Cardiomyocyte cell cycle control and growth estimation in vivo—an analysis based on cardiomyocyte nuclei

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Aims
Adult mammalian cardiomyocytes are traditionally viewed as being permanently withdrawn from the cell cycle. Whereas some groups have reported none, others have reported extensive mitosis in adult myocardium under steady-state conditions. Recently, a highly specific assay of 14C dating in humans has suggested a continuous generation of cardiomyocytes in the adult, albeit at a very low rate. Mice represent the most commonly used animal model for these studies, but their short lifespan makes them unsuitable for 14C studies. Herein, we investigate the cellular growth pattern for murine cardiomyocyte growth under steady-state conditions, addressed with new analytical and technical strategies, and we furthermore relate this to gene expression patterns.

Methods and results
The observed levels of DNA synthesis in early life were associated with cardiomyocyte proliferation. Mitosis was prolonged into early life, longer than the most conservative previous estimates. DNA synthesis in neonatal life was attributable to bi-nucleation, therefore suggesting that cardiomyocytes withdraw from the cell cycle shortly after birth. No cell cycle activity was observed in adult cardiomyocytes and significant polyploidy was observed in cardiomyocyte nuclei.

Conclusion
Gene analyses identified 32 genes whose expression was predicted to be particular to day 3–4 neonatal myocytes, compared with embryonic or adult cells. These cell cycle-associated genes are crucial to the understanding of the mechanisms of bi-nucleation and physiological cellular growth in the neonatal period.

Keywords
Mitosis • Cardiomyocytes • Hypertrophy • Bi-nucleation

1. Introduction
The amount of DNA synthesis in adult murine cardiomyocytes is disputatious. Adult mammalian cardiomyocytes are traditionally viewed as being permanently withdrawn from the cell cycle,1,2 a view supported by clinical observations—primary myocardial tumours are rarely observed in adults and significant myocardial injury results in a permanent reduction in cardiac performance. Lately, the consensus in the field has been challenged by investigators that have claimed ongoing cardiomyocyte mitosis throughout life to the extent that if arrested, the heart would be completely depleted of myocytes within 5 years in humans.1,4 Despite extensive research in the field of cardiomyocyte proliferation and mitosis, the results from mice are conflicting, suggesting either ongoing5 or absence of mitosis in the adult heart.2

Previous studies have relied heavily on histological screening of cardiomyocytes with common proliferation markers and labelling assays such as 5’-bromo-2’-deoxyuridine (BrdU), Ki-67, and triated thymidine. Many of the variations in myocyte cell cycle activity can be traced to difficulties in the identification of nuclei in histological sections, and the sensitivity of the assays used.6 Cardiomyocytes comprise only 20–30% of the cells in the heart,7,8 furthermore the varying degree of bi-nucleation, and distinguishing between cytokinesis and karyokinesis makes accurate assessment of proliferation even more demanding. To date, the most specific cardiomyocyte mitosis assay is 14C dating of cTropT+ nuclei. We and others have established

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with $^{14}$C dating that human cardiomyocytes are generated into adulthood, albeit to a low extent. However, the $^{14}$C dating has a resolution of about 1–2 years, making it unsuitable for studies in species with a short lifespan.

The uncertainties associated with previous strategies and the lack of consensus in the field motivated us to readdress this argument. Utilizing a novel fluorescence activated cell sorting (FACS)-based strategy for the purification of cardiomyocytes, based on cTropinin-T expression, we were able to remove the anxieties associated with accurate identification of cardiomyocyte nuclei for the purposes of labelling assays. Herein, we describe the turnover of myocytes at three developmentally distinctive periods: foetal, neonatal, and adult. Additionally, we have used a transgenic mouse model to establish the gene expression pattern in cardiomyocytes at these developmental time points.

2. Methods
Details of the following methods can be viewed in Supplementary material online.

2.1 Immunohistochemistry
Cell cycle activity was detected by the Ki-67 antibody (Nova Castra Laboratories) and BrdU (Sigma Chemical, St Louis, MO, USA) incorporation. For BrdU incorporation experiments, mice were injected intraperitoneally (i.p.) with 1 mg BrdU (Sigma-Aldrich) per 6 g body weight twice daily for 14 days in the case of adult time points and 4 h prior to cardiac isolation for other time points in sterile saline. Age-matched WT mice used were embryonic day 14.5 (ED14.5) (n = 7); N1 (n = 7); N7 (n = 6); 2 (n = 4); 3 (n = 7); and 12 weeks (n = 7). Heart tissue was immersion fixed in stefanini solution at 4°C, equilibration in 20% sucrose, embedded in Tissue-Tek (O.C.T. Sakura), and frozen. Cryosectioning 8–10 μm. See Supplementary material online, for detailed Methods.

2.2 Mice
Wild-type C57BL/6 mice were from Jackson Laboratories (Maine). Enhanced GFP-transgenic DBA mice with GFP expression driven constitutively by the α-MHC promoter, originally created on CrHeb/FeJ, and then back-crossed 8–9 x to DBA/2J. All mice procedures were performed with the local Ethics Committee at Lund University. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3 Flow cytometry
FACS samples were prepared in the following manner: embryonic, neonatal, and adult hearts were dissected and dissociated to single cell solution with Liberase Blendzyme 3 (0.1 mg/mL) (Roche Diagnostics), washed and spun down and resuspended in cardiomyocyte isolation buffer (130 mM NaCl; 5 mM KCl; 1.2 mM KH2PO4; 6 mM HEPES; 5 mM NaHCO3; 1 mM MgCl2; 5 mM glucose). Cells were fixed with BD Cytofix/Cytoperm solution, permeabilized and incubated with monoclonal mouse anti-troponin T, FITC conjugated (Hytest Ltd, Turku, Finland). Cells were sorted on a FACS Aria (BD Biosciences, San Jose, CA, USA). Doublets were discriminated as previously described.

2.4 BrdU and cell cycle analysis of cardiomyocyte nuclei
Age-matched WT mice ED14; N1; N4; N7; 2; 3; and 12 weeks were given an intraperitoneal BrdU injection as previously described. BrdU incorporation was evaluated by using a BrdU and 7-amin actinomycinin (7-AAD) intracellular staining kit (BD Pharmingen). Ki-67 expression was detected with anti-Ki-67-APC (BD Pharmingen) and DNA was stained with 7-AAD (Sigma-Aldrich). Cell cycle data were generated using Flowjo software.

2.5 Isolation of viable cardiomyocytes from α-MHC-eGFP mice
Cells dissociated as previously described and 7-AAD (Sigma-Aldrich Co., St Louis, MO, USA) used to exclude dead cells. Antibodies used for cell surface staining were PECAM-1-APC; APC-labelled Rat IgG2a, (BD Pharmingen). Myocytes were sorted on a BD FACS Diva or FACS Aria Cell Sorter (BD Biosciences) using 100 μm nozzle.

2.6 RNA amplification and Illumina Sentrix Beadchip array
RNA was isolated with RNeasy Mini or micro kit (Qiagen, Inc., CA, USA) following the protocol of the manufacturer and subjected to two rounds of leaner amplification. Chips were scanned in standard mode on a Bead Station 500 GX (Illumina) and raw data were extracted for statistical analysis with BeadStudio v3.1 software. (Illumina, Inc., San Diego, CA, USA). Entire array data accessible online at the NCBI Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/geo Accession number GSE17020.

2.7 Quantitative RT–PCR
PECAM-1 negative, α-MHC-eGFP positive, and GFP negative cells were FACS Aria sorted with 100 μm nozzle into RLT buffer and frozen at −80°C. RNA extraction was performed with an RNeasy Micro kit (Qiagen, Inc.) according to the manufacturers’ instructions, including on-column DNase 1 digestion. cDNA was synthesized from 46.5 ng total RNA for all sample time points. Eluted RNA samples were reverse transcribed using SuperScript II and random hexamers (invitrogen) according to protocol supplied by the manufacturer. PCR was performed using iQ5 Real-time PCR thermal cycler and iQ SYBER Green supermix (Bio-Rad). The normalized values of each biological triplicate were averaged before the calculation of fold change in expression levels between adult and neonatal myocytes in comparison to embryonic myocytes using the 2−ΔΔCt method. Expression levels in embryonic cardiomyocytes were set to 1 as a reference.

3. Results
3.1 Isolation and purification of cardiomyocytes by FACS
Staining for the intracellular cardiac isoform of Troponin (cTrop) T clearly distinguished between 20 and 30% of the cells in the heart as being positive (Figure 1A). The purity of sorted cardiomyocytes by FACS was >98% (Figure 1B and C). To enable accurate assessment of cardiomyocyte DNA synthesis and to compensate for the bi-nucleation phenomenon of cardiomyocytes, nuclei were extracted from sorted intact cells and nuclei preparations confirmed by cytosin and immunohistochemistry (Figure 1D). The condition and frequency of isolated nuclei were verified by flow cytometry, preparations of which were subsequently used for proliferation marker staining (Figure 1E).

3.2 BrdU cell cycle analysis of isolated myocyte nuclei
To address the proliferative capacity of cardiomyocytes in the heart, we performed nuclei isolation on purified populations of myocytes and subsequently FACS analysis (Figure 2A) with BrdU and 7-AAD staining, while excluding doublets as previously described (Figure 2B).

S. Walsh et al.
nuclei was 22.95 ± 0.07% (n = 37; where n = number of embryos) and predominantly uniform in DNA content (Figure 2C), whereas by neonatal day 3 (n = 10), the number of BrdU-positive cells decreased to 9.6 ± 0.62% (Figure 2D) and a heterogeneous ploidity level became apparent. The downward progression continued, by neonatal day 7 (n = 7) only 0.94 ± 0.76% of nuclei were BrdU positive (Figure 2E) and by neonatal day 14 (Figure 2F), there was very low BrdU detection in myocyte nuclei at 0.02% (n = 6). By neonatal day 21 (n = 7) through to adulthood (n = 6) (data not shown), no detectable BrdU-positive nuclei were observed, suggesting that cardiomyocyte DNA synthesis completely ceases around the 3 weeks time point in murine left ventricular tissue. This is in agreement with previous studies, which have even suggested an upper limit of 0.005% labelling index in adult mouse heart.2

3.3 Ki-67 cell cycle analysis of isolated myocyte nuclei by FACS
Myocyte turnover was also assessed by measuring the fraction of Ki-67 + nuclei from a purified population of myocytes from individual

Figure 1 Cardiomyocyte purification by FACS and nuclei isolation. Flow cytometry plots of mouse ventricular cardiomyocytes labelled with cardiac specific antibody to Troponin-T intracellular antigen are shown in (A). Cells were gated based on size (far left panel), isotype control (centre panel), and then separated into two populations with cardiac troponin T (right panel). Purity of the sorted cardiomyocyte population was verified by flow cytometry (B). Unstained control consisting of mixed cardiac cell populations (left panel, shaded) and Troponin-T stained mixed cell populations (left panel, red outline). Enrichment for cTropT was >98.8% (C). Mononucleated embryonic (D, top left panel) and bi-nucleated neonatal myocytes (D, top right panel). Cytopsins of nuclei preparations were stained with cTrop-T antibody and Hoechst and examined by microscopy. Intact sorted myocytes (D, bottom left panel) and subsequent nuclei preparation (D, bottom right panel). FACS plot of nuclei preparation yielded >99% nuclei dissociation efficiency (E).
animals (Table 1). At embryonic day 14.5 (n = 30), 49.65 ± 2.18% of nuclei were in G1/G0 and 21.15 ± 2.33% in S-phase (Figure 2G). By the early neonatal period day 1 (n = 7), the number of myocyte nuclei undergoing DNA synthesis dropped to 2.4 ± 0.42% (Figure 2H). By the middle neonatal period day 4 (n = 12), there is a marked increase in the amount of nuclei in S-phase, 8.45 ± 0.27% (Figure 2I) consistent with previous observations of DNA synthesis associated with hypertrophic growth and bi-nucleation.13,14 Late neonatal growth and proliferation was associated with a reduction in the number of myocytes undergoing DNA synthesis, 5.5 ± 1.9% during day 7 (n = 7) and 14 (n = 6), respectively (Figure 2J–K). By 3 weeks of age (n = 4), no proliferative activity was observed (Figure 2L) in the isolated nuclei, consistent with the view of adult mammalian cardiomyocytes being post-mitotic15–17 lacking the ability to undergo cell division, and consistent with our labelling indices generated by BrdU FACS staining.

3.4 Immunohistochemistry

The values reported for adult cardiomyocyte labelling indexes range across several orders of magnitude in both normal and injured rodent hearts.18–21 Herein, we have compared the labelling indices obtained from two common proliferation markers, BrdU and Ki-67 sections of mouse cardiac tissue. The percentage of BrdU+ myocyte nuclei gradually decrease from abundance at ED14.5 at 23% (Figure 3A) through to the early neonatal period 13.0%. By neonatal day 7 and 14 (Figure 3B), the frequencies of BrdU+ nuclei were 8 and 0.5%, respectively. No BrdU incorporation was detected after 3 weeks of age in contrast to some previous studies which have recorded proliferation in adult myocardium.5,19 Ki-67 was also utilized to determine accurate labelling of myocytes in cell cycle progression.

Table 1. Cell cycle characteristics of isolated myocyte nuclei

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>G1/G0 (%)</th>
<th>S-phase (%)</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED14.5</td>
<td>49.65 ± 2.18</td>
<td>21.15 ± 2.33</td>
<td>13.85 ± 0.49</td>
</tr>
<tr>
<td>N1</td>
<td>51.6 ± 4.7</td>
<td>2.4 ± 0.42</td>
<td>45.9 ± 8.1</td>
</tr>
<tr>
<td>N4</td>
<td>68.9 ± 9.1</td>
<td>8.45 ± 0.27</td>
<td>14.65 ± 0.77</td>
</tr>
<tr>
<td>N7</td>
<td>47.11 ± 4.1</td>
<td>5.5 ± 1.9</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>94.36 ± 1.07</td>
<td>1.38 ± 0.6</td>
<td>—</td>
</tr>
<tr>
<td>21</td>
<td>100 ± 0</td>
<td>—</td>
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Again the observation indicated a gradual decrease in cycling activity from foetal ED14.5 12% (Figure 3C) to 10% by neonatal day 3 (Figure 3D) and 3% by neonatal day 7. No Ki-67+ nuclei were detected after 2 weeks of age (data not shown). Ki-67 expressing cells after the 3 weeks time point were cTropT negative.

3.5 Cell cycle kinetics of sorted myocyte nuclei

The frequency of cardiomyocyte nuclei determined to be in the cell cycle was congruous regardless of the proliferation marker assessed by FACS (Figure 4A) and IHC (Figure 4B). Proliferation was undetectable after 21 days indicating terminal differentiation and cell cycle inhibition. Proliferation, however, is but one outcome of myocyte cell cycle activity, as myocytes were predominantly mononucleated in the late gestational period committed to the process of karyokinesis coupled with cytokinesis. (Figure 4C). Karyokinesis alone was evident at neonatal days 2–4 whereby the increase in DNA synthesis observed was associated with bi-nucleation. A marked increase in DNA synthesis detected in the early neonatal period was associated with bi-nucleation. Between neonatal day 5 and 8, the majority (>98%) of cardiomyocytes in the mouse heart were bi-nucleated.

3.6 Ploidity of cardiomyocyte nuclei

Polyploid cells containing more than one full set of chromosomes is uncommon in many mammalian tissues, but some including liver and heart muscle are exceptions with high percentage of polyploid cells. To separate ploidity populations, we FACS sorted cardiomyocytes based on cTropT from embryonic (ED14.5), neonatal, late neonatal, and adult time points, which then underwent nuclei isolation, and were stained with 7-AAD as an indicator of the ploidity level. Diploid (2N), tetraploid (4N), octoploid (8N), and even hexadecaploid (16N) nuclei populations were distinguishable. Flow cytometry plots of isolated nuclei from embryonic day ED14.5 (see Supplementary material online, Figure S1A); neonatal day 3 (see Supplementary material online, Figure S1B) and 12 weeks (see Supplementary material online, Figure S1C) allowed estimation of the ploidity level of individual cardiomyocyte nuclei. Cardiomyocytes exhibit a heterogeneous and variable ploidity level in the early and late neonatal periods. Both mononucleated and bi-nucleated cells can exhibit the same cell ploidity, while exhibiting different intra-nuclear ploidity, e.g. we identified mononucleated cells with 4N (see Supplementary material online, Figure S1D) and bi-nucleated 2 × 2N (see Supplementary material online, Figure S1E) populations within the early neonatal period. At ED14.5, ~98.4% of mononucleated cardiomyocytes were diploid (see Supplementary material online, Figure S1F). By neonatal day 2, the amount of diploid nuclei had reduced to ~66% and tetraploid nuclei comprised ~29% of the population. Neonatal day 7 exhibited a large increase in the amount of octoploid nuclei ~20% present similar to the late neonatal period at 21 days ~17%. Less than 1% of nuclei were hexadecaploid at 3 weeks. These data display that polyploidization of murine myocytes in the left ventricle occurs predominantly in the first week of post-natal life and becomes monotonous after 7 days as myocytes terminally differentiate.

3.7 Gene Ontology

We isolated viable myocytes from eGFP-transgenic mice with GFP expression driven by the cardiomyocyte α-MHC promoter, allowing us to examine the gene expression patterns between cardiomyocytes of embryonic, neonatal, and adult origin (Figure 5A–D). We extracted RNA from each flow-sorted population and used a high-density Illumina Sentrix Beadchip microarray to profile gene expression for each myocyte population. On the basis of the raw microarray data, a gene list was generated using one-way ANOVA (P = 0.05), whereby embryonic, neonatal, and mature cardiomyocyte stage-specific genes were identified. Genes were clustered by cell cycle function as biological description in Gene Ontology (GO). 1153 transcripts were enriched ≥2-fold in neonatal cell populations; 2990 transcripts enriched in embryonic; and 1536 transcripts enriched in mature adult cardiomyocytes (Figure 5E). Of these 906, 337, and 465 transcripts in embryonic, neonatal, and adult, respectively, were cell cycle associated. To further characterize the molecular program of hypertrophy and bi-nucleation, we categorized transcripts enriched in neonatal cardiomyocytes using GO annotations with EASE23 software identifying genes likely to function in temporally restricted processes and stage-dependent profiles. Selected transcripts that exhibited high levels of enrichment in neonatal cardiomyocytes were subjected to RT–PCR.

3.8 RT–PCR

Transcripts of the cleavage furrow interacting protein anillin, a major facilitator of cytokinesis were undetectable in the neonatal period (Figure 6A). Spindlin expression was evident at the same period but down regulated in mature cardiomyocytes (Figure 6B) and centromere protein A (Figure 6C) was down-regulated in both neonatal and adult cardiomyocytes. Transforming acidic coiled-coil-containing protein 2 (TACC2) was strongly up-regulated in neonatal, >6-fold (Figure 6D). TACC2, a microtubule-interacting protein has been identified as a product of a gene with reduced expression in metastatic breast cancer cells,24 however, TACC2 deficiency does not result in any phenotypic alterations in mammals.25

Several key cyclin kinases associated with the G1/S transition were down-regulated in neonatal and adult myocytes including cyclin-dependent kinase-2 (Figure 6E) and cyclin-dependent kinase-3 (Figure 6F), both important regulators for G1 exit and S-phase entry,26 both of which were undetectable in adult cells. Cyclin kinase 4 (Figure 6G) also involved in the G1/S transition exhibited a similar pattern of expression. Cyclin D1 was also found to be up-regulated in neonatal cardiomyocytes, capable of stimulating growth in post-mitotic cells but proliferation in cells capable of cell cycle re-entry and associated with multi-nucleation (Figure 6H).27

Cyclin kinase 10 associated with G2/M progression and proliferation was down-regulated postnatally (Figure 6I). EZF transcription factor 1 (E2F1) expressed in proliferating cells and also mediating the transition from G2 into M phase,28 and shown to induce DNA synthesis; cell cycle progression and apoptosis when overexpressed in neonatal cardiomyocytes29 was not detected in neonatal myocytes or adult myocytes (Figure 6J). WEE1, an interphase promoting factor and involved in cell growth30 was found to be expressed in neonatal myocytes (Figure 6K), inhibiting cyclin B, which results in G2/M arrest in a p53-independent G2/M checkpoint. No detectable expression was observed in mitotically active myocytes. Positive regulator of G2/M progression, CDC5L cell division cycle 5-like protein31 was down-regulated at the neonatal and adult stages (Figure 6L).

Ki-67 manifestation was restricted to embryonic cardiomyocytes, although extremely low transcript levels were detected in neonatal cells (see Supplementary material online, Figure S2A), and Cyclin I
Figure 3 Immunohistochemistry proliferation assay. Tissue sections were co-immunostained with BrdU, Pan-Cadherin, and Hoechst to quantify total nuclei number and adjust for binucleation. ED14.5 (A) and 14 days (B). IHC analysis also performed with Ki-67 assay. Tissue sections were also co-immunostained with Ki-67, Pan-Cadherin, and Hoechst. The mean percentage value of Ki-67+ cells was determined. Embryonic (C) and neonatal (D) ventricular Ki-67 activity. (E) Bi-nucleated cardiomyocyte expressing Ki-67 in both nuclei at neonatal day 3 and Ki-67 expressing cardiomyocyte at 3 weeks (F).
was found to be two-fold up-regulated in neonatal cardiomyocytes (see Supplementary material online, Figure S2B) in comparison to other time points, expression of which is associated with ischaemia and pressure overload and has been shown to decrease the frequency of apoptotic cells. Growth arrest-specific 2 protein (GAS2) implicated in increasing the threshold for apoptosis in polyploid hepatocytes and structural maintenance of chromosomes protein, necessary for sister chromatid cohesion were down-regulated (see Supplementary material online, Figure S2C and D). Topoisomerase (DNA) III beta with a role in DNA recombination, cellular ageing and maintenance of genome stability was down-regulated in neonatal cardiomyocytes (see Supplementary material online, Figure S2E). WNT1 inducible signalling pathway protein 1 was two-fold up-regulated in neonatal compared with adult, previously shown to exert both pro-hypertrophic and pro-mitogenic effects in vitro, stimulating Akt-dependent cardiomyocyte growth, as well as collagen synthesis and fibroblast proliferation (see Supplementary material online, Figure S2F).

4. Discussion

The field of cardiomyocyte expansion during development has been a major issue of dispute. Everything has been reported, in that while some studies claim that cardiomyocytes are terminally differentiated, others claim extensive mitosis. Therefore, it is demanding to draw conclusions from previous reports. The main concern is that the correct cells have been adequately identified in the assays utilized to estimate cycling activity. Herein, we re-evaluated the findings of earlier studies, with the intention of improving the specificity of the estimates of cardiomyocyte kinetics. The most specific marker of cardiomyocytes is the cardiac isoform of Troponin-T, which was exploited for the FACS-based purification of cardiomyocyte populations and subsequent nuclei analysis. We therefore consider that our estimates exclusively valuate the cell cycle activity of cardiomyocytes. Local stem or progenitor cells with myogenic potential could lack cTropT expression and thus would be neglected with our assay, excluding any cell cycling attributed to these cells. There are, however, discrepant
views pertaining to the nature of possible cardiomyocyte progenitor or stem cells,35–38 nevertheless the focus of this report was to evaluate mitotic activity in undisputable cardiomyocytes.

Our data support the notion that adult mouse cardiomyocytes do not enter the cell cycle, and neonatal myocytes undergo karyokinesis in the absence of cytokinesis resulting in bi-nucleation. It has previously been observed in skeletal myotubes that the maximum range of operation for each nucleus in a myotube is $34 \pm 31 \mu m$,39 it is therefore plausible that with an acceleration in cellular mass during hypertrophic development, the greater the requisite for bi-nucleation. This phenomenon appears to be regulated by a battery of G2/M regulators and restricted expression of structural proteins necessary for cleavage formation and cell division. Bi-nucleation frequencies in adult human myocytes are reported to be $\sim 30–65\%$,22 in contrast to virtually 100% in the mouse. However, decreasing cell cycle activity in development is accompanied by increased polyploidy in both species, observed in humans in the first 10 years of life.9 This evidence suggests that human and mouse cardiomyocytes of a similar developmental stage share common features such as neonatal polyploidization, bi-nucleation, and growth arrest in adult. However, the period of each developmental phase differs immensely between these species, e.g. although the neonatal phase of bi-nucleation and polyploidization in humans lasts for the first decade of life, this phase seems to be completed in mice within 3 weeks.

Previously several key cell cycle regulators have been manipulated in cardiomyocytes including CCND1/CDK4,40 and CDK2,41 and shown to induce proliferation in terminally differentiated cardiomyocytes. Regardless, the extent of generation in mice over-expressing CDK2 does not correlate with the estimates of basal turnover in human hearts determined by $^{14}C$ dating cardiomyocytes, suggesting that adult mice have a much lower turnover rate than their human counterparts. Our studies show that mouse cardiomyocytes are more strictly arrested at maturation than those in humans. Considering the immense difference in lifespan, this is not unexpected.

The rapid switch from hyperplasia to myocyte hypertrophy provided the natural model in which to examine the molecular regulation and mechanisms which modulate cell proliferation and hypertrophy. Our microarray confirmed that several cell cycle regulators are differentially expressed—adult cardiomyocytes differ from their neonatal and foetal counterparts in that Cdk2, Cdk3, and the cyclin D1/Cdk4 complex, are solely down-regulated. Several G1/S transition regulators were expressed during neonatal development, coupled with the down-regulation of key kinetochore structural proteins necessary for completion of mitosis, specifically the down-regulation of anillin. The defective focusing of anillin at the mid-body region at the neonatal time point is associated with the failure to undergo cellular abscission which thereby could lead to binucleation12 (Figure 6A). These expression patterns were synchronous with G2/M modulators E2F1 (Figure 6J) and WEE1 (Figure 6K), evidence of a complex

Figure 6 Cell cycle control in neonatal cardiomyocytes. Quantitative RT–PCR data of indicated genes in FACS-sorted cardiomyocytes from embryonic (ED11–12), neonatal (N3–4), and adult (10 weeks) time points. All data were normalized to the expression of GAPDH and β-actin housekeeping genes. Expression levels in embryonic cardiomyocytes were set to 1 as reference. Results represent mean (SEM) values from two independent experiments, with PCR analysis in each experiment performed in triplicates. ND = no detectable expression after 45 cycles of PCR.
multi-gene checkpoint control of cytokinesis during the switch from hyperplasia to hypertrophic growth during neonatal cardiomyocyte development. Collectively these results suggest that a multi-gene manipulation not only addressing cell cycle regulators but also involving structural proteins will be necessary in any future therapeutic approaches. Further studies will focus on characterizing the mechanisms by which these factors participate in hypertrophic growth and if isolation of cardiomyocytes with hyperplastic potential will be possible.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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