substrates, and availability of oxygen has the potential to elucidate whether heart regeneration is in fact strictly limited to ectothermic organism, or if even an endothermic creature like ourselves can be manipulated to regenerate the heart after injury.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors would like to acknowledge Lundbeckfonden (Grant# R324-2019-1470) and A.P. Møller Fonden til Lægevidenskabens Fremme (Grant# 17-L-0200) for providing funding to investigate the link between cardiac metabolism and regeneration.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

axolotl, comparative physiology, ectotherm, endotherm, heart regeneration, metabolism, zebrafish

Received: March 11, 2020
Revised: August 12, 2020
Published online: September 11, 2020

[1] J. Maienschein, Dev. Biol. 2011, 358, 278.
[2] L. Spallanzani, Prodromo di un’ opera da impressi sopra le riproduzioni animali. Giovanni Montanari, Modena. Translated in English by Maty M. 1769.
[3] J. O. Oberpriller, J. C. Oberpriller, J. Exp. Zool. 1974, 187, 249.
[4] N. B. Frohlich, C. Bickelmann, F. Witzmann, Proc. R. Soc. B Biol. Sci. 2014, 281, 20141550.
[5] Y. Yang, T. Owerkowicz, R. Elsey, C.-L. Lien, Science 2014, 345, 12513.
[6] E. L. Price, J. M. Vieira, P. R. Riley, Expert Rev. Cardiovasc. Ther. 2016, 14, 1095.
[7] H. C. Ott, T. S. Matthiesen, S.-. K. Goh, L. D. Black, S. M. Kren, T. I. Netoff, D. A. Taylor, Nat. Med. 2008, 14, 213.
[8] J. Glynn, H. Song, B. Hull, S. Winthers, J. Gelow, J. Mudd, A. Starr, R. Wampler, Artif. Organs 2017, 41, 904.
[9] M. L. Martik, Elife 2020, 9, e54665.
[10] G. R. Dagenais, D. P. Leon, S. Rangarajan, F. Lanas, P. Lopez-Jaramillo, R. Gupta, R. Diaz, A. Avezum, G. B. F. Oliveira, A. Wielgosz, S. R. Parambath, P. Mony, K. F. Alhabib, A. Temizhan, N. Ismail, J. Chifamba, K. Yeates, R. Khatab, O. Rahman, K. Zatonska, K. Kazimi, L. Wei, J. Zhu, A. Rosengren, K. Vijiakumar, M. Kaur, V. Mohan, A. Yusufali, R. Kelishadi, K. T. Teo, P. Joseph, S. Yusuf, Lancet 2020, 395, 785.
[11] S. Johansson, A. Rosengren, K. Young, E. Jennings, BMC Cardiovasc. Disord. 2017, 17, 53.
[12] S. P. Karunathilake, G. U. Ganegoda, Biomed. Res. Int. 2018, 2018, 5767864.
[13] J. I. Malik, E. Moshref, S. Siddiqi, K. M. Broughton, B. A. Bailey, N. A. Gude, M. A. Reuter, M. Heister, C. Scholz, T. Borchardt, T. Braun, J. Cell. Sci. 2006, 119, 4719.
[14] A. A. C. Killen, J. Zhao, Z. Hu, J. Riepe-saame, N. Hamilton, T. Kudoh, P. R. Riley, R. van Aarle, Y. Yamamoto, M. T. M. Mommersteeg, Cell Rep. 2018, 25, 1997.
