REVIEWS–A PEER REVIEWED FORUM

Developmental Dynamics

Myocyte Proliferation in the Developing Heart

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Regulation of organ growth is critical during embryogenesis. At the cellular level, mechanisms control-ling the size of individual embryonic organs include cell proliferation, differentiation, migration, and attri-tion through cell death. All these mechanisms play a role in cardiac morphogenesis, but experimental studies have shown that the major determinant of cardiac size during prenatal development is myocyte pro-liferation. As this proliferative capacity becomes severely restricted after birth, the number of cell divisions that occur during embryogenesis limits the growth potential of the postnatal heart. We summarize here cur-rent knowledge concerning regional control of myocyte proliferation as related to cardiac morphogenesis and dysmorphogenesis. There are significant spatial and temporal differences in rates of cell division, peak-ing during the preseptation period and then gradually decreasing toward birth. Analysis of regional rates of proliferation helps to explain the mechanics of ventricular septation, chamber morphogenesis, and the devel-opment of the cardiac conduction system. Proliferation rates are influenced by hemodynamic loading, and transduced by autocrine and paracrine signaling by means of growth factors. Understanding the biological response of the developing heart to such factors and physical forces will further our progress in engineering artificial myocardial tissues for heart repair and designing optimal treatment strategies for congenital heart disease. *Developmental Dynamics 240:1322–1334, 2011.* © 2011 Wiley-Liss, Inc.

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INTRODUCTION

At the cellular level, mechanisms con-trolling size of individual embryonic organs include cell proliferation, dif-ferentiation, migration, and cell death. All these mechanisms play a role in cardiac morphogenesis, but ex-perimental studies have shown that the major determinant of cardiac size during prenatal development is myo-cyte proliferation (Clark et al., 1989; Saiki et al., 1997; Sedmera et al., 2002). The adult heart has tradition-ally been regarded as a postmitotic organ. Although it is clear today that this is not entirely correct, there is still agreement that most myocyte proliferation occurs during prenatal development. Proliferative activity in the heart not only increases its mass to match the increasing circulatory demands of the developing embryo, but together with programmed cell death and migration is a main factor shaping the developing heart. Under most nota-ble pathological conditions, some changes in cell proliferation are usually detectable. Numerous studies using varied methodological approaches have systematically mapped cell prolifera-tion in different compartments during development. Because some of them are rather old, and in languages other than English, we here provide an over-view of these precious bits of informa-tion, both to summarize findings to date and to serve as a methodological guideline for investigators willing to analyze proliferation in specific cardiac compartments. We also put into histori-cal perspective some recent studies using computer-assisted methods to de-cipher proliferative structure of the entire organ.

METHODS OF ASSESSING CELL PROLIFERATION
DNA Labeling

One of the most common methods of measuring cell proliferation is DNA pulse-labeling. Its principle lies in incorporation of labeled nucleotide into DNA of proliferating cells, specifically those going through DNA replication (S-phase). The length of such pulse depends on proliferative activity (cell cycle and S-phase length) of the tissue under study. To simplify the counting process and minimize numbers of counted cells to ensure robust statistics, length of pulse resulting in labeling index in the order of tens of percent is desirable. If the labeling index is below 5%, large numbers of cells must be counted to detect any differences, similar to apoptotic indices in the heart reported by the Anversa group (Kajstura et al., 1996; Anversa et al., 1998). Before the development of anti-bromodeoxyuridine (BrdU) immunohistochemistry, cell nuclei in the S-phase of cell cycle were detected by labeling with [3H]-thymidine followed by autoradiography (Fig. 1). This technique retains merit today as being quantitative, yielding estimates of nuclear doubling under controlled conditions (Sedmera et al., 2003b). The earliest sampling time allowing incorporation of detectable amount of label into DNA is approximately 30 min, and in general, the labeling period is in the order of hours. The bioavailability of the label for in vivo incorporation does not commonly exceed 2 hr in mammals, but can be 1 to 2 days in the chick embryo (Yurkewicz et al., 1981) due to immature liver metabolism. This technique can be modified to "label-dilution" mode, in which the sampling is delayed by several days after label administration (Fig. 1A), thus preferentially detecting cells that stopped dividing shortly after labeling. Because this time point is commonly narrowed, or sharpened, by administration of an excess amount of unlabeled thymidine, this technique is referred to as a "pulse-chase" experiment. Methodologically easier (and cheaper) is labeling with halogenated thymidine analogs such as 5-bromodeoxyuridine or 5-iododeoxyuridine that can be distinguished immunohis-tochemically (Burns and Kuan, 2005). This technique is widely applicable in systems ranging from cultured cells in vitro to whole animal labeling in vivo (Fig. 1B,C) and allows identification of proliferating cell type by double immunohistochemistry (i.e., for DNA label and specific cell marker).

If the pulse duration is equivalent to or longer than cell cycle length, one can obtain "saturation labeling" that highlights (with suitable nuclear counterstaining) the nonproliferating cell population (Fig. 1C). This has proven useful in the study of quiescent or differentiating cell populations, such as newly committed myocardium (Sedmera et al., 2003a) or cardiac conduction system (Thompson et al., 1990a, 1995; Cheng et al., 1999). A note of caution should be added for early postnatal studies. This period is characterized by a shift from hyperplastic to hypertrophic myocyte growth, and often involves binucleation, i.e., karyokinesis without cytokinesis (Clubb and Bishop, 1984; Kellerman et al., 1992; Li et al., 1996). Additionally, DNA synthesis is often activated during development of cardiac hypertrophy in the adult heart, so interpretation of labeling studies in such cases needs to be complemented by another method (cell counts, cell volume measurements).

Intrinsic Markers of Cell Proliferation

For archival tissue or human biopsies, several endogenous cell cycle markers can be detected by immunochemistry. One popular marker used in pathology is PCNA (proliferating cell nuclear antigen, Fig. 1B), which works well but presents variable intensity of labeling in comparison with "all or nothing" nature of anti-bromodeoxyuridine. PCNA is unfortunately of limited value for quantification of proliferation in rapidly proliferating embryonic tissues where almost all cells are positive (except for detecting quiescent cell populations, see below). Other popular markers of proliferation include Ki-67 (Orlic et al., 2001b; Lynch et al., 2007), and phospho histone-H3 (Poolman and Brooks, 1998; Suzuki et al., 2005), that provides a sharper, less laborious alternative to earlier mitotic counts (Grohmann, 1961; Rychter et al., 1979). Various techniques and their principles are summarized in Table 1.

Viral and Genetic Techniques

In experimental systems, retroviral, adenoviral, or genetic labeling reveals proliferative history and clonal origin of cells within myocardium. Infection with adenovirus, which has an incremental likelihood of being lost with subsequent cell division, has been used to highlight areas of slowly dividing myocardium such as the outflow tract in the avian heart (Cheng et al., 1999; Watanabe et al., 2001). Retroviral labeling has been used to prove the existence of common progenitors of both working and conducting myocardium, firmly establishing the myogenic origin of ventricular conduction system (Gourdie et al., 1995; Cheng et al., 1999). Conceptually similar studies were performed using nlaacZ transgene under the control of the alpha cardiac actin gene in the mouse (Meilhac et al., 2003), demonstrating that patterns of myocardial growth are similar in mammals and birds. Recently, this model was used to study the origin of ventricular conduction system, and it was shown that it is also myogenic (Miquerol et al., 2010). It is distinguished from the surrounding working myocardium by notably lower proliferation rates, as can be also appreciated from saturation labeling (Fig. 1C).

DEVELOPMENTAL CHANGES IN RATES OF MYOCYTE PROLIFERATION
Embryonic Period

Let us now review the dynamics of myocyte proliferation during development. During the earliest period of heart function, before peak proliferation at embryonic days 3–4 (in the chick embryo, Fig. 2a), the tubular heart grows primarily by addition of newly committed mesenchymal cells (Moorman et al., 2010), whose high proliferative activity drops as they start to synthesize contractile proteins to become functional cardiomyocytes (Kelly et al., 2001; Sedmera et al., 2003a). In general, proliferative activity is inversely related to the
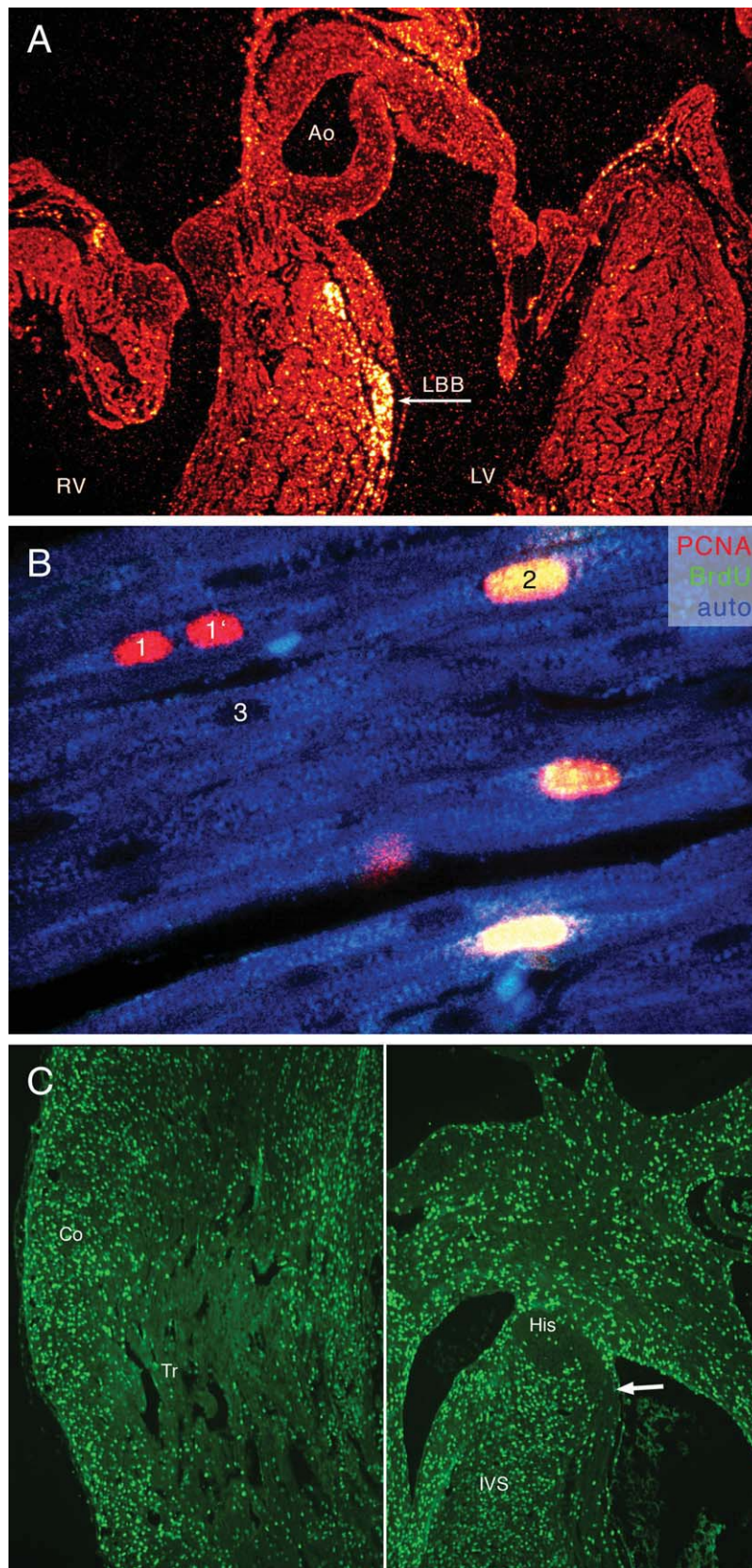
Fig. 1.

level of cell differentiation. Thus, the rates of cell proliferation are the highest at the early, less differentiated developmental stages (ballooning period of chamber formation, Moorman et al., 2010) and then taper off until birth or hatching (Fig. 2). There is a short spike in the early postnatal period, during adaptations to postnatal circulatory changes, followed by essentially permanent cell cycle arrest.

Regional Differences in Cardiac Morphogenesis

The pattern of gradual decrease of proliferative activity during development has been noted by several investigators, including Ivan Cameron in the chick (Jeter and Cameron, 1971) and Pavel Rumyantsev in the rat (Rumyantsev, 1982). Other interesting temporal patterns emerge when one follows proliferative activity over time in different cardiac compartments. The path from the simple tubular

Fig. 1.   A: Label dilution of [3H]-thymidine in chick embryonic heart. Label was applied on ED2, and the heart sampled at ED10. Most of the radiolabel detected by autoradiography is retained in the ventricular conduction system, while more rapidly dividing working myocardium diluted label through many rounds of cell division. Ao, aorta, LBB, left bundle branch, LV, left ventricle, RV, right ventricle. From Thompson et al. (2000). **B:** Illustration of different immunohistochemical techniques for myocyte proliferation. Bromodeoxyuridine labeling (secondary antibody in the green channel) gives all-or-nothing signal, and labels only cells passing through S-phase during the labeling period (2). Proliferating cell nuclear antigen (red channel) lingers in the nuclei for longer time, and labels also cells that recently completed karyokinesis or cytokinesis (1, 1'). Unlabeled nuclei (without counterstaining) appear as oval shadows in the autofluorescent cytoplasm (3). Sample from neonatal pig myocardium labeled for 2 hr with bromodeoxyuridine. **C:** Saturation labeling in the fetal heart. Sections from rat embryonic heart labeled by means of Alzet osmotic mini-pump from ED17.5 to ED18.5. While almost all nuclei are labeled in the compact myocardium (Co), the trabeculae (Tr) show much lower labeling. Central conduction system in the interventricular septum (IVS) stands out by its paucity of label. Arrow points to left bundle branch (compare with panel A, where this structure, in a different species, is also distinguished by its low proliferation). Note also that the working myocardium of the interventricular septum shows similar labeling intensity as the compact myocardium (see also Fig. 2B for comparison with fetal chick data).

TABLE 1. Overview of Commonly Used Techniques for Studying Myocyte Proliferation[a]

Short name	Target Labeled	Reference
Mitotic counts	Chromosomes in mitosis; laborious, requires patience	Grohmann, 1961; Manasek, 1968; Rychter et al., 1979
Phosphohistone H3	Nuclear protein, phosphorylated during mitosis	Poolman and Brooks, 1998; Suzuki et al., 2005
[3H]-thymidine autoradiography	DNA of nuclei in S-phase	Thompson et al., 1990b; Sedmera et al., 2002
BrdU immunolabeling	DNA of nuclei in S-phase	Sedmera et al., 2002; van den Berg et al., 2009
Ki-67	Nuclear antigen, cell proliferation marker, positive in tumors	Orlic et al., 2001b; Lynch et al., 2007
PCNA	Nuclear antigen present in proliferating cells, good for quiescent cells in the embryo or cycling cells in tumors	Thompson et al., 1990a; Thompson et al., 1995; Lin et al., 2000; Morritt et al., 2007

[a]Care should be taken to use an unambiguous (preferably nuclear) myocyte marker, as it is otherwise difficult to distinguish in vivo myocytes from non-myocytes (Dowell et al., 2003).

heart nourished by diffusion from its lumen to mature ventricle supplied from the outside by coronary vessels is not straightforward. Ventricular trabeculae first appear as a sponge-like network of well-differentiated muscle, perhaps enabling to increase myocardial mass in the absence of coronary perfusion (Minot, 1901). Early ventricular trabeculae are fenestrated myocardial sheets (Icardo and Fernandez-Teran, 1987; Sedmera et al., 1997) that appear in histological section as finger-like (or villous) protrusions from the ventricular wall (Marchionni, 1995). Trabeculae not only decrease the diffusion distance for oxygen and nutrients, but also play a role in electrical conduction (de Jong et al., 1992). In the chicken, they also contribute to ventricular septation. Dieter Grohmann (Grohmann, 1961) noticed that the forming interventricular septum in the chick has a low mitotic activity, as it arises from coalescence of the slowly growing ventricular trabeculae (Harh and Paul, 1975; Ben-Shachar et al., 1985). The difference between the ventricular compact myocardium and trabeculae (Fig. 1C) also tends to decrease with advancing development and trabecular compaction (Fig. 2D). Once septation and trabecular compaction are under way, the septal musculature adopts the kinetics of nearby working ventricular myocardium, cycling at higher rates (Fig. 2B). The developing conduction system stands out by its low proliferative activity in chick (Cheng et al., 1999), mouse (Erokhina and Rumyantsev, 1988; Sedmera et al., 2003a), and rat (Thompson et al., 1995).

Differential growth of regions within the same compartment is important for molding of the entire organ. Proliferative regions within the ventricular free walls (Fig. 3) have been highlighted by mitotic counts by Klaus Goerttler (Goerttler, 1956) and Rychter and associates (Rychterova, 1978; Rychter et al., 1979) and with BrdU labeling (Thompson et al., 1990b), beautifully illustrated by the Moorman lab using three dimensional rendering (van den Berg et al., 2009). Regional differences exist even across the ventricular wall, with the highest proliferative activity localized in the parietal compact zone (Sedmera et al., 2002), lower proliferation in the trabeculae (Fig. 2D), and virtually zero in the terminally differentiated parts of the central conduction system (Mikawa et al., 1992; Cheng et al., 1999). These local differences are confirmed by analysis of shape of clones derived from a single myocyte (Meilhac et al., 2003), which suggests that growth and shaping of the ventricular myocardium occur centripetally, i.e., by apposition of new cells (Jeter and Cameron, 1971) and by "ballooning" of the ventricular chambers in the period of cardiac chamber formation (Moorman and Christoffels, 2003).

Postnatal Myocyte Proliferation

As mentioned earlier, myocyte proliferation essentially stops early after birth or hatching, and further growth in volume of myocardial compartment occurs by increasing cell size (hypertrophy). In both chick and rat, this transition is rather abrupt (Clubb and Bishop, 1984; Kellerman et al., 1992; Kajstura et al., 1995a; Li et al., 1996). However, induced growth during this period (such as occurs after experimental changes in mechanical loading) is still based, at least in part, on cell proliferation (Sedmera et al., 2003b). It was shown recently that the early neonatal period in mouse is privileged in its myocyte regenerative capacity (Porrello et al., 2011) approaching that of lower vertebrates such as zebrafish (Lepilina et al., 2006; Kikuchi et al., 2010). This suggests that early corrective surgical interventions could potentially benefit through compensatory growth that is impossible to achieve with later interventions, thus enhancing the feasibility of such procedures as early biventricular repair for milder cases of hypoplastic left heart syndrome (Foker et al., 1999; Tchervenkov et al., 2000).

CELL CYCLE CHECKPOINTS
Postnatal Proliferative Block

What makes myocytes divide is interesting, but what makes them stop dividing is fascinating. The mitotic block in postnatal myocytes is generally attributed to inhibition of cyclins and cyclin-dependent kinases (Horky et al., 1997; Brooks et al., 1998; Burton et al., 1999; Nagahama et al.,
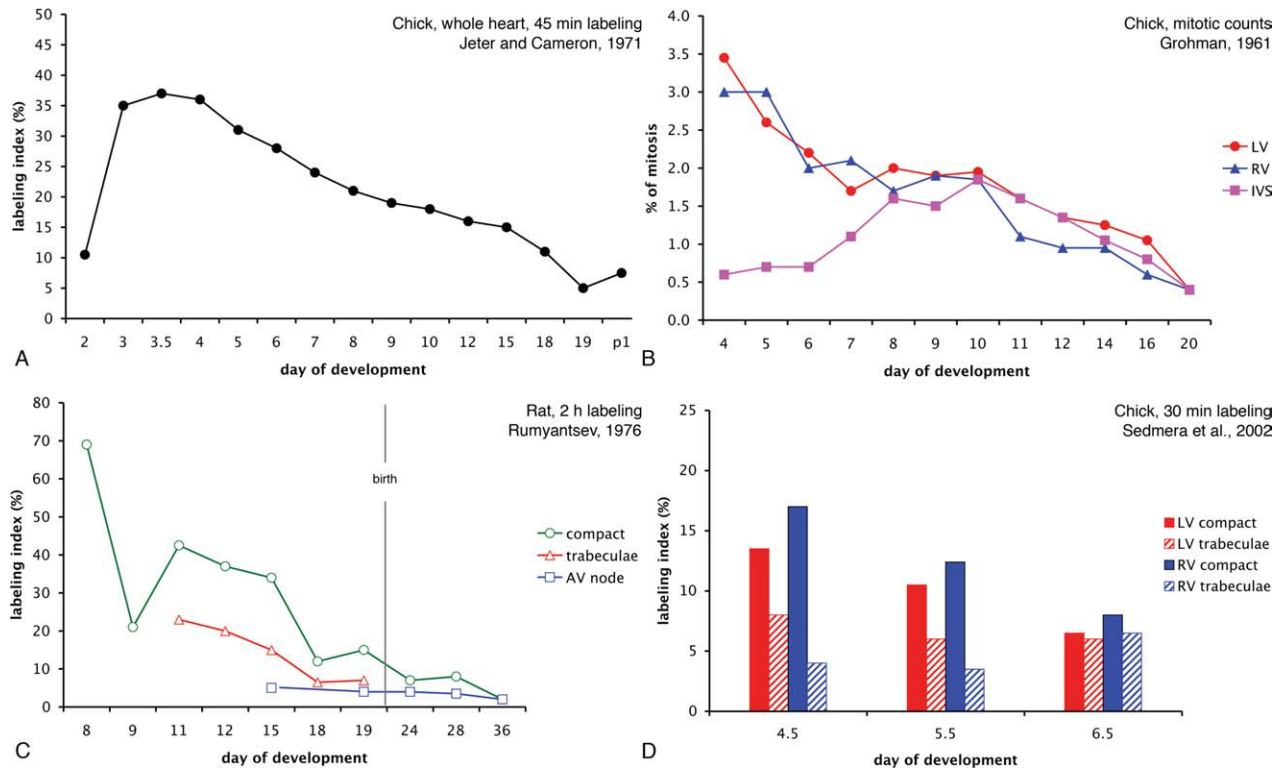
Fig. 2. Quantitative data of developmental trends in myocyte proliferation. **A:** Data from the entire chick embryonic heart (labeling with radioactive thymidine for 45 min show the peak in labeling index between embryonic day 3 and 4. **B:** Historical data of mitotic counts from chick embryonic heart showing similar declining trend from peak at embryonic day 4 toward hatching in both ventricles. This graph also shows clearly that the forming interventricular septum is distinguished by its low proliferative activity, while after completion of septation it behaves as the rest of the working myocardium. **C:** Regional differences in myocyte labeling in developing rat heart. Note the difference between the compact zone and the trabeculae as well as gradual decrease toward birth. **D:** Values from 2-hr bromodeoxyuridine labeling in the preseptation chick heart show decreasing gradient between the compact myocardium and trabeculae as well as decline in labeling with advancing development. The trends are identical in both left and right ventricle.
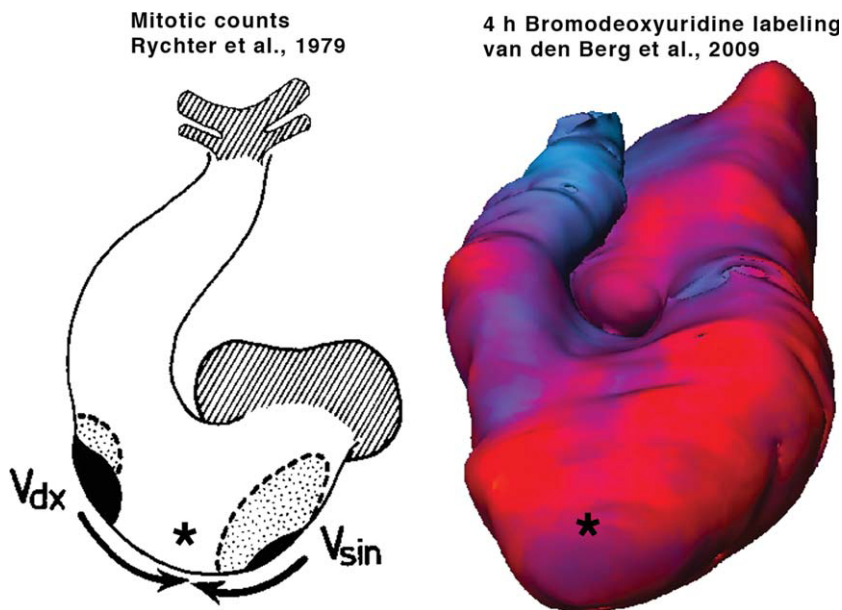

Fig. 3. Differences in myocyte proliferation in the compact zone at the organ level. Two proliferative centers (black) in the apices of prospective left and right ventricle, together with lower activity in the interventricular septum (*), were noted by Rychter et al. (1979). Similar data (high proliferative activity in red, slowly dividing septum, *), together with regions of low myocyte proliferation in the atrioventricular canal and the outflow tract (blue) were recently reported by van den Berg et al. (2009).

2001). There are other control mechanisms that might arrest cardiac myocytes in G0 phase of cell cycle, such as tumor suppressor protein p16ink associated with cell senescence (Kajstura et al., 2010), cyclin-dependent kinase inhibitor p21 (Poolman and Brooks, 1998), tumor suppressor protein p53 (Liu et al., 2010b), and its activator p14ARF, or retinoblastoma protein (Brooks et al., 1998), that must be phosphorylated by cyclin D-cdk4/6 to promote postnatal myocyte hypertrophy (Hinrichsen et al., 2008). Recent in vitro study of differentiated rat and mouse atrial and ventricular myocytes confirmed p21, p53 and 14-3-3 cell cycle regulators as key factors maintaining the myocytes in G0 phase (Zhang et al., 2010). Semi-quantitative immunohistochemistry showed their down regulation during de-differentiation of myocytes in culture and ensuing cell cycle re-entry. This study also showed that atrial myocytes, expressing smaller amounts

of those inhibitors, were more ready to renew proliferation. Notch signaling has also been shown to enable renewed cycling of quiescent embryonic stem cell-derived and neonatal ventricular cardiomyocytes through its action on cyclin D (Campa et al., 2008). Down-regulation of transcription factor C/EBPβ is associated with cell cycle reentry during exercise-induced myocardial growth (Bostrom et al., 2010). This proliferation was shown to be mediated in part by CITED4 up-regulation. A distinct control mechanism appears to be involved in maintenance of postnatal hypertrophy (Li et al., 1998; Poolman and Brooks, 1998). In contrast to this plethora of molecular players, the role of epigenetic factors, including oxygen tension, mechanical loading or metabolic changes that all show profound changes during postnatal myocyte maturation, remains underexplored.

Embryonic Cell Cycle Arrest and Development of Conduction System

What forces a precisely defined population of embryonic cardiomyocytes destined to become conduction system to dramatically slow and even stop their proliferation at a specific time point amidst their rapidly cycling neighbors is indeed puzzling. While there is slowly emerging a molecular fingerprint of genes differentially expressed in the cardiac conduction system (Mommersteeg et al., 2007; Aanhaanen et al., 2009), little attention has been focused on the link between these and myocyte proliferation. The earliest precursors of ventricular conduction tissues are derived from the inner cell layer of the tubular single ventricle; some exit the cell cycle permanently during cardiac looping (Stage 13 in the chick), well before any known extracardiac cell population reaches the heart (Thompson et al., 1990b, 2000, 2003). This suggests a local mechanism of control, perhaps higher mechanical strain of the tissue (Damon et al., 2009), or signaling from the endocardium (Gourdie et al., 2003). Although it remains difficult to separate these two possibilities in vivo, recent experiments with unloaded chick embryonic hearts in culture have shown that passive myocyte strain, rather than shear stress-dependent signaling from the endocardium, is sufficient to stimulate differentiation of the primary ring myocardium (Sankova et al., 2010). In the chicken, recruitment of working myocytes to the conduction lineages occurs throughout development, with more distal Purkinje fibers added in the last third of incubation, as their proliferation slows or stops (Thompson et al., 2000). This hypothesis of distal recruitment based upon studies in the chick embryo (Cheng et al., 1999) has been challenged by more recent results of clonal analysis of ventricular myocytes in a transgenic mouse in which LacZ is randomly activated in cardiomyocytes and resulting clone size gives an estimate of proliferative history of cells in various myocardial compartments (Miquerol et al., 2010); small clones of cells were found limited to conduction tissues, suggesting that there is a non-zero proliferative activity going on even after commitment to the conduction lineage. However, comparison of their size with much larger clones found in the working myocardium suggests that slowed proliferation is definitely a common theme in conduction system formation in both birds and mammals. Results derived from clonal analysis and proliferative history as related to cardiac growth and conduction system formation have been recently reviewed and integrated by Moorman and associates (Moorman et al., 2010).

Long-Term Kinetic of Ventricular Conduction System

Long-term follow-up studies tracked the fate of the early-exiting ventricular conduction system myocytes throughout the live of the animal. In an extension of our earlier study (Thompson et al., 2000), a group of 81 chick embryos were pulse-labeled with one microcurie of [3H]-thymidine at embryonic day 3 (Hamburger-Hamilton Stage 15) and sampled at various stages during incubation and at days 1, 10, 30, 100, and 300 of postnatal development. Invariably, heavily labeled cells (with nuclear grain counts of 15–30 grains per nucleus) were detected in the His bundle, its bifurcation and proximal bundle branches. No evidence of division, detected either by 24-hr bromodeoxyuridine labeling or decrease in grain counts was found in the most heavily labeled cells prenatally. Comparison of myocyte grain counts with those over nuclei of motor neurons in the neural tube suggested that one round of nuclear division may have occurred in labeled myocytes within 10 days after hatching. This was confirmed by the rare occurrence of BrdU-positive nuclei with heavy thymidine label, usually two nuclei in one cell, suggesting binucleation without cytokinesis. In later life (days 30–300; Fig. 4), only gradual decreases in counts of labeled cells were found in serial sections spanning the ventricular conduction system. This was likely due to apoptosis, which was found at low intensity (less than 1 in 1,000 TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labeling) -positive cells) in the myocardium including the conduction fascicles. No evidence of renewed DNA synthesis was found past day 30 in conduction system cardiomyocytes.

MYOCYTE REGENERATION POTENTIAL

The topic of heart regeneration is currently controversial, as reviewed in a recent book on the topic of cardiac development and regeneration in particular (Rosenthal and Harvey, 2010). During the past 2 decades, a paradigm shift has occurred from considering the adult myocardium as entirely postmitotic (the only population of cells capable of proliferation being fibroblasts, as evidenced by scar formation during myocardial infarction), to admitting a potentially important role of continued proliferation and differentiation of myocytes from resident stem cells for maintenance of homeostasis. It was recently postulated (Zhang et al., 2010) that the cell material for regeneration might derive from (i) resident cardiac stem cells, (ii) bone marrow-derived stem cells, and/or (iii) stem cells resultant from de-differentiation of existing cardiomyocytes. Let us further explore
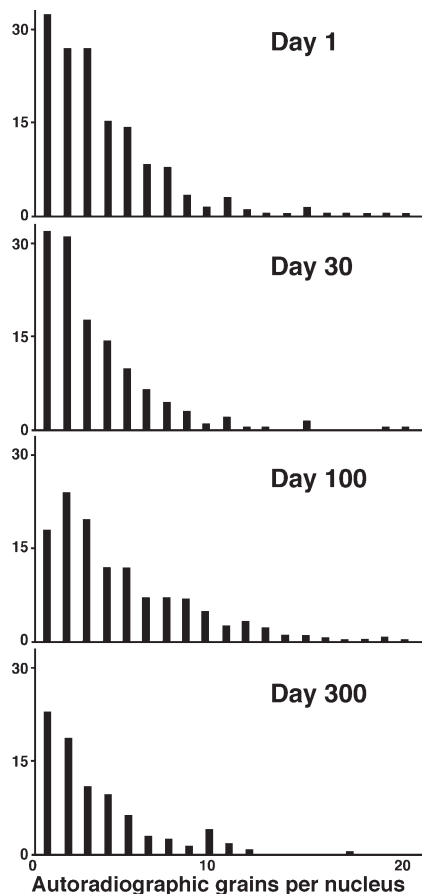
Fig. 4. Persistence of labeled cells in the ventricular conduction system in the chick. There is a gradual decrease in heavily labeled cells (over 10 grains per nucleus) with post-natal development, but both these and less intensively labeled cells can be found into adulthood, ten months after hatching. Values are means from three hearts, counting grains per nucleus in every tenth section (see Thompson et al., 2000).

these three options that are not mutually exclusive.

Cell Cycle Re-entry in Adult Myocytes

Early studies in a rat infarction model showed that there is small myocyte proliferative activity, which can increase in some compartments after experimental perturbation (Rumyantsev and Kassem, 1976). Next it was posited from rates of programmed cell death (apoptosis) in normal myocardium that cardiac muscle must somehow turnover within months or several years, depending on age and species under study, as well as the technique used for detecting dying cells (Kajstura et al., 1995b, 1996).

Cell division in bona fide cardiomyocytes has been observed in the early chick (Manasek, 1968), in pathological conditions such as heart failure (Anversa et al., 1998; Kajstura et al., 1998), and also in normal hearts, with very high numbers of cells counted (in the order of tens of thousands, for detecting both cell death and cell division, as these are indeed not very common events).

Resident Cardiac Stem Cells

Discovery of clusters of cells that are negative for traditional myocyte markers such as contractile proteins but positive for stem cell markers such as c-kit (Beltrami et al., 2003) made an important contribution to elucidation of this story. Indeed, well-differentiated binucleated working myocytes have not been found reentering the cell cycle, but those cells caught in the process of DNA synthesis appear to be new, small myocytes possibly derived from these resident stem cells. An alternative ingenious approach used radiocarbon dating of normal human tissues from before and after the era of atomic weapons testing to show that a substantial number of myocytes appear younger than the person (Bergmann et al., 2009). Calculated turnover rate was found to decrease from 1% in youth to less than half a percent in the senium, suggesting that even adult myocardium possesses a self-renewing capability. A unique study on patients treated with iododeoxyuridine for cancer showed large differences between individual patients, but this very long-term (weeks) "saturation labeling" showed rates of positive myocyte nuclei between 2.5 and 46%, suggesting rather active turnover of myocytes with half-life between 4 and 8 years (Kajstura et al., 2010). Meticulously designed control experiments exploring alternative explanations excluded increased myocyte ploidy or cell fusion as quantitatively important explanations of these observations. Despite rather atypical human population and small sample size (eight), these numbers (considerably higher than those demonstrated by radiocarbon study of Bergman and associates) clearly show that the adult mammalian heart possesses a signifi-

cant self-renewing potential. An important question remaining is whether, and how, might this potential be harnessed for regeneration of myocardium lost during myocardial infarction. Genetic fate-mapping study in mice (Hsieh et al., 2007) have shown that while the stem cells contribute to replacement of myocytes after injury, they do not play a major role in normal homeostasis and renewal during aging. This accords with the above observations showing proliferative capacity of adult cardiomyocytes sufficient to guarantee physiological renewal. Overview of studies in the adult weighting the relative contribution of differentiated cardiomyocytes and multipotent cardiovascular progenitor cells was recently provided by Sturzu and Wu (2011). Possible molecular players capable of inducing adult myocytes to re-enter the cell cycle are discussed in the next section.

Myocytes From Other Sources?

Extracardiac cell sources such as bone marrow stem cells were reported to be beneficial postinfarct 10 years ago (Orlic et al., 2001a,b,c; Dawn et al., 2005), but ensuing clinical trials in humans show that such effects, while real and sustained, are rather modest, and more likely due to paracrine factors produced by the transplanted cells rather than the addition of a significant mass of "new" myocardium from the transplant (summarized by Jiang et al., 2010). Experimental studies from other labs have shown that the cardiogenic potential of hematopoietic cells, especially in vivo, might be overstated, and suggest either cell fusion (Alvarez-Dolado et al., 2003) as a possible explanation of observations of the Anversa lab, or that the presence of circulation-derived cells in the myocardium after infarction does not have to mean that they differentiate into myocytes (Balsam et al., 2004; Murry et al., 2004). Clearly, many more basic issues need to be resolved before myocardial regeneration comparable to the zebrafish amputation model (Lepilina et al., 2006) can be seen in clinically relevant model of myocardial infarction in mammals. One step in this direction is the observation of the

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ability of 1-day-old mice to regenerate amputated apex of the ventricle, an ability lost as early as one week after birth (Porrello et al., 2011).

One of the unresolved questions we would like to pose here is the actual developmental origin of those resident stem cells: at the early tubular stages, there is no evidence of nonmyocytes being interspersed in the early myocardium; hence, it is possible that their source is extracardiac. Possible contributors could be (1) epicardium-derived cells, which were found to be capable of differentiating into myocytes in vitro (Kruithof et al., 2006), although controversy about applicability of this mechanism in vivo has been raised (Christoffels et al., 2009); (2) neural crest cells that are known to be multipotent (Yelbuz et al., 2003; Sieber-Blum et al., 2004; Kirby, 2007; Krejci and Grim, 2010) and contribute to multiple cell types in the heart; or (3) cells derived from the circulation (possibly originating in bone marrow or other hematopoietic locations), shown to preferentially invade a perivascular niche during the second half of incubation in the quail–chick parabiosis system (Zhang et al., 2006).

Alternatively, the cardiac resident stem cells can derive from myocytes by their dedifferentiation, as was shown in vitro (Zhang et al., 2010). This decision can be dictated by the local microenvironment, because physical factors such as low oxygen and nutrient availability are known to contribute to reversal of working myocyte fate. Another possibility is the contribution from second heart field cells expressing Isl1 (Cai et al., 2003; Zhou et al., 2008) that were shown to contribute to multiple cardiac lineages (myocytes, smooth muscle, endothelium, and epicardium).

MOLECULAR CONTROL MECHANISMS OF MYOCYTE PROLIFERATION

Adult Myocytes

The regulation of proliferation remains one of the major questions in developmental biology of cardiac muscle (Pasumarthi and Field, 2002), especially as related to heart repair (Oh et al., 2004). Interested readers should also consult other reviews

focused on cell proliferation after infarction (Dowell et al., 2003), during regeneration in adult animals (Rubart and Field, 2006) or during development (Moorman et al., 2010). Postnatal cell cycle arrest appears to be species-specific (Wills et al., 2008) and potentially reversible, as indicated by significant endogenous regenerative capability of fish heart in response to injury (Lepilina et al., 2006). Recent experiments in zebrafish have shed some light on the cellular nature of this process. Amputation of part of the ventricle leads to myocyte dedifferentiation and proliferation involving polo-like kinase 1 (Jopling et al., 2010). These newly generated myocytes become electrically coupled with the remainder of the ventricle within a month from injury (Kikuchi et al., 2010). Recent studies in mammals have also shown that adult myocytes can be induced to proliferate by inhibition of p38 mitogen-activated protein kinase or stimulation with fibroblast growth factor-1 (FGF1) (Engel et al., 2005), by stimulation by periostin (Kuhn et al., 2007), or through a neuregulin/ErbB signaling cascade (Bersell et al., 2009). Such studies also point out various signaling pathways regulating myocyte proliferation during normal development as well as during remodeling.

Regulation of Growth in Prenatal Development

Multiple studies, both in vivo and in vitro, agree on a pivotal role of the FGF/FGF receptor (FGFR) cascade in immature myocytes (Speir et al., 1992; Sugi et al., 1993, 1995; Kardami et al., 1995; Mima et al., 1995; Sheikh et al., 1999; Franciosi et al., 2000; Lavine et al., 2005). Among 23 members of the FGF family and 4 different FGF receptors, FGF2 and FGFR1 are the ones most often implicated. We have shown recently that exogenous addition of depleted FGF2 can rescue myocyte proliferation in the context of experimental left ventricular hypoplasia, indicating the therapeutic potential of soluble factors controlling myocyte proliferation (Dealmeida and Sedmera, 2009).

Sources of such growth factors may be multiple. They are produced by

myocytes themselves and released in response to increased stretch (Clarke et al., 1995). The epicardium is often cited as a source of signaling molecules modulating myocyte proliferation, e.g., retinoic acid (Chen et al., 2002) and PDGF (Kang et al., 2008). Perhaps not surprisingly, a variety of other molecules influence myocyte proliferation, as evidenced by often lethal phenotypes resulting from their deletion. In some cases, it is not clear whether these effects are primary or secondary, because myocyte proliferation is intimately linked with cardiac function and will of course drop before embryonic demise. Some molecules derived from the endocardium, such as endothelin or neuregulin with their signaling cascade, are required for both proliferation and differentiation into chamber myocardium (Asai et al., 2010) or trabecular formation (Liu et al., 2010a). Neuregulin1 signaling by means of its receptors ErbB2 and ErbB4, together with FGF1 and periostin, are also able to induce adult rat myocyte proliferation in vitro (Bersell et al., 2009). In Table 2, we compile evidence for growth factor signals affecting myocyte proliferation in both developing and mature cardiomyocytes.

Epigenetic Control Mechanisms

Mechanical loading is one important epigenetic factor affecting myocardial growth. Like skeletal muscle, myocardium adapts to increased functional demands, either by hyperplasia during the prenatal period (Clark et al., 1989; Saiki et al., 1997) or by hypertrophy in adulthood. This is true even for myocytes grown in culture, which show much higher rates of proliferation and differentiation if appropriately stretched (Miller et al., 2000). Even in the absence of detailed knowledge about the molecular nature of such strain sensitive signals, discussed by Damon et al. (2009), mechanical loading or use of various conditioned media could be exploited for generation of artificial myocardial constructs (Evans et al., 2003). Once the critical thickness of approximately 50–100 microns is reached, vascularization becomes necessary for further growth. Indeed, in myocardial

TABLE 2. Growth Factors Affecting Myocyte Proliferation[a]

Factor	Species	Study design	Reference
Prenatal heart			
ANF	Chick	Cell culture stimulation and inhibition	Koide et al., 1996
CT-1	Mouse	Cell culture, expression studies	Sheng et al., 1996
EGF	Human	Cell culture	Goldman et al., 1996; Hornberger et al., 2000
	Chick	Cell culture	Lau, 1993
FGF2	Chick	Soaked beads in vivo	Franciosi et al., 2000
		Adenoviral overexpression	Dealmeida and Sedmera, 2009
FGFR1	Chick	Retroviral expression of DN receptor, antisense	Mima et al., 1995
IGF	Chick	Cell culture	Lau, 1993
NT-3	Chick	Retroviral overexpression of truncated receptor	Lin et al., 2000
PDGF	Chick, mouse	Expression studies, induced by overload	Jedlicka et al., 1991; Lau, 1993; Pexieder et al., 1995
	Mouse	Alpha subunit receptor deletion	Schatteman et al., 1995
	Rat	Epicardium-conditioned media	Kang et al., 2008
	Human	Decreased in left heart hypoplasia	Burton et al., 1991
Retinoids (indirectly?)	Mouse	Targeted deletion of receptors	Kastner et al., 1994; Gruber et al., 1996; Chen et al., 2002
Postnatal heart			
EPO	Rat	Cell culture	Wald et al., 1996
FGF1	Rat	Primary culture	Engel et al., 2005
	Newt	Primary culture	Soonpaa et al., 1994
FGFs	Zebrafish	Inhibition of signaling leads to scarring instead of regeneration	Wills et al., 2008
IGF-1	Rat	Correlation of expression of ligand and receptor with proliferation	Cheng et al., 1995
	Mouse	Recombinant protein administered in vivo post-infarction	Kofidis et al., 2004
Neuregulin1	Rat, mouse	Primary culture, in vivo injection, ErbB4 receptor null or overexpression; expression studies	Zhao et al., 1998; Bersell et al., 2009
Periostin	Rat	Cell culture, in vivo infarction	Kuhn et al., 2007

[a]Cell cycle controlling factors were also summarized previously by Dowell et al. (2003).

constructs in unstirred bioreactors, hypoxia appears to be a limiting factor beyond thickness of approximately 150 microns (Radisic et al., 2005; Tobita et al., 2006).

Normally, the process of growth of the myocardium and coronary bed is tightly coupled (Tomanek et al., 1999). Interestingly, the factors controlling the growth of these two compartments are similar, with FGF2 emerging as a major player (Tomanek et al., 1996, 2008; Lavine and Ornitz, 2009). This is perhaps not surprising, because both myocardium and vascular tissues are mesodermally derived and, therefore, likely to respond to similar stimuli in similar manner.

In conclusion, myocyte proliferation in the developing heart certainly plays a critical role in growth, morphogenesis, and remodeling of the organ. It is also an important part of the equation controlling homeostasis in the adult. Because of the complexity of the patterns and pronounced differences between developmental periods, care must be taken in designing studies to measure its rate. Identification of control mechanisms, as well as those directing the exit from the cell cycle, will help in designing strategies for repair of diseased adult myocardium, including formation of artificial myocardial tissue.

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