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Cell-based therapies for the treatment of myocardial infarction: lessons from cardiac regeneration and repair mechanisms in non-human vertebrates

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Abstract
Ischemic cardiomyopathy is the cardiovascular condition with the highest impact on the Western population. In mammals (humans included), prolonged ischemia in the ventricular walls causes the death of cardiomyocytes (myocardial infarction, MI). The loss of myocardial mass is soon compensated by the formation of a reparative, non-contractile fibrotic scar that ultimately affects heart performance. Despite the enormous clinical relevance of MI, no effective therapy is available for the long-term treatment of this condition. Moreover, since the human heart is not able to undergo spontaneous regeneration, many researchers aim at designing cell-based therapies that allow for the substitution of dead cardiomyocytes by new, functional ones. So far, the majority of such strategies rely on the injection of different progenitor/stem cells to the infarcted heart. These cardiovascular progenitors, which are expected to differentiate into cardiomyocytes de novo, seldom give rise to new cardiac muscle. In this context, the most important challenge in the field is to fully disclose the molecular and cellular mechanisms that could promote active myocardial regeneration after cardiac damage. Accordingly, we suggest that such strategy should be inspired by the unique regenerative and reparative responses displayed by non-human animal models, from the restricted postnatal myocardial regeneration abilities of the murine heart to the full ventricular regeneration of some bony fishes (e.g., zebrafish). In this review article, we will discuss about current scientific approaches to study cardiac reparative and regenerative phenomena using animal models.

Keywords Myocardial infarction · Cell-based therapies · Tissue regeneration · Tissue repair · Animal models

Introduction

Cardiovascular diseases kill more people every year than cancer (http://www.who.int/classifications/icd). Among cardiovascular conditions, cardiac ischemic disease is the one with the highest prevalence in Western populations [1] (see also Fig. 1). Cardiac ischemia is the most frequent cause of myocardial infarction (MI). From a pathophysiological perspective, MI is characterized by the loss of myocardium after sustained oxygen deprivation. Such ischemic episodes are linked to coronary flow obstructive events related to coronary artery atherosclerosis [2].

Unless other vertebrates, mammals (including humans) cannot compensate for the loss of heart muscle, and therefore show very little cardiac regenerative ability. Instead of regenerating, the injured heart substitutes the dead myocardium by fibrous connective tissue secreted by activated cardiac fibroblasts (myofibroblasts). This reparative, non-contractile fibrosis, which prevents cardiac wall rupture, soon transforms into a reactive fibrosis that continuously expands at the expense of the surviving myocardium. Post-MI scar ultimately alters cardiac function, leading to heart failure [3]. Despite the many advances in the field of pharmacotherapy (including the design of new thrombolytic, beta-blocker, or antiarrhythmic drugs), the continuous surgical improvements and the use of ventricular assist devices (catheters, pacemakers, implantable defibrillators, artificial valves and stents) [4], the mortality and morbidity rates associated to MI continue growing (health costs for cardiac ischemic disease in the EU are estimated to

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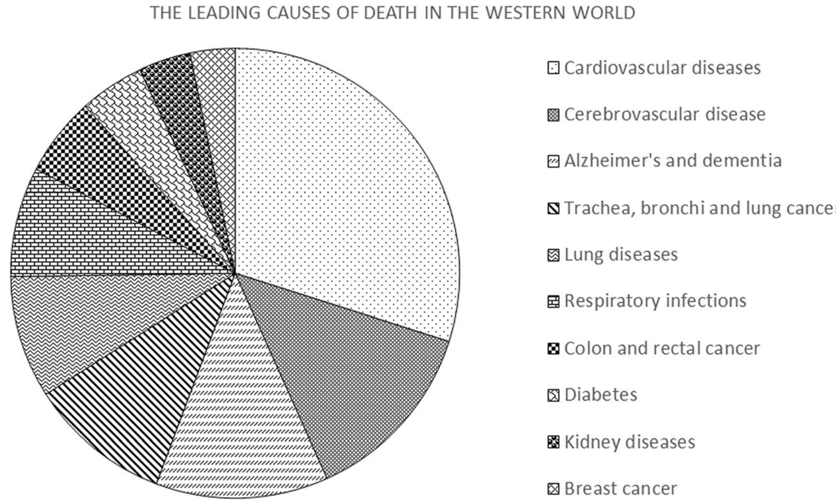

Fig. 1 Leading causes of death in the Western World (from World Health Organization, 2015). Modified from http://www.who.int/classifications/icd

be 111.000 10^6€ per year, see http://www.ehnheart.org/). Both the increasing incidence of MI and its chronification in many patients urge for the development of alternative cardiac therapies to treat the infarcted heart [5].

Post-MI ventricular remodeling disrupts heart performance

Heart responses to myocardial death are first triggered by cardiac macrophage-induced local inflammation. This primary inflammation is soon amplified through the recruitment of blood-circulating cells to the injured heart, first granulocytes and then monocytes [6]. These latter cells infiltrate into the ventricular walls to eliminate dead cardiomyocytes, but also initiate a reparative response that depends on the recruitment of cardiac resident fibroblast progenitors to the damage site [7]. These activated fibroblasts (myofibroblasts) synthetize large amounts of extracellular matrix proteins, forming a non-contractile fibrous tissue that progressively transforms into a poorly cellularized stiff scar. All these post-MI phenomena are jointly referred to as “ventricular remodeling” and are responsible for the loss of ventricular wall contractility.

Human heart repairs but does not regenerate

As discussed above, the formation of a non-contractile fibrous tissue in the infarcted human heart prevents ventricular wall rupture and cardiac tamponade. This characteristic substitution of a damaged tissue by another one displaying different functional properties is called “repair.” On the contrary, the substitution of a damaged tissue by the same one is known as “regeneration.” Regenerative phenomena are tightly regulated by complex molecular mechanisms. Unfortunately, the terms “repair” and “regeneration” are often used interchangeably, and although they can occur in the same organ, these two processes should be considered different biological entities.

Repair events based in the massive deposition of fibrotic extracellular matrix are a common animal tissue response to damage. In humans, these reparative responses can become clinically relevant when an organ cannot undergo regeneration. The adult mammalian heart is the perfect example of an organ lacking the ability to regenerate spontaneously. However, some experimental studies have shown that during a short postnatal temporal window (around a week after birth), the murine heart can undergo regeneration [8]. We do not know whether such intrinsic regenerative ability is also present in the human postnatal heart. Relevant to this discussion, it is important to mention that other researchers have reported data on the existence of different populations of adult cardiac resident stem cells (CSCs) with the ability of differentiating into cardiovascular cell types [9, 10]. Taken together, all these findings strongly suggest that the mammalian heart has an intrinsic regeneration potential that, for unknown reasons, is not effectively displayed after a severe injury. Conducting research on this specific topic may be an excellent strategy to discover new therapies to regenerate the injured heart.

Cardiac regeneration in different non-human vertebrates

The ability of regenerating tissues and organs can be present or absent in organisms of the same supraspecific taxon [11]. Remarkably, while anamniote vertebrate (fishes and amphibians) tissues have a relatively high regenerative capacity [12, 13], other groups of amniote vertebrates (reptilians, avians, and mammals) have a limited regenerative potential [14]. We believe that a comparative analysis of the regenerative properties of cardiac tissues in different animal models will allow for the identification of relevant similarities and differences in the cellular and molecular motifs that control cardiac regeneration.

Anamniote vertebrates

Recent studies have shown that different species of anamniote vertebrates such as the goldfish [15], the zebrafish [16, 17], the axolotl [18], or the newt [19] regenerate their cardiac ventricle after injury. In particular, the zebrafish, which is the most studied animal model in the field of cardiac regeneration, cardiomyocyte de-differentiation [20], and proliferation [17] are both involved in ventricular chamber regeneration and functional restoration. The formation of a blastema-like structure (a rather undifferentiated, proliferative mass of mesenchyme) is instrumental to zebrafish myocardial regeneration [21]. Moreover, zebrafish cardiomyocyte proliferation is tightly related with the mononucleated, diploid status of adult cardiac myocytes [22]. This ability is also present in other cyprinid teleostei like the goldfish [15] but seems to have been lost in other species such as medaka [23], whose main response to apical ventricular resection is extensive fibrosis of the heart walls.

Despite the reported regenerative ability of adult amphibian tissues [24–27], amphibian heart responses to induced damage are not still well understood, and certain amount of controversy remains on whether all amphibians have the same regeneration abilities. It has been recently shown that different anuran species (frogs and toads) display different cardiac responses to damage. While *Xenopus tropicalis* heart is able to regenerate after an endoscopy-based ventricular resection [28], *Xenopus laevis* fails to do so after suffering a similar loss of myocardial mass [29]. These differences can be explained by the specific methods applied to create the experimental insult [30], being the wound size a determinant feature for the initiation of the regeneration process [31]. In addition, it has been shown that external factors as environmental conditions and age could influence the regenerative properties of both the zebrafish [32] and the anuran [31] heart. Furthermore, it is not clear whether regeneration is a common response of urodeles (salamanders and newts) to cardiac injury. Although the axolotl (*Ambystoma mexicanum*) ventricle can fully regenerate without developing a scarring process [18, 33, 34], other species seem to be less prone to regeneration. Indeed, the adult myocardium of the newt *Notophthalmus viridescens* increases cardiomyocyte proliferation at the wound area after an apical amputation of the ventricle [35], but myocardial regeneration is never completed in these organisms and the prevalent response to damage remains fibrotic repair [36].

Mammals

Several studies have shown that neonatal mice submitted to apical ventricular resection can also regenerate the myocardium during the first week after birth [8, 37] (Fig. 2). This suggests that the cardiac regenerative ability of mammals progressively decreases from embryonic stages to adult life. This process implies an increase of cardiomyocyte proliferation rate combined with a marked reduction of cardiac fibrosis [38].

It is also obvious that the regenerative potential of the neonatal mouse heart depends on the extent of the damage, since a complete tissue regeneration does not occur after an experimental transmural cryodamage (i.e., an injury affecting the whole ventricular wall thickness) [39]. On the other hand, despite the high proliferation of some adult anamniote vertebrate tissues (including the myocardium), the regenerative properties of such tissues decrease in elderly animals, probably due to the aging of the cellular component of the regenerative processes [40].

In addition to the proliferative activity of cardiac tissues after myocardial infarction, it has also been argued that the presence of different populations of cardiac resident cells expressing stem cell markers is an evidence for the regenerative potential of the adult mammalian heart. Many of these cells are supposed to be real cardiac stem cells (CSCs); the majority of CSCs express the c-Kit receptor, although they also express other markers like Sca-1 [9, 41]. Interestingly, c-Kit$^+$ cells play a fundamental role in the early regenerative capacity of the neonatal (but not the adult) mouse heart, most probably through a paracrine mechanism that still needs to be characterized in detail [42].

Can we thus conclude that the mammalian heart has an endogenous regenerative potential, which is lost or actively repressed during postnatal life? This is a difficult question to answer, but all the evidences we have discussed suggest that this is the case. In this regard, a specific mouse strain (MRL line) can completely regenerate different damaged adult tissues, including the heart [43, 44]. MRL CSC populations are different from those of other mice, and their control of cell cycle is also different. Moreover, MRL mice do not produce significant scar tissue after myocardial damage. We should thus conclude that (1) the murine genetic background is relevant to the study of mammalian heart regeneration; (2) theoretically, there are no evolutionary constraints limiting mammalian heart regeneration (Fig. 2).

Cardiac vertebrate regeneration: biomedical implications

The analysis of regenerative phenomena in different animal species has shown that there are two main regeneration modes in the Animal Kingdom, both of which seem to have been preserved throughout evolution in different animal lineages. These two kinds of regeneration were already described by T.H. Morgan in 1901 [45]. The first type of regeneration

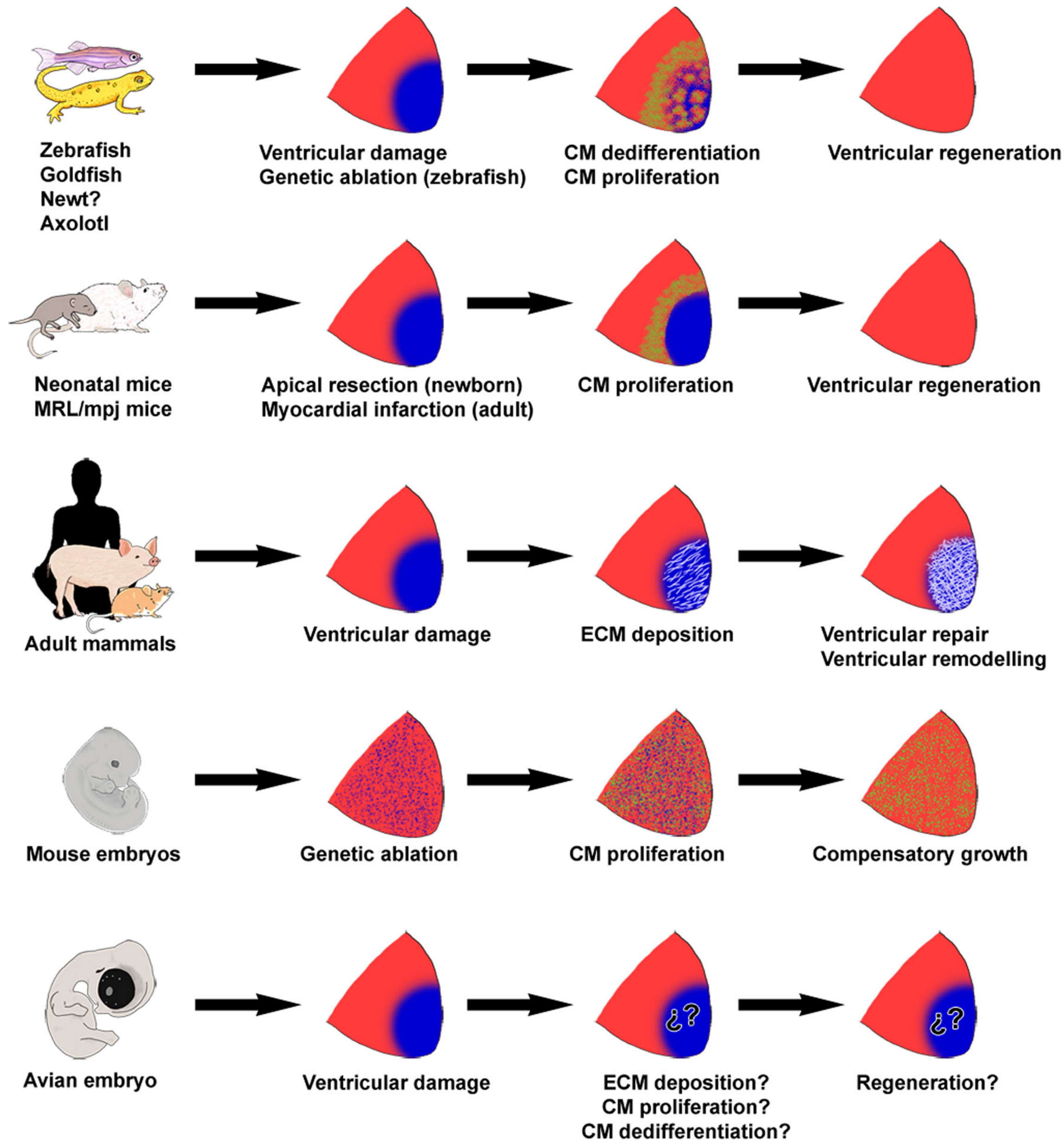

Fig. 2 Cardiac regeneration and repair in vertebrate phylogeny. Differences on the incidence of cardiac reparative or regenerative processes in different animal taxa are shown. Abbreviations: CM cardiomyocyte, ECM extracellular matrix

involves the increase of the proliferation rate of stromal cells adjacent to the damaged area, usually associated with the formation of a blastemal, i.e., a mass of highly proliferative, undifferentiated mesenchymal cells. This type of process, which is known as "epimorphic regeneration," is complex but can fully and efficiently regenerate sophisticated anatomical structures (e.g., full limbs in salamander). The second type of regeneration implies a marked change in cell size, morphology, and spatial organization which is often linked to transdifferentiation phenomena (direct differentiation of a given type of somatic cell into a different one). This process is known as "morphallactic regeneration" and is frequent in many invertebrate animals. We suggest that some characteristic responses of mammalian cells to pathologic stimuli (e.g., cell hypertrophy) could result from the activation of mechanisms involved in morphallactic regeneration [46].

According to the criteria discussed above, cardiac regeneration in vertebrates such as the zebrafish or the newt can be ranked among epimorphic regeneration events [17, 21, 47, 48], being cell proliferation the key cellular feature of the regenerating tissue. In accordance with these findings, we can conclude that epimorphic regeneration cannot take place in the adult mammalian heart (including the human one) because neither the low number of proliferating cardiomyocytes [9, 49] nor the absence of a true blastema support the formation of new cardiac tissue [50]. But, is parenchymal cell proliferation the only factor we have to take into account when dissecting epimorphic organ regeneration? In the context of heart regeneration, the formation of a primary reparative fibrotic response following mammalian cardiac muscle death is likely to interfere with regeneration phenomena [51]. It is not known how anamniote vertebrates like the zebrafish manage to avoid the formation of such fibrotic scar, allowing for the full expansion of newly differentiated cardiomyocytes. However, it is tempting to hypothesize that differences between lower (anamniote) and higher (amniote) vertebrate proteolytic machineries could underlie this divergent vertebrate heart response to injury.

In addition to epimorphic and morphallactic regeneration, many animal tissues (e.g., skin, blood) continuously renew some tissues as based in the presence of adult resident cells in various organs [52]. This process shares some features with classic epimorphic regeneration phenomena (including the presence of highly proliferative cells in the tissue) but does not involve a blastema formation. In the case of the heart, different populations of resident cardiac stem cells (CSCs) [9, 10, 53, 54] have been shown to be able to differentiate into cardiomyocytes, albeit at a very low rate [55]. The embryonic origin of these cells remains a mystery, and several cell sources including the bone marrow [56], embryonic cardiac progenitor fields [57], or the embryonic epicardium [58] have been suggested to be sources of CSC. The case of epicardial contribution to cardiac regeneration is indeed relevant, since many embryonic epicardially expressed genes are activated shortly after adult myocardial injury [48]. Recent studies have concluded that the epicardium and its derived cells do not materially contribute new cardiomyocytes to the regenerating heart, but rather secrete instructive molecules that promote cardiac regeneration and cell survival [59–61], also having an essential role in the revascularization of the wound [48]. However, further research is necessary to detail the specific roles played by this tissue in cardiac regeneration and repair. The activation of reparative fibrotic mechanisms after myocardial death seems to be the most important factor limiting cardiac regeneration, and some authors have suggested that this fibrosis interferes with CSC differentiation into new cardiomyocytes by disrupting their niche (extracellular microenvironment) [62] and severely hampers CSC activation, expansion, and differentiation.

Lessons learnt from animal models regarding cardiac regeneration can be summarized as follows. First, effective cardiac regeneration exists in some animal vertebrate species, and these regeneration events depend on the ability of pre-existing myocardial tissue to generate new functional muscle. This means that, while the proliferative properties of adult higher vertebrate cardiac tissues are restricted to a low number of adult cardiomyocytes and CSC, adult cardiomyocyte proliferation in lower vertebrate animals is robust and can easily provide high numbers of new cardiomyocytes to the injured heart. Second, the fibrosis that develops after heart damage only persists in higher vertebrates, and it interferes with the replacement of dead muscle. Therefore, to minimize post-MI fibrosis should be a major objective of novel therapeutic strategies aiming to treat the infarcted heart. Finally, the viability of any regenerative process in mammals depends on the efficacy of the neovascularization phenomena taking place in the regenerating tissues. To discover how to stabilize the primitive vascular structures that irrigate de novo formed cardiac tissues remains a scientific challenge for the field of cardiovascular regenerative medicine.

Experimental perspectives on cardiac response after damage: choosing an animal model

As it can be inferred from the previous discussion, the development of new, viable cell-based therapies to treat the infarcted heart must be grounded on extensive research using animal experimental models. Our laboratory, following a counterintuitive rationale, has been studying cardiac reparative and regenerative mechanisms in avian embryos. Our preliminary results show that the activation of cellular and molecular mechanisms related to both tissue repair (e.g., fibrosis) and regeneration (e.g., proliferation and differentiation of cardiomyocytes) are fully active in the embryonic heart. The choice of the mouse model to study adult cardiac responses to myocardial death is evident, but research on the embryonic developmental basis of mammalian cardiac repair and regeneration is challenging for various reasons. While mouse embryo genetic manipulation allows for the detailed study of cell lineage fate and the molecular control of cardiac morphogenesis, in vivo microsurgical manipulation and reincubation of the mammalian embryo does not seem an option. It is known that, in mammals, the loss of embryonic cardiomyocytes is quickly compensated by the proliferation of adjacent cardiomyocytes [63, 64]. This response, which might be reminiscent of standard regenerative processes, should be rather interpreted as the result of a compensatory growth of the developing tissue (Fig. 2). Given the experimental limitations of performing research with mammalian embryos, we would like to suggest that research using the avian embryo perfectly complements the work with mammalian ones, as *in ovo* microsurgical manipulation of the avian embryos, is an efficient experimental tool to produce experimental damage in avian embryo tissues that can then be combined with different methods to trace cell fate and differentiation (Fig. 3) [65].

The promise of the new advanced therapies to repair the damaged heart

The development of efficient cell-based therapies is the main objective of clinical laboratories performing research

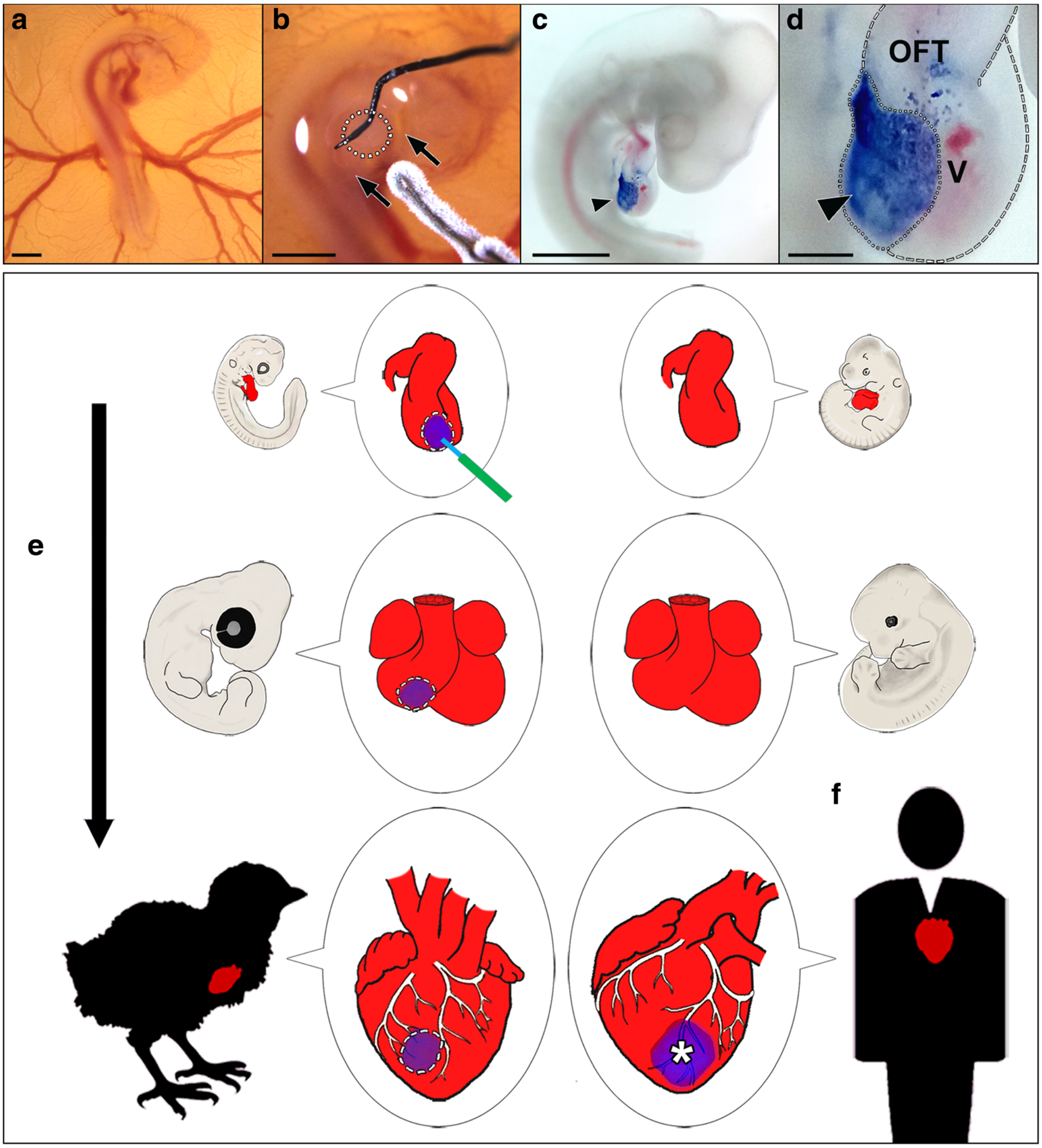

Fig. 3 The avian embryo as a model for the study of cardiac repair and regeneration. *In ovo* avian embryonic cardiac cryodamage (**a**, **b**) leads to cell death in a concrete region of the ventricle (trypan blue$^+$ cells, **c**, **d**). This procedure is used in avian embryos (**e**) to study embryonic and postnatal heart responses to damage. The avian model shows parallels with cardiac human development and responses to injury (**f**). Comparing results from these two species may be useful to study cardiac responses to myocardial death

on cardiac regeneration. Cell therapy products are considered drugs by the European Union since 2003 (medicinal products were introduced in the European legislation through Directive 2003/63/EC); in 2007, cell therapy, gene therapy, and tissue engineering products were re-defined as advanced therapy medicinal products by the Regulation (EC) No.1394/2007 [66]. The whole strategy of cell-based therapies is, in short, to substitute dead or damaged cells by new functional ones. In the case of the heart, the identification of an appropriate cell type to promote efficient and extensive heart muscle regeneration is still required.

In this context, the use of stem cells seemed to be the best option, as their developmental pluri/multipotency was expected to lead to significant cardiomyocyte differentiation. However, although pluripotent and multipotent stem cells have been shown to be able to differentiate into cardiomyocytes in vitro [67, 68], their ability to differentiate into cardiomyocytes in vivo (as well as their effective electrical coupling to the host tissue) has not been proven to be successful as yet. Bone marrow mesenchymal stem cells (MSCs) have also been used in experimental cell-based therapies because they show multipotency and are suitable for autologous transplantations. MSC may also be thought to promote cardiac regeneration through paracrine-like interactions [69]. Furthermore, MSCs have some immunomodulatory properties and display no significant genetic alterations during their early in vitro expansion (e.g., chromosomal abnormalities) [66, 70]. Unfortunately, the results from clinical trials using MSCs have shown that efficient cardiomyocyte differentiation from these cells does not occur in vivo [71].

Pluripotent stem cells (PSCs) are another alternative cell source for cardiac regeneration therapies. Embryonic and induced stem cells (ESC and iPS) are prototypical PSC examples. PSC can differentiate into cell derivatives from the three blastoderm layers cells (ectoderm, mesoderm and endoderm), are clonogenic, and display sustained self-renewal capacity [72], but the clinical use of these cells has many drawbacks: PSC autologous transplantation is only possible in the case of iPS, whose generation in the laboratory remains relatively expensive, and the risk of developing teratomas remains a fundamental disadvantage of PSC use as a medicinal product [73, 74].

In addition, in vivo reprogramming of resident non-cardiomyocytes cells like cardiac fibroblasts [75] could be an alternative treatment of the infarcted heart, offering several advantages over iPSC transplantation. The prevention of pluripotency reversion after cell grafting, the swiftness and simplicity of the process, and the low risk of contamination with immature cells are the main advantages of in vivo reprogramming methods compared with iPSC transplantation procedures [76]. Although in vitro reprogramming of human fibroblasts to cardiac-like myocytes is possible [77], the low reprogramming efficiency of this process is the main problem of using in vivo direct cardiac reprogramming as a clinical therapy.

In a similar way, some researchers have proposed the use of resident cardiac stem cells (c-Kit$^+$) [78] and cardiosphere-derived cells [79] for cell-based cardiac therapies as a plausible alternative to PSC. Both cell types would be suitable for autologous cell transplantation, but cardiomyocyte differentiation from these cells is still suboptimal for their future possible use in the clinics.

Finally, some other authors have suggested to combine cell-based therapies with microRNA and anti-microRNA to promote in vivo cardiac regeneration [80]. As a matter of fact, some experiments demonstrate that the overexpression of some of these factors, like miR-15, induces an increase in cardiomyocyte proliferation in damaged hearts [81]. Since an increase of cardiomyocyte proliferation rate is the main response to damage in the regenerating zebrafish [17] and neonatal mouse [8] hearts, the ability to control cardiomyocyte cell cycle entry in the adult mammalian heart would represent a powerful therapy to treat myocardial infarction consequences. In this way, there are some recent studies that seek the in vivo stimulation of adult cardiomyocyte proliferation to regenerate the infarcted heart [82]. Although these treatments decrease the infarcted wound size and improve cardiac function after myocardial infarction, full ventricular regeneration is not achieved. In addition, the potential of ectopic proliferation calls for caution in any tissue with strong mitotic properties, as it could lead to the generation of tumors.

Conclusions

Lessons from animal models (both during embryonic development and adult life) suggest that additional efforts must be done to study: (1) the regulation of cardiomyocyte proliferation. (2) CSC-dependent mammalian heart endogenous regenerative potential, and (3) reparative fibrotic events. All these tasks require initiating systematic research on the study of the cellular and molecular mechanisms that control cardiac regeneration in a major number of animal species, avoiding the assumption that phylogenetically close animal taxa have the same ability to regenerate tissues and organs. In order to appropriately tackle this objective, it is necessary to choose a proper preclinical animal model to then evaluate the significance of these studies in the human context (Fig. 3). In summary, extensive basic research and preclinical studies are to be accomplished before cells can be successfully used as a medicinal product to regenerate the infarcted heart.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

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