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The mouse as a model system to study cardiac regeneration

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Abstract

Cardiomyocytes exhibit robust proliferative activity during development. After birth, cardiomyocyte proliferation is markedly reduced. Consequently, regenerative growth in the postnatal heart via cardiomyocyte proliferation (including, by inference, via proliferation of stem cell-derived cardiomyocytes) is limited and often insufficient to effect repair following injury. Here we review methodologies which employ the mouse as a model system to study cardiac regeneration, and in particular cardiomyocyte replenishment, in health and disease.

Introduction

Although new medical therapies can reduce the progression to chronic heart failure in diseased hearts, once lost, replenishment of functional cardiomyocytes is limited [1,2]. As a consequence, damaged myocardial tissue is replaced by non-contractile fibrous scar tissue. This leads to adverse remodeling, diminished cardiac function and electrophysiological instability [3]. The resulting strain on the heart can trigger additional cardiomyocyte loss via necrosis and apoptosis, which triggers additional fibrosis which may ultimately progress to regional and global systolic dysfunction with a poor prognosis for the patient [4]. Therefore, regenerative strategies designed to augment myocardial tissue are warranted. These include strategies to induce cardiomyocyte proliferation, strategies to mobilize resident cardiomyogenic stem cells, and strategies to transplant cardiomyocytes or cardiomyogenic stem cells. The mouse has several technical advantages as a model system to study regenerative cardiac growth. Studies exploiting these attributes are reviewed below.

In Vitro Models

In vitro studies of regenerative cardiac growth can entail a number of experimental endpoints. For example, monitoring cell cycle activity in response to cytokines, growth factors or genetic manipulations in primary fetal or neonatal cardiomyocyte cultures is often used as a primary screen for interventions to enhance regenerative cardiac growth *in vivo* [5]. The general approach is useful, but makes the assumption that adult, terminally differentiated cardiomyocytes will exhibit the same response to the intervention as fetal or neonatal cells. Unfortunately, culture of adult cardiomyocytes from any species is difficult, making it problematic to experimentally address this important caveat. The use of mice for primary cardiomyocyte studies is not particularly advantageous compared to the use of other species; indeed, neonatal mouse cardiomyocytes are more difficult to culture than neonatal

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Conflict of Interest

None.

rat cardiomyocytes. Moreover, the cardiomyocyte yield per heart is markedly reduced in mouse vs. rat.

Assessing cardiomyogenic stem cell activity constitutes another *in vitro* experimental endpoint to screen for regenerative cardiac growth. The mouse is particularly well suited for such experiments, as numerous genetically modified lines harboring lineage-restricted reporters that facilitate isolation of progenitor populations or identification of differentiation events are available. For example, lineage tracing experiments demonstrated that the LIM-homeodomain transcription factor Islet-1 (*Isl1*) is expressed in progenitors which give rise to cardiomyocytes populating atria and the right ventricle [6]. A variation of this lineage tracing system was employed to isolate the progenitors via fluorescence activated cell sorting [7]. The *Isl1* progenitors could be amplified in an undifferentiated state when cultured on a cardiac mesenchymal feeder layer. Moreover, when co-cultured with fetal cardiomyocytes, the *Isl1* progenitors differentiated into cardiomyocytes. Thus, mice harboring lineage restricted reporters can be used to isolate progenitors for *in vitro* cardiomyogenesis studies.

Lineage reporters have also been utilized to monitor cardiomyogenic differentiation of putative progenitors *in vitro*. For example, bone marrow-derived stem cells from transgenic mice with a cardiomyocyte restricted beta-galactosidase reporter were tested for their ability to undergo cardiomyogenic differentiation when co-cultured with spontaneously differentiating embryonic stem (ES) cells [8]. The absence of reporter transgene expression suggested that bone marrow-derived stem cells lacked the ability to form cardiomyocytes, even when placed in a *bona fide* cardiomyogenic milieu.

Experiments with ES cells constitute perhaps the best utilization of the mouse as a model system to screen for cardiac regeneration *in vitro*. ES cells are derived from the inner cell mass of pre-implantation embryos. When cultured transiently in suspension under appropriate conditions, they form aggregates known as embryoid bodies (EBs) which are able to spontaneously differentiate *in vitro* into derivatives of all three germ layers, including cardiomyocytes. Early studies established that cells with attributes of atrial, ventricular and conduction system cardiomyocytes were present in spontaneously differentiating cultures [9,10], and that the period of sustained cardiomyocyte reduplication in the ES system was temporally similar to that observed during *in vivo* development [11].

The ability of ES cells to produce *bona fide* cardiomyocytes *in vitro* has facilitated numerous studies related to cardiac regeneration. For example, transgenes using lineage-restricted promoters to drive expression of cell cycle regulatory genes markedly enhanced cardiomyocyte proliferation in spontaneously differentiating ES cultures [12]. Manipulation of cell media constituents can also enhance cardiomyocyte proliferation in ES-derived cultures [13]. ES cultures have also been used to identify factors which promote cardiomyogenic differentiation. A striking finding was that co-culture of mouse EBs and chick pre-cardiac endoderm/mesoderm markedly enhanced cardiomyogenic differentiation [14]. The use of ES cells as a high throughput platform to screen for natural and synthetic factors which induce cardiomyogenic differentiation has recently been reviewed [15].

ES cells have also been used to isolate and characterize cardiomyogenic precursors. For example, a precursor population identified by transient expression of Flk-1 (the Vascular Endothelial Growth Factor receptor), gave rise predominantly to cardiomyocytes, vascular smooth muscle cells, and vascular endothelial cells when cultured [16]. Similar multipotent cardiac precursor populations (each showing the ability to give rise to multiple cardiac cell lineages) were isolated from differentiating ES cultures based on expression of *Isl-1* [17] or *Nkx2.5* [18] reporter transgenes.

One difficulty encountered with the use of ES-derived cardiomyocytes is the relatively low content of cardiomyocytes in spontaneously differentiating cultures. Several of the strategies employed above markedly enhanced cardiomyocyte yield, although they would be difficult to use for large scale production of cells. An alternative approach is to utilize lineage restricted promoters to drive expression of gene products which permit isolation of the desired cell type (Figure 1). In one example, ES cells were transfected with a transgene which used the cardiac myosin heavy chain promoter to drive expression of a gene encoding resistance to the antibiotic neomycin. Following spontaneous differentiation, the cultures were treated with a neomycin analogue. Cardiomyocytes survived drug treatment because they expressed the transgene, while non-cardiomyocytes died. This strategy resulted in cardiomyocyte cultures which were greater than 99% pure [19]. The selected cardiomyocytes were able to engraft the myocardium following transplantation into adult hearts. Other studies utilized a transgene which employed a cardiac restricted promoter to target expression of enhanced green fluorescent protein (EGFP). Fluorescence activated cell sorting of spontaneously differentiating ES cultures carrying this transgene resulted in a similar degree of cardiomyocyte purity [20].

The drug selection protocol has proven to be particularly useful for large scale production of ES-derived cardiomyocytes. In one example, a stirred bioreactor was seeded with a single cell suspension of undifferentiated ES cells [21]. Stirring conditions were optimized to generate high density cultures of differentiating EBs. After 9 days of differentiation, drug selection was initiated and the cells were cultured for an additional 9 days. The protocol resulted in the generation of essentially pure cardiomyocyte cultures, with a total yield of greater than 10^9 from a single 2 L bioreactor run.

ES cells have traditionally been derived from pre-implantation embryos. More recently, it has been demonstrated that cells with characteristics of ES cells can be derived via genetic manipulation of differentiated adult cells. For example, retroviral transfer of four genes which are typically expressed in ES cells resulted in the reprogramming of mouse fibroblasts into cells which were able to contribute to the germ line following injection into blastocysts [22]. When differentiated *in vitro*, these induced pluripotent stem (iPS) cells gave rise to cardiomyocytes with molecular, structural, and functional attributes which were very similar to those for ES cell-derived cardiomyocytes [23]. iPS cells thus provide the potential to generate autologous donor cells for transplantation-based cardiac regeneration studies.

In Vivo Models

Given the ease of genetic manipulation, the mouse is a particularly useful model system with which to study cardiac regeneration *in vivo*. Experimental endpoints for regenerative growth include monitoring cardiomyocyte proliferation and monitoring *de novo* cardiomyocyte formation. The use of reporter transgenes can greatly facilitate these analyses.

A priori, assessment of cardiomyocyte proliferation should be a straightforward task. Many studies have relied on immune histologic approaches to score the co-localization of markers for cell cycle progression (i.e., tritiated thymidine incorporation, bromodeoxyuridine incorporation, Ki67, PCNA, etc) and markers for cardiomyocyte identification (i.e., structural proteins like cardiac troponin or myosin heavy chain). Given their size, cardiomyocytes comprise more than 90% of the mass of the adult heart. However, they represent less than 20% of the total number of cells. Consequently, non-myocyte nuclei vastly outnumber cardiomyocyte nuclei. In any given histologic section, non-myocyte nuclei often overlie cardiomyocyte cell bodies. Given this, assays which rely simply on co-localization of a nuclear and cytoplasmic signal (i.e., anti-Ki67 and anti-myosin immune reactivity) are subject to considerable counting error [24]. Counting error in diseased hearts

is further increased, as the ratio of non-myocytes to cardiomyocytes is often dramatically increased.

Genetically modified mouse lines have been developed to eliminate potential subjectivity in the identification of cardiomyocyte nuclei. One example utilizes the cardiomyocyte-restricted myosin heavy chain promoter to drive expression of a nuclear localized beta-galactosidase reporter (MHC-nLAC mice, see Figure 2A). Virtually all cardiomyocytes in these animals exhibit nuclear beta-galactosidase activity, which can be detected in histologic sections by reacting with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactosidase (X-GAL, Figure 2B). Non-cardiomyocytes do not express the transgene and thus their nuclei do not stain with X-GAL.

Given the specificity of the transgene reporter, cardiomyocyte cell cycle activity in MHC-nLAC mice can be scored non-subjectively simply by examining co-localization of X-GAL signal and a *bona fide* proliferation marker. For example, the animals can be injected with tritiated thymidine. Proliferating cells will incorporate the radioisotope in their replicating DNA, and can be subsequently identified in histologic sections via simple autoradiography (that is, silver grains are detected over the nuclei of cells which were replicating during tritiated thymidine injection). The presence of silver grains over an X-GAL positive nucleus is indicative of a proliferating cardiomyocyte (Figure 2C). When using this approach, the cardiomyocyte renewal rate in uninjured adult mouse hearts was observed to be 0.0005% following a single injection of tritiated thymidine and a 4 hour chase period [25]. The cardiomyocyte renewal rate with this assay increased to 0.008% at the border zone in infarcted mouse hearts. This assay does not distinguish between cell cycle induction in pre-existing cardiomyocytes vs. proliferation of nascent stem-cell derived cardiomyocytes.

Reporter transgene systems have also been developed to specifically monitor cardiomyocyte renewal from stem cells. This genetic fate map strategy utilizes a sophisticated binary reporter system which is subject to both spatial and temporal regulation. The first transgene is designated Z/EG [26]; the Z/EG transgene uses the beta-actin promoter to target ubiquitous expression of a conditional reporter (Figure 3). The reporter consists of a cytoplasmic beta-galactosidase flanked by two lox-P recombination sites, followed by sequences encoding EGFP. In the absence of cre recombinase (which catalyzes recombination between lox-P sites) the Z/EG transgene targets expression of beta-galactosidase. In the presence of cre recombinase, the beta-galactosidase sequences are eliminated and the Z/EG transgene will encode EGFP. The second transgene used a cell lineage-restricted promoter to target expression of cre recombinase (with either constitutive or conditional activity).

This system was recently used to monitor cardiomyocyte renewal in normal and injured adult mouse hearts [27]. Z/EG mice were intercrossed with MerCreMer mice, which carry a transgene that utilizes the cardiac-restricted myosin heavy chain promoter to target expression of a tamoxifen-inducible Cre recombinase fusion protein. In the absence of tamoxifen, no cre activity is present in MerCreMer mice. However, in the presence of tamoxifen, cardiomyocyte restricted cre activity is induced (Figure 3). Adult MerCreMer-Z/EG double transgenic mice were treated with tamoxifen, which promoted cre-mediated recombination of the Z/EG reporter in approximately 80% of the cardiomyocytes (thus, 80% of the cardiomyocytes exhibited EGFP fluorescence due to recombination of the Z/EG reporter, while the remaining 20% exhibited beta-galactosidase activity as the Z/EG reporter was not recombined). The percentage of EGFP and beta-galactosidase expressing cardiomyocytes did not change during a 12 month follow-up period. Since the MerCreMer transgene would not be transcriptionally active in progenitor cells during the tamoxifen treatment, the absence of an increase in beta-galactosidase expressing cardiomyocytes

suggested that there was no stem cell-derived myocardial renewal during the aging process (within the limits of the assay's sensitivity).

Tamoxifen-treated MerCreMer-Z/EG double transgenic mice were then subjected to myocardial injury. Myocardial infarction and pressure overload both resulted in an increase in the number of beta-galactosidase expressing cardiomyocytes, and a concomitant reduction in the number of EGFP expressing cardiomyocytes (roughly a 15% change 3 months post-injury). These results suggest that stem-cell derived cardiomyocytes contribute to myocardial renewal following injury. Although this assay is quite powerful, the incomplete penetrance of cre-mediated Z/EG reporter transgene recombination warrants some caution in interpreting the results. Even very slight differences in viability or cell cycle activity in EGFP vs. beta-galactosidase expressing cardiomyocytes could impact the cell type ratio over a 3 month period. This caveat notwithstanding, this study supports the presence of post-injury myocardial renewal.

Reporter transgenes have also been employed to reveal the presence of cell fusion events in cardiac regeneration studies. For example, marrow reconstitution studies were performed using donor hematopoietic stem cells from mice carrying a ubiquitously active cre transgene and recipient mice carrying a ubiquitous cre-dependent beta-galactosidase reporter. The presence of cre-mediated activation of the beta-galactosidase in cardiomyocytes was indicative of fusion with marrow-derived cells [28] In another study, skeletal myoblasts carrying a ubiquitously expressed EGFP reporter were engrafted into the myocardium of mice carrying the MHC-nLAC reporter transgene described above. The presence of myocytes with a green cytoplasm and nuclear beta-galactosidase activity was indicative of myoblast/cardiomyocyte fusion events [29].

Model Comparison

The mouse enjoyed an early advantage as a model system for *in vitro* studies of cardiac regeneration. This stemmed in part from the fact that mouse was the first species from which pluripotent ES cells were derived. This advantage has now been eclipsed somewhat by the ability to generate ES cells (and perhaps more importantly, iPS cells) with cardiomyogenic activity from a number of species. Although the use of the mouse for primary cardiomyocyte culture studies suffers from low myocyte yield, use of this species is advantageous from the standpoint that many genetically modified lines are available which can be used to facilitate progenitor isolation, enhance cardiomyogenic yield, and/or monitor cardiomyogenic differentiation. Importantly, many *in vitro* protocols impacting cardiac regeneration which were developed in mouse have been readily transferred to other species. The mouse retains a marked advantage for *in vivo* studies of cardiac regeneration. This is due in part to the availability of powerful reporter models which can faithfully track myocardial renewal. In addition, the relative ease of manipulation of the mouse genome facilitates studies to identify genetic pathways which are able to effect regenerative cardiac growth. No other mammalian species permits such analyses.

Model Translation to Humans

The mouse models for cardiac regeneration described above translate well to humans. For example, methods which relied on transient expression of the Flk-1 receptor to isolate multipotent mouse ES-derived cardiac progenitors [16] were readily translated to human ES cells [30]. Cardiomyocytes, vascular smooth muscle cells, and endothelial cells from human ES-derived progenitors readily engrafted following transplantation into hearts of immune compromised mice. The mouse ES-derived cardiomyocyte selection protocol [19] was also readily translated to human cells [31]. Cultures comprised of more than 99% cardiomyocytes were routinely generated; importantly these highly purified cell populations

did not induce teratomas following transplantation into immune compromised hosts. The enhanced cardiomyogenic differentiation observed in co-cultures of mouse ES cells and pre-cardiac endoderm/mesoderm cells [14] was largely recapitulated in co-cultures of human ES cells and visceral endoderm-like cells [32]. Moreover, treatment of human ES cells with activin A (a major growth factor elaborated by pre-cardiac endoderm/mesoderm) and BMP4 resulted in a cardiomyocyte content of approximately 30% [33]. Finally, genetic manipulation which resulted in reprogramming of mouse fibroblasts to ES-like cells with cardiomyogenic activity [22,23] can also be employed to produce human iPS cells with cardiomyogenic potential [34]. Thus, most of the *in vitro* mouse models for cardiac regeneration are transferable to humans.

It is also possible to ascertain how faithfully one of the *in vivo* mouse models for cardiac regeneration translates to humans. It has recently been reported that in the human myocardium, there is an annual cardiomyocyte turnover of 1% at the age of 20, and that this rate decreases to 0.3% at the age of 75 [35]. These calculations were based on quantitating the level of ^{14}C (radiocarbon) integration into DNA in individuals who were alive during nuclear bomb tests during the Cold War. One can compare these results to normalized data obtained with acute tritiated thymidine incorporation in MHC-nLAC mice described above [25], where 0.0005% of the cardiomyocytes exhibited DNA synthesis during a 4 hour tritiated thymidine labeling period. If normalized to a 24 hour labeling period (x6) and projected for the duration of a year (x365), the predicted annual cardiomyocyte turnover in adult mice is 1.095%, a value which is remarkably close to that obtained in the human studies.

Conclusions

The studies reviewed here demonstrate that the mouse is a viable model to study cardiac regeneration. *In vitro* studies are facilitated by the availability of numerous genetic models which can be used to generate cells for primary culture experiments. The mouse has a major advantage for *in vivo* studies as the availability of reporter models can greatly facilitate quantitation of cardiac regeneration. Given the observation that at least some of these reporter systems translate well to human cardiac regeneration, coupled with ease at which the mouse genome can be experimentally modified, makes this the ideal species with which to model cardiac regeneration *in vivo*.

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Figure 1. Promoter based selection of ES cell derived cells

(A) DNA construct carrying a selectable gene under control of a tissue or lineage specific promoter. (B) Scheme for selection of lineage specific cell lines from ES cells: ES cells stably transfected with the aforementioned construct are allowed to differentiate. The desired lineage specific cell population could be selected based on the presence of the selectable gene.

Figure 2. Quantification of cardiomyocytes DNA synthesis

(A) MHC-nLac mice carry the β -galactosidase gene under control of the cardiomyocyte specific α -MHC promoter enabling the detection of CM nuclei. (B) Low-power view of a heart section from a MHC-nLac transgenic mouse. The section was stained with the chromogenic substrate X-GAL, which reveals a blue signal in >99.5% of all cardiomyocytes of the heart. (C) High-power photomicrograph of a histologic heart section derived from a double transgenic MHC-nLac x MHC-CyclinD2 mouse. Prior to sacrificing, the mouse received a single injection of tritiated thymidine, which is incorporated into replicating DNA. Thereafter, the hearts were processed for beta-galactosidase activity and autoradiography. Shown is an example (arrow, silver grains) of DNA synthesis in a cardiomyocyte (blue nucleus).

Figure 3. Genetic lineage tracing based on the Z/EG switch reporter

Z/EG mice (upper left) express the β -galactosidase gene driven by a constitutive β -Actin promoter, marking all cells blue after X-Gal staining. Before recombination, expression of GFP is prevented by a stop codon after the nLac transcript flanked by two flox sides. In MerCreMer mice (lower left) conditional activation of the Cre-recombinase driven by the cardiac specific α -MHC promoter can be induced by tamoxifen treatment. By crossing these two mice (MerCreMer-Z/EG mice), conditional recombination results in the loss of the nLac transcript including the stop codon between the two flox sides resulting in GFP expression specifically in cardiomyocytes. In principle, this approach could be transferred to track the fate of any cell population of interest by replacing the α -MHC promoter with a lineage specific promoter and combining the Z/EG “switch reporter”.