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Cardiomyocyte nuclearity and ploidy: when is double trouble?

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Abstract
Considerable effort has gone into investigating mechanisms that underlie the developmental transition in which mammalian cardiomyocytes (CMs) switch from being able to proliferate during development, to essentially having lost that ability at maturity. This problem is interesting not only for scientific curiosity, but also for its clinical relevance because controlling the ability of mature CMs to replicate would provide a much-needed approach for restoring cardiac function in damaged hearts. In this review, we focus on the propensity of mature mammalian CMs to be multinucleated and polyploid, and the extent to which this may be necessary for normal physiology yet possibly disadvantageous in some circumstances. In this context, we explore whether the concept of the myonuclear domain (MND) in multinucleated skeletal muscle fibers might apply to cardiomyocytes, and whether cardio-MND size might be related to the transition of CMs to become multinuclear. Nuclei in CMs are almost certainly integrators of not only biochemical, but also—because of their central location within the myofibrils—mechanical information, and this multimodal, integrative function in adult CMs—involving molecules that have been extensively studied along with newly identified possibilities—could influence both gene expression as well as replication of the genome and the nuclei themselves.

Keywords Cardiomyocyte · Skeletal muscle fiber · Binuclearity · Multinuclearity · Ploidy · Cell cycle · Regeneration · Proliferation · Myonuclear domain · Troponin

Introduction

Vertebrate animals rely on contraction of the heart to circulate blood—supplying the body's tissues with oxygen and nutrients—as was recognized by William Harvey in the seventeenth century (Harvey 1628). Today, genetics, lifestyle and environmental factors combine to elevate the risk for heart disease and cardiac arrest. For example, in the U.S. almost 40% of adults and nearly 20% of youths are considered obese, and $<40\%$ of youths and $<25\%$ of adults achieve levels of physical activity that are considered optimal, increasing the likelihood of negative consequences for cardiovascular health (Benjamin et al. 2019). Furthermore, mutations in cardiac myofilament proteins may be associated with cardiomyopathy (Garfinkel et al. 2018; Gonzalez-Martinez et al. 2018; Maron and Maron 2013; Marques and de Oliveira 2016; Martins et al. 2015; Parvatiyar et al. 2010; Willott et al. 2010; Yotti et al. 2019) in as many as 1 out of every 200 individuals (Semsarian et al. 2015).

Regardless of cause, insult to the myocardium such as that associated with non-fatal myocardial infarction can reduce contractile function of the heart due to irreversible loss of cardiomyocytes (CMs). The re-establishment of the damaged or failing myocardium to its original functional capacity through regenerative processes is of great interest to researchers and clinicians, not to mention the affected patients and their families (Broughton and Sussman 2017). However, the adult mammalian heart does not naturally possess the ability to regenerate myocardium. This deficiency in regenerative capacity results from most adult CMs being unable to re-enter the cell cycle and complete cell division (Carvalho and de Carvalho 2010).

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In contrast to mammalian cardiac muscle, skeletal muscle has innate regenerative capacity. This is mainly due to the presence of satellite cells that participate in repairing the damaged tissue (Pannérec et al. 2012), but there are also fascinating and relevant distinctions between nuclei of skeletal and cardiac myocytes (Fig. 1). In skeletal muscle of adult mammals, typical fibers are multinucleated (for developmental reasons) and the nuclei are located on the periphery of the cell (Fig. 1); central location of nuclei within skeletal fibers due to regeneration or improper migration of the nuclei may be a sign of myopathy (Collins et al. 2017; Folker et al. 2011; Jungbluth and Gautel 2014; Mazzotti and Coletti 2016). On the other hand, most CMs in adult mammals are either mono- or bi-nucleated (Fig. 1), with only a small proportion of myocytes that have more than two nuclei (Laflamme and Murry 2011; Rumyantsev 1991). The dominant proportion of mono- or bi-nucleated CMs depends on species: for example, humans have a greater proportion of mono- than bi-nucleated CMs, while rodents have more binucleated CMs. Nuclei are typically located centrally in adult CMs (Fig. 1), which means that the nuclei are embedded within the myofilament lattice where they would be expected to experience the force associated with systolic contraction along with variations in intranuclear Ca^{2+} that accompany the cytoplasmic Ca^{2+} transient (Wu and Bers 2006). In addition to localization, the significance of the number of CM nuclei and ploidy are not known in healthy myocardium, but they appear to have significance for not just normal development but also for the possibility of cardiac regeneration in cardiac disease (Broughton and Sussman 2017; Laflamme and Murry 2011; Leone et al. 2018; Mohamed et al. 2018).

Considering the potential significance for human health if myocardial regeneration could be triggered, and the definitive significance of the nucleus in regeneration, we summarize in this review what is known about multinuclearity[1] of CMs as well as nuclear ploidy, and their relationship to cardiac development and physiology.

[1] We have chosen to use "nuclearity" to refer to the number of nuclei in a cell, paralleling its definition in chemistry. Note that "nucleation" has been used more commonly in the cell biology literature to refer to the number of nuclei in a cell and also the process of increasing the number of nuclei in a cell, while it has different meanings in chemistry and biochemistry. In this review, we retain the limited use of "binucleation" to refer to the *process* of increasing the number of nuclei in a cell from one to two. Furthermore, to avoid confusion with the class of blood cells termed "mononuclear," the commonly used, related terms "mononucleated" (instead of mononuclear), "binucleated," "tetranucleated" and "multinucleated" are used to describe cells with one nucleus, two nuclei, four nuclei, or more than one nucleus, respectively.

Ploidy

Role of nuclear ploidy in cardiomyocyte function

Diploid cells—the norm for most human cells—contain two complete sets of homologous chromosomes, while cells that possesses more than two copies of the haploid genome are considered polyploid. In addition to diploid and polyploid cells, there also exists an exception to the typical diploid status of normal human cells on the lowest end of the ploidy spectrum, i.e., cells with no nucleus: red blood cells normally lose their nuclei following differentiation and thus have no chromosomes when circulating in the cardiovascular system. The focus of this review is on cells—myocytes—that not only retain their nuclei but can become polyploid through fusion or initiation of DNA replication without the completion of nuclear division and cytokinesis. CMs become polyploid through failure of cytokinesis and endoreduplication, in contrast with skeletal muscle myocytes that become polyploid through cell fusion (Fig. 1) (Orr-Weaver 2015). Proposed biological advantages for polyploid cells consist of resistance to apoptosis, and genomic protection against mutations due to the presence of multiple copies (Hassel et al. 2014; Orr-Weaver 2015). Polyploidization occurs widely throughout nature and contributes to organogenesis through cellular hypertrophy, associated with increasing DNA content (Orr-Weaver 2015). However, ploidy number can be highly variable depending on developmental stage and species. Examples of normal, polyploid cell types include mammalian CMs, hepatocytes, and megakaryocytes. Interestingly, megakaryocytes are classified as obligate polyploid cells with one multi-lobulated nucleus, with continuously increasing DNA content as part of their normal life cycle and function of producing blood platelets (Zimmet and Ravid 2000).

Polyploidization is a normal part of development in many species, and in most mammalian species the transition from diploid to polyploid coincides with the decline of regenerative capacity. In CMs, polyploidization is associated with increased cell size postnatally and also with the blunted response to cardiac tissue damage. The degree of ploidization and abundance of polyploid CMs differs among species. During embryogenesis and early neonatal development, the majority of mammalian CMs are diploid and are capable of proliferation (Laflamme and Murry 2011). Transition to polyploid nuclei occurs at different points of development, depending on species. In mice, CMs withdraw from the cell cycle between postnatal days 4 and 21, resulting in an increased number of binucleated and polyploid cells (Fig. 1) (Soonpaa et al. 1996). Approximately 96% of adult mouse CMs are binucleated, and 30–60% of the mononucleated CM population

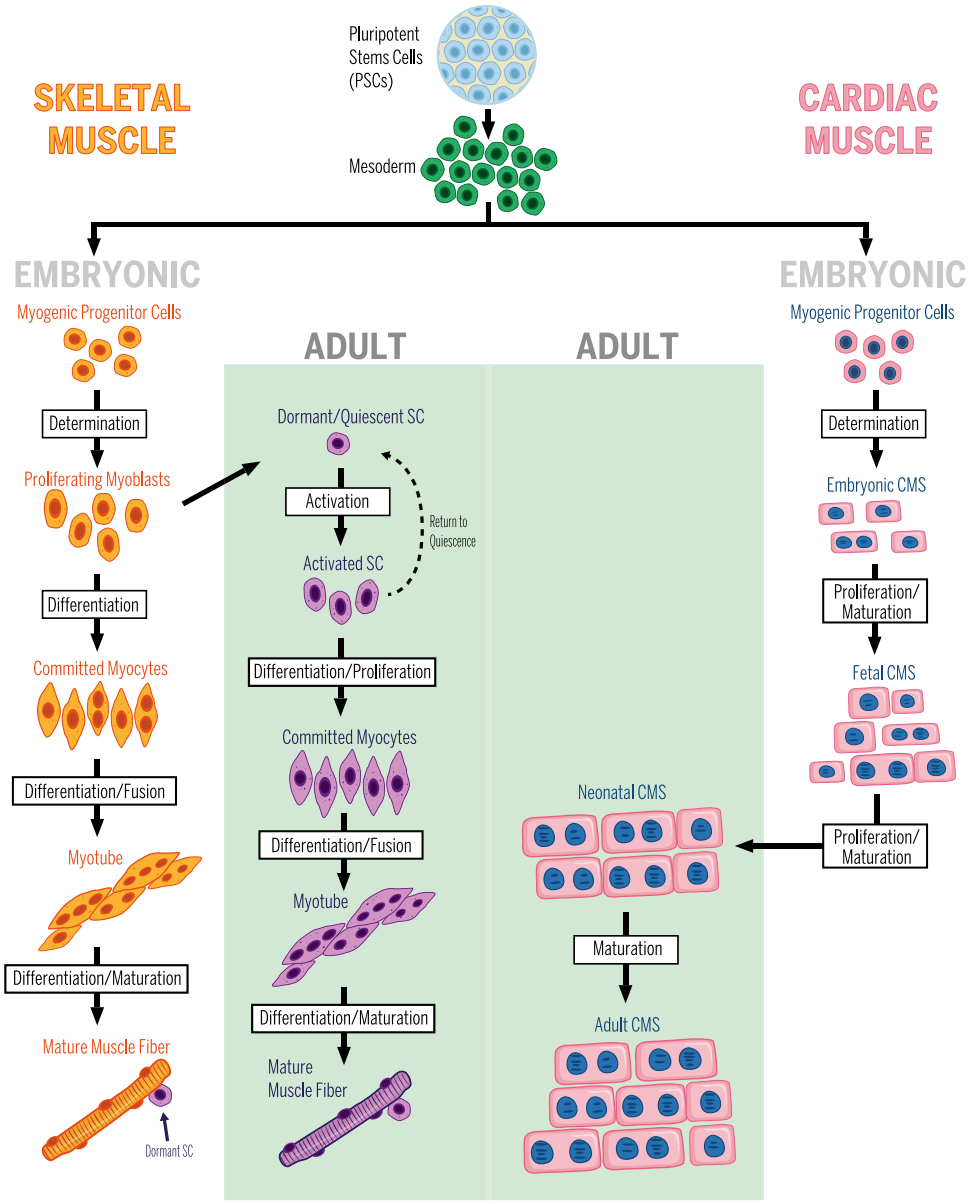

Fig. 1 Overview of skeletal and cardiac muscle myogenesis. Following the development of primordial germ lines (top), mesoderm-originated myogenic progenitor cells give rise to striated muscle cells through different processes. In regard to skeletal muscle development (left half of figure), myogenic progenitor cells undergo myogenic commitment, originating proliferative myoblasts that later differentiate into fusion-competent myocytes. The newly formed myocytes exit the cell cycle and initiate a fusion process including cell alignment and rearrangement of actin cytoskeleton at contact sites followed by membrane fusion, resulting in a multinucleated cell called a myotube. Myotube nuclei are initially located in the center of the cell. As contractile filaments are organized into sarcomeres, the nuclei are displaced from the center to the peripheral region of the cell. Addition of myocytes to the myotubes followed by a cascade of molecular events finally result in a mature myofiber. During myogenesis, part of the myoblast population does not proceed to cell fusion and differentiation, each remaining in the tissue as a quiescent satellite cell (SC) located outside of the sarcolemma, e.g., within the muscle cell's basement membrane. Upon muscle damage, these cells can be reactivated and differentiated into newly formed, fusion-competent myocytes. In regard to cardiac muscle development (right half of figure), myogenic progenitor cells commit to the CM cell fate through temporal expression of different transcription factors and epigenetic regulators. During the embryonic phase, CMs show robust proliferative capacity and are usually small, mononucleated, and have myofibrils that are sparse and irregularly organized. Embryonic CMs proliferate and mature into fetal CMs, which become elongated with well-developed myofibrils and sarcomeres. After birth, neonatal CM proliferative capacity significantly decreases. Depending on the species, it is around this time that a majority of CMs exit the cell cycle (transitioning from hyperplasia to hypertrophy), becoming binucleated and polyploid. Maturation of neonatal CMs results in adult CMs with densely packed and well-organized bundles of myofibrils with clearly defined sarcomeres

is tetraploid (Leone and Engel 2019). This transition coincides with the loss of regenerative potential that occurs within the first week of postnatal life in mice (Porrello et al. 2011). Almost all human CM cells are diploid during the first years of life (Bergmann et al. 2015). In the second decade of life there is a switch from cytokinesis to polyploidization, resulting in an increase in the amount of DNA content per nucleus (Bergmann et al. 2015). Cardiac regeneration in humans is very limited after birth and is insufficient to restore contractile dysfunction after cardiac damage. Withdrawal from the cell cycle results in 60% of adult human CM nuclei being tetraploid (Adler and Costabel 1975). During the second decade of life, nuclear ploidy of CMs in the left ventricle increases by 1.7-fold, and those in the right ventricle by 1.6-fold (Bergmann et al. 2015). DNA synthesis can also be re-initiated in response to cardiac stress and overload, including hypertension, myocardial infarction, and post-surgery (Anatskaya and Vinogradov 2007; Orr-Weaver 2015).

Expression of different cell cycle regulators has been shown to influence the process of polyploidization in CMs. For instance, the expression of cyclin-G1 during postnatal development in mice correlates with the onset of increased ploidy, and overexpression of cyclin-G1 resulted in increased DNA synthesis and delayed mitosis (Liu et al. 2010). Many investigators have also noted the transition of CMs from diploid to polyploid often coincides with the decline of regenerative capacity. Although adult mammalian CMs do not possess the innate ability to proliferate, zebrafish maintain efficient CM regeneration throughout their lifespan through proliferation of preexisting CMs (Jopling et al. 2010). CM nuclear ploidy number is one noteworthy difference among species. Interestingly, adult zebrafish CM cells are mostly mononucleated and diploid, and also retain the ability to proliferate (Matrone et al. 2017; Wills et al. 2008). Recently, Gonzalez-Rosa et al. (2018) provided evidence that polyploidization of CMs impaired regenerative properties. The group developed a transgenic zebrafish model with increased CM ploidy by overexpressing a regulator of cytokinesis and proto-oncogene, Ect2, which is phosphorylated by Cdk1. The polyploid zebrafish CMs have a diminished ability to proliferate in response to tissue damage. When the group induced the expression of Ect2 in all CMs and injured cardiac tissue, there was formation of scar tissue and the CMs were no longer capable of proliferation. Similarly, Patterson et al. (2017) identified *Tnni3k* as a gene that impacts the ploidy and proliferation of CMs. They developed a *Tnni3k* knockout mouse strain, which resulted in CMs that were mononucleated and diploid. The *Tnni3k* knockout mice displayed increased CM proliferation after injury. These results provide compelling evidence that polyploidization is likely to be an important factor in the loss of regenerative potential in hearts.

The relation between cell ploidy and regeneration are cell-type specific. For instance, the liver contains a mixture of hepatocytes with cells that are diploid or polyploid. Hepatocytes contribute to the remarkable regeneration of damaged liver segments through proliferation with or without ploidy reversal (Duncan et al. 2010). Like CMs, hepatocytes become polyploid postnatally through failure to complete cytokinesis and endoreduplication (Margall-Ducos et al. 2007). While hepatocytes have an innate regenerative capacity, it has been suggested that the polyploid state has little effect on liver regeneration, metabolism or function (Zhang et al. 2018). Instead, increases in ploidy number of hepatocytes were associated with tumor suppressive functions (Zhang et al. 2018). Uncovering the roles of ploidy in different cell types and tissues provides insight into the potential roles and barriers DNA content might pose in cell proliferation.

We should also consider the normal roles that different cell types carry out, and the consequences of cell division on function. CMs are responsible for the contractile function of the heart, and their division (Senyo et al. 2013) would require disassembly of sarcomeres—structures that are required for contraction. This energy-consuming disassembly of the contractile proteins and myofibrils takes place in two steps: first the Z-disk and actin filaments disassemble, then the M-bands and myosin filaments break down (Ahuja et al. 2004). After cell division has occurred, the myofibril proteins return to their cross-striated patterns (Ahuja et al. 2004). The disassembly of the sarcomeric structure during CM division could potentially compromise force generation and contraction. Therefore, elevated ploidy number in CMs may be beneficial because it allows for CM growth while still maintaining contractile function. It has been suggested that polyploidization that occurs around the switch from CM proliferation to hypertrophy after birth is an adaptation to increase the transcriptional output during a period that requires energy and growth for organogenesis of the heart (Vivien et al. 2016). Further research into the role of ploidy in cardiac regeneration will improve our understanding of the impact of DNA content in proliferation. It could turn out to be important to devise therapeutic methods that will amplify the scarce population of diploid CMs if that stimulates heart regeneration in different disease states.

Nuclearity

Cardiomyocyte nuclearity throughout development

Although binucleated cells are commonly observed in tissues such as liver only under pathological conditions, the normal heart contains not only mononucleated CMs but also binucleated CMs and a smaller percentage of multinucleated

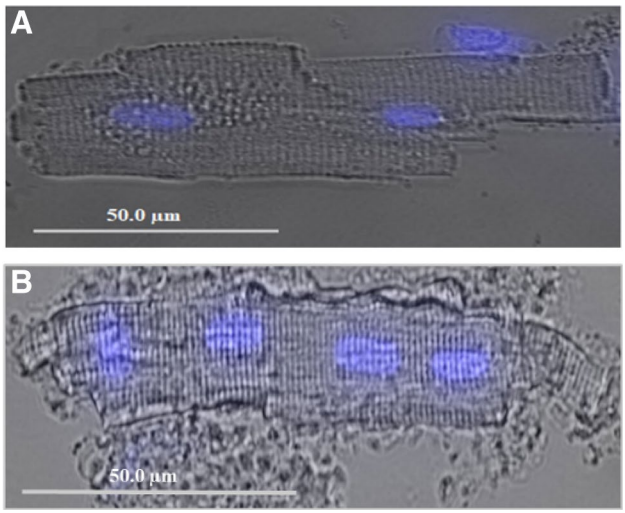

Fig. 2 Single **a** binucleated and **b** multi- (tetra-) nucleated cardiomyocytes (CMs) isolated as described (Martins et al. 2015) from wild-type mouse hearts by Karissa Dieseldorff Jones and Jennifer Le Patourel. CMs were fixed with formalin, permeabilized with IGEPAL (0.5% in relaxing buffer), and stained with NucBlue (Invitrogen). Brightfield (striations) and DAPI fluorescence (purple nuclei) images for each CM were obtained sequentially at the same calibrated magnification and without moving the sample, and merged digitally. Imaging was performed using an Olympus BX61 fluorescence microscope with Olympus DP71 color digital camera in the Biological Science Imaging Resource (BSIR) in Florida State University's Department of Biological Science. Note that the extra nucleus in **a** (top, right) was from another CM. Also note that sarcomere length of the CM in **b** is shorter than physiological because the preparation had been subjected to thermal cycling; this suggests that the length of the living CM would have been almost twice as long. (Color figure online)

CMs with more than two nuclei (Fig. 2). The number of nuclei has little or no effect on the functionality of hepatocytes (Tormos et al. 2015) or CMs. During human heart development, the majority of the CMs are mononucleated and exhibit high proliferative patterns (Paradis et al. 2014). These embryonic CMs arise from cardiac precursors that underwent cell differentiation and later the division of existing CMs (Fig. 1) (Foglia and Poss 2016). To compensate for the continuously increasing blood flow demand, the preexistent CMs undergo hyperplasia, in which CMs successfully complete both karyokinesis and cytokinesis. (Paradis et al. 2014). However, briefly after birth, CMs initiate a final cell cycle completing only karyokinesis, but fail to complete cytokinesis. This uncoupling of cytokinesis from karyokinesis results in an increased number of binucleated CMs that have exited the cell cycle and became terminally differentiated (G_0) (Clubb and Bishop 1984; Elhelaly et al. 2016; Li et al. 1996; Paradis et al. 2014; Rumyantsev 1977; Soonpaa et al. 1996; van Amerongen and Engel 2008). In rodents, binuclearity occurs only within the first 14 days of life as a consequence of mitosis without cell division, instead of fusion of two individual mononucleated CMs (Clubb and Bishop 1984; Li et al. 1996). The presence of more than one nucleus in CMs is widely considered to be an indicator of a transition from hyperplastic to hypertrophic growth and is directly related to CM hypertrophy associated with sarcomere addition that enhances cardiac hemodynamics (Clubb and Bishop 1984; Oparil et al. 1984; Soonpaa et al. 1996; Zebrowski and Engel 2013). Coincident with CMs becoming binucleated and withdrawing from the cell cycle, these cells lose their proliferative capability and thus organ size increases through hypertrophy of existing CMs (Bugaisky and Zak 1979; Clubb and Bishop 1984; Elhelaly et al. 2016; Li et al. 1996; Oparil et al. 1984; Soonpaa et al. 1996; Walsh et al. 2010). Altogether, the results demonstrate that binucleated CMs no longer proliferate even though mononucleated CMs continue to exhibit proliferative ability (Miko et al. 2017; Paradis et al. 2014).

In adult mammalian hearts, the ratio of binucleated to mononucleated CMs varies from one species to another. Of the CM population in adult rodent hearts, 90% are binucleated while in human adult hearts this percentage is markedly lower, varying from 25 to 60% (Botting et al. 2012; Paradis et al. 2014). Mononucleated CMs in adult rodent hearts are able to re-enter the cell cycle, producing either mono- or bi-nucleated CMs (Bersell et al. 2009). Interestingly, binucleated CMs isolated from chicken heart can become tetranucleated CMs after both nuclei undergo karyokinesis (Li et al. 1997a). The population of mononucleated CMs in human adult heart exhibits low or no regenerative capacity, while in adult zebrafish, 99% of CMs are mononucleated and exhibit high regenerative capacity (Gonzalez-Rosa et al. 2018). Hypoxia, which can be a major stressor during development, has been linked to early binuclearity in rodent CMs, which increased apoptosis (Bae et al. 2003) and negatively affected proliferation (Tong et al. 2013). In contrast, in zebrafish, hypoxia induces proliferation which suggests that hypoxia could play a dual role regarding cell proliferation (Jopling et al. 2012; Paradis et al. 2014). Studies on fetal sheep suggested that hypertension, induced by intravascular plasma infusion into the fetal circulation, influences CM proliferation in the fetal heart (Jonker et al. 2007). After 4 days of fetal hypertension, the rate of CM proliferation was increased relative to control, as was the population of mononucleated CMs. After 8 days, however, the number of binucleated CMs increased and proliferative capacity was lost (Jonker et al. 2007).

DNA synthesis

DNA synthesis has central and obvious importance for cell cycle completion. It was previously reported that CM DNA synthesis in the fetal mouse heart is exclusively related to cell proliferation, although it becomes associated with binuclearity soon after birth (Soonpaa et al. 1996). To explain the

observation that hypertrophied tissues typically have greater DNA content, Miko et al. (2017) suggested that duplication of the nucleus could be more energetically favorable than generating a daughter cell after successfully undergoing mitosis since, under some circumstances, there may be functional advantages for larger cells compared to a greater number of small cells. Increasing DNA content could be explained by the high protein synthesis demand, and associated high metabolic activity, of CMs undergoing hypertrophy and adding more sarcomeres, expanding their dimensions in both length and volume (Fig. 1) (Ahuja et al. 2007b; Frawley and Orr-Weaver 2015; Lee et al. 2009; Miko et al. 2017; Orr-Weaver 2015). Furthermore, CMs can undergo DNA endoreplication as part of a response to cellular stress; such a mechanism would be expected to improve the overall cellular response and enhance cell survival through restoration of function (Anatskaya and Vinogradov 2007; Meckert et al. 2005).

Molecular events associated with cardiomyocyte binucleation

While considerable effort has been dedicated to identifying the functional significance of CM binuclearity, the molecular mechanisms that lead to this outcome are not well understood. A variety of explanations for CM binucleation have been suggested. Ahuja et al. (2007a) reported that binucleation could be the result of downregulation of components involved in actomyosin ring formation. Li et al. (1997b) observed that the presence of intact myofibrils in the equatorial cortex could physically impair furrowing of the cell membrane by the actomyosin ring. Engel et al. (2006) showed mislocalization of anillin due to its incorrect recruitment to the cortex and subsequently to the midbody caused actomyosin ring disassembly, which led to CM binucleation. Leone et al. (2018) associated CM binucleation with alterations in cellular localization and recruitment of IQGAP3, non-muscle myosin IIB, and RhoA. Additionally, they also observed mitotic microtubule apparatus disorganization, diffuse localization/patterns of γ-tubulin and PCM1—indicating alterations in mitotic MTOC—and disorganized EB1 pattern at the central spindle, which is an indicative of loosely packed microtubules. Thus, these alterations were associated with defective cleavage furrow ingression with absence or formation of a one-sided cleavage furrow, consequently leading to CM binucleation (Leone et al. 2018). Finally, cell senescence regulator SIRT1 has been shown to protect CMs from apoptosis and regulate CM binucleation. Inhibition of SIRT1 activity was found to decrease CM binuclearity in rats during cardiac development; in contrast, overexpression of SIRT1 increased overall CM size (Shin et al. 2018; Sundaresan et al. 2011).

Studying cell division in binucleated cells is experimentally challenging. Aurora B-kinase is often used as a marker for cell division because of its role in connections between centromeres and the mitotic spindle. However, aurora B-kinase is not an unambiguous marker for cell division in CMs because it also localizes to midbodies between the nuclei in binucleated cells (Hesse et al. 2018a). Therefore, one possibility for studying cell division could be to focus on the location of the midbody: in binucleated cells, the midbody is asymmetrically positioned between the two nuclei, while during authentic cell division, the midbody is found midway between the daughter nuclei (Hesse et al. 2018a). It was also suggested that measuring the distance between adjacent nuclei provides a more precise distinction between binucleation and cell division because the nuclei in binucleated cells are closer to each other compared with the larger distance between the nuclei of two individual cells (Hesse et al. 2018a).

Does the myonuclear domain concept in striated muscle fibers apply to cardiomyocytes?

The concept of the myonuclear domain (MND) is related to the volume within a myocyte that is governed by an individual myonucleus within a multinucleated cell. In contrast with cardiomyocytes (Fig. 2), mature skeletal muscle fibers can have a large number of nuclei. The origin of this high degree of multinuclearity in skeletal muscle stems from fusion of mononuclear myoblasts during development, and retention of those functional nuclei throughout maturation of the skeletal fiber (Fig. 1). Additional nuclei can be added to mature skeletal fibers, e.g., during tissue repair, by fusion of so-called satellite cells (Fig. 1) which are a type of stem cell residing within the muscle cell's basement membrane (Yablonka-Reuveni 2011). Helen Blau and colleagues demonstrated that many products from each nucleus of a skeletal muscle cell remain in the region around that myonucleus, which reinforces the idea of a single myonucleus supporting its surrounding area (Pavlath et al. 1989). Experimentally, average MND size for a skeletal muscle fiber can be simply quantified as the volume of the fiber divided by the number of nuclei in that fiber; alternatively, individual MNDs can be defined and assessed by using an image analysis algorithm to rigorously estimate the boundaries between adjacent nuclei in a fiber. Experimentally measured MND is likely to be an underestimate of the true, functional MND because there are no fixed compartments separating adjacent MNDs in a skeletal muscle fiber and product distribution within the cell is limited by diffusive and active transport; thus the volume that is actually influenced by a nucleus will overlap with MNDs of nearby nuclei. Even with this caveat, the MND concept is useful for helping us to appreciate the volume

of a myocyte that an individual nucleus can support and maintain.

A comparative study by Lars Larsson and colleagues quantified MND size in single skeletal muscle fibers from six adult mammalian species covering a wide range of body mass: mouse, rat, human, pig, horse and rhinoceros (Liu et al. 2009). Within a species, MND size is highly dependent on muscle fiber type, with the largest MND typically found in fibers that express the fastest myosin isoforms. There is also a general correlation of MND with body mass, although there was little or no difference among MNDs in the same fiber type in mice, rats and humans. It is not clear what regulates MND size in skeletal muscle. Myosin isoform expression and mitochondrial proteins—both related to fiber type—are correlated with MND size (Liu et al. 2009). MND size may not be absolutely and rigidly determined for a given muscle as there is variation that occurs during skeletal muscle hypertrophy (Murach et al. 2018). It is not known if enlargement of cytoplasm due to hypertrophy leads to incorporation of new myonuclei or if the pre-existent nuclei are able to increase their protein synthesis supporting larger surrounding area. Resolution of this apparent "chicken and egg" (i.e., what comes first?) problem in skeletal muscle, however, should eventually provide insight into the molecular mechanisms that control MND size (Teixeira and Duarte 2011).

We wondered if the MND size concept from skeletal muscle might be relevant to mono- versus bi-nuclearity in CMs. It is possible that the greater proportion of binucleated CMs in adult rodent hearts, in comparison with adult humans, could be directly associated with the faster heart rate and higher overall metabolic rate (and protein turnover). Even if we do not know the molecular mechanisms that control MND size in skeletal muscle, it seems possible—and perhaps likely—that a single nucleus might not able to accommodate the high cellular demand of most CMs in adult rodent heart, while a single nucleus may be sufficient for the lower cellular demand of most adult human CMs. The quantitative data in Table 1 of Bensley et al. (2016) allow us to compare CM volumes among mono- versus bi-nucleated CMs in juvenile (or fetal) versus adult mouse, rat, rabbit and sheep hearts. Their data are highly supportive of the notion that the MND concept from skeletal muscle might be applicable in the heart, albeit on a smaller scale (i.e., smaller numbers of nuclei per myocyte). First, all of the average volumes of mononucleated CMs reported in Bensley et al. (2016) are smaller than average MND size from slow skeletal muscle fibers reported in Fig. 5 of Liu et al. (2009). Second, all of the average volumes of binucleated CMs are larger than those of mononucleated CMs from the same hearts. As shown in Fig. 3, average volumes of mono- and bi-nucleated CMs measured by Bensley et al. (2016) from the same hearts are highly and linearly

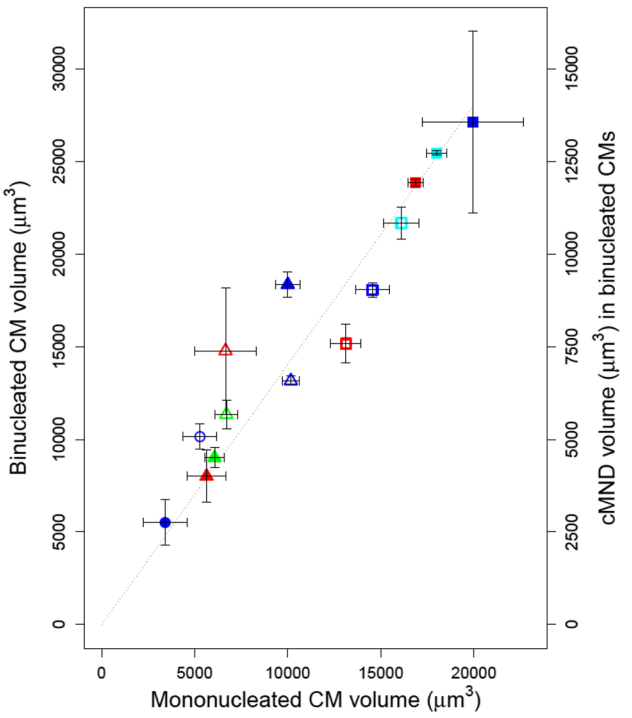

Fig. 3 Relationship between mono- and bi-nucleated CM volumes in ventricular tissue samples from mouse (red), rat (cyan), rabbit (green) and sheep (blue). Data were plotted from Table 1 in Bensley et al. (2016) with consent of Drs. Bensley and Black; their original work (Bensley et al. 2016) was published under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0). Average values from left ventricle (filled symbols) and right ventricle (open symbols) were plotted separately. Error bars represent SEM. Symbol shape indicates age: circle (fetal, sheep); triangle (weanling, mouse and rabbit; 9-week-old, sheep); square (adult, mouse, rat and sheep). The line is the unweighted, linear least squares regression, constrained to pass through the origin, on all of the data combined ($y = 1.40x$; $R^2 = 0.869$). The right vertical axis shows the cardio-myonuclear domain (cMND) size for binucleated CMs (half the CM volume on left vertical axis); note that cMND size for mononucleated CMs is equal to CM volume (horizontal axis). (Color figure online)

correlated ($R^2 = 0.869$), and unweighted linear least squares regression indicates that binucleated CM volume is larger by ~40% (this value applied to the three samples from adult left ventricles, 36–42%); in five of the 14 samples (all fetal or juvenile), binucleated CM volume is larger by approximately twofold (62–122%). Third, both the average volume and percentage of binucleated CMs increased with developmental stage for each organism, although for the three animals studied, the minimum percentage of binucleated CMs was 80% and thus none of the samples examined by Bensley et al. (2016) are comparable to the lower percentage of binucleated CMs reported in humans.

A very simple possibility is that two nuclei are necessary to support the higher metabolic requirements of CMs that reach a certain size—as suggested by experiments on yeast

(Zhurinsky et al. 2010)—and that the cardio-myonuclear domain (cMND) size (Fig. 3) may be slightly smaller than, but overall not very different from MND size found in slow skeletal muscle cells (Liu et al. 2009). This might suggest that a single nucleus is sufficient to support the entire volume of most CMs in larger mammals such as humans where heart rate, and thus metabolic needs, are lower even if CM size is similar to that in smaller mammals. An alternative, although not necessarily mutually exclusive possibility is that one nucleus in a binucleated CM is "dominant" over the other nucleus. At one extreme, the non-dominant nucleus might be almost inactive; at the other end of the spectrum of possibilities, the role of the dominant nucleus might be something along the lines of coordinating the two nuclei rather than supporting the entire volume of a binucleated cardiomyocyte. The possibility that binucleated CMs have a dominant nucleus, and its role, should be examined. Furthermore, it appears clear to us that it would be worthwhile to rigorously test whether CM volume and cMND size, along with CM metabolic rate (Gude et al. 2006) and mechanical factors that would increase with CM size such as force generation, could be significant determinants—albeit probably not the only ones—of whether a CM remains mononucleated or becomes multinucleated. In doing so, it should be instructive to compare the structures and metabolic needs of cMND—where the nucleus is embedded within myofibrils and the cardiomyocyte contracts periodically—with skeletal muscle MND—where the nucleus is located at the edge of the myocyte and myofilament activation is, in most instances, sporadic.

Cell cycle and regeneration

At different developmental stages in various vertebrates, the regenerative properties of CMs differ and thus the heart responds differently to cardiac lesion. Regenerative capability per se is only observed in mammalian hearts during the embryonic stage (Hesse et al. 2018b; Porrello et al. 2011). Neonatal rodent hearts maintain some regenerative capacity strictly during the first week of life, and after an insult exhibit minimal signs of scar tissue formation which would otherwise indicate incomplete regeneration or compensatory growth. Through genetic fate-mapping studies, it was observed that preexisting CMs proliferate and partially regenerate the damaged area, gradually restoring the majority of cardiac morphology (compensatory growth) as well as number of cells (hyperplasia). CMs located farther from the border zone (i.e., the border delimiting tissue affected by an insult from adjacent, healthy tissue) also exhibited cell cycle activity which suggests that there could be some form of communication among the CMs (Hesse et al. 2018b; Porrello et al. 2011). The ability of CMs to duplicate or proliferate ceases during late fetal life as the expression levels of genes associated with these events are dysregulated (Soonpaa and Field 1997, 1998). The irreparable loss of CMs after cardiac injury in adult hearts is clinically relevant because of loss of contractile function of the heart generates collagen-containing scar instead of CMs (Hesse et al. 2018b; Virag and Murry 2003). Unlike adult zebrafish CMs—which can de-differentiate, re-enter the cell cycle and successfully complete cytokinesis in response to damage—adult mammalian CMs re-enter the cell cycle but do not complete cytokinesis (Heineke and Molkentin 2006; Jopling et al. 2010; Mercola et al. 2011). Upon re-entering the cell cycle, mononucleated mammalian CMs become either mononucleated and polyploid due to endoreduplication, or binucleated and polyploid. Despite these effects on CMs in the mammalian border zone, neither structure of the heart nor number of cells are fully restored (Hesse et al. 2018b).

During late stages of neonatal life, pro-mitotic regulators such as cyclins and cyclin-dependent kinases (CDKs) are downregulated. On the other hand, during adulthood, anti-mitotic regulators such as cyclin inhibitors p21, p27, and p57 are upregulated (Hesse et al. 2018b; Kang et al. 1997; Poolman et al. 1998; Soonpaa et al. 1996; Tane et al. 2014). Dysregulation of several pro- and anti-mitotic genes associated with the cell cycle plays an important role in CM terminal differentiation, which contributes to the transition phase hyperplasia-to-hypertrophy followed by cell cycle arrest (Matrone et al. 2017; Paradis et al. 2014). Leone et al. (2018) have summarized a number of hypotheses that could explain cell cycle arrest in CMs such as downregulation of proteins known for regulating cell cycle, DNA damage caused by the oxygen-rich postnatal environment, and developmental alterations in centrosome integrity.

In a clever approach to investigate CM turnover in the mature human heart, Bergmann et al. (2009) used ^{14}C dating—from radionuclides generated by above-ground nuclear testing during the Cold War and incorporated into CM DNA—to estimate CM age in hearts from normal, mature individuals who died at ages from 19 to 74 years old. They estimated that CM turnover rates are ~ 1%/year at age 25, and the rate further decreases to ~ 0.45%/year around 75 years old. Consequently, it can be estimated that almost one half (~ 45%) of CMs in the human heart are replaced over the normal human's adult lifespan (Bergmann et al. 2009, 2015). Studies on mice indicate that new CMs result from division of existing CMs in adult hearts (Senyo et al. 2013).

Conclusions and future directions

Our title asks when multinuclearity and/or elevated ploidy of a CM limit the cell's capabilities beyond contractile function. The information discussed herein points toward greater

amounts of chromatin being beneficial for maintaining CM metabolism, which is a key requirement for contractile function. But—adversely for repair and renewal—the preponderance of evidence also indicates a relationship between "extra" DNA and a reduced ability for CM replication. To be able to repair damaged and failing hearts—a major part of the "trouble" referenced in the title—it is clearly important to understand how cardiomyocytes in mammals transition from having the ability to readily replicate during development, to essentially having lost that ability at maturity. The interrelationship among age, CM size, the average number of nuclei per CM and the typical number of chromosome copies—and their variation among species—is central to understanding this significant transition. While there is not yet a definitive answer to the question posed in our title, it is possible that "extra" chromatin is not as problematic as postulated previously—correlation does not always demonstrate causality—and multinuclearity and/or elevated ploidy of CMs could actually be beneficial for some aspects of cardiac repair (Broughton and Sussman 2017; Leone and Engel 2019). Several molecules such as those involved in cell cycle regulation have been identified that could provide control of this transition, although no single master regulator has been found. It could turn out that some aspects of the "trouble" are simpler in nature, and we suggest that causal factors associated with cell size such as metabolic load and force generation could also, through more general signaling pathways, predispose a CM to undergo such a transition that places an upper limit on cardiomyocyte myonuclear domain size.

One set of mechanochemical factors that have not yet been extensively explored, but could play a role in the changes associate with maturation of CMs, are sarcomeric proteins that localize to the nucleus, including troponin, tropomyosin and SUMOylated actin (Asumda and Chase 2012; Chase et al. 2013; Hofmann et al. 2009; Johnston et al. 2018; Serebryannyy et al. 2016), along with various isoforms of myosin including non-muscle isoforms (de Lanerolle and Serebryannyy 2011; Keeling et al. 2017). While the role(s) of sarcomeric proteins in the nucleus are not known, they should be considered for possible roles in establishing the number of nuclei and perhaps even ploidy of a nucleus. Actomyosin networks within the nucleus, along with lamins (Brayson and Shanahan 2017), are involved in nuclear mechanics (Keeling et al. 2017), and in principle they could be regulated by nuclear Ca^{2+} in CMs (Resende et al. 2013; Wu and Bers 2006). Furthermore, the localization of CM nuclei within the myofibrils (in contrast to skeletal muscle nuclei that are located peripherally) enables them to be integrators of both Ca^{2+} and tension generation. Where might this all lead? We note that the troponin T (TnT) subunit of the Ca^{2+} regulatory protein troponin has been shown to be involved in gene regulation by an epigenetic mechanism in CMs (Cole et al. 2016; Wu et al. 2015) and by interaction with a specific DNA sequence in skeletal muscle (Nunez Lopez et al. 2018; Xu et al. 2017) which has startling implications for skeletal muscle function (Pinto et al. 2017; Zhang et al. 2016). We look forward to seeing the resolution to these tantalizing questions.

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