INTERNAL MEDICINE GRAND ROUNDS
November 13, 1997

How to mend a broken heart?

Cell cycle control in the terminally differentiated myocyte: a platform for myocardial repair?

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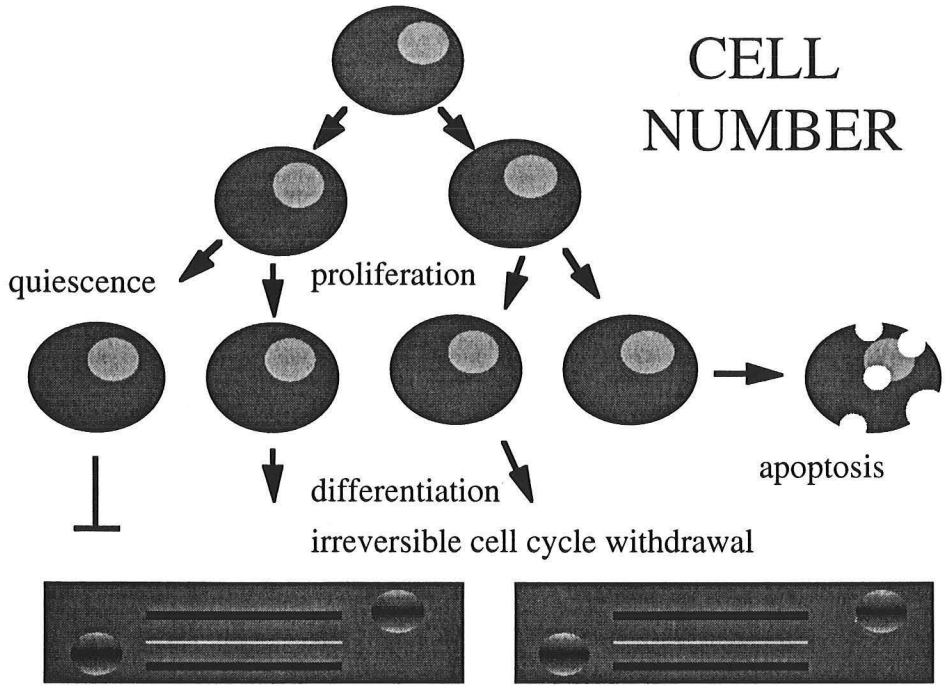

Disclosure: The speaker, R. Sanders Williams, has no financial interests or other relations with commerical concerns related directly or indirectly to this program.

Synopsis

Current therapeutics applied to patients with heart failure, with the exception of cardiac transplantation, fail to address the quantitative deficiency of cardiomyocytes that compromises ventricular performance in many of these individuals. A successful biotechnological strategy to add new cardiomyocytes to the damaged heart would meet a major clinical need. Cardiovascular investigators are exploiting advances in our fundamental understanding of stem cell biology, cardiogenic differentiation, and cell cycle control to promote proliferation of otherwise post-mitotic cardiomyocytes or engraftment of transplanted cardiogenic cells into the myocardial wall of intact animals. While formidable problems remain to be solved before clinical applications can be considered, myocardial repair has become an increasingly interesting focal point for both basic and translational research efforts.

Clinical background

Heart failure is both common and deadly. In the United States alone, approximately half a million individuals present with newly diagnosed heart failure each year, and over 4 million persons are currently afflicted. The majority of the most severely affected individuals suffer from dilated cardiomyopathy arising from coronary artery disease or of unknown cause. Mortality is high, with annual death rates approaching 50% in certain subsets. Alarmingly, mortality from heart failure has been increasing over the past two decades, an apparent anomaly within a period of declining overall death rates from cardiovascular disease. Morbidity from heart failure, in the words of Dr. Claude Lenfant[46], Director of the National Heart, Lung and Blood Institute, remains "staggering". The need for innovative approaches to improve care of these patients is apparent.

Currently available pharmaceuticals ameliorate secondary consequences of heart failure (Fig. 1) and can diminish symptoms and reduce mortality rates to some degree. Certain drugs may delay maladaptive morphological changes in the myocardial wall (remodeling) that lead to progressive deterioration of contractile performance over time[16, 24]. Only cardiac transplantation, however, provides a definitive solution to the irreversible loss of cardiomyocytes in the failing heart. Unfortunately, the limited availability of donor hearts leaves the vast majority of afflicted patients at need.

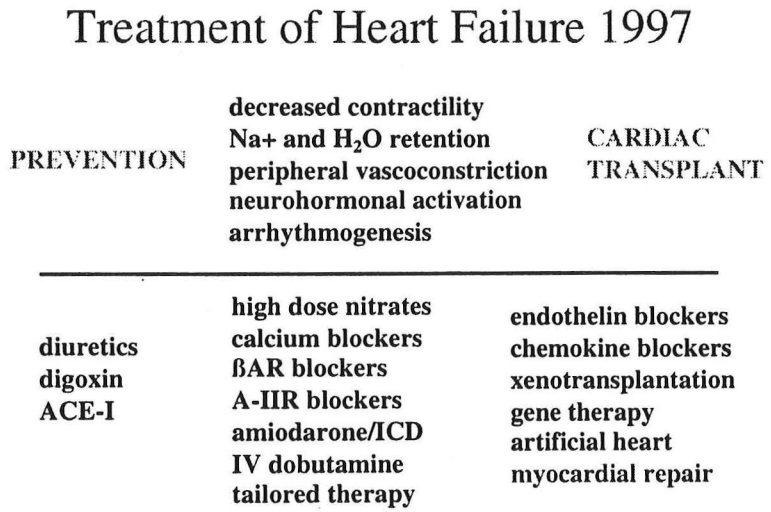

Fig. 1. Current, emerging, and potential strategies for clinical management of heart failure.

Cell death in cardiomyopathy

A quantitative deficiency of cardiomyocytes is an important feature of many of the disease processes that culminate in dilated cardiomyopathy[5]. This relationship is intuitively obvious in myocardial infarction, direct toxic damage to the heart, and in assault by infectious pathogens or autoimmune processes. Though less obvious, loss of myocytes also accelerates the deterioration of contractile function at some point in the course of cardiomyopathy caused by inherited gene defects or hemodynamic overload resulting from valvular abnormalities or hypertension[47].

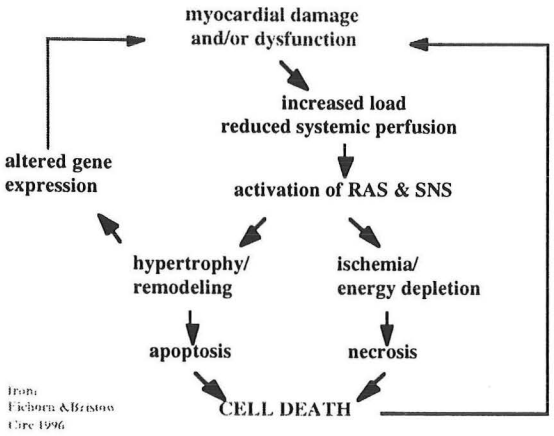

Fig. 2. Factors promoting progressive death of cardiomyocytes in the failing heart.

Destruction of cardiomyocytes (Fig. 2) may result from necrosis -- direct and irreversible damage to critical cellular proteins or membranes -- or from apoptosis -- programmed cell death mediated by an evolutionarily conserved pathway that terminates in activation of a specific family of death-promoting proteases[86]. Numerous recent reports draw attention to the importance of apoptosis in cardiomyopathic processes [6,36,53]. Irrespective of the mechanism of cell death, however, loss of more than a small percentage of the cardiomyocyte population may have catastrophic consequences.

Cardiomyocytes suffering lethal injury are not replaced. The adult myocardium can respond to injury only by local scarring and by cellular hypertrophy within undamaged segments of the heart. These responses are, at best, temporarily compensatory in maintaining contractile function. At worst, interstitial fibrosis, hypertrophy, and misalignment of surviving myocytes (ventricular remodeling) are maladaptive and promote distortion of ventricular geomentry and further deterioration of systolic or diastolic performance. The remaining cardiomyocytes have lost the capability to proliferate, and no pool of undifferentiated precursor cells is available to replenish the population of myocytes that have been lost.

Proliferative growth of cardiomyocytes in heart development

Soon after gastrulation, precursor cells that ultimately give rise to cardiomyocytes are specified within lateral plate mesoderm by inductive signals from adjacent regions of the vertebrate embryo. Paired cardiogenic regions fuse in the midline to form the linear heart tube, the outer layer of which is comprised of spontaneously contractile cardiomyocytes. Differentiation of clearly defined cardiomyocytes within the heart tube is one of the earliest events in mammalian organogenesis, and the establishment of a fetal circulation preceeds the complex series of subsequent steps (looping morphogenesis, formation of valves,

selective growth and gene expression in specific regions, establishment of the specialized conduction system, innervation) that ultimately establish the structure of the mature heart[57].

The physical growth of the heart during fetal life is driven by proliferation of cardiomyocytes that populate the heart tube. Thus, the initial differentiation of this cell type is not fundamentally incompatible with cell cycle progression, as may be the case in skeletal myofibers and certain other terminally differentiated cells. Decisions that ultimately determine the number of cardiomyocytes within the mature heart, and interesting distinctions between the heart and skeletal muscles, are illustrated in Figure 3. A diversity of intracellular and extracellular signaling molecules are involved in regulation of cell proliferation within the myocardial layer, as revealed by the hypoplastic heart phenotype of mice with null mutations in genes encoding components of several different signaling pathways [8,33,77].

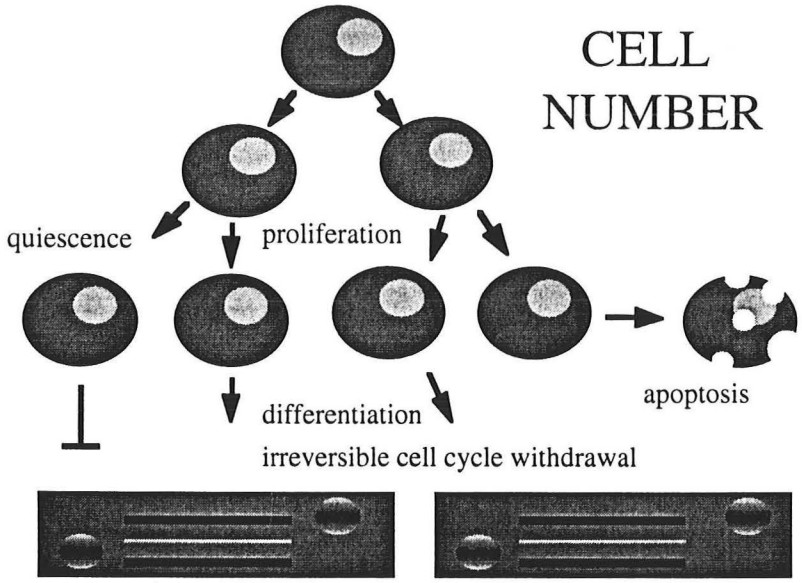

Fig. 3. Control of cell number during myocyte differentiation. During embryogenesis, proliferating progenitor cells destined to become cardiac or skeletal myocytes migrate to their appropriate positions. Cell proliferation is terminated by one of three outcomes: terminal differentiation and irreversible cell cycle withdrawal, apopotosis (programmed cell death), or withdrawal to an undifferentiated, quiescent state. The extent of mitotic proliferation of precursor cells, and the relative frequencies of these three outcomes, determine cell number in the mature organ. In the heart, differentiated cardiomyocytes continue to proliferate during fetal life, but lose this capability about the time of birth. No quiescent cardiogenic stem cells are established and injured cardiomyocytes cannot be replaced. During skeletal myogenesis, differentiation and cell cycle withdrawal are contemporaneous events, but undifferentiated, quiescent myogenic stem cells (satellite cells) persist in adult muscles, and can be mobilized by muscle injury to restore myocyte number.

About the time of birth, however, mammalian cardiomyocytes irreversibly exit from the cell cycle, and thereafter lose the capacity to replicate DNA and proliferate. Fetal cardiomyocytes have a single nucleus, but cytokinesis does not follow karyokinesis in the terminal cell cycle that ends the proliferative growth phase, such that most adult cardiomyocytes are binucleate. The impressive physiological growth of the heart that takes

place between neonatal life and adulthood, and the massive increases in cardiac mass associated with certain pathological states, are driven almost exclusively by myocyte hypertrophy, accompanied by variable increases in non-myocytes and interstitial fibrous tissue.

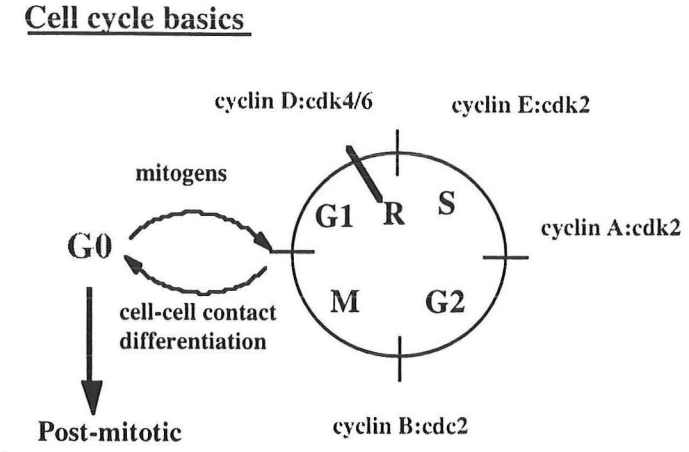

Fig. 4 Basic features of the mammalian cell cycle.
The mammalian cell cycle is driven by the sequential activation of specific cyclin:cyclin dependent kinase pairs. DNA replication (S phase) and mitosis (M phase) are separated by periods characterized by unreplicated (G_1) or replicated (G_2) DNA. In the absence of mitogenic stimuli, or under the influence of dominantly acting signals that suppress cell growth, the cell cycle stops -- a condition characterized as G_0. The restriction point (R) is a point in G_1 after which the decision to replicate DNA no longer requires the continued presence of mitogenic growth stimuli. Adult cardiomyocytes and certain other specialized cell types become resistant to mitogens (post-mitotic) and appear to be locked permanently in G_0.

Blocks to cell cycle progression in cardiomyocytes

Discrete stages of the cell cycle are illustrated in Fig. 4. Most cell types are capable of withdrawing temporily from the cell cycle into a quiescent state known as G_0 [71]. Re-entry into the cell cycle is driven by extracellular mitogens acting through cell surface receptors linked to intracellular signal transduction pathways that ultimately promote transcription of genes encoding proteins of the cell cycle machine. The responsiveness to mitogens also is regulated by cell-cell interactions (contact inhibition). Entry into, and progression through, the cell cycle is regulated at a series of discrete steps, primarily by a complex series of protein phosphorylation events mediated by cyclin dependent protein kinases. Of these steps, the decision to replicate DNA is particularly critical. Once cells pass a boundary known as the restriction point, they most often will complete the remainder of the cell cycle even if extracellular mitogens are withdrawn. A substantial body of evidence has revealed fundamental features of the control mechanisms that govern the G1-S transition (Fig. 5)[68,81]. Somatic or inherited mutations in proteins of this pathway are a common feature of malignantly transformed cells [69], highlighting their importance in governing proliferative cell growth.

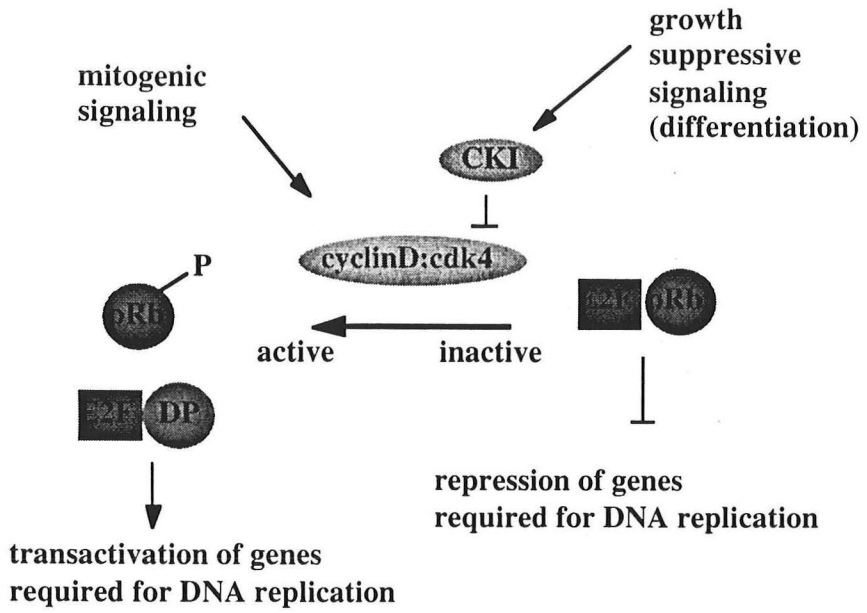

Fig. 5 Control of G1/S progression in mammalian cells.
A number of specific proteins have been shown to exert important regulatory controls over the decision to initiate DNA replication. The dysregulated cell growth of tumor cells frequently is based, at least in part, on mutations of proteins in this pathway[69]. A nodal point of this control mechanism is centered on the activity of cyclin D and its cyclin dependent kinase partners cdk4 or cdk6. Under conditions of growth suppression, cyclin D:cdk4 is inactive, either because cyclin D is absent or because the activity of this enzyme is repressed by specific cyclin dependent kinase inhibitors (CKI). In this situation, members of the retinoblastoma family of tumor suppressor proteins (pRb) are hypophosphorylated and form complexes with E2F transcription factors to repress transcription of several downstream genes required for DNA replication. When cells respond to mitogenic signals, the inhibitory effects of CKIs are overcome, and cyclin D:cdk4 is activated to phosphorylate Rb proteins. Hyperphosphorylation of pRb frees E2F proteins to dimerize with their coactivator DP1, thereby activating transcription of downstream genes that promote entry into S phase.

In the terminal steps of fetal development of the heart, cardiomyocytes exit from the cell cycle into a state resembling G_0, but different in some fundamental respects that render the decision irreversible. Some investigators have presented evidence for induction of DNA replication within the adult heart by certain stresses [37, 63] but other studies using highly rigorous methods indicate that DNA replication by adult mammalian cardiomyocytes occurs as an exceedingly rare event[40,73]. All investigators agree, however, that the degree of cardiomyocyte proliferation, if it occurs at all, is inadequate to serve as an effective repair mechanism.

What molecular mechanisms account for the essentially irreversible cell cycle withdrawal of cardiomyocytes? Several potential mechanisms can be postulated (Fig. 6). Maturation of cardiomyocytes in postnatal life may be accompanied by loss of cell surface receptors for mitogenic growth factors, or loss of components of intracellular signaling pathways that are required for receptor-mediated cell proliferation. Genes encoding essential proteins

required for DNA replication and cell cycle progression may be repressed and unresponsive to intracellular signals that otherwise would promote cell proliferation, even if the upstream components of mitogenic signaling pathways are present and active. Differentiated myocytes may elaborate inhibitors of cell proliferation that prevent entry into the cell cycle, even though essential components of the cell cycle machinery are present. Any or all of these potential blocks to mitogenic growth could result from anti-proliferative effects of myogenic determination factors that specify the cardiomyocyte phenotype.

Establishing and maintaining the post-mitotic state

- **loss of growth factor receptors or signaling components**
- **anti-proliferative effects of myogenic determination genes**
- **irreversible loss of cell cycle proteins and/ or DNA replication machinery**
- **elaboration of anti-proliferative factors**

Fig. 6. Potential mechanisms leading to irreversible withdrawal of myocytes from the cell cycle.

In reality, a complex combination of molecular mechanisms serves to maintain the post-mitotic state of adult cardiomyocytes, but no single event has been shown to be paramount. The rarity of malignant transformation of cardiac myocytes by comparison to most other cell types supports the viewpoint that redundant and powerful negative control mechanisms are in place. Current evidence permits only a rudimentary understanding of the process, but gives a few clues as to why cell cycle block becomes essentially irreversible in these cells.

Cell surface receptors that recognize certain mitogens are down-regulated in differentiated cardiac myocytes by comparison to fetal cells[11], but others remain present and are linked to downstream kinase cascades and other intracellular events that trigger proliferative growth of other cell types [65,66]. Some of the receptor-mediated events that promote proliferation in other cell types contribute to hypertrophic growth of the heart, but fail to promote entry into the cell cycle. Certain components of the biochemical machinery required for DNA replication and cell cycle progression are absent from cardiac myocytes, while others may be present [63]. Differentiated myocytes express negative regulators of cell proliferation, including inhibitors of cyclin dependent kinases that act in a dominant manner to arrest the cell cycle [30,38,45,60], though cardiac growth has not been markedly perturbed by disruption of the genes encoding several of these proteins in transgenic mouse models[22]. The exuberant proliferation of contracting fetal cardiomyocytes indicates that myogenic determination genes that specify the cardiac lineage do not act *pari passu* to block the cell cycle, though adult myocytes may express a different repertoire of factors to maintain, as opposed to establish, the myocyte phenotype. The precise mechanisms that maintain cardiomyocytes in a post-mitotic state, resistent to proliferation-promoting effects of mitogenic signals, remain far from clear.

Regeneration mechanisms in skeletal muscle

Unlike the heart, other tissues have a capacity for self-repair. By what strategies do they acquire a regenerative capability that is lacking in the myocardium? Injury to the liver

promotes a proliferative response from surviving hepatocytes that restores cell number. Likewise, blood vessels can be replaced by proliferative growth of remaining endothelial and smooth muscle cells. In these cell types, terminal differentiation is not associated with irreversible withdrawal from the cell cycle. In contrast, mature erythrocytes or absorptive epithelial cells of the intestinal mucosa have no proliferative capability. If red blood cells are lost through bleeding or hemolysis, however, they are replaced by expansion of erythroid precursors derived from a self-renewing stem cell pool in the bone marrow. Likewise, injury to the intestinal mucosa can be repaired by expansion of precursor cells derived from a stem cell pool within the crypts of Lieberkühn.

Skeletal myofibers share a number of important characteristic with cardiomyocytes. Both cell types are irreversibly post-mitotic in the adult; they express identical or closely related contractile proteins; certain myogenic determination factors are important for specifying the differentiated phenotype in both lineages[58]. Adult skeletal muscles, however, retain a pool of undifferentiated stem cells that can be mobilized by muscle injury to replace damaged skeletal myofibers[25,67]. This capability, as we have already noted, is absent in the heart. Regeneration of skeletal muscle is based on mobilization of satellite cells, an interesting and enigmatic cell type of which we have only a rudimentary understanding.

Properties of satellite cells

- **committed to a myogenic fate**
- **differentiation arrested at an early stage (known myogenic determination genes are not expressed)**
- **quiescent in adult muscles**
- **activated by myofiber injury to proliferate and recapitulate primary myogenesis**
- **self-renewing**

Fig. 7. Satellite cells of skeletal muscle are small mononuclear cells that share a common basal lamina with the much larger, multinucleated skeletal myofibers. Satellite cells are commited to a myogenic cell fate, but do not express any known myogenic determination genes that serve as markers for muscle precursor cells in the embryo. Following injury to mature skeletal myofibers, satellite cells are stimulated to enter the cell cycle and to express myogenic determination genes that specify a muscle cell fate.

Expansion of this precursor cell population is followed by cell fusion and differentiation into skeletal myofibers. This series of events, by and large, recapitulates primary myogenesis in the embryo. The satellite cell population is self-renewing, and a residual pool of satellite cells capable of supporting additional rounds of regeneration is reestablished after each discrete episode of muscle injury. This capacity for self-renewal, however, is finite, and exhaustion of the myogenic stem cell pool may contribute to the clinical deterioration observed with advancing age in human patients with muscular dystrophy, and perhaps to the loss of muscle mass observed in normal aging. A schematic representation of the regeneration process in skeletal muscle is shown in Figure 8.

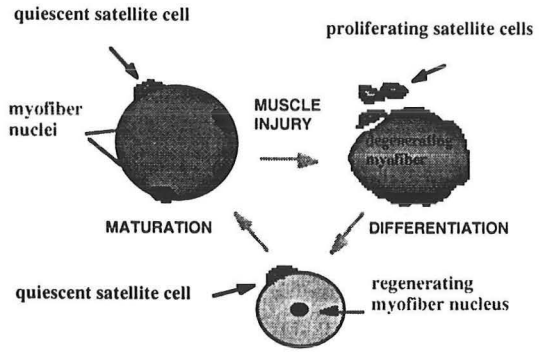

Fig. 8. Myocyte regeneration in skeletal muscles. Adult skeletal muscles contain a population of myogenic stem cells (satellite cells) that share a common basal lamina with differentiated, post-mitotic, multinucleated myofibers[28]. In response to lethal injury to the myofibers, quiescent satellite cells are stimulated to re-enter the cell cycle. Proliferation of satellite cells, followed by their differentiation, regenerates myofibers. Immature, regenerating myofibers can be recognized histologically by the central location of their nuclei, which move to the periphery of the cell as the myofibers mature. The satellite cell population is self-renewing, and capable of supporting multiple rounds of regeneration.

How is this pool of quiescent, myogenic stem cells established during development of skeletal muscle and maintained following muscle injury while other myogenic precursor cells are undergoing differentiation? Why is the heart devoid of an analogous stem cell population that would confer a capacity for tissue repair? Satisfying answers to these questions are not currently available, but my laboratory and others are attacking these problems with great enthusiasm.

Recently, we identified two proteins termed MNF-α [3]and MNF-ß[85] that promise to provide novel insights into features of satellite cell biology. The primary amino acid sequence of the DNA binding domain of these MNF proteins identified them as members of the forkhead/winged helix family of transcription factors[14]. Other members of this extended gene family exert important regulatory controls over cell proliferation and pattern formation during development in tissues and organs other than muscle[2, 84], but MNFs are the first members of this family suspected to have specific functions in muscle tissues.

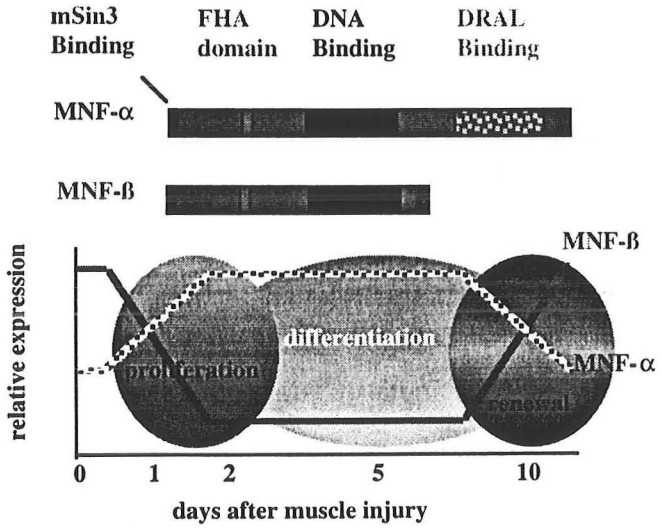

Fig. 9. Schematic structure of MNF proteins and their reciprocal expression during stages of muscle regeneration. Domains of MNF proteins conserved in other forkhead family members (DNA binding or FHA) or required for interactions with other proteins (mSin3 or DRAL) are shown.

In adult skeletal muscles, MNF protein is expressed selectively in satellite cells [28]. Preliminary studies in our lab indicate this to be the MNF-ß isoform. Following muscle injury, as satellite cells are stimulated to enter the cell cycle, MNF-ß is down-regulated, while there is a reciprocal up-regulation of the MNF-α isoform. A developmentally regulated alternative splicing event controls the switching of MNF isoforms, since both proteins are produced as products of a single gene. As muscle repair proceeds, myoblasts derived from satellite cells differentiate, a process dependent on myogenic determination genes of the MyoD family[50]. Expression of MNF declines as regenerating myofibers mature, and high levels of MNF (probably MNF-ß) remain primarily in the population of satellite cells that is re-established. MNF proteins also are expressed in the fetal heart[28]. They are detectable at the earliest stages of heart tube formation but disappear as cardiac morphogenesis is completed during fetal life. Unlike skeletal muscles, cells expressing immunoreactive MNF are not detectable in the post-natal heart.

Other experiments indicate clearly that MNF-ß functions as a transcriptional repressor[85]. We speculate, based on the reciprocal pattern of isoform expression, that MNF-α may have an opposing function to activate gene transciption, but this effect is most likely to require a co-activator molecule that has yet to be identified. The promoters of several genes necessary for cell cycle progression contain evolutionarily conserved binding sites for MNF proteins[85], suggesting a direct mechanism whereby MNFs may modulate proliferative cell growth (Fig. 10). We have knocked out the MNF gene in transgenic mice, and will soon report the phenotypes observed in these animals.

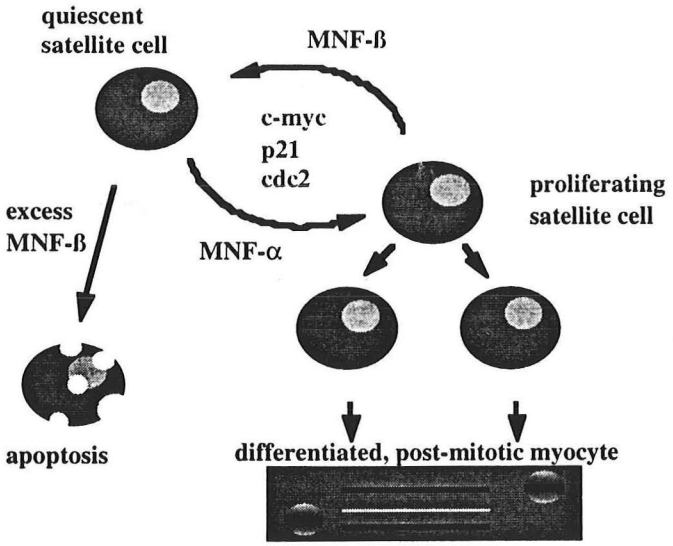

Fig. 10. Hypothetical functions proposed for MNF proteins during muscle regeneration. High affinity binding sites for MNFs are conserved in transcriptional control regions of several genes involved in cell cycle regulation (c-myc, p21, cdc2).

Other laboratories have identified a specific growth factor and its cognate receptor that are essential for satellite cell activation following muscle injury [1]. Intriguingly, this satellite cell mitogen proved to be hepatocyte growth factor, a protein that also plays an important role in liver regeneration. Hepatocyte growth factor (also known as scatter factor) is the extracellular ligand for the c-met receptor. Further studies of this specific signaling pathway should increase our understanding of the mechanisms and limits to tissue regeneration in both liver and skeletal muscles.

Cardiogenic stem cells

It is possible to define specific regions of the early embryo that comprise a cardiogenic field, meaning that cells destined to form the myocardium arise from this location. Also,

pluripotent embryonic stem cells in culture are capable of differentiation into contractile cardiac myocytes. Thus, populations of cells with cardiogenic potential, admixed with other cell types, can be identified and isolated. It has not been possible, however, to isolate pure populations of undifferentiated, proliferating cells committed to a cardiogenic fate in a manner resembling the satellite cell population of skeletal muscles. Though we know several of the extracellular signals and intracellular transcription factors that participate in specifying the cardiogenic cell lineage, this information so far has not defined a specific and unique marker by which an expandable population of cardiogenic stem cells can be identified and separated. Current knowledge also is inadequate to ascertain why the pool of undifferentiated cardiogenic stem cells present in the embryo is exhausted completely during development, without retention of a remnant population that could promote repair of this tissue following injury.

Potential strategies for myocardial regeneration

Since nature has not endowed the myocardium with an endogenous capacity for self-repair, and since a deficiency of cardiomyocytes contributes to hemodynamic insufficiency in the most prevalent forms of heart failure, efforts to develop biotechnological strategies to repair the injury myocardium are both rational and needed.

Strategies for cardiac repair

- **cell transplantation**
  - **skeletal myoblasts**
  - **fetal cardiomyocytes**
  - **cardiogenic stem cells**
- **myogenic trans-differentiation**
- **proliferation of endogenous cardiomyocytes**

Fig. 11. Several potential approaches are under investigation at this time (reviewed in [72]). Each strategy is supported by preliminary experiments that establish biological plausibility, but each presents biological, technical or ethical limitations that must be overcome before application to human patients can be considered seriously.

Cell transplantation to the myocardium

Transplantation of isolated, dispersed cells into the heart has been performed in animal models by several groups. As with whole organ transplantation, the issues of immunological rejection and availability of donor cells must be addressed. In addition, a cellular transplantation strategy presents additional problems that are not pertinent to organ transplantation as it is currently practiced. Specifically, donor cells must engraft within the target tissue in sufficient numbers; they must acquire the appropriate characteristics of the cell population they are meant to replace; and they must arrange themselves in a manner consistent with the normal supracellular organization of the tissue. This is a challenging proposition.

Skeletal myoblasts propagated in cell culture have been injected directly into the myocardial wall [12,41,52,79]. A potential advantage of using skeletal myoblasts for cardiac repair is that large numbers of immunologically autologous cells can, in principle, be produced from satellite cells derived from the same individual. At least a small proportion of cells introduced in this manner survive and complete the differentiation process. Thus, the myocardial wall provides a microenvironment permissive for myogenic differentiation.

The differentiated myocytes generated in this manner, however, retain the properties of skeletal rather than cardiac myocytes, and fail to couple electrically with adjacent cardiomyocytes. The ultimate prospects for the use of skeletal myoblasts in myocardial repair are limited by important biological differences between skeletal and cardiac myocytes with respect to both electrical and contractile physiology.

This latter problem is obviated if cardiogenic cells, rather than skeletal myoblasts, constitute the donor cell population. As discussed previously, differentiated fetal cardiomyocytes retain a capacity for proliferation, and they have the potential to mature into cells that are morphologically and functionally indistinguishable from surviving cardiomyocytes within the recipient heart. It is, in fact, possible in rodent or canine models to engraft fetal cardiomyocytes into the myocardial wall of adult animals[21,43,74]. Thus, the biological hurdles that limit application of this approach are reduced by comparison to strategies that employ skeletal myoblasts. The problem of donor cell availability, however, becomes more formidable, and the ethical issues more complex. By comparison with the potential use of fetal cells for treatment of neurological diseases, where engraftment of small numbers of cells may be adequate for therapeutic benefit, cardiomyocyte transplantation is likely to require millions of new cells to be efficacious, and continued proliferation of engrafted cells cannot be expected to expand the population removed from the donor embryos. These biological problems would be lessened if further research findings make it possible to isolate cardiogenic precursor cells at an earlier developmental stage where they have a greater capacity for clonal expansion, but ethical barriers to the use of human fetuses as a source for medical materials would remain.

Deriving cardiomyocytes from embryonic stem cells

Loren Field and colleagues have approached this issue in a unique and creative manner using murine embryonic stem cells[40]. These cells, by definition, are pluripotent, meaning that they can give rise to daughter cells capable of differentiation into any of the multitude of specific cell types specified at later stages of development. This pluripotentiality can be demonstated in the intact organism, through generation of chimeric animals following microinjection of embryonic stem cells at the blastocyst stage of embryonic development, a procedure that forms the basis for gene knockout experiments in mice. The pluripotent nature of embryonic stem cells also can be demonstrated in cell culture. If exogenously supplied growth factors that maintain their undifferentiated state in culture are withdrawn, embryonic stem cells spontaneously form structures known as embryoid bodies in which differentiated cell types of ectodermal, endodermal, and mesodermal origin are present. Spontaneously contractile cardiac myocytes, admixed with many other cell types, can be generated in this manner. Field and colleagues transfected into embryonic stem cells a transgene that confers resistance to a toxic drug under the control of a cardiac specific promoter. When embryoid bodies derived from these stably transfected embryonic stem cells were exposed to the toxic antibiotic, only cardiac myocytes were able to survive. These cells were harvested and injected into the myocardial wall of adult mice, where they engrafted and formed appropriate cell-cell contracts (intercalated discs with desmosomes and gap junctions) with endogenous cardiomyocytes while maintaining a morphologically differentiated state.

This strategy is appealing for several reasons. It becomes possible, in principle, to generate large numbers of donor myocytes, and the ethical problems of extracting cells from aborted fetuses are muted somewhat. Even the ethical objections that may be raised to isolation of embryonic stem cells from a human fetus could be overcome if embryonic carcinoma cell lines, which also exhibit a multipotent differentiation potential, could be safely employed for this purpose.

Even in the most hopeful future case, where problems of cell source become completely non-limiting, this particular strategy may still be limited by obstacles relating to immunological rejection, techniques of cell delivery, or unphysiological pattern formation by the engrafted cells. Nevertheless, further research efforts in this area seem to be well justified.

Myogenic transdifferentiation

Progress in identifying the effector molecules and pathways that control cardiogenic differentiation may ultimately make it possible to convert fibroblasts or endothelial cells that survive within an injured myocardium into cardiomyocytes, thereby adding contractile units and improving the function of the failing heart. The spectacular and dominant ability of skeletal myogenic determination genes of the MyoD family to activate muscle-specific programs of gene expression in diverse non-muscle cell backgrounds[83] provided the original impetus to this idea. As yet, however, no single protein has been shown to divert non-muscle cells to a cardiogenic cell fate, even though a variety of transcription factors [7,58,51] have been identified that exert important regulatory controls over genes expressed selectively in cardiac myocytes. Even in skeletal muscle, where molecular mechanisms of myogenic commitment and differentation are more completely characterized, combinatorial effects of multiple regulatory proteins are necessary to drive the full repertoire of downstream genes that define the fully differentiated phenotype[59]. On a conceptual basis, the strategy of using a single master regulatory gene sufficient to promote transdifferentiation probably will yield to more complex strategies that involve specific combinations of regulatory proteins applied at a successive series of nodal points to specify the cardiogenic cell fate.

Prospects for clinical applications of myogenic transdifferentiation schemes to the treatment of heart failure are limited at this time, not only by our incomplete knowledge of the proteins that control cardiogenic differentiation, but by the inadequacies of current gene transfer techniques[80]. Significant advances on both fronts, however, could invigorate this approach to myocardial repair.

Proliferation of endogenous cardiomyoctyes for myocardial repair

A biotechnological strategy to coax adult cardiomyocytes to reenter the cell cycle and to replicate themselves in the manner of regenerating hepatocytes, or of their own forebears within the fetal myocardium, would represent an intuitively attractive platform for myocardial repair. The first problem faced by such a scheme, of course, is the apparent irreversibility of the post-mitotic state of the mature cardiomyocyte. Even when isolated and dispersed in culture, so as to remove any growth-suppressive effects of contact inhibition, these cells are resistant to the mitogenic effects of extracellular growth factors, and remain firmly anchored in G_0. Even if extracellular mitogens fail to induce cell cycle entry in cardiomyocytes, however, it may be possible to exploit our increasing knowledge of molecular mechanisms of cell cycle control to overcome the blockade to proliferative growth exhibited by these cells.

The forced expression of viral transforming genes such as SV40 large T antigen and adenovirus E1A protein can promote DNA replication and stimulate proliferative growth of cardiomyocytes in culture, or in hearts of transgenic mice. Hearts of mice expressing an SV40 large T antigen transgene under the control of the atrial naturetic peptide or α-myosin heavy chain promoters are enlarged, hypercellular, and manifest focal cardiac tumors[26]. Cell lines isolated from these animals can be maintained by serial transplantation in mice, and have been transplanted to the myocardial wall[42]. These cells continue to express contractile proteins and display certain features of the differentiated state even after serial

passage through many cell doublings. Expression of adenovirus E1A in cardiomyocytes also promotes DNA replication[39,49]. Entry into the cell cycle forced by E1A, however, provokes programmed cell death, a response that can be inhibited by the product of the adenoviral E1B gene[31]. Viral oncoproteins drive cells into S phase, at least in part, through protein-protein interactions that compromise the growth-suppressive functions of proteins of the Rb family and stimulate the growth-promoting functions of E2F transcription factors[20,55], as illustrated in Fig. 3. The consequences of unrestrained E2F activation may include apoptosis, even in the absence of viral proteins[27].

The ability of SV40 large T antigen and adenovirus E1A to promote DNA replication in cardiomyocytes establishes two important principles: the post-mitotic state of the mature cardiomyocyte is not irreversible; and proliferation of cardiomyocytes does not require complete dedifferentiation. If viral oncoproteins can drive cardiomyocytes to replication, can this be accomplished by manipulation of endogenous cell cycle regulators? Proteins that act in the major pathway controlling progression across the G1-S boundary (Fig. 3) or that are known to promote oncogenic transformation in native or modified form are the most likely candidates.

In one of the first experiments to address this point, a transgenic mouse line that expressed the c-myc proto-oncogene to high levels in the heart was noted to have cardiomegaly, based not on hypertrophy of cardiomyocytes, but on hypercellularity[34]. Myocytes from these hearts did not, however, exhibit sustained proliferative growth over time. This phenotype was different from that observed subsequently in the SV40 large T antigen transgenics, and appeared to result from a single extra round of cell division during fetal development. This experiment proved that dysregulated expression of endogenous proteins can perturb cell cycle regulation during cardiac development, but did not address whether this could be accomplished in the post-natal heart.

Driving DNA replication in post-natal cardiomyocytes with native mammalian proteins

Recently, forced expression of a cyclin D1 transgene using the α-myosin heavy chain promoter was shown to stimulate DNA replication in post-natal cardiomyocytes[75]. As shown in Fig. 3, cyclin D serves as the regulatory component of cyclin-dependent kinases (cdk4 or cdk6) in controlling entry of mammalian cells into S phase. Cyclin D isoforms are down-regulated in G_0 cells and any cyclin D:cdk4 complexes that are formed are rendered inactive by high levels of cyclin dependent kinase inhibitors (CKI; Fig. 3) in quiescent or post-mitotic cells. Forced expression of cyclin D to supraphysiological levels, however, may titrate out the pool of CKIs and generate active cyclin D:cdk4 complexes that phosphorylate Rb family members[10] and promote transactivation of downstream genes controlled by E2F transcription factors, leading to DNA replication.

A rigorous and quantitative analysis was applied to define the cardiac phenotype with respect to DNA replication in cyclin D1-transgenic animals. First, the cardiomyocytes were marked with a second transgene expressing a nuclear localized ß-galactosidase gene under the control of the α-myosin heavy chain promoter. This is an important maneuver, since it permits cardiomyocytes to be distinguished unequivocally from other cell types present in histological sections or in populations of dispersed cells. The forced expression of cyclin D1 provoked >100-fold increase in the percentage of cardiomyocytes that incorporated [^{3}H]-thymidine, a standard marker for DNA replication, in animals assessed at 3 months of age. In addition, there was a significant increase in the proportion of cardiomyocytes with greater than 2 nuclei per cell, as assessed by flow cytometry. It is instructive to review the actual numbers of replicating cardiomyocytes: 1/270,000 in wildtype animals and 112/270,000 in the transgenic hearts. Thus, this genetic perturbation of the normal

mechanisms that arrest growth in the heart is by no means complete, and the majority of cardiomyocytes appear to remain restrained from cell cycle entry, or to cycle at very slow rates. Nevertheless, these data provide proof-of-principle that endogenous cellular proteins, and not only viral transforming genes, can be manipulated to promote DNA replication in post-natal cardiomyocytes. Cyclin D activity inhibits differentiation of skeletal myotubes in cell culture[70], but normal levels of expression of several cardiac-specific genes were observed in hearts of these transgenic mice. This finding suggests that stimulation of DNA replication is not associated with de-differentiation in this model.

Other transgenic models indicate that cardiomyocytes may remain susceptible to supraphysiological levels of certain extracellular mitogens, at least during an early period of post-natal life. Forced expression of transgenes expressing either IGF-1 or the angiotensin II type 1 receptor also can lead to myocardial hypercellularity[32, 64].

Advances in identifying novel proteins that exert critical regulatory functions over DNA replication and cell cycle progression may add new and potentially superior candidates for manipulation in biotechnological strategies to stimulate proliferation of cardiomyocytes. Our laboratory has focused recently on initiation factors: proteins that act directly to establish and fire origins of DNA replication in mammalian cells. A framework for understanding the initiation of DNA replication (Fig. 12)[54,76] has been developed from studies of unicellular eukaryotes (budding yeast: Saccharomyces cerevisiae; and fission yeast: Saccharomyces pombe) where genetic approaches can be exploited most efficiently in concert with biochemical studies.

As established in yeast models, origins of DNA replication are specified by binding of a protein called ORC (Origin Recognition Complex)[4]. Binding of ORC is essential for DNA replication, but not regulatory. ORC subunits are expressed at near constant levels throughout the cell cycle and ORC binding to DNA is reestablished immediately after the origin itself has been replicated. The assembly of additional proteins necessary to fire the origin is regulated by Cdc6[15,48]. (The Cdc6 homologue in S. pombe is called Cdc18[56]. For reasons of simplicity, in this discussion I will refer to both proteins as Cdc6.)

Cdc6 binds to ORC[44,48], and this interaction promotes recruitment of additional proteins called MCMs[23,78], of which 6 are known to be important for replication initiation. Like ORC, MCM proteins are essential but not regulatory in this process. They are abundant within the cell during all phases of the cell cycle. Cdc6, in contrast, is an unstable protein with a half life of less than 5 min. Expression of Cdc6 is regulated across the cell cycle both by transcription and by protein stability[62]. The protein is expressed late in mitosis and throughout G1 up to the G1/S boundary. If G1 is prolonged (by nutrient withdrawal), a second peak of Cdc6 synthesis preceeds entry into S phase. After a replication origin has fired, Cdc6 is inactivated or degraded in a manner controlled by cyclin dependent kinase activity[9,35], an event thought to be important in preventing re-firing of replication origins within a single cell cycle (the once-and-only-once rule necessary to preserve genetic uniformily in daughter cells)[19].

Unique and distinctive regulatory functions of Cdc6 in yeast have been revealed by the observation that forced overexpression (S. pombe) or point mutations within the protein (S. cerevisiae) promote over-replication -- repeated rounds of DNA replication without mitosis [56]. On the other hand, a deficiency of Cdc6 blocks initiation and drives cells prematurely into mitosis with unreplicated DNA [62]. Thus Cdc6 is involved in several important decisions that govern cell cycle progression: forming pre-replication complexes that render origins competent to initiate DNA synthesis; restricting replication of each segment of DNA to once in each cell cycle; recognizing that replication has been completed before mitosis can begin.

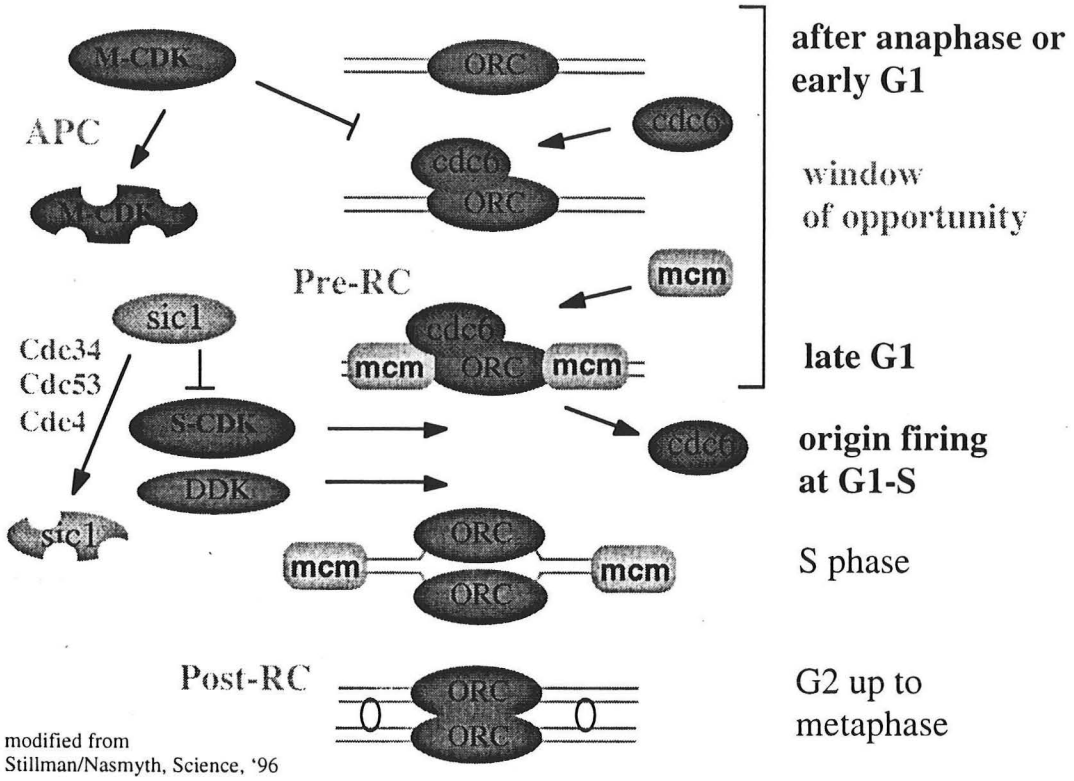

Fig. 12 Initiation of DNA replication in yeast. Initiation of DNA replication in eukaryotic cells requires the stepwise assembly of a prereplication complex (Pre-RC) at the chromosomal sites where replication will subsequently begin. Origins of replicationare specified by binding of ORC, a protein consisting of six subunits. Binding of ORC is essential but not regulatory, since ORC remains bound throughout all phases of the cell cycle. Formation of the prereplication complex is regulated by Cdc6, which binds to ORC and promotes the subsequent binding of MCM proteins. Replication origins are fired, meaning that DNA synthesis begins, by the activity of S-phase promoting cyclin dependent kinases (S-CDK & DDK), which act on as yet undetermined substrates within the prereplication complex to recruit DNA polymerase and the other accessory proteins of the replication machine. After replication begins, post-replication complexes (Post-RC)containing ORC remain bound to DNA, but Cdc6 is inactivated or degraded, and formation of Pre-RCs is inhibited until cyclin dependent kinases active in M-phase (M-CDK) are degraded by the anaphase promoting complex (APC). Degradation of a CDK inhibitor (Sic1) via a ubiquitination-proteosome pathway (Cdc34, Cdc53, Cdc4) is necessary for S-CDK activity. This model is based primarily on studies of budding and fission yeasts[76], but available data indicate that fundamental features of this model may be conserved in mammalian cells.

While the assembly of the prereplication complex at potential origins is regulated by Cdc6, phosphorylation events driven by S phase promoting cyclin dependent protein kinases also are necessary to fire the origin[61]. The critical substrates for S phase promoting kinases have not yet been defined, but may include sites on Cdc6 itself. Likewise, the manner in

which the activated prereplication complex leads to unwinding of DNA and recruitment of the many additional protein components of the replicative apparatus has not been defined.

Initiation mechanisms in mammalian cells

Do mammalian cells utilize fundamentally similar or completely divergent molecular mechansims to initiate DNA replication? Until the past 2-3 years, this question was unanswerable. It has proven difficult to identify specific DNA sequences from mammalian chromosomes that specify replication origins in the manner of the ARS (autonomously replicating sequence) elements of yeast, but origin mapping techniques have localized sites of replication initiation to defined zones of kilobase size at specific sites in the human genome. It is evident now, moreover, that proteins very closely related to the replication initiation factors defined in yeasts (ORC, Cdc6, MCMs) are conserved in humans and other vertebrates[13,18,29,82], suggesting that fundamental principles of origin control may be similar in all eukaryotes.

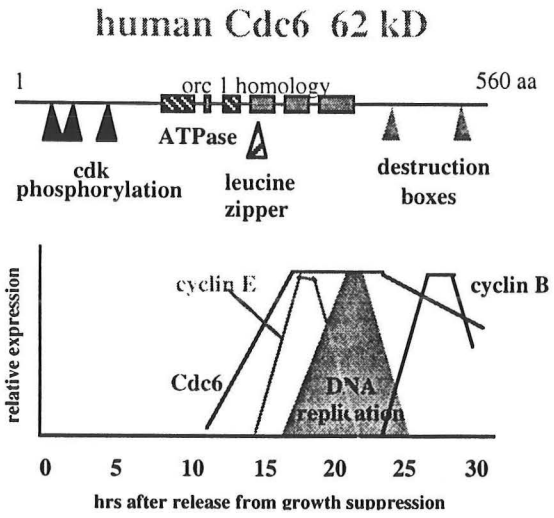

Fig. 13. Structure and expression of human Cdc6. Cells of humans and other mammals express a protein closely related to Cdc6 from yeast. The primary amino acid sequence determined from cloned cDNA suggests the presence of several putative structural and functional domains within the protein, including consensus sites for phosphorylation by cyclin dependent kinases, a leucine zipper likely to mediate interactions with other proteins, a nucleotide binding/ATPase domain, destruction boxes similar to sequences that promote degradation of other proteins at specific stages of the cell cycle, and several blocks of amino acids similar to sequences found in ORC1, the largest subunit of the ORC protein. Neither Cdc6 protein nor mRNA is detectable in quiescent cells, but expression of human Cdc6 is activated by mitogens prior to entry into S phase[82].

My laboratory group has focused on mammalian Cdc6 proteins, the human form of which (Fig. 13) we identified and cloned [82] collaboratively with Dr. Bruce Stillman at the Cold Spring Harbor Laboratory. We also have characterized frog and mouse sequences encoding Cdc6-related proteins. We mapped the human Cdc6 gene to chromosome 17q21 and identified a functional promoter segment upstream of the transcriptional start site that is regulated by E2F transcription factors. This finding identifies human Cdc6 as a downstream target of the major pathway that controls progression into S phase (Fig. 14).

Accordingly, expression of Cdc6 mRNA and protein is extinguished when cells exit from the cell cycle[82]. We are currently studying the effects of forced expression of Cdc6 and other putative human initiation factors on DNA replication in cultured cells and in tissues of transgenic animals, including the heart.

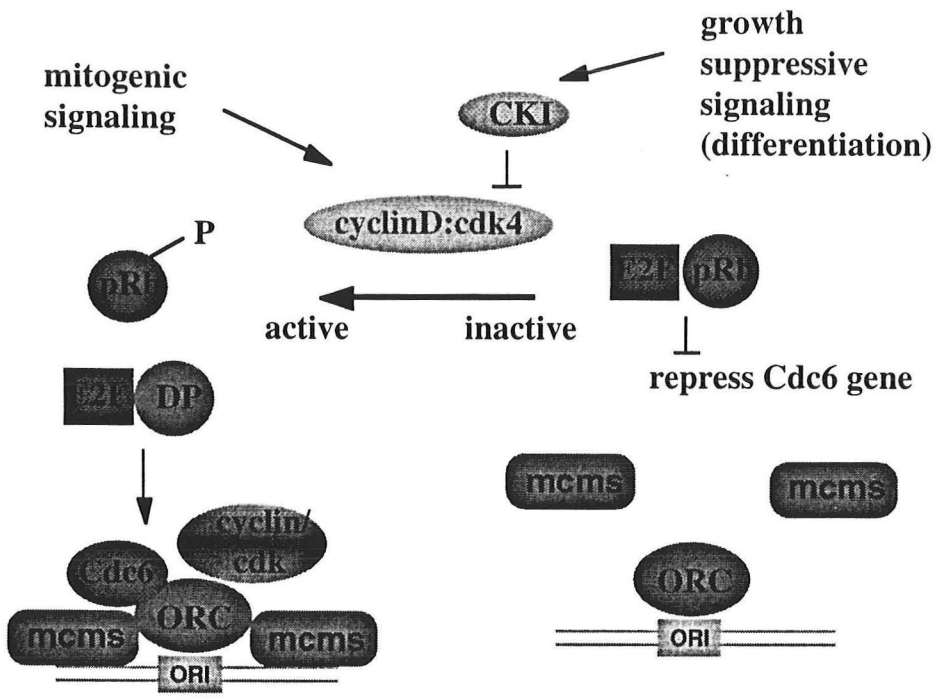

Fig. 14. Pathway for control of replication licensing in mammalian cells.

Prospects for clinical applications

Cardiomyocytes can be induced to reenter the cell cycle by expression of transgenes encoding viral oncoproteins or endogenous cellular proteins that participate in cell cycle control. Fetal cardiomyocytes isolated from live embryos or derived in culture by differentiation of embryonic stem cells can be engrafted into the myocardium. Species-specific responses to these manuevers are always a possibility, and advancing age or disease may reduce the responsiveness of cardiomyocytes to cell cycle activators or the receptivity of the myocardium to engraftment of foreign cells. Otherwise, there are no compelling reasons to think that human cardiomyocytes would respond in a manner fundamentally different from their rodent counterparts. Thus, it is possible that currently available technologies could be employed to add functioning contractile units to failing hearts of human patients.

The approaches available for doing so, however, have by no means been optimized, and the evidence that one could actually improve contractile performance in this manner is lacking. Likewise, the potential to do harm by unforeseen cellular responses, by maladaptive effects on supracellular organization within the myocardium, or by arrhythmogenic effects, is real and uncharacterized . It seems imprudent, therefore, to propose clinical testing using the existing biotechnological platforms.

The long term prospects for myocardial repair, in contrast, are more promising, and both basic and translational research pertinent to this goal merits aggressive support, as recommended by a Special Emphasis Panel convened recently by the National Heart, Lung, and Blood Institute[17]. Our fundamental understanding of stem cell biology, cardiogenic differentiation, and cell cycle control -- the areas of basic science most relevant to the problem -- is advancing rapidly, and improvements in gene transfer techniques reasonably

can be anticipated. It should become possible to gain a greater degree of control over initiation of DNA replication and cell cycle progression in cardiomyocytes through regulated expression of transgenes, and to avoid the unregulated growth that has characterized some of the early experiments.

Issues and problems for cardiac repair

- **genes vs. cells vs. drugs**
- **limitations and risk of delivery procedure**
- **stopping and starting the process**
- **tissue architecture (pattern formation)**
- **vascular supply**
- **proarrhythmic effects**
- **underlying disease process**

Fig. 15. Beyond the cell cycle and cardiomyocyte proliferation: obstacles to clinically effective myocardial repair.

Predictions of biotechnological progress are hazardous, since seminal breakthroughs most often arise from unexpected sources. I would hazard a guess, however, that replenishing the myocardium with a sufficient number of new cardiomyocytes soon will present less of an obstacle to clinically efficacious myocardial repair strategies than the problem of correct pattern formation -- arranging and orienting these cells with respect to pre-exisiting myocytes, to the fibrous skeleton of the heart, and to the microvasculature, while avoiding electrical instability.

Finally, there is always the possibility that competing medical technologies will eliminate the clinical need for myocardial repair. Dramatic breakthroughs in cardiac xenotransplantion could provide a virtually unlimited source of donor hearts. Longstanding efforts to develop a completely artificial heart could finally succeed to a level that would meet the clinical demand. Even stunning successes in these other fields are unlikely, however, to dampen enthusiasm for a truly efficacious myocardial repair strategy that could be more cost effective and less invasive.

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