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Cardiomyocyte Polyploidy and Implications for Heart Regeneration

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Abstract

In mammals, most cardiomyocytes (CMs) become polyploid (they have more than two complete sets of chromosomes). The purpose of this review is to evaluate assumptions about CM ploidy that are commonly discussed, even if not experimentally demonstrated, and to highlight key issues that are still to be resolved. Topics discussed here include (*a*) technical and conceptual difficulties in defining a polyploid CM, (*b*) the candidate role of reactive oxygen as a proximal trigger for the onset of polyploidy, (*c*) the relationship between polyploidization and other aspects of CM maturation, (*d*) recent insights related to the regenerative role of the subpopulation of CMs that are not polyploid, and (*e*) speculations as to why CMs become polyploid at all. New approaches to experimentally manipulate CM ploidy may resolve some of these long-standing and fundamental questions.

INTRODUCTION

CM: cardiomyocyte

1x4n: having one tetraploid nucleus

2x2n: having two diploid nuclei

Typical cells are diploid; i.e., they have exactly two complete sets of chromosomes. In contrast, almost all adult ventricular cardiomyocytes (CMs) in mammals are polyploid. Polyploidy in CMs includes cells that have a single tetraploid (or higher ploidy level) nucleus, multiple diploid nuclei, or multiple tetraploid (or higher) nuclei. Polyploidy is distinct from situations such as trisomy or aneuploidy of individual chromosomes, which have a different cause and a different biological significance.

CM polyploidy arises by a process known as endoreplication (1, 2), in which the genome of a diploid cell is replicated in the S-phase of the cell cycle but followed by interruption in mitosis. Interruption can occur either before nuclear division (before karyokinesis), resulting in a single tetraploid nucleus (this state is designated as 1x4n; n is the haploid chromosome complement), or after karyokinesis but before completion of cytokinesis, resulting in a binucleated 2x2n state (one cell with two diploid nuclei). Further cycles of endoreplication increase the number of nuclei or the number of chromosome sets per nucleus in the same manner (**Figure 1**). The polyploid state is not unique to CMs; a number of other cell types have a high frequency of polyploidy that is reached by endoreplication in the same manner (e.g., adult hepatocytes and fetal extraembryonic trophoblasts; selected cell types in the lung, kidney, and pancreas are also polyploid but at lower frequency). Polyploidy is also seen in a variety of cell types in invertebrate animals and in plants (1). Endoreplication is distinguished from cell fusion, such as occurs in skeletal muscle, which also results in a polyploid (multinucleated) state.

Mammalian species differ as to which specific polyploid state is more common in normal (healthy young adult) CMs. Mouse and rat CMs, for example, are primarily 2x2n (3–6), whereas human CMs are more typically 1x4n (7–9). CMs with four or more nuclei are present at a low

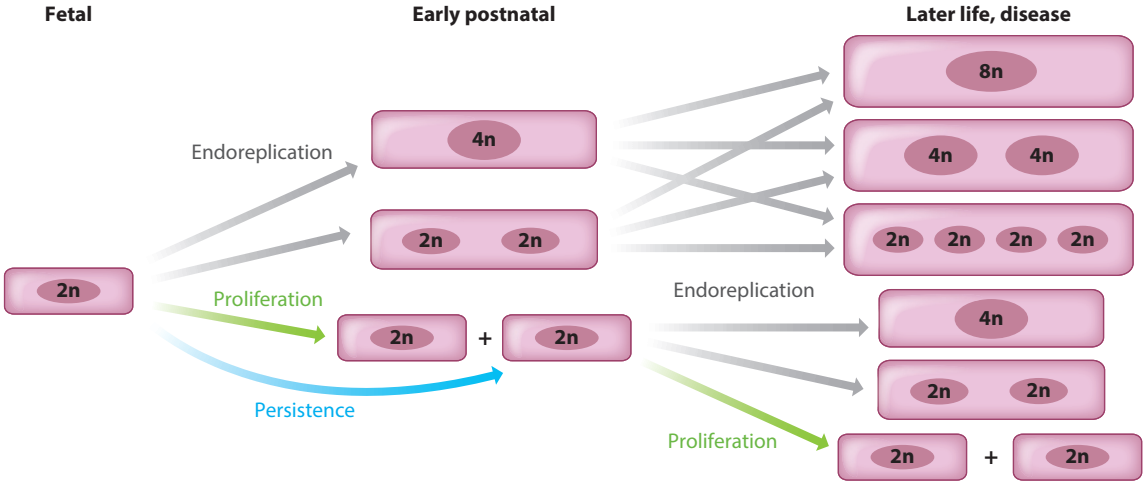

Figure 1

Timing and consequences of events that modify mammalian cardiomyocyte (CM) ploidy. Essentially all late fetal CMs are mononuclear and diploid (1x2n). In the early postnatal period, most CMs undergo endoreplication to become either 1x4n or 2x2n; a small percentage either replicate their DNA and complete mitosis to generate additional 1x2n CMs (i.e., proliferate) or persist without DNA replication as 1x2n CMs. Throughout postnatal life, a variety of circumstances induce CM cell cycle entry. Endoreplication results in CMs with higher levels of ploidy or in newly polyploid CMs. Completion of mitosis results in new 1x2n CMs (proliferation, which in an injured heart is equivalent to regeneration). Not illustrated here (but described in the text) are circumstances in which a previously polyploid cell divides into new 1x2n CMs or events that result in an uneven distribution of chromosomes (aneuploidy) in daughter nuclei.

to moderate frequency in mouse and human hearts, whereas normal pig (10) and rabbit hearts have a high proportion of CMs with as many as 8–16 nuclei. This variation across species suggests that the common feature of adult mammalian ventricular CMs is to be polyploid, regardless of the exact manifestation that this assumes. The observation that the natural ratio of mononuclear tetraploid and binucleated CMs varies across mammalian species, and even varies within a species, such as among different inbred mouse strains (11), demonstrates that the propensity of an endoreplicating CM to arrest either before or after karyokinesis is subject to genetic control. It remains unknown if there are any significant differences between these different subtypes of polyploid CMs. Regardless, these variations of polyploid CMs are distinguished as a group from the small percentage of adult ventricular CMs that are mononuclear and diploid (1x2n).

1x2n: having one diploid nucleus

E: embryonic day

P: postnatal day

CM polyploidy has been recognized for decades as the typical state of mammalian ventricular CMs, yet the specific features that regulate this process in CMs and how they interact with the cell cycle and mitotic machinery are only just becoming apparent. Moreover, with new technologies and insights, the biological significance of CM polyploidy is now at least amenable to direct experimental evaluation, even if a full understanding is still not in hand. The goal of this review is to summarize key selected features related to CM polyploidy, including its relationship to CM proliferation and heart regeneration, to critically evaluate some of the assumptions about CM ploidy that have been commonly discussed even if not formally demonstrated and to highlight some of the key issues that are still to be resolved.

THE TIMING AND EXTENT OF CARDIOMYOCYTE POLYPLOIDIZATION

CMs do not begin in development as polyploid cells. In all species that have hearts, very early in development, mononuclear diploid mesodermal progenitors differentiate into an early pool of mononuclear diploid CMs (12). Following this early differentiation phase, further heart growth occurs by the proliferation of preexisting CMs, rather than by continued progenitor differentiation. In mice, progenitor differentiation is completed by embryonic (E) day E10, and rapid CM proliferation ensues over the next several days, particularly in the ventricle wall. During this period, all CMs are 1x2n (mononuclear diploid). Thus, the fetal 1x2n CM state is associated with proliferation and growth. Measurement in mouse embryos of total CM cell numbers (13), as well as quantification of the CM proliferative index by incorporation of a nucleotide analog such as BrdU (14, 15), suggests that at least the majority of fetal CMs at this stage are mitotic with a cycle time of approximately 1 day. In other mammalian species, it is likely that a similar phase of rapid CM proliferation occurs, as in all cases the embryo itself is growing robustly during the corresponding period of heart development, and growth of the heart is required to maintain a progressively growing embryo (16). Thereafter (in mice starting from around E14), the rate of CM proliferation declines. In mice (6) and probably in most mammalian species, the rate of CM proliferation has dropped to minimal levels before or by the time of birth.

In mice and rats, although some binucleated CMs can be detected before birth, the vast majority become polyploid (mostly binucleated) midway during the first postnatal week (5, 6). Specifically, there is reactivation of cell cycle entry, with DNA replication peaking [in mice, at postnatal (P) day P4] and then declining. Because almost all mouse fetal CMs prior to birth are 1x2n, and almost all are polyploid by P7–P10, the majority of mouse CMs therefore appear to undergo endoreplication during this first postnatal week. Only a small percentage of CMs after P10 and for the remainder of life are 1x2n; it is still uncertain if these never entered cell cycle, or if these are the daughter cells of CMs that entered cell cycle, replicated their DNA, and then completed both karyokinesis and cytokinesis.

The timing of CM polyploidization is different in different mammalian species. In mice and rats, this occurs during the first postnatal week. Human and pig hearts both appear to initiate endoreplication during the last period of gestation [i.e., both are approximately 10% binucleated at term (17, 18)] with further polyploidization after birth (8). In lambs, polyploidization primarily occurs during the last few weeks of gestation with little additional polyploidization occurring after birth (19).

The number of mouse CMs that are in S-phase of the cell cycle is at minimal levels at P7 and beyond (6, 20), and because cell cycle activity is not further activated thereafter (at least at appreciable levels in normal biology), the adult number of 1x2n and polyploid CMs in mice can be measured accurately by P10 (M Patterson, H Sucov, unpublished observations). In rats, the adult number of polyploid CMs is reached by P12 (5). Because some CMs with >2 nuclei can be detected in neonatal mice as early as P3 (the earliest time we have examined; M Patterson, H Sucov, unpublished observations), and with increasing numbers of nuclei over the next week and beyond (20), it is likely that mouse and rat CMs can undergo multiple rounds of endoreplication in the weeks after birth. However, most CMs undergo only one round and then persist as such into later life. In adulthood, several disease states, including those associated with acute CM loss (myocardial infarction and ischemia-reperfusion) as well as those associated with gradual and progressive CM loss (e.g., several forms of heart failure) are accompanied by an increase in ploidy level in at least some of the remaining CMs (21, 22). This indicates that the capacity to reinitiate cell cycle and undergo further endoreplication remains present in adulthood, although it probably requires a stimulus associated with a pathological environment to cause this to occur.

Polyploid CMs are abundant in all adult mammals, although the ratio of 1x2n to polyploid CMs varies considerably; some mammalian species, for example, are reported to have over half of adult CMs as 1x2n (23), while the 1x2n level in others is as low as just 2% (11). Avians are similar in showing a moderately high level of polyploid CMs across species, in most cases also with a substantial 1x2n composition (24). In contrast, zebrafish and newt hearts have very few polyploid CMs and are overwhelmingly (>95%) composed of 1x2n CMs (25).

Because of the relevance of CM ploidy to heart regeneration (discussed below) and the major clinical impact of adult ventricular injury to impaired heart function, most attention in this subject has been focused on ventricular CM ploidy. In contrast, there has been relatively little attention given to the ploidy or regenerative status of atrial CMs. Atrial and ventricular CMs differ substantially in a number of parameters (e.g., ion channels and electrophysiology, gene expression and contractile components, etc.), and although based on only limited analyses, they may also have a widely different degree of overall ploidy (26, 27). To the extent that CM polyploidy has emerged as a timely and relevant topic, the relative absence of attention to atrial CMs stands as a significant gap in knowledge. It should be clear in the following discussion that unless stated otherwise, the subject is ventricular CM ploidy.

MAYBE NOT QUITE BINARY: TECHNICAL ISSUES IN DEFINING THE DEGREE OF POLYPLOIDY

With heart cells dissociated by enzymatic digestion and adhered to microscope slides, it is relatively easy to count the number of nuclei per cell, usually using a DNA stain such as DAPI, and numerous ways of visualizing CMs from non-CMs. A critical feature of adult CM biology that has been overlooked in some studies is their large and asymmetric size (>100 microns in length and >20 microns wide, for adult CMs) and thus their proclivity to become sheared into smaller pieces after mechanical manipulation (by pipetting or in passage under pressure through a small-bore nozzle in flow cytometry). A CM that was initially binucleated with two diploid nuclei (2x2n,

the most common ventricular CM in rodents) that becomes sheared into two smaller pieces, each retaining one nucleus, could be inappropriately scored as two 1x2n cells. Some discrepancies in reported numbers of mononuclear and multinuclear CMs are almost certainly based on this artifact. In our studies (11), we isolate living heart cells and include a fixable Live-Dead stain so that we only score cells that were alive at the time of fixation and immediately prior to plating on microscope slides. In an alternate procedure, heart tissue is first fixed and then dissociated into a single cell preparation by alkali digestion (28). Even fixed large cells can become broken into what appear to be smaller but still normal cells. For CMs, markers that are localized to intercalated discs and can be visualized at both ends of an isolated CM can help to define intact cells.

Several methods have been employed to define CM nuclear ploidy, and each has advantages and limitations. Probably the most common is based on nuclear fluorescence signal intensity. In this approach, isolated cells are stained with a DNA-intercalating dye (e.g., DAPI) and the fluorescence signal captured in a digital photograph and its intensity quantified using commonly available software (such as ImageJ). CM nuclear staining intensity is normalized to non-CM nuclei that are presumed to be diploid. A chief advantage of this method is its ease of evaluation and, generally, clustered intensity values are obtained that allow assignment as diploid versus tetraploid or higher. Nonetheless, there is always a range of signal intensity for both CM nuclei and non-CM nuclei, which may be real (e.g., if a cell is midway through S-phase DNA replication, which would be very rare for adult CMs, or if there exist varying degrees of unrecognized aneuploidy) but can also originate from measurement variation inherent in the assay. In typical studies, therefore, one window of signal is defined as diploid and the other as tetraploid, and intermediate values are either assigned to their closest side or discarded from analysis. Notably, this strategy is analogous to how flow cytometry has been used for decades to measure cell cycle in non-CM cell types.

A variant approach is to isolate bulk tissue nuclei and evaluate nuclear ploidy by flow cytometry, again using a fluorescent DNA intercalating dye. Here, identification of CM nuclei is necessary; a genetic marker or antibodies recognizing the CM-specific nuclear proteins PCM-1 or TnI have been used in past studies. Diploid versus tetraploid and higher-ploidy nuclei can be identified by this method, but as described above, the analysis is subject to signal variability. A much more significant limitation is that the source of the CM nucleus cannot be identified; a diploid (2n) nucleus could have been from a 1x2n or a 2x2n CM.

The alternative to DAPI staining of nuclei in cell preparations is fluorescence in situ hybridization (FISH), in which one or more labeled probes each corresponding to an individual chromosome is prepared and hybridized to permeabilized cells. Each probe binds to its cognate target chromosomes in the nucleus, which are visible as fluorescent spots, and thus the number of spots indicates the number of the chromosomes detected per nucleus. In our analysis using this approach (11), we used two probes simultaneously (recognizing mouse chromosomes 3 and 15). An advantage of FISH is that each positive signal can be counted directly and unmistakably as an individual chromosome. A disadvantage is that only a subset of chromosomes is counted (in our case, only two of the 20 mouse chromosomes), so that any fluctuation in other chromosomes is invisible to analysis. A larger issue specifically related to CM FISH analysis is that CMs are particularly difficult to prepare in a way that allows efficient hybridization of the FISH probes. It is necessary to partially digest the cells with proteinase to make the chromatin accessible, and too much or too little digestion may lead to variable hybridization, perhaps even within subdomains of the same nucleus. For example, in our analysis, we observed instances in which one probe gave 4 spots, whereas the other gave only 3 (e.g., see figure 1*c* in 11). We score this as a tetraploid nucleus, but we cannot say if this is fully tetraploid and the missing spot was missing for technical reasons, or perhaps this nucleus indeed only had 3 copies of the corresponding chromosome.

Some studies have attempted to measure CM ploidy in tissue sections, usually cleared so as to be optically transparent and then stained with a cell membrane marker and a DNA marker. This is problematic for several reasons. First, the tissue sections have to be fairly thick so as to include entire CMs, which raises issues of reagent penetration. Second, in our experience it can be difficult to accurately distinguish borders of adjacent cells in tissue sections. Third, quantitation of fluorescence signal is complicated in tissue sections by light scattering (especially for short wavelength DAPI signal). Another approach is to approximate nuclear ploidy by nuclear size (specifically, nuclear area in an image), under the assumption that more DNA requires more volume. In tissue sections or isolated cells, CM nuclei can be round or highly elongated and can have a two-dimensional area in an image that is not proportional to its real volume in three dimensions. We feel that both approaches can provide an approximation of nucleation status and ploidy, but because of these limitations, they are not optimal for accurate measurement.

As noted above, it is assumed that CM nuclei are diploid (2n), tetraploid (4n), or higher multiples of a 2n state. This assumption has not been subjected to rigorous evaluation and thus may not be correct. Although mostly hypothetical at the moment, several steps in endoreplication might cause CMs and individual CM nuclei to have an imbalanced chromosome content. If there is an imbalance in chromosome number or in chromosome attachment to the mitotic spindles, this could activate the spindle assembly checkpoint (29). In other types of dividing cells, this checkpoint serves to delay mitosis until all chromosomes are properly paired at the metaphase plate and attached to mitotic spindles prior to karyokinesis. If cell cycle arrest is too long, in some cell types this can trigger cell death via a process known as mitotic catastrophe (30). If mitosis eventually proceeds with unbalanced chromosome segregation, the daughter nuclei are not diploid for all chromosomes, which in dividing cell types can lead to aneuploidy and cancer. CMs are not actively dividing, but the nuclei in a 2x2n CM might have unequal levels of each chromosome if an analogous process occurs in CMs as they first become polyploid. Furthermore, after the first instance of endoreplication, polyploid CMs are able to reenter cell cycle with further rounds of endoreplication, but now starting with twice as many chromosomes. This provides an even greater likelihood for imbalance of chromosome number prior to or after karyokinesis. An additional parameter that is likely to be relevant is the presence of extra centrioles. Normal cells have one centriole that is duplicated prior to mitosis, with one allocated to each daughter cell. At least in some contexts, CMs that arrest at either the 1x4n or 2x2n state retain the second centriole. Then in a new round of endoreplication, they have three or four centrioles, with the likelihood that karyokinesis with multiple spindle poles can result in an even more asymmetric allocation of chromosomes to the new daughter nuclei (31). All of these situations would result in nuclei with an unequal number of chromosomes, and potentially this serves as one reason why most adult CMs are postmitotic. It remains unclear how frequently these events occur; by the standard methods of evaluation (DAPI intensity, FISH), these would be measured to be 2n, 4n, etc., even if they do not have a full complement of each chromosome. As mentioned above, we have seen cases in FISH ploidy evaluation that could indicate an imbalance in chromosome content, although we cannot confirm that these were not the result of a technical artifact. Additional supporting evidence is that individual nuclei in CMs with a large number of nuclei (i.e., after multiple rounds of endoreplication) tend to be more asymmetric in size (10), suggesting they have more or less total DNA.

A variety of efforts to force cell division in adult CMs have been attempted, including both genetic strategies (e.g., forced expression of mitotic activators) and chemical or protein treatment (e.g., with pathway modulators or growth factors). In just one example (of many in the literature) involving transgenic overexpression of cyclin D2, there was a mild basal phenotype of increased heart size but with significantly improved outcome after adult infarction (32). However, if too many CMs are forced into cell cycle, such approaches might result in CMs with even greater

disparity in chromosome content, perhaps to the point of inducing cell death. Indeed, this appears to be the explanation for the phenotype of mice lacking glycogen synthase kinase-3 activity, which die with dilated cardiomyopathy marked by progressively increasing nucleation, irregularity of nuclear size (i.e., asymmetry in DNA content), and CM cell death by mitotic catastrophe (33). Similarly, in cultured primary CMs, overexpression of factors that override the G2/M checkpoint promoted cell division (cytokinesis) but was followed by cell death (34). In this latter study, cell death was prevented if CMs also expressed G1 checkpoint factors. Thus, approaches to stimulate CM proliferation in adult hearts may be beneficial or detrimental, presumably based on the specific cell cycle pathways in CMs that are activated, how strongly these are activated, and perhaps how they affect chromosome imbalance.

INDUCERS OF POLYPLOIDIZATION AND THEIR RELATION TO CARDIOMYOCYTE MATURATION

In all mammals, CM polyploidy is initiated after a period of mitotic inactivity, indicating that this is an induced process. Thyroid hormone has emerged as one endogenous trigger of polyploidization. In sheep and in mice, thyroid hormone levels rise coincident with the prenatal or neonatal onset of polyploidy, respectively. Thyroid hormone treatment can prematurely activate polyploidization, and interference in thyroid hormone signaling delays and reduces the extent of polyploidization (23, 35, 36).

In mice and rats, most CMs become polyploid shortly after birth, whereas this occurs prenatally in sheep (19) and extends well into the postnatal period in humans (8). Thus, the circumstances that surround the act of being born per se cannot be the sole trigger of CM cell cycle entry or interruption in all mammals. However, by virtue of being coincident in timing, the abrupt transition from fetal to postnatal life might contribute to these events in newborn rodents and perhaps also in many other species. Some of the changes that occur immediately with birth include the loss of exposure to placental or maternal hormones, increased neuronal activity of the sympathetic and parasympathetic systems (on the heart and on peripheral vasculature), and a substantial anatomical reorganization of the circulatory system (closure of the atrial septum, closure of the ductus arteriosus, and increased pulmonary blood flow) that is associated with considerable hemodynamic changes in blood pressure and heart wall strain. How these circumstances might affect the process of polyploidization is unknown.

In mice, a compelling case has been made that an increase in reactive oxygen species (ROS) associated with birth and postnatal life is a cause of cell cycle arrest (37), which is in principle equivalent to the onset of endoreplication and polyploidization. While excessive ROS (oxidative stress) is pathological and causes cell death, physiological levels of ROS are not damaging but rather serve as necessary intracellular signals. ROS increase after birth for several reasons. First, preterm life is relatively hypoxic because of limitations of placental transfer of maternal oxygen to fetal circulation and because of dilution of oxygenated blood in the umbilical vein with deoxygenated systemic blood before reaching the left ventricle. After birth and with the establishment of neonatal respiration and pulmonary circulation, blood oxygen saturation rises substantially (38). The presence of more oxygen leads to the generation of more cellular ROS, particularly in highly metabolically active tissues such as the heart. Second, there is a profound shift in nutrition before and after birth. Glucose is the primary fuel of fetal life; there is little fat transported across the placenta or made in the embryo (39). In contrast, nutrition after birth is based on maternal milk, which is rich in fat and in rodents is particularly low in lactose and other sugars (40). In all mammals, CMs after birth and for the remainder of normal life rely disproportionately on fat rather than glucose metabolism (41), and fatty acid oxidation is associated with greater generation

of ROS (42, 43). Human milk has a higher sugar concentration (primarily lactose) and lower fat content than rodent milk (44), which in comparison to rodents might contribute to the extended time frame of polyploidization in humans. To our knowledge, there has not yet been any experimental alteration of maternal milk composition in any species that would allow a direct evaluation of the relevance of milk composition to CM polyploidy, although in principle such work could be done using genetic or perhaps pharmacological interventions in mice.

Although not yet confirmed, it is likely that thyroid hormone is also associated with ROS. Thyroid hormone is classically known to boost metabolism and does so by promoting fatty acid oxidation and other aspects of mitochondrial function (45), all of which increase ROS. The increase in ROS might therefore be a unifying mechanism to explain the onset of polyploidy in all mammals: In rodents it may be driven by synergy between thyroid hormone, oxygenation, and nutrition, whereas in sheep it is perhaps driven primarily by thyroid hormone alone. It is still to be shown whether ROS are the downstream effector of thyroid hormone in fetal sheep polyploidization; this is particularly relevant given that the low oxygen level of fetal sheep is similar to other mammals (46, 47). For all mammals, whether thyroid hormone has additional consequences in this process that are independent of ROS and metabolism is not possible to resolve at this point.

Broadly stated, polyploidization requires two major events: cell cycle entry as indicated by S-phase DNA replication and interruption of mitosis. ROS might serve in both functions. Thus, in certain contexts, ROS stimulate CM proliferation (48, 49) and in others ROS stimulate CM cell cycle arrest (37). Much attention has been given to the expression and function of specific components of the cell cycle machinery that are directly involved in CM cell cycle entry and progression (50); these are not reviewed further here. Recent insights have revealed how alterations in the cytokinesis machinery result in binucleation in neonatal rodent CMs (31, 51). Connecting ROS and their presumed upstream activators such as nutrition, oxygenation, and thyroid hormone to these cell cycle components, and how CM cell cycle entry and cell cycle interruption are both modulated by ROS (if indeed this occurs), are important subjects of further investigation.

In our work (11), we have used natural variation among inbred mouse strains to identify genes that influence the adult level of mononuclear diploid (1x2n) versus polyploid CMs. One such gene is the CM-specific kinase *Tnni3k*; more 1x2n CMs result when expression of this gene is eliminated. Although not fully confirmed, *Tnni3k* does not seem to impact how many CMs enter cell cycle (in the normal neonate or in the injured adult heart) but rather influences how many complete cytokinesis or arrest in mitosis. Although we still do not know how *Tnni3k* functions in this role, an interesting speculation is that it does so via modulation of oxidative stress (ROS level) or oxidative stress response, as *Tnni3k* is implicated in this pathway in an unrelated adult disease context (adult heart ischemia-reperfusion injury) (52). How *Tnni3k* might have a selective role in cell cycle arrest is uncertain, but with approaches like these, it might be possible to disentangle the complex features that account for CM cell cycle entry from those that control CM cell cycle completion or interruption.

A large number of changes occur in CMs coincident with the acquisition of a polyploid state. A partial list of these includes a transition in metabolism from glucose utilization to fatty acid oxidation, extensive replacement of CM mitochondria, cell enlargement (hypertrophy), assembly of longer and more highly structured sarcomeres, assembly of T-tubules (invaginations of the cell membrane juxtaposed to the sarcoplasmic reticulum that facilitate calcium dynamics), changes in gene expression at both the transcriptional and RNA splicing levels, changes in epigenetic patterns, and acquisition of adult-type ion channel electrophysiology. These changes (including polyploidization) are collectively called maturation (53).

An open question is the extent to which any one of these aspects of CM maturation depends on others and in turn influences others. The combination of oxygen availability, high-fat nutrition

in maternal milk, and exposure to thyroid hormone is an obvious contributor to the metabolic changes associated with CM maturation and, as summarized above, the increase in ROS associated with this metabolic change might in turn initiate polyploidization. In organoid culture with human pluripotent stem cell–derived CMs, addition of fatty acid (palmitate) to low-glucose culture media induced a number of changes associated with maturation, including contractile properties and gene expression profile (54). In cell culture, treatment of immature CMs with thyroid hormone and glucocorticoid induced formation of higher-structured sarcomeres and T-tubules (55) and, in a different study, glucocorticoid treatment alone induced CM binucleation in addition to other changes associated with CM maturation (56). Polyploidy may in turn be a prerequisite for hypertrophy, in that polyploid CMs are almost always larger than 1x2n CMs, and experimentally induced polyploidy in zebrafish caused the resulting cells to be larger (57). These types of experimental approaches provide an opportunity to dissect the events that underlie the many individual features of CM maturation, including CM polyploidization, and to address their interrelatedness.

THE RELATION OF CARDIOMYOCYTE PLOIDY TO REGENERATION

In all species and at all stages of life, the ability of the heart to grow or regenerate after injury is an indication of the proliferative capacity of its CMs. The embryonic heart grows during development by CM proliferation, with a rate that peaks and then declines as the proper number of CMs is reached. In mammals, CM proliferation is low by the time of birth, and in normal heart biology, it remains minimal throughout the remainder of life. The rate of CM turnover (loss and replacement) in both humans and mice is estimated at only approximately 1% per year (58, 59). Rigorous evidence that new CMs arise from preexisting CMs, even at this very low level, was reached in mouse studies using independent methodologies (58, 60). A limitation of these studies is that the specific CM subtype that accounts for proliferation could not be identified: The observed results could be explained by a special rare subpopulation of CMs that uniquely has this ability, or this could be a general property of adult CMs that is only rarely active.

Adult zebrafish and newts retain the capacity to reactivate robust CM proliferation in the adult and can thereby fully regenerate their hearts after injury (61, 62). For a brief period of time after birth, the neonatal mammalian heart can regenerate after injury also by reactivation of CM proliferation; this has been observed in rats (63), mice (64), and pigs (65, 66) and is likely also true in humans (67, 68). Shortly after birth, however, the ability to reactivate CM proliferation in mammals is no longer present, at least at high levels. In mice, this occurs during the first postnatal week (37) and likely occurs within only a few days after birth (69, 70). Consequently, in the adult mammalian heart, injury does not reactivate sufficient CM proliferation to support regeneration. This has major clinical significance. A common cause of human heart injury is atherosclerotic coronary artery occlusion leading to myocardial ischemia or infarction and consequent CM death in the impacted region. Instead of regeneration, adult heart injury is followed by scarring and permanent loss of myocardial function, which can progress to heart failure.

In all species, CMs are actively proliferative or are potentially regenerative when they are mononuclear and diploid (1x2n), e.g., in mammals during fetal and early neonatal life. The time when the mammalian heart loses regenerative capacity is coincident with the time when a majority of CMs have become polyploid. Zebrafish and newt CMs do not become polyploid, and these species retain the ability to reactivate CM proliferation and to regenerate after adult heart injury. These correlations led to the hypothesis that the state of being polyploid may cause most CMs to be irreversibly postmitotic, either unable to enter or complete the cell cycle. Conversely, 1x2n CMs may retain proliferative capacity even in the adult mammal, just as they are proliferative in the embryonic heart. A variety of further observations also implicate mononuclear CMs in

proliferation (71, 72). An interesting test of this correlation would be neonatal sheep, as sheep undergo CM polyploidization before birth (i.e., earlier than most mammals that have been studied). Fetal sheep prior to the time of polyploidization accomplish full heart regeneration (73), although the same analysis has not yet been conducted in late fetal or newborn sheep. Similarly, a broad survey implicated many mammalian species as having a very high adult 1x2n CM level (23), but these species have not yet been tested for regeneration.

In mouse, the level of 1x2n CMs in the normal adult heart is typically measured as approximately 2%. In our work (11), rather than assuming this to be a fixed feature of the adult heart, we showed that the percentage of 1x2n CMs in the adult heart is surprisingly variable between inbred mouse strains, being around 2% in many but reaching as high as 10% in some. We showed that strains with more of these had better regeneration at the functional and cellular levels after adult heart injury. As noted above, we identified one gene, *Tnni3k*, as having naturally occurring variants that influence the 1x2n CM percentage, and there are clearly others that also contribute to variation in this trait. Direct manipulation of this one gene in a controlled inbred strain background resulted in the predicted increase in 1x2n CM percentage and a consequent improvement in CM cellular regeneration.

By extension to human biology, different people presumably also have different percentages of 1x2n CMs in their hearts by virtue of their different genetic constitutions and could have different degrees of regeneration after adult heart injury. Indeed, patients with initially similar degrees of infarction are known to have widely different extents of ventricular remodeling and long-term outcomes (74, 75), and some of this divergence might be explained by variable extents of regeneration. If the same pathways and mechanisms control CM polyploidization in humans as in mice, naturally occurring *TNNI3K* variants in the human population (76, 77) would be predicted to cause a higher percentage of 1x2n CMs to be present, just as the corresponding alleles do in mice.

Zebrafish CMs do not naturally undergo the same progression to become polyploid as occurs in mammals, and zebrafish hearts remain regenerative throughout adult life. In our work (11), after finding that mutation of *Tnni3k* in mice raised the number of 1x2n CMs and increased regeneration, we also overexpressed *Tnni3k* in zebrafish. This increased the percentage of polyploid CMs and compromised the ability of adult transgenic zebrafish to regenerate. A study from a different lab working on zebrafish reached a similar conclusion. In this analysis (57), transient transgenic expression of a dominant negative version of the cytokinesis component Ect2 caused a high degree of CM polyploidy, and much later (after transgenic Ect2 was no longer present, but CMs were still polyploid), adult fish were consequently not able to regenerate effectively.

The significance of the two studies together is in their use of direct experimental approaches, rather than observational correlations, to conclude that 1x2n CMs are proliferative and regenerative, and conversely that the polyploid state of CMs is nonproliferative and nonregenerative. Conceptually, this breaks the dogma that the adult mammalian heart is nonregenerative and replaces this with a more nuanced paradigm that regeneration occurs but is based either exclusively or primarily on the presence of 1x2n CMs. In most mammalian species and in most individuals within a species, there might be relatively few of these, but in some cases there could be more, and perhaps many more, of these potentially regenerative CMs.

Because the focus of both studies was 1x2n CMs, neither study negates the possibility that some polyploid CMs might also be able to support regeneration. Clearly though, most polyploid CMs do not. As noted earlier, many CMs in all mammals naturally reach higher levels of polyploidy than simply 1x4n or 2x2n, which can only be explained by repeated cycles of CM endoreplication. Similarly, the degree of CM ploidy increases in various disease states, again implicating repeated cycles of CM endoreplication. Therefore, the barrier that prevents regeneration by most adult

polyploid CMs is probably not their inability to enter cell cycle, but rather their inability to complete it and survive thereafter. 1x2n CMs appear to not be subject to this constraint and can thereby accomplish regeneration.

As described above, after polyploidization, it is possible that CM nuclei do not carry an exact chromosome complement, which might underlie or contribute to mitotic arrest and possibly to mitotic catastrophe. At the same time, some fraction of polyploid CMs may not be compromised in mitotic progression, and when appropriately stimulated may be able to rereplicate their DNA and then divide into daughter cells without triggering cell cycle arrest. Binucleated hepatocytes are able to divide efficiently, and perhaps some small fraction of binucleated CMs also have this ability. This might explain why some reports have shown videos of individual binucleated CMs in culture that undergo complete cell division (78, 79). Clearly though, most polyploid CMs do not have this ability, based on direct observation in culture (71) and on the general lack of regeneration after adult mammalian heart injury. Reciprocally, the 1x2n CM population, which appears to be the primary CM subpopulation that is capable of proliferation and of supporting regeneration, may itself be heterogeneous, such that only a subset is proliferative. If so, the proliferative subset would appear to be a large subfraction of the total 1x2n population. In our studies in injured adult mice (11), we calculated that approximately half of cell cycle entry events by 1x2n CMs culminated in cytokinesis and formation of new 1x2n CMs (the remainder became either 1x4n or 2x2n), but this depended prominently on strain background and *Tnni3k* gene status, and was minimal in a nonpermissive genetic context.

One challenge in studying 1x2n CMs, in general and specifically their regenerative potential, is the current lack of molecular markers with which to identify these cells. It is easy enough to distinguish these in single cell preparations by virtue of having one diploid nucleus, but in terms of most other features of CM cell or molecular biology, these CMs appear to be the same as all other CMs (one exception is their trend to be smaller than polyploid CMs). One possible insight is the observation that a reporter system that identifies CMs that are hypoxic (at least in part because their microenvironment has a lower capillary density) and that preferentially labels newly regenerated CMs after injury is preferentially (although not exclusively) expressed in mononucleated CMs (of undefined ploidy) (80). This analysis gives additional support to the premise that 1x2n CMs are (or include) the regenerative CM population of the adult heart, and furthermore supports the premise that oxidative stress (which is lower in hypoxic cells) may be one condition that causes CMs to become polyploid.

CONSIDERATIONS OF REASONS WHY CARDIOMYOCYTES MIGHT BECOME POLYPLOID

The linkage between CM polyploidy and general loss of proliferative capacity explains why efficient heart regeneration does not occur in adult mammals. Clearly in mammals, unlike zebrafish and other highly regenerative species, maintaining adult heart regenerative capability was not subjected to positive evolutionary selection. A very low 1% per year ventricular CM replacement rate is all that is needed for maintenance of the human heart for 100 years or longer, so preservation of a higher level of proliferative ability is clearly not needed for a normal lifetime of full heart function. Coronary artery occlusion and other forms of heart disease occur relatively late in mammalian life and generally past the time when reproduction would be impacted (and thus influence evolutionary selection), except only in very recent human history. Thus, although highly relevant in human biology for its clinical impact, the loss of regenerative ability associated with CMs becoming polyploid is more likely to be a neutral feature and not one that involved any real degree of evolutionary compromise.

In principle, though, the acquisition of polyploidy had beneficial consequences that were subjected to evolutionary selection. Several possible reasons to explain why mammalian CMs become polyploid have been discussed. Although it is impractical to determine why nature evolved this feature of heart biology to the state in which we find it today, various ideas can be considered in light of current understanding.

One commonly mentioned idea is that CM polyploidy arose specifically to prevent CM proliferation. For a cell to divide, it must break its attachment to its neighbors (and for a CM, to also at least partially disassemble its sarcomeres so as to allow cytokinesis), and because CMs exist in long myofibers, if one undergoes cell division, the entire fiber could lose contraction. According to this model, if too many CMs were to proliferate at the same time, there would be too much impairment of heart function, so becoming polyploid is one way to prevent this from happening. We raise two counterarguments to this model. First, we note in the fetal heart (at least as studied at midgestation in mouse) that the rate of CM proliferation is robust and perhaps such that all CMs are proliferative at its peak. This occurs while the heart is actively and productively beating. Clearly then, widespread proliferation per se is not incompatible with heart contraction (although, the embryonic heart may be subject to different contractile requirements and constraints than the adult heart). Second and more compelling, this model conflates the capacity for proliferation with actual proliferation. In the neonatal mammalian heart prior to polyploidization, there is little active CM proliferation even though there is great capacity for regeneration after injury. Thus, it is hard to reconcile how a latent capacity for much greater proliferation in the adult heart would be detrimental so long as it were only called into action at the minimal rate of 1% per year.

Another oft-stated reason for CM polyploidy is rooted in the observation that CMs become larger as part of the maturation process. A larger cell involves a greater amount of turnover of cellular components, which are more easily replaced when more chromosomes are available from which to express relevant genes. The linkage between cell size and ploidy is supported by observations that CMs with more nuclei tend to be larger and that both CM size and CM ploidy increase in many disease states that involve CM loss. Furthermore, in zebrafish, experimentally forced polyploidy resulted in the increased size of the polyploid CMs (57). Nonetheless, at least in uninjured normal heart biology, we question whether there is any benefit of having a smaller number of larger polyploid cells or a greater number of smaller diploid cells, as these both seem to sum to an equivalent amount. For the same reason, another hypothesis to explain polyploidy as providing a more rapid transcriptional response to changing physiological requirements (i.e., expression from four genomes instead of two) also seems lacking, as the ratio of genomes to cytoplasm is the same in a smaller diploid or a larger tetraploid cell. One parameter that might be sensitive to the dynamic of CM size is the strength of contraction within versus between cells (i.e., a fiber with fewer CMs each having longer sarcomeres might contract better than one with more cells but shorter sarcomeres). Another is the propagation of depolarization that initiates each heart contraction; this might occur too slowly if it is forced to pass through the gap junctions between more cells. We are not aware of specific studies that have demonstrated either of these differences. We note also that the mammalian heart has many ways by which to increase or decrease both contractile force and impulse propagation based on physiological need and that both parameters differ markedly among different mammalian species even though all presumably have a high CM polyploidy level. Thus, if there were a reason to maintain a heart with more 1x2n CMs, one could imagine ways in which this could be accomplished, although as noted above there may simply not have been an evolutionary benefit in this.

Another speculation is that polyploidy provides protection against DNA damage that is associated with ROS generation in highly metabolically active CMs. Accordingly, CMs that have more

genes can suffer more DNA damage before all copies of a critical gene become compromised, which would then lead to either a noncontractile cell or to apoptosis. DNA damage in the myocardium is slow but measurable; one analysis concluded a ninefold increase in DNA damage in wild-type mice under standard housing conditions between 2 and 39 months (81). Even if slow, this process might be relevant to the onset of idiopathic heart disease in older individuals. This might also be relevant to individuals who are heterozygous for inherited loss-of-function gene mutations that are associated with adult-onset heart disease; gene inactivation by ROS-induced DNA damage would occur twice as fast in a heterozygous individual. A similar rationale has been invoked to explain polyploidy in hepatocytes as providing protection against the mutagenic challenge of liver biology (82). It is interesting that in many tissues, the solution to the problem of accumulation of DNA damage is to eliminate the damaged cell by apoptosis and have it replaced by regeneration. Perhaps polyploidy in CMs is an alternate solution that delays the significance of DNA damage long enough that regeneration is not required at a level higher than 1% per year.

In heart development, the appearance of polyploid CMs coincides with other aspects of maturation (e.g., mitochondrial metabolism and higher ROS generation, higher-order sarcomere structure, T-tubules, etc.) that typify adult CMs. Between species, the presence of polyploid CMs coevolved with a number of changes in organismal physiology, including a high-pressure circulatory system, thermoregulation, and even immune cell composition (reviewed in 83). In different avian species, CM polyploidy relates to adult growth and activity (24, 84). Thermoregulation (endothermy) has received recent renewed attention for its connection with thyroid hormone signaling (23). These correlations may explain why CMs become polyploid, but such speculations all rest on teleology (logic), with few direct experiments to date that can address them. We suggest that a test of these, particularly in the context of mammalian physiology, is within sight. Our work in mice demonstrates that the percentage of 1x2n CMs in the normal healthy adult mouse heart ranges as high as 10% among the natural inbred strains we have tested (11). We used a forward genetic strategy to uncover natural gene variants that impact this frequency, and others have used a candidate gene approach and also environmental perturbation to test for impacts on CM ploidy. If it is possible to increase the number of diploid CMs in adult mice in a controlled experimental manner, the basal rate of CM proliferation, the extent of regeneration after adult heart injury, any change in other features of CM maturation and CM function, and any pathology that might emerge under basal or challenged physiology could be determined. This might reveal if there is something fundamental about diploid and polyploid CMs in mammalian heart physiology that accounts for the high frequency of the latter.

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