Cell

Cardiomyocyte Cell-Cycle Activity during Preadolescence

Earlier studies (Soonpaa et al., 1996) revealed a rapid drop-off of ventricular cardiomyocyte cell-cycle activity at birth in mice, followed by a burst of DNA synthesis during the first week of postnatal life which contributed to the formation of multi-nucleated cardiomyocytes by postnatal day 10 (PN10). It has recently been suggested that a second burst of cardiomyocyte cell-cycle activity occurs during preadolescence, between PN14 and PN18, resulting in a 40% increase in cardiomyocyte number (Naqvi et al., 2014). Since there was no overt change in mono- versus bi-nuclear cardiomyocyte content and no change in cardiomyocyte nuclear ploidy between PN14 and PN18, a 40% increase in ventricular cardiomyocyte number during preadolescence should result in newly synthesized DNA in 57% of the cardiomyocyte nuclei.

To characterize this putative burst of preadolescent cell-cycle activity, MHC-nLAC mice, expressing a nuclear-localized β-galactosidase reporter in cardiomyocytes and maintained in a DBA/2J background (Soonpaa et al., 1994), were implanted with BrdU-containing osmotic mini-pumps. Cumulative ventricular cardiomyocyte DNA synthesis was quantitated by co-localization of β-galactosidase and BrdU immune reactivity (Figure 1A) as described (Reuter et al., 2014). Only low levels of cardiomyocyte DNA synthesis were detected in mice carrying pumps from PN10 through PN19 (2.96% ± 0.55%) or from PN12 through PN19 (1.09% ± 0.33%; see also Table S1A). BrdU was detected in small intestine crypt cells by 24 hr postimplantation, and at the end of the labeling period (Figure 1B), confirming continuous infusion. Cumulative preadolescent cardiomyocyte DNA synthesis was also quantitated in C57Bl/6J inbred mice (the strain used by Naqvi and colleagues); S-phase cardiomyocytes were identified by nuclear BrdU immune reactivity in dispersed cell preparations (Figure 1C). Only low rates of cardiomyocyte DNA synthesis were detected (Table S1B). Mice receiving a single BrdU injection on PN14.5, PN15, or PN16 and analyzed on PN19 also had little labeling (Table S1C), arguing that BrdU cytotoxicity and/or the presence of the osmotic mini-pump per se were not confounding factors.

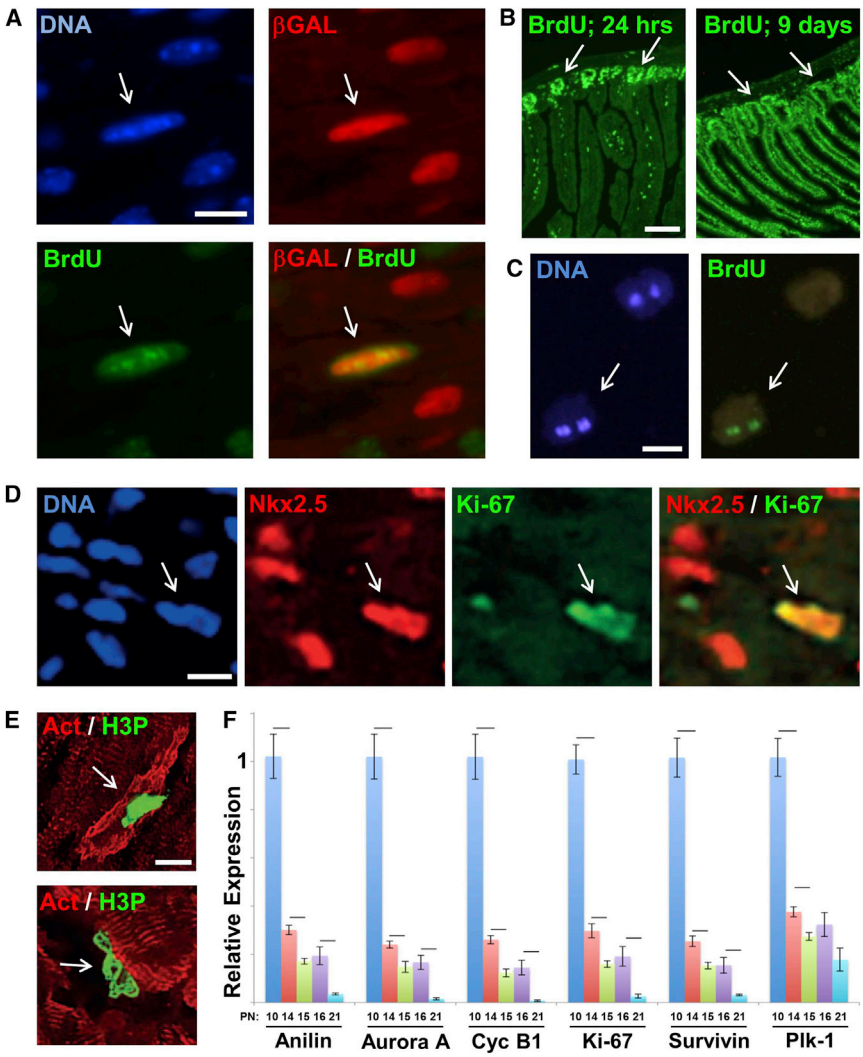

Figure 1. Characterization of Cardiomyocyte Cell-Cycle Parameters during Preadolesence

(A) Example of cardiomyocyte DNA synthesis (arrow) in the heart of an MHC-nLAC mouse carrying a BrdU mini-pump from PN10 through PN19. Panels show Hoechst staining of DNA (blue signal), beta-galactosidase immune reactivity (red signal) and BrdU immune reactivity (green signal). Scale bar, 10 microns.

(B) BrdU immune reactivity (green signal) in the small intestine of an MHC-nLAC mouse carrying a BrdU mini-pump for 24 hr (left) or 9 days (right). Arrows, villi crypts. Scale bar, 200 microns.

(C) Example of cardiomyocyte DNA synthesis (arrow) in dispersed cells from a C57Bl/6J mouse heart carrying a BrdU mini-pump from PN10 through PN19. Panels show Hoechst staining of DNA (blue signal) and BrdU immune reactivity (green signal). Scale bar, 20 microns.

(D) Example of an S-phase cardiomyocyte nucleus in a PN15 heart as evidenced by co-localization of Nkx2.5 (red signal) and Ki-67 (green signal) immune reactivity (arrow). Scale bar, 10 microns.

(E) Example of cardiomyocyte (upper panel) and non-cardiomyocyte (lower panel) H3P immune reactivity (green signal, arrow) in postnatal hearts. Cardiomyocytes were identified by α-actinin immune reactivity (red signal). Scale bar, 10 microns.

(F) Cardiac expression of mitosis-related genes on PN10, PN14, PN15, PN16, and PN21 in C57BL/6J mice (mean ± SEM) relative to their level at P10. mRNA levels were quantitated and normalized to 18S as described (Livak and Schmittgen, 2001). Significance was tested using unpaired, 2-tailed Student's t tests with Bonferroni correction for multiple testing; bars indicate $p < 0.05$ versus subsequent time point.

Cardiomyocyte cell-cycle activity was also quantitated via co-localization of Nkx2.5 and Ki-67 immune reactivity (Figure 1D); Ki-67 is expressed from G1 phase to anaphase and thus provides a very good estimate of the fraction of a given cell population with cell-cycle activity (Lopez et al., 1994). The cardiomyocyte nuclear Ki-67 labeling never exceeded 1% in C57Bl/6J ventricles between PN12 and PN16 (Table S1D), in agreement with the BrdU incorporation data. Phosphorylation of histone H3 on serine 10 (H3P), which labels cells from G2/M through early anaphase (Hendzel et al., 1997), was also used to monitor cardiomyocyte cell-cycle activity. Since cardiomyocyte mitosis is characterized by sarcomere disassembly (Engel et al., 2006), mitotic ventricular cardiomyocytes can be identified by the presence of H3P signal and sarcomere disassembly (Figure 1E). No mitotic cardiomyocytes were observed after PN12 (Table S1E).

Ventricular expression levels of a panel of mitosis-related genes (encoding anilin, aurora A, polo-like kinase 1, survivin, cyclin B1, and Ki-67) were measured by qPCR in C57Bl/6J mice, and the relative levels of expression were normalized to that of 18S rRNA as described (Liu et al., 2015). No changes in transcript levels supporting the presence of a proliferative burst between PN14 and PN16 were detected (Figure 1F). Collectively, the low rates of cardiomyocyte DNA synthesis (analyzed by M.H.S. and L.J.F.), the low levels of cardiomyocyte Ki-67 and H3P immune reactivity (analyzed by D.C.Z. and F.B.E.) and the absence of transient increases in mitotic transcripts (analyzed by C.P and A.R.) during preadolescence are consistent with the pattern of gradual postnatal ventricular cardiomyocyte cell-cycle withdrawal reported earlier using single injections of tritiated thymidine to monitor cardiomyocyte cell-cycle activity (Soonpaa et al., 1996).

Potentially trivial factors such as the method for defining postnatal age, strain differences, BrdU cytotoxicity, cell identification in histologic samples, and the sequences of the PCR primers used, were controlled for and thus cannot explain the differences in the results presented here and those of Naqvi and colleagues. Whether subtle differences in animal husbandry can explain the differences in the measured parameters between the two studies remains unclear and worthy of further investigation. Indeed, the current study does not rule out the possibility that a preadolescent burst in cardiomyocyte cell-cycle activity can exist, assuming that as of yet undefined optimal conditions of litter size, nutrients, etc., are met. However, the data presented here clearly indicate that preadolescent development did occur in the absence of a burst of cardiomyocyte proliferation over multiple litters in three independent breeding colonies. Consequently, a burst of preadolescent cell-cycle activity is not required for normal cardiac development.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one supplemental table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.10.037.

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