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Musings on intrinsic cardiomyocyte cell cycle activity and myocardial regeneration

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Abstract

Although the myocardial renewal rate in the adult mammalian heart is quite low, recent studies have identified genetic variants which can impact the degree of cardiomyocyte cell cycle reentry. Here we use the compound interest law to model the level of regenerative growth over time in mice exhibiting different rates of cardiomyocyte cell cycle reentry following myocardial injury. The modeling suggests that the limited ability of S-phase adult cardiomyocytes to progress through cytokinesis, rather than the ability to reenter the cell cycle per se, is a major contributor to the low levels of intrinsic regenerative growth in the adult myocardium.

Keywords

Cardiomyocyte renewal; growth modeling; cardiac regeneration

Perspective:

The rate of cardiomyocyte proliferation is dramatically reduced after birth in animal models [1-5] and in humans [6, 7]. Studies examining tritiated thymidine incorporation in mice [8] and atmospheric radioactive carbon incorporation in humans [6] revealed low but surprisingly similar levels of cardiomyocyte cell cycle reentry in young adult hearts in the absence of injury (calculated to be approximately 1% per year). Although myocardial injury is associated with a localized increase in cardiomyocyte S-phase activity [1, 8-11] the rate of cell cycle induction is modest as compared to the non-cardiomyocyte population. Thus,

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Disclosures

None.

cardiomyocyte loss in postnatal hearts frequently results in fibrotic repair (as opposed to renewal of the damaged myocardium), with a concomitant reduction in cardiac function.

Our group has a long-standing interest in quantitating cardiomyocyte cell cycle induction using modified nucleotides to detect S-phase activity. Over the years we have used immune cytology or morphological criteria in dispersed cell preparations, immune histology in tissues sections, and histologic analysis of genetically modified mice with cardiomyocyte-restricted cytoplasmic or nuclear reporters, to identify cardiomyocyte-specific cell cycle events. Most of our studies utilized mice in an inbred DBA/2J (D2J) background; adult D2J mice consistently exhibit very low levels of cardiomyocyte S-phase activity under baseline conditions, with an induction following injury [4, 8, 12-21].

Naturally occurring genetic variants can impact cardiomyocyte cell cycle activity. For example, approximately 50% of the commonly used inbred mouse strains harbor a SNP variant at nucleotide 154,875,123 in the troponin I-interacting kinase (*Tnni3k*) gene. This variant, which is present in D2J mice, results in aberrant splicing, which in turn introduces a frame-shift mutation, premature translation termination, and nonsense-mediated decay of *Tnni3k* transcripts [22]. Although the precise physiologic role of TNNI3K in cardiac homeostasis remains elusive, its expression is associated with adverse remodeling under conditions of cardiac stress [22-24]. We have recently shown that mice expressing TNNI3K, as for example C57Bl6N (B6N) mice, have elevated levels of cardiomyocyte S-phase activity in the remote myocardium and in the infarct border zone following permanent coronary artery ligation, as compared to mice lacking TNNI3K expression, as for example D2J mice; interestingly cardiomyocyte S-phase levels within the infarct zone itself were not statistically different between the two strains [25]. There is some debate as to the degree to which the presence or absence of TNNI3K expression impacts further cell cycle progression in adult hearts [25, 26]. Regardless of that debate, the surprisingly high levels of post-injury cardiomyocyte cell cycle reentry observed in mice with normal TNNI3K expression has prompted us to model the potential impact that intrinsic cardiomyocyte cell cycle activity might have on cardiac structure and function following injury.

The first step of this modeling entails consideration of regional cardiomyocyte nuclear number following injury. Towards that end we have used transgenic mice which express a nuclear-localized β-galactosidase (β-GAL) reporter exclusively in cardiomyocytes (MHC-nLAC mice) [12]. Cardiomyocyte nuclei in heart sections prepared from MHC-nLAC mice are readily identified and quantitated based on the presence of β-GAL immune reactivity (Figure 1A) as has been described previously [4, 25, 27, 28]. We retrospectively analyzed a data set prepared from 4 adult MHC-nLAC mice which were subjected to permanent coronary artery ligation (an injury model commonly used in studies examining cardiac regeneration) and implantation of a 14-day osmotic pump containing Bromodeoxyuridine (BrdU). The hearts were harvested two weeks later, a time point when cardiomyocyte cell loss has subsided and the resolution of inflammation, scar formation, and neovascularization are completed [29]. The hearts were sectioned, and the sections were then processed for β-GAL immune reactivity to identify cardiomyocyte nuclei, BrdU immune reactivity to identify S-phase activity and Hoechst staining to identify all nuclei. Figure 1B shows a color-combined image of the border zone from a coronal section of one of the hearts;

the transition from viable myocardium to scar/granulation tissue is easily identified by the precipitous reduction in nuclear β-GAL immune reactivity (which appears violet in the color-combined image due to the overlay of red anti-β-GAL and blue Hoechst fluorescence).

To parse the heart into different zones post-injury, a line was drawn defining the position of the transition from healthy to infarcted myocardium on the color-combined image (see the white lines in Figure 1C). The border zone was then defined as the band comprising an area 500 microns on either side of the white lines (i.e., the tissue within the white tracing, Figure 1D). The infarct was defined as the region apical to the border zone (which is largely devoid of cardiomyocytes) while the remote myocardium was defined as the remaining ventricular tissue (a region containing the left and right ventricular walls and the septum, and which is comprised largely of viable cardiomyocytes; the atria were excluded from the analyses). In the section shown, the border zone contained 454 cardiomyocyte nuclei, the infarct contained 178 cardiomyocyte nuclei, and the remote myocardium contained 8,519 cardiomyocyte nuclei. To determine an average value for the number of cardiomyocyte nuclei surviving in the border zone and infarct, the data set was screened to identify sections containing 8,519 ± 500 cardiomyocyte nuclei in the remote myocardium zone (i.e., sections which were anatomically similar to the example shown in Figure 2C). Sixteen sections were identified, yielding an average cardiomyocyte nuclear number of 599 for the border zone, 184 for the scar, and 8,489 for the remote myocardium.

To estimate the number of cardiomyocyte nuclei surviving in each region following infarction, a coronal section from a non-infarcted adult MHC-nLAC heart sampled at a comparable anatomic plane to those studied from the infarcted animals was analyzed. The section was processed as described above, and had a total of 14,410 cardiomyocyte nuclei. Next, a 1,000-micron wide band was defined (Figure 1E and F) in an analogous anatomical location to the border zone band in the infarcted heart and then finely repositioned such that the cardiomyocyte nuclear number in the area corresponding to the remote myocardium in the non-infarcted heart and that in the infarcted hearts were very similar (8,499 vs. 8,489 cardiomyocyte nuclei, respectively). The region of the non-infarcted heart corresponding to the border zone contained 1,242 cardiomyocyte nuclei and the region corresponding to the infarct contained 4,669 cardiomyocyte nuclei. Regional cardiomyocyte survival was estimated by dividing the regional cardiomyocyte nuclear number in the infarcted hearts by that in corresponding regions in the non-infarcted heart. This analysis indicated that on average 48% (i.e., 599/1,242) of the border zone myocardium and 4% (i.e., 184/4669) of the infarct myocardium survived 2 weeks following permanent coronary artery ligation.

The second step of this modeling entails consideration of the extent of regional myocardial regeneration. Towards that end we employed the compound interest law, which is typically used by financial institutions to calculate compounded interest over time. Blackman provided one of the earliest descriptions of the use of this law to model growth in multicellular organisms [30]. We have employed this law to model the increase in cardiomyocyte nuclei over time as a surrogate readout for myocardial regeneration. Cardiomyocyte S-phase activity was used to estimate the increase in nuclear number, and in our initial hypothetical models it was assumed that each S-phase cardiomyocyte nucleus

would divide within 24 hours to give rise to two daughter nuclei. The law assumes that growth is compounded every nuclear division and is given by:

$$[1 + \text{nCMN}\,\% \ / \ 100]^{n}$$

where nCMN is the increase in cardiomyocyte nuclei per day (in percent) and n the number of days of observation.

We used the law to predict the level of regional myocardial regeneration over time in (B6NxD2J)-F1 mice (which carry one wildtype *Tnni3k* allele and express the protein) to that in D2J mice (which carry two mutant *Tnni3k* alleles and do not express the protein). We have previously quantitated the cumulative regional cardiomyocyte S-phase levels in these mice following myocardial infarction and implantation of a 14-day osmotic pump containing BrdU; S-phase positive cardiomyocyte nuclei are identified by co-localization of BrdU and β-GAL immune reactivity [25]. An example of an S-phase cardiomyocyte from this work is shown in Figure 1G. nCMN (see Table 1) was calculated from the cumulative regional cardiomyocyte S-phase levels using formula #2 (derivation provided in the Appendix) which takes into account the lag in pump activation which is encountered post-implantation, the impact of compounded growth over the labeling period, and the semiconservative nature of DNA synthesis which results in BrdU incorporation in both daughter nuclei. Once again, the initial prediction assumed that each S-phase event resulted in a karyokinetic event, and that each daughter nucleus could re-initiate S-phase in 24 hours after the initiation of S-phase.

Under these assumptions, the compound interest law predicts that the cardiomyocyte nuclear number in the border zone of infarcted (B6NxD2J)-F1 mice would increase from 48% of that present in non-infarcted animals at day 0 post-injury to 100% at day 1,200 (Figure 2A, solid red line). Thus, the intrinsic level of cardiomyocyte cell cycle reentry in animals expressing a single TNNI3K allele could lead to significant regenerative growth during this time frame if each cardiomyocyte exhibiting S-phase activity was able to progress through karyokinesis and cytokinesis. Given that current reperfusion therapies result in marked myocardial salvage [31], such levels of cardiomyocyte renewal would likely be clinically relevant (importantly, TNNI3K is expressed in human hearts [32]).

Unfortunately it is clear that, in the absence of intervention, most cardiomyocyte S-phase events in the adult mouse heart result in the formation of polyploid nuclei. If one assumes that only those S-phase events which gave rise to 2N cardiomyocyte nuclei (as calculated in Table 1) resulted in a karyokinetic event, and that each daughter cell could reenter the cell cycle within 24 hours after the onset of S-phase, the predicted increase in cardiomyocyte nuclear number in the border zone of (B6NxD2J)-F1 mice at 1,200 days post-injury is negligible (Figure 2A, dotted red line). Thus, it appears that the lack of meaningful myocardial renewal in TNNI3K-expressing hearts in regions with significant cardiomyocyte survival post-injury is not due to the absence of cardiomyocyte cell cycle entry per se, but rather is due to the inability of those cells entering the cell cycle to progress beyond polyploidization.

In D2J mice (which do not express TNNI3K), the percentage of border zone cardiomyocyte nuclei entering the cell cycle per day is roughly 7-fold lower than that in mice carrying a single functional *Tnni3k* allele (see Table 1). With this level of cell cycle activity, and assuming that each S-phase event resulted in a karyokinetic event, cardiomyocyte nuclear number in the border zone would only increase from 48% at day 0 post-injury to 54% at day 1,200 (Figure 2A, solid blue line). Thus, the decrease in the rate of cell cycle entry due to the loss of TNNI3K expression severely compromises the potential for meaningful myocardial renewal in regions with significant cardiomyocyte survival post-injury. Moreover, if only those S-phase events which gave rise to 2N cardiomyocyte nuclei resulted in a cytokinetic event, the predicted increase in cardiomyocyte nuclear number in the border zone of D2J mice at 1,200 days post-injury is negligible (Figure 2A, dotted blue line). Although cardiomyocyte S-phase levels within the infarct zone itself were similarly elevated in (B6NxD2J)-F1 and D2J mice following permanent coronary artery ligation (Table 1), the predicted level of myocardial renewal over 1200 days is negligible due to the low number of surviving cardiomyocytes (only 4% of that in non-injured hearts, see Figure 2B).

It is also informative to use the compound interest law to calculate what rate of cardiomyocyte renewal would be required to restore cardiomyocyte nuclear number in the border zone and infarct over a fixed period of time. In the case of the border zone, 0.8% of the surviving cardiomyocyte nuclei would have to renew each day to reverse the damage in 3 months, while daily renewal rates of 0.4% and 0.2% would be required to reverse the damage in 6 and 12 months, respectively (Figure 3A). These daily cardiomyocyte nuclear renewal rates would correspond to 14-day cumulative BrdU labeling levels of 19.5%, 10% and 5%, respectively (calculated with Formula #2, and assuming all S-phase events gave rise to karyokinesis within 24 hours of S-phase entry). Due to the low number of cells surviving in the infarct zone, considerably higher renewal rates would be required to restore cardiomyocyte nuclear number over similar timelines. Specifically, 3.9%, 1.92% and 0.95% of the remaining cardiomyocytes would have to renew each day to reverse the damage in the infarct in 3, 6 and 12 months, respectively (Figure 3B), corresponding to 14-day cumulative BrdU labeling levels of 79%, 44% and 23% (again, assuming all S-phase events gave rise to karyokinesis within 24 hours). When employed in this context, the compound interest law affords an invaluable “reality check” for cardiac regeneration studies, as it provides a convenient means to determine if the magnitude of the increase in cardiomyocyte cell cycle activity following intervention is consistent with magnitude of the improvement in cardiac structure and function. A discordant result would suggest that any beneficial impact of a given intervention is due to a salutary effect (or effects) of the intervention independent of its impact on cardiomyocyte renewal.

Several factors should be considered when using the compound interest law to model regenerative growth of the heart. In the projections above it was assumed that cardiomyocyte renewal rates are constant over the temporal window of the prediction. If changes in cardiomyocyte cell cycle activity are detected over time, the compound interest law formula can still be used to model regenerative growth simply by performing iterative calculations over the temporal windows exhibiting discrete renewal rates. It was also assumed that cardiomyocytes would progress through cytokinesis and be able to contribute to the proliferative pool 24 hours after entering S-phase. If a longer duration is required for *de*

novo cells to enter the proliferative pool (which can be determined empirically), the formula can readily be modified to take the additional time into account. The formula can also be modified to take into account cardiomyocyte loss associated with long-term adverse remodeling, if needed. In instances where renewal rates are based on S-phase values, one would need to determine if the resulting cells were mononucleated, binucleated and/or multinucleated as that would influence the net increase in cell numbers predicted by the formula. Finally, factors which are crucial for the accurate scoring of cardiomyocyte cell cycle activity also need to be considered [33-35], as the data from those assays are required to program the formula.

In summary, the compound interest law employed here provides a convenient way to predict the extent of cardiomyocyte renewal over time. This law predicts that, if those cardiomyocytes entering S-phase were able to progress through cytokinesis, the intrinsic rate of cardiomyocyte cell cycle reentry in mice with a single intact *Tnni3k* gene would be sufficient to completely regenerate a regional loss of 52% of the myocardial nuclear number within 1,200 days following permanent coronary artery ligation. Given that reperfusion therapy can result in substantial regional cardiomyocyte survival, and given that human cardiomyocytes express TNNI3K, the modeling presented here suggests that modulation of pathways which facilitate cardiomyocyte cell cycle progression beyond S-phase may be sufficient to promote meaningful regional regeneration.

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Appendix:

The Compound Interest Law assumption says that Cardiomyocyte Nuclei Number on cell cycle n as denoted by A_n is given by:

$$A_n = A_0(1 + nCMN)^n \quad \{formula\ \#1\}$$

where nCMN is the Cardiomyocyte Nuclei Growth Rate and A_0 is the initial Cardiomyocyte Nuclei Number.

Here we wish to estimate nCMN given estimates for A_0 and A_n for a specific n. We utilized cumulative cardiomyocyte S-phase levels (determined by BrdU incorporation) to calculate the nCMN and in our initial hypothetical models it was assumed that each S-phase cardiomyocyte nucleus would divide within 24 hours to give rise two daughter nuclei.

By taking the natural log of both sides we have:

$$\ln\left(\frac{A_n}{A_0}\right) = n * \ln(1 + nCMN)$$

or

$$\frac{1}{n}\ln\left(\frac{A_n}{A_0}\right) = \ln(1 + nCMN)$$

or

$$(1 + nCMN) = exp\left[\frac{1}{n}\ln\left(\frac{A_n}{A_0}\right)\right]$$

or

$$nCMN = exp\left[\frac{1}{n}\ln\left(\frac{A_n}{A_0}\right)\right] - 1$$

Employing this in Table 1 where n=13 cell cycles (assuming 1 cell cycle per day over 13 days post pump activation) we can write an explicit solution for nCMN as:

$$nCMN = exp\left[\frac{1}{13}\ln\left(\frac{A_{13}}{A_0}\right)\right] - 1$$
$$= exp\left[\frac{1}{13}\ln\left(\frac{Total\ CM\ nuclei}{Total\ CM\ nuclei - \frac{BrdU\ CM\ nuclei}{2}}\right)\right] - 1 \quad \{formula\ \#2\}$$

where we have taken A_n = *Total CM nuclei* and A_0 = *Total CM nuclei – BrdU CM nuclei/2* (given the nature of semi-conservative DNA replication, the total number of BrdU CM nuclei is divided by two in order to obtain the net gain of CM nuclei number due to cell cycle activity).

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Highlights:

- The intrinsic rate of cardiomyocyte renewal in the adult mammalian heart is low
- Recent studies have identified genetic variants which can impact the ability of cardiomyocytes to enter the cell cycle post-injury
- Modeling using the compound interest law indicates that the rate of intrinsic cell cycle entry in some lines of mice could lead to meaningful regeneration provided that the cardiomyocyte which reenter the cell cycle are able to progress through cytokinesis.

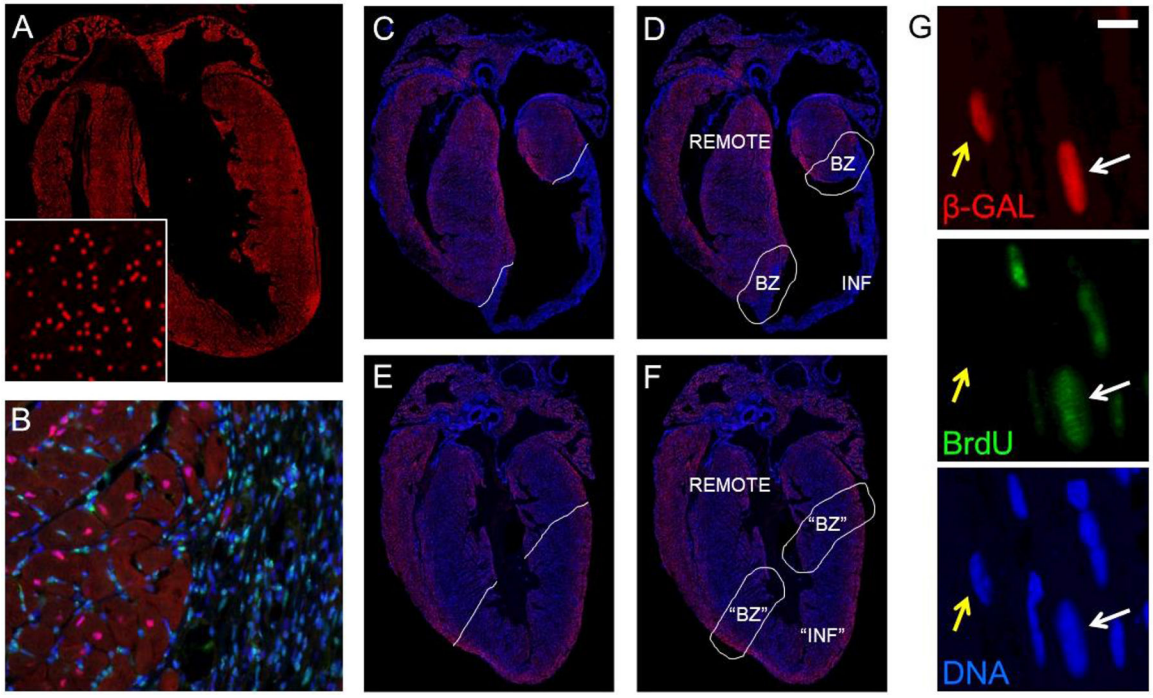

Figure 1.

Use of the MHC-nLAC reporter transgene to quantitate cardiomyocyte nuclear number in infarcted and normal mouse hearts. (A) Image of an entire coronal section from an MHC-nLAC heart processed for β-GAL immune reactivity (red signal). Inset shows a high power image of nuclear β-GAL immune reactivity. (B) Color-combined image of the border zone region in the ventricular septum of an MHC-nLAC mouse heart subjected to myocardial infarction and 14 days of BrdU infusion. The heart was then sectioned, processed for β-GAL (red) and BrdU (green) immune reactivity and then counterstained with Hoechst to mark all nuclei (blue). (C) Red and blue color-combined image of an entire coronal section from an infarcted MHC-nLAC heart with the boundary between the healthy myocardium and the infarct defined by white lines. (D) The same image, but with the infarct border zone (BZ) defined by white tracing (the zone encompasses the band residing 500 microns above and 500 microns below the white lines in Panel C). The infarct (INF) is located apical to the border zone. (E) Red and blue color-combined image of an entire coronal section of a non-infarcted MHC-nLAC with white lines analogous to those drawn for the infarcted heart in (C) positioned on the non-infarcted heart. (F) The same image, but with the pseudo-infarct border zone ("BZ") defined by white tracing (the zone encompasses the band residing 500 microns above and 500 microns below the white lines in Panel E). The position of the pseudo-infarct ("INF") is indicated. (G) Example of an S-phase positive (white arrow) and an S-phase negative (yellow arrow) cardiomyocyte nucleus from an infarcted (B6N x D2J)-F1 MHC-nLAC mouse. The upper panel shows β-GAL immune reactivity (red), the middle panel shows BrdU immune reactivity (green) and the lower panel shows DNA content (blue) from the same field. S-phase positive and S-phase negative non-cardiomyocyte are also present in the image. Bar = 10 microns.

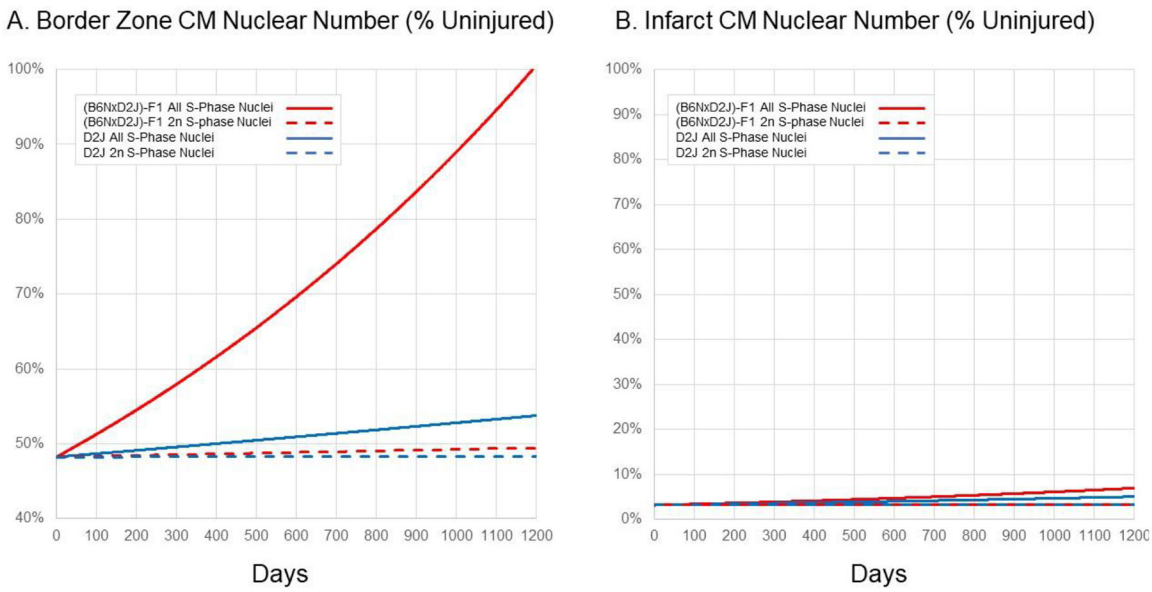

Figure 2.

Changes in cardiomyocyte nuclear number in the border zone and infarct over time as predicted by the compound interest law. The predicted cardiomyocyte nuclear number was plotted from 1 day through 1,200 days post-infarction for the border zone (A) and for the infarct (B) in (B6NxD2J)-F1 (red lines) and D2J (blue lines) mice.

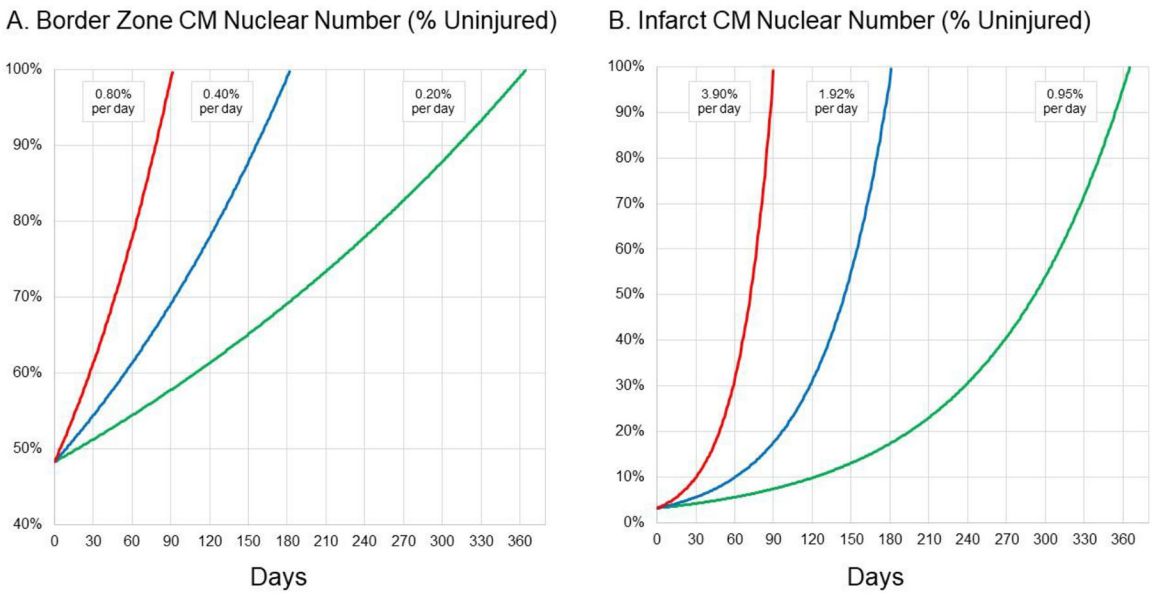

Figure 3.

Cardiomyocyte nuclear renewal rates required for complete regeneration of the (A) border zone or (B) infarct over 3, 6 or 12 months (red, blue and green traces, respectively).

Table 1.

Estimating the percentage of new cardiomyocyte nuclei per day (nCMN) for D2J and (B6NxD2J)-F1 mice following permanent coronary artery ligation. Mice were infarcted, implanted with BrdU-containing osmotic pumps, sacrificed 14 days later, and the level of cumulative regional S-phase activity was determined previously [25]. nCMN was calculated from the cumulative regional BrdU incorporation level using formula #2, the derivation of which is provided in the Appendix. The cardiomyocyte ploidy-corrected nCMN was calculated using data generated by analysis of nuclear DNA content in dispersed-cell preparations from EdU-labelled hearts as described [25].

Strain:	Cardiomyocyte Nuclear parameters:	Border Zone Nuclei:	Infarct Nuclei:
(B6NxD2J)-F1	14-day cumulative BrdU level	1.585	1.687
	nCMN, calculated via formula 2	0.06122	0.06518
	nCMN, corrected for 2N DNA content (3.4%)	0.00208148	0.00221612
D2J	14-day cumulative BrdU level	0.235	0.997
	nCMN calculated via formula 2	0.00904	0.03845
	nCMN, corrected for 2N DNA content (0.5%)	0.0000452	0.00019225