Regulation of Cardiomyocyte Proliferation and Myocardial Growth During Development by FOXO Transcription Factors

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Abstract—Cardiomyocytes actively proliferate during embryogenesis and withdraw from the cell cycle during neonatal stages. FOXO (Forkhead O) transcription factors are a direct target of phosphatidylinositol-3 kinase/AKT signaling in skeletal and smooth muscle and regulate expression of the Cip/Kip family of cyclin kinase inhibitors in other cell types; however, the interaction of phosphatidylinositol-3 kinase/AKT signaling, FOXO transcription factors, and cyclin kinase inhibitor expression has not been reported for the developing heart. Here, we show that FOXO1 and FOXO3 are expressed in the developing myocardium concomitant with increased cyclin kinase inhibitor expression from embryonic to neonatal stages. Cell culture studies show that embryonic cardiomyocytes are responsive to insulin-like growth factor 1 stimulation, which results in the induction of the phosphatidylinositol-3 kinase/AKT pathway, cytoplasmic localization of FOXO proteins, and increased myocyte proliferation. Likewise, adenoviral-mediated expression of AKT promotes cardiomyocyte proliferation and cytoplasmic localization of FOXO. In contrast, increased expression of FOXO1 negatively affects myocyte proliferation. In vivo myocyte-specific transgenic expression of FOXO1 during heart development causes embryonic lethality at embryonic day 10.5 because of severe myocardial defects that coincide with premature activation of p21cip1, p27kip1, and p57kip2 and decreased myocyte proliferation. Transgenic expression of dominant negative FOXO1 in cardiomyocytes does not obviously affect heart development at embryonic day 10.5, but results in abnormal morphology of the myocardium by embryonic day 18.5 along with decreased cyclin kinase inhibitor expression and increased myocyte proliferation. These data support FOXO transcription factors as negative regulators of cardiomyocyte proliferation and promoters of neonatal cell cycle withdrawal during heart development. (Circ Res. 2008;102:0-0.)

Key Words: heart development ■ FOXO1 (FKHR) ■ Cip/Kip cyclin kinase inhibitors

Normal heart morphogenesis and development are dependent on highly controlled differential regulation of cell proliferation in specific populations of cardiomyocytes during embryonic, fetal, and neonatal stages.1 Embryonic cardiomyocytes throughout the primitive heart tube rapidly proliferate to provide sufficient cell numbers to build the working myocardium.2 At fetal stages, proper formation of the ventricular trabeculae, compact zone, and interventricular septum is dependent on more localized temporal and spatial regulation of cardiomyocyte proliferation.3,4 Immediately before birth, cardiomyocytes throughout the myocardium undergo a hyperplastic to hypertrophic transition in which cell division slows and cell growth increases.5 After birth, neonatal cardiomyocytes withdraw from the cell cycle, and growth of the myocardium into adulthood occurs primarily by hypertrophy.1 The molecular mechanisms that control cardiomyocyte maturation, proliferation, and resultant myocardial growth are fundamental to normal heart development, but they are not yet well defined. Likewise extensive efforts to induce postmitotic cardiomyocytes to reenter the cell cycle have not been very successful. Therefore identification of the signaling pathways, transcriptional effectors, and downstream target genes that control normal cardiomyocyte proliferation during prenatal development and neonatal cell cycle withdrawal could provide new strategies for manipulation of the cardiomyocyte cell cycle in older individuals.

The Forkhead O (FOXO) family of transcription factors has important roles in cell proliferation, metabolism, and aging in a variety of cell types.6 FOXO1 and FOXO3 have been implicated in the regulation of skeletal and smooth muscle differentiation and cell size regulation and proliferation, but their roles in the developing heart have not been determined.7 Loss of FOXO1 function in mice leads to embryonic lethality before heart morphogenesis, and mice lacking FOXO3 are apparently normal at birth but develop cardiac hypertrophy and heart failure later in adult life.8,9 Therefore, alternative strategies are necessary to determine the functions of FOXO transcription factors in cardiac cell development.

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proliferation and growth. FOXO transcriptional activity is regulated by a variety of signaling mechanisms including phosphatidylinositol-3 kinase (PI3K)-mediated activation of AKT, which directly phosphorylates FOXO at serine 256, leading to nuclear export and inactivation. In the absence of negative regulation by AKT, FOXO factors remain in the nucleus and induce transcription of a variety of downstream target genes that collectively inhibit proliferation and induce cell cycle withdrawal. Direct downstream targets of FOXO1 include the members of Cip/Kip family of cyclin kinase inhibitors (CKIs), p21CIP1 and p27KIP1, which have also been implicated in cardiomyocyte cell cycle withdrawal after birth. FOXO factors remain in the nucleus and inactivate cyclin kinase inhibitors.6 Direct downstream targets of FOXO1 during heart development provide in vivo evidence of a critical role for FOXO factors in the control of cardiomyocyte proliferation and myocardial growth via transcriptional regulation of CKIs.

Growth factors, including insulin-like growth factor (IGF)1, activate PI3K/AKT signaling in the heart to promote growth of the myocardium during development and after birth. In mice, loss of IGF1 causes hypoplasia of the myocardium and overall retarded growth, whereas cardiogenic control embryos. Chromatin immunoprecipitation of FOXO1 and FOXO3 are expressed in the developing myocardium in parallel with p21CIP1, p27KIP1, and p57KIP2. A, RT-PCR analysis of FOXO1, FOXO3, and FOXO4 expression in the developing heart at indicated stages. E15 liver, brain, and forelimb tissues were also examined for FOXO expression. L7 is included as a control for RNA input and samples lacking reverse transcriptase (−RT) were used as negative controls. B, FOXO1 and phosphorylated FOXO1 (Ser256) protein is expressed in E14 to adult heart lysates. C, p21CIP1, p27KIP1, and p57KIP2 are expressed in the developing heart from E14 to adult stages. GAPDH serves as a loading control for all protein lysates. Neo indicates neonatal stage.

Materials and Methods

Collection of cardiac tissue as well as procedures for RNA isolation and immunostaining were performed as previously described. Embryonic cardiomyocytes were enzymatically dissociated from E14-5 hearts of FVBN mice for primary cultures, which were treated pharmacologically or infected with recombinant adenoviruses expressing FOXO or AKT. Protein lysates for Western blotting were obtained from stage-specific cardiac tissue, as well as cultured primary cardiomyocytes. βMHC-FOXO1 transgenic embryos were generated using the β-myosin heavy chain (MHC) promoter driving expression of wild-type (WT), constitutively active (CA), or dominant negative (DN) FOXO1. Cardiomyocyte proliferation indices were calculated and quantitative real-time RT-PCR was performed on tissue isolated from hearts of transgenic and nontransgenic control embryos. Chromatin immunoprecipitation of p21CIP1 promoter sequences bound to FOXO1 was performed using embryonic and neonatal cardiomyocyte samples. An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

FOXO Transcription Factors Are Expressed in the Developing Heart

To determine the developmental expression patterns of FOXO transcription factors in the heart, mRNA and protein expression of FOXO family members was examined at various stages of heart development. Expression of FOXO1 and FOXO3 transcripts was detected by RT-PCR in the developing heart at E11, E15, and neonatal stages (Figure 1A). In the adult heart, FOXO1 expression was very low in comparison with earlier stages, whereas the expression of FOXO3 was not observed. FOXO4 was detected in the heart during embryonic and neonatal stages, at much lower levels than FOXO1 and FOXO3, and was not detected in the adult heart. In addition to cardiac tissue, FOXO1, FOXO3, and FOXO4 are expressed in E15 liver, brain, and forelimbs during embryogenesis. Protein expression studies confirmed expression of FOXO1 in heart tissue lysates from E14, E18, neonatal day 1 (N1) and N7 (Figure 1B). The expression of phosphorylated FOXO1 (pFOXO1-Ser 256) was also examined and compared with total FOXO1 expression. Densitometric analysis of bands revealed that at E14, the level of pFOXO1 was equivalent to the level of total FOXO1. However, at E18 the ratio of pFOXO1 to total FOXO1 was decreased by 80% and continued to decline in neonatal...
stages. The relative increase in dephosphorylated FOXO1 is suggestive of increased nuclear localization and transcriptional activity. Thus, the expression of FOXO transcriptional targets was also examined at the protein level. Differential expression of p21cip1, p27kip1, and p57kip2 was detected throughout the developmental time course of heart tissue lysates (Figure 1C). Expression of p21cip1 and p27kip1 protein was observed to increase at N7 and E18, respectively. In contrast, expression of p57kip2 protein was detected in the myocardium at E14 and perinatal stages but was expressed at a much lower level at N7.

The spatial localization of FOXO and p21cip1 protein expression was examined in the developing heart at midgestation and perinatal stages. FOXO1 protein was detected in the myocardium at E12.5 (Figure IA in the online data supplement) and was observed to become progressively nuclear by N3 (supplemental Figure IB). At E18.5, FOXO1 protein is predominantly localized to the nuclei of cardiomyocytes of both the trabeculae and compact layer (Figure 2A), consistent with increased total FOXO1 compared with pFOXO1 in immunoblot assays at this time point (Figure 1B). At N7, relatively fewer myocytes express FOXO1 and nuclear FOXO1 is found primarily within myocytes of the compact layer (Figure 2B). Quantification of FOXO1 positive nuclei revealed a peak of expression at E18 that declines in neonatal stages (Figure 2C). The FOXO transcriptional target p21cip1 was predominantly expressed in trabecular myocytes at E18.5 (Figure 2D). In cultured cardiomyocytes, FOXO1 is found within the cytoplasm and nucleus at E14 (Figure 2E) and is predominantly nuclear by N1 (Figure 2F), where it colocalizes with p21cip1 (Figure 2G). Together, these studies suggest that during the perinatal period (E18 to N3), FOXO1 protein is expressed in the heart at the proper developmental stages to direct transcriptional activation of target genes such as CKIs. Moreover, the spatial distribution of nuclear FOXO1 and p21cip1 protein expression at E18.5 in the myocardium correlates to the previously characterized pattern of decreased cardiomyocyte proliferation in trabecular versus compact layer myocytes.

PI3K/AKT Signaling Affects Subcellular Localization and Phosphorylation of FOXO Factors in Embryonic Cardiomyocytes

In contrast to neonatal cardiomyocytes, primary cardiomyocytes isolated from E14.5 embryos are highly proliferative in culture (20% versus 5% by 5-bromodeoxyuridine (BrdUrd) incorporation, data not shown) and serve as a useful model to study active cardiomyocyte proliferation. Therefore the ability of the growth-promoting IGF1 and PI3K/AKT signal transduction pathway to affect FOXO transcription factors was examined in this system. To stimulate PI3K/AKT signaling, embryonic cardiomyocyte cell cultures were placed in serum-free media and then treated with IGF1. Proliferation of cardiomyocytes increased by 34% after treating the cultures with 50 ng/mL IGF1 in comparison with nontreated controls (Figure 3A). In contrast, treatment with LY294002, a PI3K-specific inhibitor, decreased proliferation 56% compared with the nontreated control (Figure 3A). To ensure a cardiomyocyte-specific assessment of proliferation, sarcomeric myosin marker MF20 was used to identify cardiomyocytes in combination with BrdUrd immunoreactivity for DNA synthesis. Only BrdUrd+/MF20+ cells were counted via confocal analysis for calculation of cardiomyocyte proliferative indices (Figure 3B).

In multiple cell types, PI3K/AKT signaling stimulated by IGF1 regulates FOXO transcription factor function via phosphorylation and nuclear export. To determine whether similar regulation of FOXO factors exists in embryonic cardiomyocytes, the subcellular distribution of FOXO1 was examined in IGF1-treated cardiomyocyte cultures. The intensity of bands indicating FOXO1 expression was used to
determine a cytoplasmic/nuclear ratio of FOXO1 subcellular distribution. IGF1 treatment of cardiomyocyte cultures resulted in a 36% increase in cytoplasmic localization of total FOXO1 protein as compared with the nontreated control (Figure 3C).

FOXO1 is phosphorylated at serine 256 in response to increased PI3K/AKT signaling and pFOXO1(Ser256) was detected in the cytoplasmic fraction of nontreated, serum-treated, and IGF1-treated cultures. However, inhibition of PI3K activity by LY294002 treatment resulted in a striking decrease in pFOXO1(Ser256) as well as increased cytoplasmic localization of FOXO1. To determine whether the inhibitory effect on FOXO1 phosphorylation was PI3K-specific, cultures were also treated with PD98059, a mitogen-activated protein kinase–specific inhibitor, which increases the cytoplasmic localization of FOXO1, and does not affect FOXO1 phosphorylation at Ser256. These studies demonstrate that the proliferation of embryonic cardiomyocytes is increased by IGF1 via PI3K and that this signaling cascade promotes phosphorylation and cytoplasmic localization of FOXO1.

Recombinant adenoviruses expressing WT, DN, or CA forms of AKT were used to assess the direct effects of altered AKT function on cardiomyocyte proliferation as well as subcellular localization of FOXO1 and FOXO3. Infection of embryonic cardiomyocyte cultures with WT AKT resulted in cytoplasmic localization of FOXO1 and FOXO3, whereas

![Figure 3. IGF1-treated E14.5 cardiomyocytes have increased proliferation and loss of nuclear FOXO1. A, Cardiomyocyte proliferation measured by BrdUrd significantly increases by 34% with treatment of 50 ng/mL IGF1 in comparison with nontreated control and decreases by 56% with a PI3K-specific inhibitor, LY294002. Significance (†) determined by Student’s t test (P<0.05, n=3). B, Example of BrdUrd/MF20 cardiomyocyte (arrow) (green, MF20; red, BrdUrd; blue, ToPro3). C, Immunoblot of treated cardiomyocyte culture lysates demonstrate relative FOXO1 nuclear (nuc) expression decreases in response to stimulation of cultures with IGF1 as indicated by 1.36-fold increase in the cytoplasmic (cyt) fraction of FOXO1 protein. The ratio of cytoplasmic:nuclear fraction of total FOXO1 determined densitometrically is indicated for each group. Nontreated cultures (NT) displayed a 1:1 ratio of cytoplasmic to nuclear FOXO1. Phosphorylated FOXO1 (pFOXO1) is significantly decreased in cultures treated with PI3K inhibitor LY294002 vs pFOXO1 expression in other samples.

Figure 4. Increased AKT activity affects FOXO subcellular localization and increases proliferation, whereas increased FOXO1 results in decreased proliferation of cultured cardiomyocytes. A, Immunoblot analysis for total FOXO1 and FOXO3 protein expression in cytoplasmic and nuclear fractions of E14.5 cardiomyocytes infected with WT, DN, or CA AKT recombinant adenoviruses. Infection of E14.5 cardiomyocyte cultures with WT AKT results in localization of FOXO1 and FOXO3 to the cytoplasmic fraction, whereas both FOXO factors are present in the nuclear and cytoplasmic fractions in DN AKT or nontreated (NT) cultures. Expression of CA AKT results in cytoplasmic localization of FOXO3, and does not affect FOXO1. B, Infection of myocyte cultures with WT or CA AKT adenovirus results in significantly increased proliferation by 47% and 40%, whereas DN AKT did not significantly affect proliferation in comparison with control. C, Increased WT or CA FOXO1 via adenoviral infection of cardiomyocyte cultures results in decreased proliferation by 35% and 31%. Infection with DN FOXO1 did not significantly affect proliferation in comparison with control. Significance (*) determined by Student’s t test (P<0.05; n=4).
infection with DN AKT did not change subcellular localization of FOXO1 in comparison with controls (Figure 4A). Interestingly, CA AKT resulted in increased cytoplasmic localization of FOXO3, but not FOXO1 (Figure 4A). Increased WT and CA AKT resulted in a 40% to 47% increase in proliferation of cardiomyocytes, whereas DN AKT did not significantly affect proliferation in comparison with control cultures (Figure 4B). To examine the direct effects of altered FOXO1 activity on cardiomyocyte proliferation, WT, DN, and CA FOXO1 adenoviruses were used. Increased WT or CA FOXO1 function resulted in a 31% to 35% decrease in proliferation of cardiomyocytes (Figure 4C). DN FOXO1 did not significantly affect proliferation compared with control cultures (Figure 4C). Together, these observations provide evidence that activated AKT promotes FOXO cytoplasmic localization concomitant with increased cell proliferation in embryonic cardiomyocytes. Furthermore, increased FOXO1 function in cardiomyocytes leads to decreased cardiomyocyte proliferation, consistent with an inhibitory role in cell cycle regulation in the developing heart.

**βMHC-FOXO1 Transgenic Mice Display Aberrant Cardiomyocyte Proliferation Consistent With Altered CK1 Expression**

To examine FOXO1 function in cardiomyocyte proliferation in vivo, transgenes that express WT, DN, and CA FOXO1 from the β-MHC promoter were used for expression in cardiomyocytes beginning at E8.5 and extending throughout heart development. The WT FOXO1 transgene encodes FOXO1 protein that remains susceptible to AKT-mediated regulation via phosphorylation. In contrast, the CA FOXO1 transgene encodes a protein that cannot be phosphorylated by AKT and thus remains nuclear and able to transcriptionally activate target gene expression. The DN FOXO1 transgene encodes a truncated version of the FOXO1 protein that is capable of binding FOXO recognition sites on DNA but is not capable of transcriptional activation. Because genetic deletion of FOXO1 results in embryonic lethality at E10.5, transgenic FOXO1 founder (F0) embryos were first examined at E10.5 to identify possible cardiac phenotypes. Transgene expression in the myocardium was confirmed by immunoreactivity for hemagglutinin (HA) tag linked to the ectopically expressed FOXO1 proteins (data not shown).

Increased FOXO1 function in WT and CA FOXO1 transgenic embryos resulted in embryonic lethality at E10.5 with obvious heart failure, indicated by pericardial edema, thin myocardium, and overall reduced size (n=13 for WT FOXO1; n=7 for CA FOXO1; Figure 5D and 5E). Comparable cardiovascular defects and embryonic lethality were observed in WT FOXO3 transgenic embryos at E10.5 (data not shown). Immunohistological analysis of transgenic hearts with sarcomeric myosin marker MF20 shows the gross morphological defects of the differentiated myocardium in the WT FOXO1 E10.5 transgenic hearts versus nontransgenic littermates (Figure 5C and 5F). The defective myocardium of WT and CA FOXO1 transgenic embryos was observed to be contractile during the initial harvest of transgenic embryos, which indicates primary differentiation of cardiomyocytes. However, the number of myocytes in the compact layer of transgenic myocardium is reduced, and there is a lack of trabeculation in these hearts, which demonstrates the reduction in myocardial growth consistent with a primary defect in cardiomyocyte proliferation. Because the β-MHC promoter is cardiomyocyte-specific at E10.5, the retarded growth observed in WT and CA FOXO1 transgenic embryos must be secondary to the cardiac phenotype. Interestingly, WT FOXO1 transgenic embryos exhibited a variable phenotype in that not all transgenic embryos had the defective cardiac phenotype (n=8/13 total embryos), whereas CA FOXO1 transgenic embryos consistently exhibited a severe hypomorphic cardiac phenotype (n=7/7). In contrast, all of the DN FOXO1 transgenic embryos were indistinguishable from nontransgenic littermates (n=5, Figure 5A and 5B) at E10.5, indicating that inhibition of FOXO1 transcriptional activity at this stage does not affect proliferation or myocardial growth. Murine cardiomyocytes are beginning the process of cell cycle withdrawal at E18.5, thus additional DN FOXO1 transgenic embryos were generated to investigate the potential role of FOXO1 during the perinatal period. The DN FOXO1 transgene is capable of disrupting the transcriptional...
activity of all endogenous FOXO transcription factors; thus, DN FOXO1 transgenic embryos should have decreased FOXO1 and FOXO3 function during cardiomyocyte cell cycle withdrawal. At E18.5, DN FOXO1 transgenic embryos displayed grossly abnormal myocardial growth and mild pericardial edema as compared with nontransgenic littermates (n=11; Figure 6A and 6B). Histological analysis showed proper formation of ventricular chambers and interventricular septum; however, an increase in the thickness of the myocardial wall was observed in DN FOXO1 transgenic embryos in comparison with hearts of nontransgenic littermates (Figure 6C and 6D). Immunohistological analysis of DN FOXO1 hearts revealed an increase in the number of proliferative cardiomyocytes as measured by pHH3 immunoreactivity of cells in M phase of the cell cycle (Figure 6E and 6F). Thus, during the perinatal period, inhibition of FOXO function in cardiomyocytes results in abnormal growth of the myocardium and increased proliferation.

Cardiomyocyte proliferation was quantified in the defective hearts of both the E10.5 WT FOXO1 and E18.5 DN FOXO1 transgenic hearts in comparison with nontransgenic littermate controls. A significant decrease (3.3 fold, P=0.017) in cardiomyocyte proliferation was observed in the E10.5 WT FOXO1 transgenic myocardium (Figure 7A), whereas a significant increase (2.5-fold, P=0.013) was observed in the E18.5 DN FOXO1 transgenic myocardium as measured by pHH3 immunoreactivity (Figure 7C). In the normal nontransgenic hearts, the expected decrease in cardiomyocyte proliferation was observed with 3.4% myocytes in M phase at E10.5 compared with 1.0% at E18.5. Note that mitotic indices determined by pHH3 immunoreactivity are ~10-fold lower than those determined other methods, which is consistent with previous analyses in cardiomyocytes. Nuclear FOXO1 also has been associated with increased apoptosis; therefore, cardiomyocyte apoptosis was measured in E10.5 WT FOXO1 and E18.5 DN FOXO1 transgenic hearts by cleaved caspase-3 immunoreactivity and TUNEL assay (data not shown). No significant differences in the number of apoptotic myocytes were observed in WT FOXO1 or DN FOXO1 transgenic embryos relative to nontransgenic littermate controls. Therefore, the cardiac phenotypes apparent in WT FOXO1 transgenic embryos at E10.5 and of DN FOXO1 embryo hearts at E18.5 are primarily attributable to altered rates of cell proliferation.

To further investigate the direct effects of altered FOXO1 function on cardiomyocyte proliferation, the expression of FOXO target genes that inhibit proliferation was examined in the hearts of FOXO1 transgenics and nontransgenic littermates at E9.75 for WT FOXO1 and E18.5 for DN FOXO1. Embryos were analyzed at E9.75 before the occurrence of obvious secondary effects on overall embryonic morphology attributable to compromised heart development. Expression of p21\textsuperscript{cip1} and p27\textsuperscript{kip1} cell cycle inhibitors demonstrated to be transcriptional targets of FOXO1 in cell culture, and the related gene p57\textsuperscript{kip2} was examined. In normal hearts, p21\textsuperscript{cip1}, p27\textsuperscript{kip1}, and p57\textsuperscript{kip2} are expressed at very low levels at E9.75 and are increased at E18.5 when cardiomyocytes are actively withdrawing from the cell cycle (Figure 7B and 7D). In the E9.75 WT FOXO1 transgenic hearts, the expression of p21\textsuperscript{cip1}, p27\textsuperscript{kip1}, and p57\textsuperscript{kip2} was significantly increased relative to hearts of nontransgenic littermates (Figure 7B). The observation that p57\textsuperscript{kip2} expression is increased in the E9.75 WT FOXO1 embryos, coincident with increased expression of p21\textsuperscript{cip1} and p27\textsuperscript{kip1}, is evidence that p57\textsuperscript{kip2} is also directly regulated by FOXO1. In striking contrast, p21\textsuperscript{cip1}, p27\textsuperscript{kip1}, and p57\textsuperscript{kip2} expression was significantly decreased in the DN FOXO1 transgenic hearts at E18.5, consistent with the observed increase in cardiomyocyte proliferation at in these embryos (Figure 7C and 7D). Moreover, chromatin immunoprecipitation experiments confirm endogenous FOXO1 binding to p21\textsuperscript{cip1} promoter sequences at neonatal but not embryonic stages (Figure 7E). Together, these studies support a key role for FOXO transcription factors in the negative regulation of cardiomyocyte proliferation through activation of cell cycle inhibitors during heart development.
In this report, we demonstrate that FOXO transcription factors are functionally regulated by IGF1/PI3K/AKT signaling in cultured cardiomyocytes and that altered FOXO function in vivo affects cardiomyocyte proliferation. Specifically, increased FOXO1 function at E10.5 leads to decreased cardiomyocyte proliferation, increased expression of p21cip1, p27kip1, and p57kip2, heart failure, and embryonic death. Transgenic expression of a DN FOXO1 in cardiomyocytes at E18.5 results in thickening of the myocardium, increased cardiomyocyte proliferation, and decreased expression of p21cip1, p27kip1, and p57kip2 at E18.5. Together, these analyses support a model for FOXO function in cardiomyocyte proliferation and growth (Figure 8). In actively proliferating cardiomyocytes, growth factors including IGF1 activate PI3K and AKT, which directly phosphorylates FOXO, leading to nuclear exclusion and inactivation. During cardiomyocyte cell cycle withdrawal, growth factor/PI3K/AKT signaling is decreased, and FOXO factors are localized to the nucleus, where they activate the expression of CKIs, thereby inhibiting cell cycle progression. Combined, these data provide new evidence that FOXO transcription factors, antagonized by IGF1/PI3K/AKT signaling, are critical regulators of cardiomyocyte cell cycle withdrawal during the perinatal period.

Previous studies have demonstrated that increased IGF1 or AKT signaling can prolong cardiomyocyte cell cycling in neonates. Here we provide evidence that inhibition of FOXO function can also enhance cardiomyocyte proliferation. FOXO transcription factors can alter the expression of a spectrum of genes that cumulatively result in decreased proliferation and cell cycle arrest. In established cell lines, FOXO transcription factors can activate cell cycle inhibitors, such as p130 and cyclin G2, as well as inhibit cell cycle activators including the D-type cyclins, making them powerful regulators of cell cycle progression. The altered cardiomyocyte proliferation observed with DN FOXO1 as well as increased FOXO1 function in cultured cardiomyocytes during heart development is associated with aberrant cardiomyocyte proliferation and dysregulated expression of p21cip1, p27kip1, and p57kip2. A, Cardiomyocyte proliferation was significantly decreased in E10.5 WT FOXO1 transgenic hearts in comparison with nontransgenic littermates as measured by percentage of pH3+ cells per total MF20+ cells (n=3, P<0.017). B, FOXO1 WT hearts displayed increased expression of p21cip1, p27kip1, and p57kip2 at E18.5 compared with nontransgenic littermates (n=3), as determined by real-time RT-PCR. C, Significantly increased cardiomyocyte proliferation determined by percentage of pH3+ cardiomyocytes was observed in E18.5 DN FOXO1 hearts (n=3, P=0.013). D, Expression of p21cip1, p27kip1, and p57kip2 was decreased in DN FOXO1 hearts compared with nontransgenic littermates at E18.5 (n=3), as determined by real-time RT-PCR. Significance (*) was determined by Student’s t test (P<0.05; n=3). E, Chromatin immunoprecipitation was performed using chromatin from embryonic (E14) and neonatal cardiomyocytes and either FOXO1, acetyl histone H3 (positive control), or normal rabbit IgG (negative control). Precipitated DNA was then amplified by PCR using primers specific for the p21cip1 promoter, which contains FOXO binding sites. Preferential binding of FOXO1 to the p21cip1 promoter was observed in neonatal vs embryonic cardiomyocytes (n=2).
cytes and in transgenic embryos is strong evidence that cardiomyocyte cell cycle control is regulated by FOXO function.

Inhibition of FOXO function in the perinatal heart results in decreased expression of cell cycle inhibitors in the Cip/Kip family concomitant with increased cardiomyocyte cell cycling. Normally, expression of \( p21^{\text{cip1}} \) and \( p27^{\text{kip1}} \) increases with the decline in cardiomyocyte proliferative capacity during postnatal stages.\(^4\) In mice lacking \( p27^{\text{kip1}} \), the cardiac cell cycle is prolonged and proliferation is extended in neonatal stages, which results in developmental hyperplasia and increased heart size.\(^3\) However, it is important to note that eventually even the cardiomyocytes lacking \( p27^{\text{kip1}} \) withdraw from the cell cycle, which indicates possible redundancy within the Cip/Kip family or other regulatory mechanisms that limit cardiomyocyte proliferation after birth. The involvement of the Cip/Kip family of CKIs in the regulation of cardiomyocyte proliferation is well documented\(^5\); however, their transcriptional regulation during heart development is not well defined. Here, we show that increased FOXO1 in cardiomyocytes results in altered expression of \( p21^{\text{cip1}} \) and \( p27^{\text{kip1}} \) as well as \( p57^{\text{kip2}} \), which has not previously been associated with neonatal cardiomyocyte cell cycle withdrawal. In addition binding of FOXO1 protein to \( p21^{\text{cip1}} \) promoter sequences was observed in neonatal but not E14 cardiomyocytes. The observations that increased cardiomyocyte cell cycling occurs in mice lacking specific CKIs and in E18.5 embryos with DN FOXO1 expression indicates that the regulation of CKIs by FOXO transcriptional activity is an important feature of neonatal cardiomyocyte cell cycle withdrawal.

In the adult heart, FOXO factors are not normally expressed, which suggests that their primary involvement in cardiomyocyte cell cycle regulation is during late heart development and neonatal cell cycle withdrawal. However, the IGF/AKT/FOXO pathway may also have important functions in adult cardiac hypertrophy and pathogenesis associated with heart disease. In support of a role for FOXO in the mature heart, loss of FOXO3 results in cardiomyocyte hypertrophy in adult mice.\(^9\) In addition, cardiac FOXO1 protein expression is increased in human heart failure patients, which is suggestive of increased FOXO function in the diseased myocardium.\(^3\) FOXO transcription factors also have been implicated in stem cell renewal and homeostasis in the hematopoietic lineages.\(^3\) Therefore induction of FOXO in the adult heart could serve a related role in cardiomyocytes if mobilization of cardiac stem cells becomes a viable therapeutic avenue. Together these observations provide initial evidence for FOXO transcription factor function in cardiac hypertrophy and heart failure, but further studies are necessary to determine the precise role of FOXO function in these processes.

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None.

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ONLINE SUPPLEMENT

MATERIALS AND METHODS

**RT-PCR/Quantitative real time RT-PCR**

Total RNA was isolated from heart, brain, forelimb, or liver tissue of E11, E15, N7, or adult mice for RT-PCR analysis. Alternatively, total RNA was isolated from hearts of βMHC-FOXO1 transgenic F0 embryos and non-transgenic littermates for quantitative real time RT-PCR analysis. 3μg of total RNA was included in Superscript II first strand synthesis reactions (Invitrogen) in preparation for RT-PCR as previously described\(^1\).

Oligonucleotide primer sequences were designed to amplify transcripts from murine FOXO1, FOXO3, and FOXO4: FOXO1- 5’- TTTCTAAGTGCTGCGAGT-3’ and 5’-CACAGTCCAAGCGCTCAATA-3’; FOXO3- 5’-AGCCGCTGACTGTGGGCTT-3’ and 5’-CCACATTCAAACCAACAACG-3’; FOXO4 – 5’-CAAGAGGAAAGTCTGTCC-3’ and 5’-TGCTGTGACTCAGGGATCTG-3’. Primer sequences were designed to amplify transcripts from p21cip1, p27kip1, and p57kip2: p21cip1 - 5’-CACAGCTCAGTGGACTGGAA-3’ and 5’-CCACCACCACACACCATA-3’; p27kip1 - 5’-CCGAGGAGGAAGATGTCAAA-3’ and 5’-TCCAGGGGCTTATGATTCTG-3’; p57kip2 - 5’-TTTAGAGGCTAACGGCCAG-3’ and 5’-GCTTTACACCTGGGACCAG-3’. Amplification reactions were performed with 30 cycles of (94° for 1 min; 57° for 1.5 min; 72° for 3 min), and amplification of the ribosomal protein L7 was performed in parallel as a loading control and for normalization\(^2\). Normalized threshold cycle values of samples amplified in triplicate
served as an indicator of relative gene expression levels. Statistical significance of observed differences was determined by Student’s t-test.

**Protein isolation, Subcellular fractionation, and Western blotting**

Protein lysates were obtained from the ventricles of embryonic hearts at specific developmental time points. Ventricles were dissected into ice-cold lysis buffer [Celllytic MT (Sigma), Protease inhibitorcocktail (Pierce), Phosphatase inhibitor (Pierce)] and homogenized. For nuclear/cytoplasmic fractionation, cultured ventricular cardiomyocytes were fractionated into nuclear and cytoplasmic lysates using PARIS kit from Ambion following manufacturer’s instructions. For western analysis of protein lysates, samples (30µg) were loaded onto Tris/HCL gels (BioRad) and separated by SDS-PAGE then transferred to immunoblot PVDF membrane (BioRad). Membranes were blocked with Starting block T20 (Tris-buffered saline) blocking buffer (Pierce) for at least 2 hours. After blocking, membranes were incubated with primary antibody in blocking buffer overnight at 4°C on a rocker, then washed five times with Tris-buffered saline with 0.1% Triton-X (TBST), and followed with an horseradish-peroxidase (HRP) conjugated secondary antibody (Pierce) [1:5000] in blocking buffer and subsequent washes with TBST. Immunoblots were developed using SuperSignal West Femto Maximum sensitivity substrate (Pierce) and bands were detected via photographic film development. Densitometric analysis of bands was performed with Gel-doc software by comparing the relative densities of bands normalized to the volume of the band (BioRad). Antibodies used for immunoblot analysis included: FOXO1 (Sigma) [1:7000]; phospho-FOXO1 (Ser 256) (Cell Signaling) [1:2000]; FOXO3 (Sigma) [1:7000]; phospho-FOXO3
Immunostaining, Confocal analysis, and cell counts

Cardiac tissue was obtained at specific developmental time points, fixed in 4% paraformaldehyde, and embedded as previously described. Deparaffinized sections (7µm) were treated with 0.25% Triton-X/phosphate-buffered saline (PBS) and blocked using 5% bovine serum albumin (BSA)/PBS for at least 1 hour at room temperature. Primary antibodies were incubated in blocking buffer (2% BSA/PBS) overnight at 4°C. Washes were conducted with PBST (0.1% Triton/PBS). Detection of antibody reactivity was visualized using diaminobenzidine (DAB) substrate (Pierce) or fluorescent-labeled secondary antibodies (Molecular Probes) for confocal analysis. Antibodies used for immunohistochemistry included: FOXO1 (Sigma) [1:100]; FOXO1 (Upstate) [1:100]; phospho-FOXO1 (pFOXO1, Ser 256) (Cell Signaling) [1:100]; p21 (BD Pharmingen) [1:100]; phospho-Histone H3 (pHH3, Ser10) (Upstate) [1:100]; Bromodeoxyuridine (BrdU) (Zymed); MF20 (Developmental studies hybridoma bank, University of Iowa) [1:500]; Cleaved caspase 3 (Asp 175) (Cell Signaling) [1:100]. Apoptosis was measured by TUNEL assay using the in situ cell death detection kit, fluorescein (Roche) per manufacturer’s instructions. Immunofluorescence was detected using a Zeiss LSM 510 confocal microscope. Images were obtained using Zeiss LSM version 3.2 SP2 software and analyzed using Meta imaging series Metamorph version 7.0r3 software. For counting, nuclei were counterstained with a 1:1000 dilution of TO-PRO 3 iodide (λ=642) (Molecular Probes) along with secondary antibody incubation. The percent of pHH3 or
BrdU positive myocyte nuclei (identified by MF20 immunoreactivity) was calculated by dividing the number of pHH3 or BrdU positive myocyte nuclei by the total number of TO-PRO 3 labeled myocyte nuclei per microscopic field. In at least three independent experiments, a total of 10 fields containing at least 200 cells each were counted for each treatment group for cultured myocytes. For analysis of tissue sections, at least 500 myocytes were counted per section of transgenic versus non-transgenic littermates (n=3). At least 3 sections were analyzed for each embryo. Statistical significance of observed differences in cell counts was determined by Student’s t-test.

Embryonic cardiomyocyte cultures, treatments, adenoviral infection

Mouse embryonic (E14.5) cardiomyocytes were isolated and cultured as previously described4. D. Accili (Columbia University) generously provided recombinant adenoviral constructs of wild type (WT), dominant negative (DN), and constitutively active (CA) FOXO15. WT, DN, and myristylated (MYR) AKT adenovirus was purchased from Vector Biolabs. For growth factor assays, culture media containing serum (DMEM/10% fetal bovine serum (FBS)/penicillin-streptomycin) was replaced with serum-free media (DMEM/pen-strep) 24 hours prior to treatment. One hour prior to treatment, cultures were treated with LY294002 (Cell Signaling) [50nM] or PD98059 (Sigma) [10µM]. Cultures were then treated with recombinant human insulin-like growth factor (IGF1) (R&D systems) [10-100ng/ml] or placed in culture media containing 10% FBS. After 30 minutes of incubation, cultures were washed with PBS and protein lysates were taken. Alternatively, Bromodeoxyuridine (BrdU) labeling solution was added to the media [1:100; Zymed] and the cells were incubated for 2 hours.
and then fixed with 70% EtOH for immunostaining. Adenoviral infections were performed 48 hours post-isolation. Cardiac myocyte cultures were infected with 10^8 plaque-forming units of the FOXO1 or AKT virus in serum-free culture media. Treatment with CMV β-galactosidase (βgal) virus was used as a control. Cells were incubated with the infection media for 5 hours, which was then replaced with fresh culture media. After 24-48 hours, cultures were washed with PBS and protein lysates were taken or cultures were processed for immunostaining. Infection efficiency of each FOXO1 and AKT adenovirus was determined to be greater than 90% in cardiac myocyte cultures via detection of viral proteins using anti-HA antibody (Novus) [1:100] for immunohistochemistry and confocal analysis. In addition, western blotting was used for confirmation of viral protein production.

*Generation of βMHC-FOXO1 cardiomyocyte-specific transgenic embryos*

Murine FOXO1 wild type (WT), dominant negative (DN), and constitutively active (CA) constructs were generously provided by D. Accili, (Columbia University)\(^5\). FOXO1 WT, DN, and CA coding sequences with hemagglutinin (HA)-tag sequence were isolated and ligated into the β-myosin heavy chain promoter vector clone 32\(^6\). The resultant DNA was linearized and purified prior to pronuclear micro-injection to generate transgenic mouse founder (F\(_0\)) embryos analyzed at E9.75, E10.5 or E18.5\(^7\). Transgenic F\(_0\) embryos were identified via PCR genotyping of yolk sac DNA for the human growth hormone (HGH) sequence in the transgene using the oligonucleotide primer set (5’-GTCTGACTAGGTGTCCTTCT-3’) and (5’-CGTCCTCCTGCTGG-3’)\(^6\). Embryos were fixed 2-6 hours in Methcarn fixative (6 MeOH: 3 Chloroform: 1 glacial acetic acid) at
4°C, then stored in 70% MeOH at -20°C in preparation for histological analysis.

Cardiomyocyte-specific transgene expression was confirmed by detection of HA-tagged protein via immunohistochemistry of sections from paraffin-embedded transgenic and non-transgenic embryos. Alternatively, total RNA was isolated from dissected hearts of E9.75 WT FOXO1 (n=3), which was pooled, or E18.5 DN FOXO1 (n=3) individual transgenic embryos and non-transgenic littermates as previously described for use in quantitative real time RT-PCR.

**Chromatin Immunoprecipitation Assay**

Cultured cardiomyocytes were crosslinked for 10 minutes by directly adding formaldehyde to the culture medium for a final concentration of 1%. The fixed cells were lysed with lysis buffer (EZ ChIP, Upstate) and sonicated 2 times for 3 seconds with output 5 (Virsonic 60; Virtis) and 2 minute refractory period in between. For immunoprecipitation, cell lysates were incubated with an antibody against FOXO1 (5 ug; Sigma) and incubated overnight at 4°C. Reagents provided in the EZ kit include positive (anti-acetyl histone H3) and negative (normal rabbit IgG) controls. The protocol was performed according to the manufacturer’s instructions, with the exception that Protein A agarose beads were used instead of those supplied. The resulting DNA was subjected to PCR using the following primers: 5’–aactcacagctctctcaagagg- 3’ and 5’–catgtatgaagccaggagttggat-3’ for p21cip1 promoter sequences containing FoxO1 binding sites and 5’–gaagatggtggtgctcc- 3’ and 5’–aaggcggtctccgatt- 3’ for murine GAPDH, which was used as another positive control.
References

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**FIGURE 1.** FOXO1 is expressed in the developing myocardium. A&B) Immunohistochemistry of FOXO1 protein demonstrates expression in the E12.5 and N3 mouse heart specifically in the ventricular myocardium (*). Note punctate nuclear expression in (B). C) Confocal analysis of FOXO1 nuclear localization in the neonatal myocardium (asterisk) (red = FOXO1, green = MF20, blue = ToPro III). D) FOXO1 is expressed in the myocardium (asterisk) and not in the valves (MV).