Biochimica et Biophysica Acta 1833 (2013) 799–803

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Challenges measuring cardiomyocyte renewal ☆

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ARTICLE INFO

Article history:
Received 14 September 2012
Received in revised form 25 October 2012
Accepted 28 October 2012
Available online 7 November 2012

Keywords:
Cardiac regeneration
Cardiomyogenesis
Cardiomyocyte proliferation

ABSTRACT

Interventions to effect therapeutic cardiomyocyte renewal have received considerable interest of late. Such interventions, if successful, could give rise to myocardial regeneration in diseased hearts. Regenerative interventions fall into two broad categories, namely approaches based on promoting renewal of pre-existing cardiomyocytes and approaches based on cardiomyogenic stem cell activity. The latter category can be further subdivided into approaches promoting differentiation of endogenous cardiomyogenic stem cells, approaches wherein cardiomyogenic stem cells are harvested, amplified or enriched *ex vivo*, and subsequently engrafted into the heart, and approaches wherein an exogenous stem cell is induced to differentiate *in vitro*, and the resulting cardiomyocytes are engrafted into the heart. There is disagreement in the literature regarding the degree to which cardiomyocyte renewal occurs in the normal and injured heart, the mechanism(s) by which this occurs, and the degree to which therapeutic interventions can enhance regenerative growth. This review discusses several caveats which are encountered when attempting to measure cardiomyocyte renewal *in vivo* which likely contribute, at least in part, to the disagreement regarding the levels at which this occurs in normal, injured and treated hearts. This article is part of a Special Issue entitled: Cardiomyocyte biology: Cardiac pathways of differentiation, metabolism and contraction.

The regenerative potential of the adult myocardium in cold-blooded animals is well established. Indeed, the initial observation suggesting regenerative growth following injury, in frogs, was reported in 1875 [1]. A substantive body of work, largely by the Rumyantsev (summarized in [2]) and Oberpriller [3–7] laboratories in the 1970s–1980s, further characterized this phenomenon, providing evidence that cardiomyocyte proliferation was a major component of the phenomenon. More recent work established a similar regenerative phenotype in zebrafish [8], a particularly important observation given the suitability of that species for mutational screens. Importantly, the magnitude of cardiomyocyte renewal in lower invertebrates and reptiles is sufficiently high so as to be readily detected by simple histologic analysis at various time points post-injury.

More recent studies examining potential therapeutic interventions to promote cardiac regeneration speak of challenging "the old dogma" that the adult mammalian heart lacks regenerative potential. In fact, the notion of ongoing cardiomyocyte renewal in the mammalian heart is not a new idea. Many studies have examined cardiomyocyte cell cycle activity in normal and injured adult hearts, and the presence of ongoing cardiomyocyte renewal has been long accepted. Rather, the debate in the literature resides in the magnitude at which this occurs. For example, in the uninjured adult rat heart, values for ventricular cardiomyocyte cell cycle activity (which is often used as a surrogate marker for cardiomyocyte renewal) varied from 0 to 3.15% (reviewed in [9]). This variation likely reflects a number of factors, including the duration of the cell cycle marker being scored. For example, the duration of S-phase is much greater than that of M-phase, and as such assays monitoring cardiomyocyte DNA synthesis (i.e., tritiated thymidine or bromodeoxyuridine incorporation) would by default give rise to a proportionally higher renewal rate than assays monitoring the presence of mitotic figures. Other markers (such as Proliferating Cell Nuclear Antigen or Ki67 immune reactivity) which are expressed throughout much of the cell cycle would give rise to even higher renewal rates.

Another factor contributing to the high level of variation reported for baseline cardiomyocyte cell cycle activity is the accuracy with which cardiomyocytes (and in particular cardiomyocyte nuclei) are identified. It has been argued that the use of confocal microscopy, in combination with immune fluorescence visualization of a cytoplasmic marker (as for example, Troponin T) and a cell membrane marker (as for example, wheat germ agglutinin or laminin) can be used to unequivocally identify cardiomyocyte nuclei in tissue sections [10]. In practice, the *z*-axis resolution of confocal microscopy is insufficient to resolve non-cardiomyocyte nuclei in close (i.e., <0.5 microns) proximity to cardiomyocyte cytoplasm [11,12], thus precluding accurate cardiomyocyte nucleus identification in some instances. This is

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http://dx.doi.org/10.1016/j.bbamcr.2012.10.029

an important consideration, since although cardiomyocytes comprise more than 90% of the mass of the heart, they constitute only 20% of the total cell number.

This caveat was nicely illustrated in experiments using a transgenic mouse wherein the cardiomyocyte-restricted alpha myosin heavy chain promoter [13] targeted expression of a nuclear localized beta-galactosidase reporter (the transgene is depicted in Fig. 1A). These mice (designated MHC-nLAC mice [14]), can be used to identify cardiomyocyte nuclei in tissue sections by simple reaction with a chromogenic beta-galactosidase substrate (i.e., X-GAL; Fig. 1B, blue signal) or by beta-galactosidase immune fluorescence (Fig. 1C, green signal). Sections from these hearts were processed for beta-galactosidase (to identify cardiomyocyte nuclei) and Troponin T (to identify cardiomyocyte cytoplasm) immune reactivity, and stained with wheat germ agglutinin (to identify cell membranes) and Hoechst (to all identify nuclei). The sections were then imaged via confocal microscopy using a high numerical aperture objective, and *Z*-axis stacks comprising 42 steps at intervals of 0.24 μm for each imaged volume were generated. Observers were supplied with image volumes containing the Troponin T, wheat germ agglutinin and Hoechst signals, and were asked to identify the origin (i.e., cardiomyocyte or non-cardiomyocyte) of each nucleus. These results were then directly compared to those obtained with beta-galactosidase immune fluorescence within the same tissue volume. Significant error rates were observed [15], underscoring the subjectivity of traditional confocal microscopic analyses for cardiomyocyte nuclear identification.

We have used the MHC-nLAC mice to monitor cardiomyocyte cell cycle activity in normal adult hearts. For these experiments, the mice received a single injection of tritiated thymidine, and were sacrificed four hours later. The hearts were harvested, sectioned, and processed for X-GAL reaction (to identify cardiomyocyte nuclei) and autoradiography (to identify S-phase nuclei). A typical cardiomyocyte in S-phase is shown in Fig. 2A; using this approach only 0.0005% of the cardiomyocyte nuclei in adult hearts were thymidine positive [16]. While this is a relatively low rate of cell cycle activity, it is important to note that it represents only those cardiomyocytes which were synthesizing DNA during the 4 h labeling session. If one assumes a linear relationship, multiplying by 6 would provide a daily cell cycle rate (i.e., 24 h/4 h), and multiplying further by 365 would provide a yearly rate. This would predict a cardiomyocyte renewal rate of 1.09% per year in the uninjured adult mouse heart, assuming that the DNA synthesis events culminated in cytokinesis. This value is remarkably close to the human cardiomyocyte renewal rate calculated by the Frisén laboratory [17]. These later experiments utilized a carbon dating approach that exploited the spike in atmospheric radioactive carbon resulting from numerous above-ground nuclear tests in the 1950s and 1960s, in combination with a cardiomyocyte nuclear-specific marker, and predicted an annual cardiomyocyte renewal rate of 1% per year in young adults.

Monitoring cardiomyocyte DNA synthesis *per se* does not necessarily discriminate between renewal via proliferation of pre-existing cardiomyocytes vs. cardiomyogenic differentiation of stem cells. For example, the human experiment was cumulative in nature, and could detect DNA synthesis events in pre-existing cardiomyocytes as well as in stem cells which subsequently differentiated into cardiomyocytes. In contrast, the labeling period in the mouse experiment was too short to detect *de novo* cardiomyogenic events; it could only detect DNA synthesis events in pre-existing cardiomyocytes. The fact that the normalized mouse data agreed well with the cumulative human data provides circumstantial data suggesting that the primary mechanism

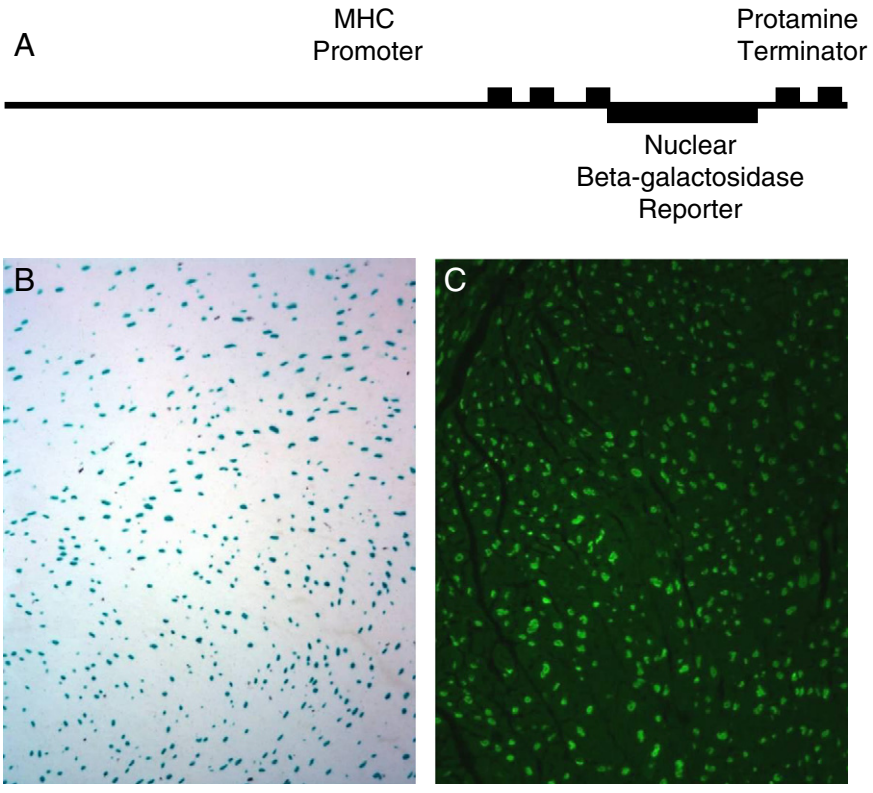

Fig. 1. Detection of cardiomyocyte nuclei in MHC-nLAC transgenic mice. A. Schematic depiction of the MHC-nLAC transgene. The MHC promoter consists of 4500 bp of 5′ flanking sequence plus Exons 1, 2 and the non-coding region of Exon 3. The protamine terminator was inserted down-stream of the beta-galactosidase sequence to ensure proper processing of transgene-encoded transcripts. B. Low power image of a section from an adult MHC-nLAC transgenic heart following reaction with X-GAL, a chromogenic beta-galactosidase substrate. Cardiomyocyte nuclei appear blue. C. Low power image of a section from an adult MHC-nLAC transgenic heart processed for beta-galactosidase immune reactivity. Cardiomyocyte nuclei are identified by green fluorescence.