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Changes in Cardiomyocyte Cell Cycle and Hypertrophic Growth During Fetal to Adult in Mammals

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ABSTRACT: The failure of adult cardiomyocytes to reproduce themselves to repair an injury results in the development of severe cardiac disability leading to death in many cases. The quest for an understanding of the inability of cardiac myocytes to repair an injury has been ongoing for decades with the identification of various factors which have a temporary effect on cell-cycle activity. Fetal cardiac myocytes are continuously replicating until the time that the developing fetus reaches a stage of maturity sufficient for postnatal life around the time of birth. Recent reports of the ability for early neonatal mice and pigs to completely repair after the severe injury has stimulated further study of the regulators of the cardiomyocyte cell cycle to promote replication for the remuscularization of injured heart. In all mammals just before or after birth, single-nucleated hyperplastically growing cardiomyocytes, 1X2N, undergo ≥1 additional DNA replications not followed by cytokinesis, resulting in cells with ≥2 nuclei or as in primates, multiple DNA replications (polyploidy) of 1 nucleus, 2X2(+)N or 1X4(+)N. All further growth of the heart is attributable to hypertrophy of cardiomyocytes. Animal studies ranging from zebrafish with 100% 1X2N cells in the adult to some strains of mice with up to 98% 2X2N cells in the adult and other species with variable ratios of 1X2N and 2X2N cells are reviewed relative to the time of conversion. Various structural, physiologic, metabolic, genetic, hormonal, oxygenation, and other factors that play a key role in the inability of post-neonatal and adult myocytes to undergo additional cytokinesis are also reviewed.

Key Words: cell proliferation ■ cell-cycle ■ heart ■ mammal ■ myocyte

Systolic heart failure, resulting from cardiomyocyte loss, is the leading cause of heart failure,[1] which costs the US government >$39 billion in health-care expenses annually.[2] The molecular and cellular basis for the progressive severe left ventricular dysfunction is the result of the inability of damaged and apoptotic myocytes to be replaced. The core pathophysiology of heart failure is the inability of the mammalian cardiomyocyte to regenerate. Instead, lost myocytes are replaced by fibrous tissue, which results in progressive left ventricular remodeling, dilatation, contractile dysfunction, and congestive heart failure that is irreversible.

In contrast to lower vertebrates,[3–7] cardiomyogenesis after injury in adult mammals is limited[8–12] and insufficient for restoring normal cardiac function. Studies in the late 1990s elegantly mapped the DNA synthesis and cell cycle dynamics of rodent hearts during late fetal development and the early postnatal period,[13–15] showing that DNA synthesis drops significantly around birth. Cardiomyocyte DNA synthesis in the early neonatal period was thoroughly reviewed by Rumyantsev in 1977[13] and by others more recently.[14] In rats and mice, there is a renewed increase in DNA synthesis from postnatal day 4 (P4) to P10 as cardiomyocytes become binucleated and then drops to low levels.[14–16] In swine, and possibly other large mammalian species, DNA synthesis continues for several weeks after birth along with the development of multiple nuclei.[17] In rodents, cardiomyocytes activate the DNA synthesis temporarily around P5 to P7, which remains low for a few days afterward,

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Nonstandard Abbreviations and Acronyms

CDK	cyclin dependent kinase
P1	postnatal day 1
Rb	retinoblastoma protein

and then becomes undetectable. Despite the important implications of human heart disease, it is entirely unclear whether larger mammals or humans have this neonatal cardiac regenerative potential, a phenomenon that has been a hot topic for the last 2 decades. Deciphering mechanisms that control the conversion of fetal 1X2N proliferating myocytes to non-proliferating 2X2N cells after birth in mammals, would have significant biological and therapeutic implications. To date, there are only anecdotal reports based on human congenital heart disease literature that suggest that there may be a window of myocardial regeneration in postnatal humans.[8–12] However, the lack of any time-course assessment of cardiomyocyte viability precludes conclusions about true regeneration in large mammals. Moreover, specific details about the regenerative potential of large mammalian hearts are simply unknown. We recently developed a large mammalian model examining the cardiac regenerative potential of neonatal hearts. Our discovery that the newborn porcine hearts are capable of regenerating from injury for only the first 2 days of life[18,19] and that the regeneration is associated with cardiomyocyte proliferation, presumably from single-nucleated pre-existing cardiomyocytes, provides an opportunity for identifying the regulators of cardiomyocyte proliferation/differentiation and neonatal heart regeneration in large mammals. The findings of this study, as well as the recent case report of a newborn patient whose heart was completely recovered according to the clinical assays from a myocardial infarction that occurred shortly after birth[20] suggest that an early postnatal window for significant myocardial regeneration exists in large mammalian hearts, which provides highly impactful insight into the regenerative properties of human hearts.

From the molecular and cellular perspective, heart failure occurs because of the loss of the contractile ability or the total cell unit of the left ventricle: cardiomyocytes. Therefore, understanding the regulators of myocyte cell cycle could have a highly significant impact on the management of heart failure. Identification of a regenerative window in large mammalian hearts is a crucial step toward understanding myocardial remuscularization in humans and may provide a platform for preclinical studies of devastating heart conditions in infants and adults with congestive heart failure. The many important clinical implications of understanding the key regulators of myocyte cell cycle could include the design of novel techniques for pediatric heart surgery, new guidelines for when the surgery should be performed, and the development of new therapeutic modalities for patients with congestive heart failure. Importantly, as we begin to better understand the mechanisms that regulate the drastic early postnatal decline in cardiomyocyte proliferation, we may be able to manipulate these mechanisms to promote myocardial regeneration.

As the primary mechanism of myocyte regeneration because of injury in early postnatal mammals appears to involve the proliferation of pre-existing cardiomyocytes,[18,21] here we review the myocyte hyperplastic growth and exit cell cycle shortly before or after birth in mammals of different species including human, and are hoping this review could provide directions for future studies aiming at characterizing the mechanisms of cardiomyocyte cell cycle regulation, and generate new interventions to manipulate the cardiomyocyte cell cycle for myocardial repair.

CARDIOMYOCYTE CELL-CYCLE REGULATION CHANGES DURING FETAL TO NEONATAL GROWTH

The cardiomyocyte cell-cycle is driven mainly by cyclin families and CDK (cyclin-dependent kinase) families. The cardiomyocyte cell-cycle activates when cyclins bind to CDKs to promote the cycle. CDKs are also regulated by cyclin-dependent kinase inhibitors, such as INK4 (inhibitor of cyclin-dependent kinase 4) families and Cip/Kip (cyclin dependent kinase interacting protein/kinase inhibitory protein) families. Besides, CDKs activities are regulated by phosphorylation, such as CDK-activating kinase, Cdc25,[22] and Wee1.[23] In fetal mouse cardiomyocytes, the major drivers of cell-cycle, cyclins, and CDK expression peak at embryonic day 16. Hereafter, their expression levels are continuing to decline until birth. In neonatal cardiomyocytes, cyclins and CDK expression increase once again at P5. After that, their expression continues to decline and stay low in adults. These cyclins/CDKs expression patterns are also correlated with cardiomyocyte DNA synthesis, which initiates the last cell-cycle at around P5 resulting in binucleation because of cytokinesis failure.[15,24] Recent studies suggested that oxygen transition from fetal hypoxia to postnatal normoxia environment,[25] expression of Jumonji, suppressor of cyclin D1 in the postnatal cardiomyocytes,[26] and loss of centrosome integrity are also associated with mammalian cardiomyocyte cell-cycle arrest.[27]

Cardiomyocytes cytokinesis failure also occurs by abnormal localization of RohA, small GTP binding

protein,[28] IQGAP3, GTPase activating protein,[28] and Anillin, actin-binding protein during cytokinesis, which resulted in cardiomyocytes binucleation. In contrast, Aurora B, serine/threonine-protein kinase localization during cytokinesis showed no error.[29] A recent report also suggested that Lamin B2 expression levels regulate the polyploidization of cardiomyocytes. Inactivation of Lamin B2 decreased metaphase progression resulted in polyploidization of cardiomyocytes. In contrast, overexpression of Lamin B2 in neonatal mouse hearts promoted M-phase entry and cytokinesis that resulted in cardiomyocytes proliferation from cardiac injury. Lamin B2 expression plays a pivotal role in karyokinesis of mammalian cardiomyocytes.[30]

Rb (retinoblastoma protein), tumor suppressor, is known to inhibit cell-cycle progression by binding the E2F complexes with recurring histone deacetylases or Jumonji.[31] Rb expression is almost undetectable by E12.5 in mouse cardiomyocytes but is upregulated in neonatal and adult periods.[32] Cardiac specific Rb and p130 inducible double-knockout mice showed expression of cell-cycle genes and proliferation of adult cardiomyocytes.[33]

Recent studies suggested that overexpression of cell-cycle drivers activated cardiomyocytes cell-cycle. Overexpression of cyclin D2 in human induced pluripotent stem cell-derived cardiomyocytes engraftment to myocardial infarcted mice significantly improved cardiac function through the proliferation of cardiomyocytes.[34] Overexpression of CDK1/cyclin B1 and CDK4/cyclin D1 combination efficiently activates the cell-cycle in post-mitotic cardiomyocytes. However, when Aurora B added in the combinations resulted in cell death, probably immature cell entry of mitosis.[35] Therefore, overexpression of cell-cycle drivers may be a useful strategy to proliferate cardiomyocytes for clinical therapy.

INHIBITION OF POLYPLOIDIZATION IN LOWER VERTEBRATES

The number of 2x2N cells in non-mammalian vertebrates is much less than in mammals. Birds still have many 2x2N cells but retain a high proportion of 1x2N cells.[36] Zebrafish and newts have virtually all 1x2N cells, and are able to replicate their damaged heart tissue throughout adult life.[6,37] In adult zebrafish treated to overexpress the gene *Tnni3k*, which plays a role in promoting polyploidization, increased numbers of 2x2N cells impaired the ability of the heart to regenerate after injury.[38] Overexpression of the cytokinesis component Ect2 in zebrafish also produced a higher degree of polyploidy and prevented regeneration.[6,39] Therefore, the inhibition of polyploidization might play a critical role on heart regeneration in lower vertebrates. In contrast, the polyploidy might pose a barrier for cardiomyocyte proliferation in adult mammals. However, the conditions responsible for cardiomyocytes entering the non-proliferative phase of growth are complex and involve other factors such as the retention of centrioles in continuously dividing zebrafish and newts, and the loss of centrioles in postnatal non-dividing cardiomyocytes.[27,40–42] Further, sarcomere and cellular structural integrity including sarcomere M-bands, T-tubules, sarcoplasmic reticulum, and mature intercalated discs, as well as increasing extracellular connective tissue elements have been shown to inhibit cytokinesis.[40,43] Blood pressure is low in the 2- and 3-chambered hearts of fish and newts, requiring minimal workload on the heart and allowing numbers of cardiomyocytes with disorganized cell structure to undergo cell division, while in the 4 chambered heart of mammals, pressures are elevated by late fetal period or soon after birth depending on species, requiring considerable work load on the heart.[44] The increased cardiac work load in mammals versus non-mammalian vertebrates would play a significant role in the cessation of cell division, requiring cells to become unable to perform contractile function.

CONVERSION TO BINUCLEATION IN VARIOUS SPECIES

In all mammalian animals studied to date, just before or after birth there is a conversion from the hyperplastic growing cardiomyocytes with 1 nucleus, 1X2N, in the fetal stage to cells containing 2 nuclei, 2X2N, which are no longer able to complete cytokinesis. These cells with ≥2 nuclei, each with the normal diploid chromatin content, increase in cytoplasmic volume in proportion to the gain in heart and body weight to the adult stage of the animal.[16,44,45] This conversion from 1X2N to 2X2N cells occurs around the time of birth but varies considerably among species. In rats and mice, the first appearance of binucleated myocytes is between 4 and 6 days postnatal,[46,47] and in dogs up to 10 to 12 days postnatal.[48] In larger mammals with longer gestation periods including humans,[49] 2X2N cells first appear in the late fetal period. In sheep, the first appearance of 2X2N cells occurs ≈30 days before the 145-day gestation period with 70% of 2X2N cells at postnatal day 1 (P1).[50–52] In pigs[53] and humans,[49] 10% of P1 myocytes are 2X2N. In all species, following the initial conversion to hypertrophically growing myocytes, the adult number of 2X2N cells is achieved within 1 to 2 weeks.

In the pig with increasing age, additional numbers of nuclei, up to ≥16 are a common feature,[17,54] and in primates, including humans, multiple copies of DNA occur in 1 nucleus, polyploidy.[55,56] In humans, cardiac

hypertrophy attributable to a variety of pathological conditions, including aging, results in a marked increase in polyploidy of cardiomyocytes.[57–59] In normal neonatal dogs and rats about 1% to 2% of cells contain 4 nuclei,[16,48] and in sheep, quadranucleated cells represent up to 7% of left ventricular cells and 2.9% of right ventricular cells by 8 weeks of age.[51] With increased pressure load on the heart in neonatal spontaneously hypertensive rats versus Wistar Kyoto rats, a small number of myocytes, up to 4%, have 4 nuclei.[16]

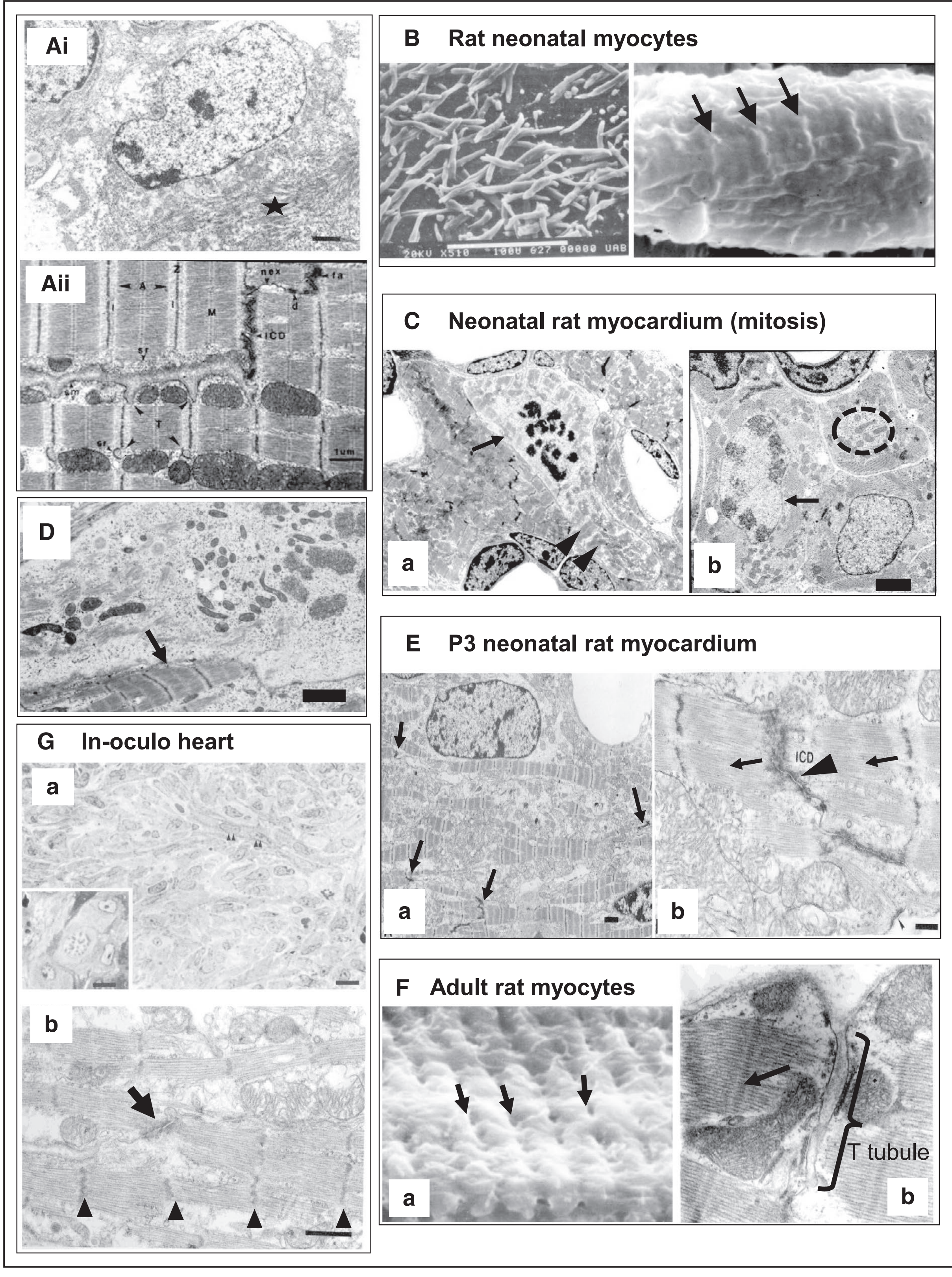
An interesting question is the role of increased nuclear DNA in binucleated, multinucleated, or polyploid cardiomyocytes. As the number of nuclei increases, cytoplasmic volume increases in proportion to the number of nuclei,[45,48,60,61] suggesting that the increased DNA in multinucleation or polyploidy supplies additional transcription capacity. However, Derks[44] has reviewed the literature related to polyploidy in non-cardiac cells including liver and vascular smooth muscle cells reporting only limited changes in the transcriptomes of polyploid cells. A recent report has also reported that RNA content did not differ between mono- and binucleated cardiomyocytes.[62] Thus, the role of multinucleation or polyploidy in the metabolism of the cardiomyocyte remains open.

Since only 1X2N cells appear to have the capacity of proliferation, the number of these cells retained in the adult may have some bearing on the ability of the heart to repair by the formation of new myocytes. In adult rats and mice, depending on strains, 80% to 98% of myocytes are 2X2N,[16,38,47] 87% in the cat,[63] ≈50% in the dog,[48] 60% in the pig,[53] and 25% in humans,[49,60,64] the remainder in non-primate species being 1X2N. In the human heart increase in DNA is predominantly in single-nucleated cells, increasing from adolescent to adult and old age with 50% to 60% of cells having multiple copies of DNA (polyploidy).[49,60] Factors responsible for this variation in polyploidy among species are unknown. In both mice[65,66] and for the human heart[8,60] it has been estimated that ≈1% of cells are reproduced annually after ≈20 years of age in humans, slightly higher in younger humans and decreasing to about 0.3% with increasing age. Using these calculations Bergman et al determined that over a full human life span, about 40% of cardiomyocytes are renewals, the remaining 60% original from the neonatal period.[60] This slow rate of cardiomyocyte renewal is insufficient to replace injured myocardial tissue. However, individual proteins including actin and myosin within growing and adult myocytes do turnover regularly.[67]

MORPHOLOGIC CHANGES OF CARDIOMYOCYTES FROM FETAL TO NEONATAL GROWTH

Among the many maturational changes which occur during the conversion from single-nucleated dividing cells to polynucleated non-dividing cells are the morphologic changes in myocyte internal structure. The sarcomere, the contractile unit of muscle, is composed of several separate protein structures.[68] The principal structures are the Z-discs at either end of the sarcomere composed of sarcomeric α-actinin and titin, cardiac α-actin attached to the Z-disc, a central A-band of myosin which interacts with actin for contracture, and a central M-band composed of myomesin connecting the actin fibrils as well as several other proteins. In early fetal development, some of these cannot be visualized by electron microscopy but these and others can be identified by immunohistochemistry.[43] At the early stage in gestation when cardiac cells first initiate contractions, cells have a rounded shape and the internal structure is loosely arranged with incomplete sarcomere structures and incomplete cell junctions (Figure A [top])

Figure. Structural alterations during fetal to neonatal and adult myocardial growth.
A, (**Top**) Electron micrograph of embryonic day 12 rat myocyte with sparse poorly organized myofibrils (star); bar=1 μm. (**Bottom**) Electron micrograph of adult rat myocyte with, A, Z, I, M-bands of the sarcomere and other structures as indicated. **B**, Low (left bar=100 μm) and high (right) magnification scanning electron micrographs of isolated rat postnatal day 2 neonatal myocytes. Note spindle shape and lack of T-tubule openings on the surface. Immature sarcomere Z-bands are evident (arrows); Z-band spacing=1.6 μm. Compare with Figure **Fa**. **C**, Electron micrographs of postnatal day 3 neonatal rat myocytes. (**Left**) Mitosis (arrow) and incomplete sarcomeres with absence of M-bands (arrowheads). (**Right**) Cross-section with mitosis (arrow). Mitochondria (dashed circle) are clustered centrally and myofibrils peripherally; bar=2 μm for both. **D**, Electron micrograph of postnatal day 1 canine myocyte in mitosis. Myofibrils are dispersed with cytoplasmic clearing. Note sarcomeres in adjacent myocyte with no M-band (arrow); bar=2 μm. **E**, Electron micrographs of postnatal day 3 neonatal rat. (**Left**) Cells have central loose cytoplasmic space with myofibrils at the periphery. Several cell junctions (arrows) are present; Bar=2 μm. (**Right**) Myofilaments connect to an intercalated disc (arrowhead). Note the absence of M-bands (arrows); bar=1 μm. Reprinted from Bishop et al[70] with permission. Copyright ©1990. Wolters Kluwer Health, Inc. **F**, Electron micrographs of adult rat isolated myocyte and myocardium. (**Left**) Scanning electron micrograph of adult rat isolated myocyte. Note T-tubule openings at Z-bands (arrows). (**Right**) Trassmission electron micrograph of adult myocardium with T-tubule at Z-line. Note M-band (arrow). **G**, Embryonic day 12 fetal heart 2 weeks after implant on the iris of an adult rat. (**Top**) Light photograph. Note disoriented myocytes with some binucleated cells (double arrowheads); bar=10 μm. Inset: Cell in mitosis; bar=10 μm. (**Bottom**) Electron micrograph. Side to side intercalated discs (arrow) and incompletely formed sarcomeres between Z-bands (arrowheads), absence of M-band and poorly aligned fibrils; bar=1 μm. Reprinted from Bishop et al[70] with permission. Copyright ©1990, Wolters Kluwer Health, Inc. d indicates desmosome; fa, filament attachment area; ICD, intercalated disc; nex, nexus; sm, sarcoplasmic membrane; sr, sarcoplasmic reticulum; and T with arrows, T-tubules.



compared with the adult myocardium with fully developed sarcomeres containing M-bands and fully organized intercalated discs (Figure A [bottom]). As the heart continues to increase in size and nearing the end of gestation or the early neonatal period, myocytes have become elongated in a spindle shape (Figure B). By electron microscopy, these rat P1-P3 neonatal sarcomeres have clearly defined Z, H, A,

and I bands, but no M-band. However, by immunostaining, proteins for these sarcomere bands are clearly identified as early as during midterm E12–14 fetal development.[43] Using immunostaining techniques, Ahuja[43] has also demonstrated that during disassembly of the sarcomere during cytokinesis, the Z-band is the first to break down, followed by the actin and myosin filaments and only in late telophase by the breakdown of myomesin M-band proteins. Myofibrils are mainly aligned around the periphery of the cell and are only loosely connected laterally. Mitochondria are spherical and clustered in the center of the cell around the nucleus (Figure C). T-tubules and sarcoplasmic reticulum are absent in fetal and early neonatal cardiomyocytes. Intercellular intercalated disc connections are simple and located along the periphery of the cell. Mitotic figures that indicate nuclear division only, not necessarily followed by cell division, are frequent and during nuclear mitosis, sarcomeres are dispersed and the cytoplasm is cleared (Figure D). Immediately after the conversion to 2X2N myocytes, both single and binucleated cell sarcomeres become well aligned but incomplete, still lacking structurally well defined M-bands (Figure E). In the rat neonatal heart, as the myocytes become binucleated between P5 and P12, structural M-bands appear in the sarcomere. With an increase in cell size, sarcomeres become fully mature, cell junctions move toward the ends of cells, and T-tubules with sarcoplasmic reticulum appear by 11 days in the rat heart[69–71] resulting in increased calcium availability and contractile force[72,73] (Figure F). The cell volume of double-nucleated cells is double that of single-nucleated cells, and cells with 4 nuclei have 4 times the single-nucleated cell volume.[48,50,52,74] Myocyte volume increases proportionally with increasing heart size, which in most species increases in proportion to body weight.[45,48,60,61] As we try to decipher the mechanisms that regulate the early postnatal cardiomyocyte exit cell cycle, we will briefly review the drastic physiological changes at birth.

PHYSIOLOGICAL CHANGES DURING FETAL TO NEONATAL GROWTH

In addition to these morphologic changes during the transition from fetal 1X2N cardiomyocytes to neonatal 2X2N cardiomyocytes, several maturational physiological alterations are occurring as well during the conversion from fetal to neonatal growth. Blood oxygen levels increase dramatically from the fetal placental oxygen supply to pulmonary oxygenation after birth. The lungs become functional and supply oxygen to blood now directly entering the left side of the heart with the closure of the ductus arteriosus and foramen ovale.[75] Pressures being equal on both left and right ventricles in the fetus, the ventricles have equal weights at birth, but following birth, left ventricular pressure is greater than in the right ventricle, resulting in more rapid growth of the left ventricle after birth.[76,77]

Currently there is no clear evidence in adult animals or humans that binucleated or polyploid single-nucleated cells are able to undergo cytokinesis and provide a significant number of functional cardiomyocytes. However, recent studies using isolated cardiomyocytes from P3 mice with newly formed and still ultrastructurally incompletely formed binucleated cardiomyocytes maintained in culture for 3 days stimulated with either 10% FBS or fibroblast growth factor1/p38i were able to undergo a low rate of cell division.[40,41] These cell culture studies, as well as earlier studies of serum or fibroblast growth factor-stimulated isolated cells from mid-gestational rat fetuses[43] and from P3 neonatal rats[29] using immunohistochemical procedures were able to identify contractile proteins during induced dissociation and cellular division. Some of these proteins, notably myomesin and others in the M-band are not visualized by electron microscopy until a few days after the initiation of binucleation in neonatal animals. Thus, with the full structural maturation of the sarcomere including M-bands, mature intercalated discs, interstitial matrix development, and vascular growth, further cell division does not occur. While these in vitro cell culture studies provide provocative evidence that terminally differentiated cardiomyocytes may be able to be stimulated to further divide in vitro with proper stimulation, whether this can occur in fully structurally organized cardiomyocytes in adult animals is still unclear.

HEMODYNAMIC CHANGES DURING FETAL TO NEONATAL GROWTH

Fetal arterial pressures gradually increase during gestation, to 50 to 70 mm Hg in goats and sheep.[78–80] In dogs, ventricular pressure was reported at 50 mm Hg.[80] In animals born in a less mature state, pressures in late fetal rabbit and cat were 20 to 30 mm Hg.[80] No fetal pressures for rat are available but P1 Wistar Kyoto and spontaneously hypertensive rat arterial pressure is 20 and 25 mm Hg, respectively.[16] Few data are available for fetal or term human fetuses, but in 1 study using 16 to 29 week fetuses during planned termination of pregnancy, right and left ventricular pressures were equal, ranging from 15 mm Hg at 16 weeks to 35 mm Hg at 29 weeks.[81] In another study using noninvasive Doppler-derived fetal aortic blood flow, pressure ranged from 28 mm Hg at 20 weeks to 45 mm Hg at 40 weeks of gestation.[82] Presumably, left ventricular pressures would continue to rise to ≈70 mm Hg before birth.

OXYGENATION CHANGES DURING FETAL TO NEONATAL GROWTH

The oxygenation state in the hearts of all mammals changes during the transition from fetus to newborn. The mammalian fetal circulation is shunt-dependent with the mixing of arterial and venous blood. Therefore, fetal arterial oxygen levels are relatively hypoxic. In sheep, fetal arterial oxygen partial pressures are at 16 to 22 mm Hg, and rapidly rising to adult levels of 90 to 100 mm Hg after birth.[83] Previous mouse studies from the Sadek group have shown that reactive oxygen species levels increase significantly in cardiomyocytes from P1 to P7, which contributes significantly to the cardiomyocyte exit cell cycle.[25] Oxidative stress and the DNA damage response may contribute to cell cycle regulation in neonatal hearts. Mitochondrial reactive oxygen species are generated as electrons leak from the electron transport chain,[84,85] which damages proteins, lipids, or DNA, and the various types of DNA damage (eg, oxidized bases and single- or double-strand breaks) activate the cellular DNA damage response, leading to cell-cycle arrest or cellular senescence.[25,86–88] Interestingly, a hypoxia fate-mapping study showed that a rare population of cycling adult mouse cardiomyocytes is located in a hypoxic niche and protected from oxidative damage.[89] Also exposure to severe hypoxia after induction of myocardial infarction induced a robust cardiomyocyte proliferation with an improvement of left ventricular systolic function in the adult mouse.[90] These reports suggested that the oxygen partial pressure in circulation plays a pivotal role in adult cardiomyocyte cell cycle arrest.

Some reports have also suggested that this rapid change in oxygenation after birth is a major stimulus for cell-cycle arrest of cardiomyocytes resulting in the conversion from hyperplastic to hypertrophic growth.[15,24,25] We have recently shown that newborn porcine hearts can regenerate from myocardial infarction injury during the first 2 days of life. Interestingly, this regeneration is associated with the induction of cardiomyocyte proliferation and is lost when cardiomyocytes exit the cell cycle shortly after birth. This limitation of regenerative capacity also may coincide with oxygenation change from fetal to the neonatal environment even in large mammals.

HORMONAL CHANGES DURING FETAL TO NEONATAL GROWTH

Thyroid hormone levels in sheep and mice increase in late fetal and early neonatal period and stimulate the conversion from 1X2N cells to 2X2N cells and hypertrophic cell growth.[91,92] In neonatal rats treated with thyroxine for 12 days, both heart weights and cell volumes were increased but total cell numbers were decreased because of accelerated conversion to hypertrophically growing 2X2N cells.[93]

Glucocorticoid exposure to preterm piglets had a stimulatory effect on cardiac growth, accelerating the conversion to 2X2N cells and also moderately increasing the rate of apoptosis.[94]

EXPERIMENTAL INTERVENTIONS AFFECTING FETAL AND NEONATAL CARDIOMYOCYTE PROLIFERATION

Several earlier studies have demonstrated that impairment of fetal oxygen or pressure overload results in stimulated hyperplastic growth. Nutritional anemia in fetal rats resulted in volume overload and increased hyperplastic growth of the heart.[95] Aortic constriction in fetal lambs,[96] neonatal dogs,[48] near term fetal guinea pigs,[97] and in young rats with renal hypertension[98] resulted in enlarged hearts because of increased hyperplastic growth of myocytes. Reduction in fetal blood oxygen by carbon monoxide exposure to rats during pregnancy and after birth results in a volume overload response with increased heart weight and myocyte cell number because of accelerated hyperplastic growth of 1X2N cells in the fetal and postnatal period.[99,100] However, in a more recent study in late-term fetal sheep with pulmonary artery constriction, the increased right ventricular pressure and hypertrophy was attributable to a combination of hyperplastic and hypertrophic cell growth.[78] In addition, anemia in fetal sheep also produced larger and more mature cardiomyocytes.[101] Further, recent contrasting studies of maternal hypoxia in fetal rats resulted in an increase in size and binucleation of cardiomyocytes.[102–104] The explanation for these contrasting studies is not clear. Fetal and early neonatal rats, guinea pigs, and dogs have 100 % single-nucleated cells while late fetal sheep myocytes are already 50% to 70% binucleated. The ratio of 1X2N to 2X2N cells may affect the response to various experimental injuries.

In a study to evaluate the role of proto-oncogenes in cardiac development, we studied a model of transgenic mice with overexpression of the *c-myc* oncogene in which the transgenic mice underwent 1 additional cell division during the fetal period. In the transgenic mice, there was a 50% to 100% increase in heart weight at birth because of twice the normal number of cells in the heart and only a slight increase in body weight compared with wild-type littermates.[105,106] In this transgenic model, the extra cell division occurred sometime during fetal growth perhaps indicating an earlier development of cardiac maturity in the transgenic mice.

In another study with neonatal spontaneously hypertensive rats, the conversion to hypertrophic growth

also started 4 to 5 days earlier than in Wistar Kyoto control rats in the presence of elevated blood pressure and heart weight in spontaneously hypertensive rats versus Wistar Kyoto rats.[16] These studies suggest that early maturation of the heart in both mice and rats is a trigger for earlier conversion from hyperplastic to hypertrophic cell growth.

PROGRAMMED CONVERSION FROM HYPERPLASTIC TO HYPERTROPHIC CELL GROWTH

Several older and recent reviews have explored the various morphological, physical, metabolic, biochemical, and other factors occurring during the conversion of fetal single-nucleated rapidly dividing cardiomyocyte to the binucleated non-dividing cells growing by hypertrophy.[14,32,42,107–110] The role of the centrosome in the ability of cells to continue to divide has received considerable attention. In newts and zebrafish, centrosomes are maintained allowing the cells to continuously divide, while in mammals after birth, when the ability to divide is lost, centrosomes are lost.[27] The centrosome, with its tubulin structure, plays an important role in the process of nuclear DNA division and subsequent cellular division. The loss of centrosomes in mammalian neonatal hearts plays an important role in the replication ability of neonatal mammalian cardiomyocytes.[41] Zebrowski[27] has shown that the tubular components of the centrosome move to the nuclear envelope and may play an important role in providing structural components in the post-mitotic cell. While these factors play an important role in the maturational development of fetal myocytes to adult myocytes, there still remains the issue of what determines the time for the conversion to occur. Clarification of this issue will play an important role in the quest to reactivate cardiomyocyte reproduction in the injured heart.

One possibility to consider is that there may be a programmed number of cell divisions that can occur in the fetal period before the conversion from 1X2N cells to 2X2N cells, indicating the conclusion of hyperplastic cell growth and conversion to hypertrophic cell growth. In the neonatal rat and mouse, this conversion occurs from 4 to 6 days after birth, in the dog up to 10 days after birth, but in pigs and sheep and humans, the conversion occurs before birth. In these different species, developmental maturation occurs at different times relative to birth. Mice, rats, cats, and dogs are relatively immature at birth while larger animals including pigs, sheep, and goats are more mature at birth, fully covered with hair, eyes open, able to stand, walk, and suckle without maternal assistance. These data all suggest that fetal cardiac myocytes in each species are programmed by an intrinsic program for a definitive number of divisions, programmed to end at a certain stage of maturity. Studies of cultured embryonic rat cardiomyocytes from E12 to E20 by Burton et al[111] have similarly shown that even in culture, under appropriate stimulation, fetal cells exhibit an intrinsic program and continue to divide for the required number of days equivocal to the timing of the cells in vivo.

The intrinsic program in cardiomyocytes may also contribute to the conversion of 1X2N to 2X2N cells during the period around birth. One study in our laboratory in an attempt to address this issue involved the implantation of a 12-day fetal rat heart on the iris of the eye of an adult rat, an in-vivo model of tissue culture.[70,112] The embryonic myocytes continued to grow, were innervated and vascularized, but had no hemodynamic pressure other than the normal capillary blood flow. Interestingly, the cells continued to grow by hyperplasia, the cells remaining single nucleated with immature sarcomeres until at 12 to 14 days *in oculo*, at a time comparable with newborn rats, the myocytes became binucleated and further growth was by the increase in cell size. At 2 weeks *in-oculo* single-nucleated myocytes had incompletely organized sarcomeres, lacking M-bands and incomplete alignment of fibrils (Figure G). By 5 weeks in oculo equivalent to a 3 weeks old neonate, sarcomeres of binuclear cells were fully formed including M-bands though there was the misalignment of fibrils. These *in oculo* studies as well as the cell culture studies of Burton[111] suggest that some internal clock might be responsible for the conversion to hypertrophic cell growth in the absence of any heart or body weight or maturational factors and normal maternal oxygen.

SUMMARY

The loss of the ability to repair an organ after lethal injury of the major functional cell component as with cardiac myocytes remains an important and unsolved question in pathology and clinical medicine. The majority of postnatal heart cardiac myocytes having completed the allotted number of embryonic and fetal or early neonatal divisions and assumed a more highly structured arrangement of organelles and replication of DNA by multinucleation or polyploidy appear to be unable to undergo any further cell division. In attempts to provide evidence that binucleated cells are capable of division, in vitro studies of isolated binucleated cells obtained from first generation binucleated cells from P3 rats with still incomplete ultrastructural maturity have been able to promote cytokinesis when properly stimulated in culture.[40,41] The completion of the structural nature

of the sarcomere following the conversion to binucleated cells including the formation of M-bands consisting of proteins stabilizing other myofibrillar proteins, is an important feature in the cessation of cytokinesis. All further growth is by an increase in cell volume, hypertrophy. Whether these highly organized cardiomyocytes in adult animals with multiple sets of DNA can be induced to divide has received much recent attention. Several efforts to evaluate this problem have been reviewed by Zebrowski et al[113] and Leone and Engel.[40] Additional questions remain relative to whether the population of single-nucleated 1N but highly structurally organized cells can be induced to divide. While many questions remain about factors which control the ability of myocytes to reenter the hyperplastic phase and play a role in the recovery of lethal myocyte injury, the use of experimental animals has provided some insight relative to naturally occurring conversion from hyperplastic to hypertrophic myocyte growth.

During the conversion from fetal to postnatal cardiac growth, there are a number of maturational factors including physiologic, metabolic, structural, genetic, hormonal, growth factor, nutritional, and other yet to be discovered factors that occur. While experimental manipulation of these factors has been shown to have a minor, but temporary effect on the conversion rate from hyperplastic to hypertrophic myocyte growth, the question of what actually controls the normal developmental change in the manner of growth remains unexplained. The stage of maturation of the developing fetus appears to be one regulating factor, as seen in the early neonatal conversion animals such as mice, rats, dogs, and cats, which reach a stage of maturity after birth. Another explanation may be that each species has a genetically programmed number of cardiomyocyte cell divisions required to enable post fetal whole body growth. The *in oculo* experiment of in vivo cell growth of the developing fetal rat heart as well as the in vitro cell culture studies of Burton[111] demonstrated that conversion to binucleated cells occurred at the appropriate time irrespective of location outside the chest of a developing animal.

Acute myocardial infarction and its associated congestive heart failure is one of the most significant problems from both the public health and economic perspectives. With the recent findings of significant remuscularization of damaged myocardium in an extremely short window of the early neonatal period using small-, large-animal models, as well as the observation of pediatric human patients, there are renewed scientific interests in deciphering the mechanism of cardiomyocytes exiting the cell cycle. Should we be able to demonstrate and intervene in the regulators that control the myocytes hyperplastic growth exit cell cycle, we would be able to "turn back the clock" of the myocyte cell cycle, and consequently remuscularize the ventricle of patients suffering from the acute myocardial infarction, and save lives. The replication of DNA happens during the process of karyokinesis as well as during the process of multinucleation or polyploidy. Therefore, from the technologic perspective, better technologies of imaging and molecular signature proteins in demonstrating myocyte dividing are urgently needed.

AFFILIATION INFORMATION

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Disclosures

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