Temporally controlled overexpression of cardiac-specific PI3Kα induces enhanced myocardial contractility—a new transgenic model

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Yano N, Tseng A, Zhao TC, Robbins J, Padbury JF, Tseng YT. Temporally controlled overexpression of cardiac-specific PI3Kα induces enhanced myocardial contractility—a new transgenic model. Am J Physiol Heart Circ Physiol 295: H1690–H1694, 2008. First published August 22, 2008; doi:10.1152/ajpheart.00531.2008.—The phosphatidylinositol 3-kinase (PI3K) signaling pathway regulates multiple cellular processes including cell survival/apoptosis and growth. In the cardiac context, PI3Kα plays important roles in cardiac growth. We have shown that cardiac PI3K activity is highly regulated during development, with the highest levels found during the fetal-neonatal transition period and the lowest levels in the adult. There is a close relationship between cardiomyocyte proliferation and cardiac PI3K activity. In adult transgenic mice, however, the prolonged constitutive activation of PI3Kα in the heart results in hypertrophy. To develop a strategy to allow temporally controlled overexpression of cardiac PI3Kα, we engineered a tetracycline (tet) transactivator tet-off controlled transgenic mouse line with a conditional overexpression of a cardiac-specific fusion protein of the SH2 domain of p85 and p110α. Cardiac PI3K activity and Akt phosphorylation were significantly increased in adult mice after transgene induction following the removal of doxycycline for 2 wk. The heart weight-to-body weight ratio was not changed, and there were no signs of cardiomyopathy. The overexpression of PI3Kα resulted in increased left ventricular (LV) developed pressure and the maximal and minimal positive values of the first derivative of LV pressure, but not heart rate, as assessed in Langendorff hearts. Mice overexpressing PI3Kα also had increases in the levels of Ca2+-regulating proteins, including the L-type Ca2+ channels, ryanodine receptors, and sarco(endoplasmic reticulum Ca2+-ATPase 2a. Thus the temporally controlled overexpression of cardiac PI3Kα does not induce hypertrophy or cardiomyopathy but results in increased contractility, probably via the increased expression of multiple Ca2+-regulating proteins. These distinct phenotypes suggest a fundamental difference between transgenic mice with temporal or prolonged activation of cardiac PI3Kα.

Akt; Ca2+-regulating proteins; Langendorff; tet-off; phosphatidylinositol 3-kinase

THE CLASS I PHOSPHATIDYLINOSITOL 3-KINASES (PI3Ks) are lipid kinases regulating many important and diverse cellular processes including cell proliferation, differentiation, survival, adhesion, and motility. The mammalian class IA PI3Ks are heterodimers of a 110 KDa catalytic subunit (p110α, p110β, or p110δ) and a regulatory subunit of 85 or 55 KDa (p85/p55), whereas the class IB PI3K (PI3Kγ) is composed of a p110γ catalytic subunit and a p101 regulatory subunit (21). These kinases phosphorylate the 3’-OH on the inositol ring of phosphatidylinositol 4,5-bisphosphate, the main substrate in vivo, and convert it to phosphatidylinositol 3,4,5-trisphosphate (PIP3). In most systems, the basal level of PIP3 in cells is low and only rises sharply upon cellular stimulation, which in turn activates downstream signaling proteins including Akt and p70S6K. Past results suggest the class IA PI3Ks are activated by receptor tyrosine kinase pathways, whereas the class IB PI3K (PI3Kγ) is coupled to G protein-coupled receptors (GPCRs). This has been studied most extensively in leukocytes (5, 14). In cardiomyocytes prepared from mutant mice with deletion of cardiac PI3Kγ, there is an increase in basal cAMP (2). The notion that classes IA and IB PI3Ks can only be activated by receptor tyrosine kinase pathways and GPCRs, respectively, is not definite since PI3Kβ is sensitive to Gβγ (9, 12). In addition, we have recently shown that PI3Kα is functionally linked to β-adrenergic receptor stimulation in vivo (22).

Among the class IA PI3Ks, the PI3Kα isoform has been shown to play important roles in the regulation of cell growth. Mutations in PIK3CA, the gene coding for PI3Kα, are associated with oncogenic transformation (7). In the mutant-transformed cells, there is a constitutive phosphorylation of Akt, p70S6K, and 4E-binding protein 1 (7). Moreover, a common phenotype seen in transgenic mice with an overexpression of constitutively active PI3Kα is cardiac hypertrophy (16). Several Akt transgenic mouse models have been reported. These include the cardiac-specific overexpression of constitutively active Akt, E40K (1) and T308D/S473D (17), membrane-targeted Akt (myr-Akt) (10), and nuclear-targeted Akt (19). Except for the nuclear-targeted model, the overexpression of Akt results in cardiac hypertrophy. On the other hand, these models display distinct phenotypes. For example, an increase in contractility is observed in Akt (E40K) but not in myr-Akt mice (8). Extensive interstitial fibrosis was found in animals overexpressing constitutively active Akt (T308D/S473/D) (17). In an inducible model, short-term (2 wk) myr-Akt overexpression induced reversible hypertrophy without causing interstitial fibrosis (18). In contrast, the sustained overexpression (6 wk) of the same transgene induced extensive hypertrophy, interstitial fibrosis, and contractile dysfunction (18).

Indirect evidence suggests that PI3Kα induces physiological hypertrophy, as the cardiac expression of a dominant negative PI3Kα blunts exercise-induced hypertrophy but not pressure overload-induced hypertrophy (11). This transgenic model, however, cannot be used to examine the immediate short-term effects in the heart because the expression of transgene is

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manifest throughout fetal, postnatal, and adult life. We have shown that the expression of cardiac PI3Kα is tightly regulated with the highest level seen during the fetal-neonatal transition period and low levels in the adult (20). The goal of the current study was to explore the effects of the temporal activation of PI3Kα in the heart. To achieve this, we engineered a conditional mouse line, which allows the temporally controlled overexpression of PI3Kα. Using this inducible system, we were able to define the immediate short-term effects of PI3Kα activation in the heart before the induction of cardiac hypertrophy.

MATERIALS AND METHODS

DNA constructs. The tetracycline (tet)-controlled (tet-off) conditional transgenic mouse line required two separate transgenic mouse lines: the effector line expressing tet transactivator (tTA)-virion protein 16 (13) and the responder line expressing the target gene iSH2-p110α (3). Both constructs are under the control of the mouse α-myosin heavy chain (MHC) promoter, a cardiomyocyte-specific promoter. The empty tet-responsive α-MHC promoter construct had three GATA sites and two thyroid response elements removed, leaving other cis-acting elements critical for cardiac-specific expression left intact (13). There are seven repeats of the tetO sequences adjacent to the TATA box, and the human growth hormone polyadenylation signal follows the unique cloning site. This construct has been shown to be functional in prior studies (13). A pCMV-6-iSH2-p110α-MT vector containing the target gene (a generous gift from Drs. Seigo Izumo and Thomas Franke) was digested with SalI (5′ end) and BamHI (3′ end) to release the transgene. Because there is an internal SalI site within the transgene sequence, we performed partial digest of the vector and were able to isolate the full-length 4.4 kb iSH2-p110α transgene fragment with the internal SalI site intact. To insert the transgene into the tet-responsive α-MHC promoter containing vector, we first opened up the vector in the multiple cloning region with SalI (5′ end) and HindIII (3′ end) double digestion. A linker DNA with a BamHI overhang was then ligated to the HindIII end, thus creating matching ligation sites (SalI-BamHI) within the tet-responsive α-MHC promoter containing vector for the iSH2-p110α insert (Fig. 1). After ligation, the final construct was purified, sequenced, and verified by restriction enzyme digestion and sequencing.

Transgenic mice. All animal handling and procedures, adhering to the APS Guiding Principles in the Care and Use of Animals, were approved by the Institutional Animal Care and Use Committee. The responder transgene construct was delivered by pronuclear microinjection into FVB/n mouse eggs (the transgenic mouse laboratory at Children’s Hospital Research Foundation of Cincinnati). Oocytes were then transferred to pseudopregnant mice. The effector transgenic mouse line has been well established and does not induce cardiomyopathy (13). The crossing of the empty tet-responsive promoter into FVB/n mouse eggs (the transgenic mouse laboratory at Children’s Hospital Research Foundation of Cincinnati) yielded one male and two female mice carrying the iSH2-p110α transgene. Breeding of each of the three responder transgenic mice (PI3Kα overexpressing) with the effector transgenic mice (tTA overexpressing) resulted in some offspring with both transgene types of TTA and PI3Kα transgenes as well as an essential myosin light chain as an internal control (ELC1v) by PCR. The following primer pairs were used: ELC1v, forward, 5′-AGCCTATTAGAGCTGTTAATGGAG-GTC-3′, and reverse, 5′-GTCGTAATATGCGCGCACTACTATC-3′; PI3Kα, forward, 5′-GATCTTTTACGGCGCGCATATA-3′, and reverse, 5′-CCAAGAGCACAACCTCAATG-3′; and ELC1v, forward, 5′-ATCGAGTTCAACCTGAAACAGATTG-3′, and reverse, 5′-CCAGACCGAGACCTCTG-3′. Double transgenic mice were maintained with doxycycline (Dox; 0.5 mg/ml) water to repress transgene expression. This relatively low dosing regimen of Dox has been used in the inducible Akt transgenic model with no cardiovascular side effects (18). Removal of Dox for 2 to 3 wk resulted in the overexpression of cardiac-specific PI3Kα.

PI3K activity and Western blotting. Cardiac tissue lysates were prepared as described (22). PI3K activity was determined by immunoprecipitation (IP) in vitro lipid kinase assay. An antibody (1 μg) specific for phospho-tyrosine (pY; clone 4G10; Millipore) or PI3Kα (sc-7174; Santa Cruz Biotechnology) was added to 20 μl of packed protein G-spheres (7–0618; GE Healthcare) in 0.5 ml buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 10 mM Na3PO4, 2 mM Na2VO4, 100 mM NaF, 1% (vol/vol) nonyl phenoxylpolyethoxylethanol-40, 1 μM pepstatin A, 1 μM leupeptin, 1 μM aprotinin, and 1 μM PMSF and incubated with rotation at room temperature for 1 h. After centrifugation (14,000 g) at room temperature for 1 min, the pellet was washed three times in IP buffer. Excess buffer was removed, and cardiac tissue lysates (0.5 mg) were added to the beads and rotated overnight at 4°C before subjected to lipid kinase assay as described (22). The expression of Akt was assessed with Western blotting using phospho-specific antibodies against phospho-tyrosine (Ser-473 and Thr-380; Cell Signaling Technology) and an antibody against total Akt as described (20). The expression of several Ca2+-regulating proteins, including sarco(endo)plasmic reticulum Ca2+-ATPase 2a (SERCA2a; Biomol SA-209), ryanodine receptor (RyR) type 2 (SC-8170; Santa Cruz Biotechnology), and the L-type voltage-gated Ca2+ channel (Ca1.2; ACC-003; Alomone Labs), was also examined.

Histological analysis. Mouse hearts were collected after removing Dox for 2 wk. After briefly perfused on a Langendorff apparatus, the hearts were quickly embedded in embedding carrier filled with Triangle Biomedical Sciences tissue freezing medium (Fisher Scientific). Hearts were then dipped into isopentane, which had been cooled with liquid N2 to form a thick slurry, frozen for 20 s, and stored at −80°C. Cross sections (15 μm), obtained using a cryotome, were fixed in 3.65% (vol/vol) buffered formaldehyde for 15 min at room temperature and stained with hematoxylin and eosin or Masson trichrome. Sections were visualized with a Nikon ECLIPSE 80i microscope equipped with a SPOT Insight camera.

Measurement of myocardial function. Baseline ventricular function in adult (3 to 4 mo old) mice was measured by using the Langendorff sistemically isolated perfused mouse heart preparation (a nonworking heart preparation) as previously described (23). We recorded left ventricular (LV) developed pressure (LVDP), heart rate, coronary flow, and the maximal and minimal positive values of the first derivative of LV pressure (LVEDP/dP/dtmax and LVPd/dP/dtmin, respectively).

Statistical analysis. Results are presented as means ± SE. The difference between PI3Kα overexpressing (Dox-off for 2 to 3 wk) and littermate control (Dox-on) groups was analyzed using Student’s t-test. A probability of P < 0.05 was considered to represent a significant difference.

RESULTS AND DISCUSSION

Establishing the conditional cardiac-specific PI3Kα transgenic mouse line. For generation of the responder mouse line, we identified one male and two female mice carrying the iSH2-p110α transgene. Breeding of each of the three responder transgenic mice (PI3Kα overexpressing) with the effector transgenic mice (tTA overexpressing) resulted in some offspring with both

Fig. 1. Engineering of a responder transgene construct. A schematic of the tetracycline (tet)-responsive α-myosin heavy chain (MHC) promoter construct expressing iSH2-p110α is shown. The full-length α-MHC promoter has 3 GATA sites and 2 thyroid response elements deleted with other cardiac-specific cis-acting elements intact. There are 7 repeats of the tet operator (tetO) sequences adjacent to the TATA box. The human growth hormone (hGH) polyadenylation (polyA) signal was immediately downstream of iSH2-p110α.
genes. The final double transgenic mice were confirmed by PCR and genotyping. Of the three double transgenic lines generated, lines 1 and 3 showed robust transgene expression upon the removal of Dox and, except in Fig. 2A, line 1 mice were used exclusively for all subsequent experiments. All mice were maintained with Dox water throughout development to suppress the overexpression of cardiac-specific PI3Kα. Adult mice (2–6 mo old, depending on the scope of the experiment) were then switched to regular drinking water for 2 to 3 wk to initiate transgene overexpression (PI3Kα group). Littermates of the same gender continued to receive Dox drinking water (control group). After 2 wk of transgenic expression, there was a significant increase in cardiac PI3K activity in the PI3Kα group compared with control littermates (Fig. 2A). These assays were performed using an antibody against either pY or PI3K activity in the IP step. This increase in PI3K activity was cardiac specific since assays performed on hepatic and pulmonary tissue lysates did not show any differences between the control and PI3Kα littermates (data not shown). We next measured Akt phosphorylation, an indicator of signaling activity downstream of PI3K. As expected, after Dox was removed for 2 wk, there was a significant increase in Akt phosphorylation in PI3Kα-overexpressing mice compared with control littermates (Fig. 2B). These data indicate that the conditional transgenic mouse displays the appropriate temporal activation via Dox treatment and that elevated levels of cardiac-specific PI3Kα in the adult, which normally has very low measurable activity (20), can be achieved.

**Temporally controlled overexpression of cardiac-specific PI3Kα does not induce hypertrophy or cardiomyopathic changes.** To examine whether the temporal cardiac-specific overexpression of PI3Kα induces hypertrophy or cardiomyopathic changes, adult male (3 to 4 mo old) double transgenic mice were maintained with regular drinking water for 2 wk. The microscopic examination of cross sections revealed no evidence of necrosis or myocyte disarray in PI3Kα mice compared with littermate controls (Fig. 3A, top). Masson trichrome stain also showed no difference in interstitial fibrosis between these animals (Fig. 3A, bottom). To further examine whether the temporal overexpression of PI3Kα resulted in cardiac hypertrophy, mice aged 2, 4, or 6 mo old were maintained with or without Dox water for 2 wk. This overexpression regimen did not induce cardiac hypertrophy, since the ratio of heart (ventricle) weight (milligrams) to body weight (grams) was not significantly different between control and PI3Kα littermates (Fig. 3B). In the inducible (tet-off) myr-Akt transgenic model, the removal of Dox for 2 wk induces Akt activation as well as cardiac hypertrophy, which can be reversed to normal size 2 wk after restarting Dox treatment (18). When the overexpression is extended to 6 wk, however, the resulting hypertrophy became only partially reversible, and there were extensive interstitial fibrosis and contractile dysfunction (18). By comparison, we show that 2 wk overexpression of PI3Kα does not induce hypertrophy. It appears that although PI3K and Akt belong to the same signaling pathway, transgenic animals overexpressing Akt may have a more direct impact on cardiac growth than do transgenic animals overexpressing PI3Kα.

The phenotypic characteristics of the transgenic mice expressing constitutively active PI3Kα include cardiac hypertrophy (16). In essence, both the constitutively active transgenic model and our model result in the induction of the same transgene: iSH2-p110α. The main difference is that our model allows the temporally controlled expression at select developmental stages. We chose to induce cardiac PI3Kα only in the adult and only for 2 wk, thus
on a Langendorff apparatus. We found that the temporal overexpression (after 2 wk transgene induction) mice were subjected to analysis and examination whether the temporal overexpression of cardiac PI3K induces alterations in ventricular function, control and PI3K activation.

In contrast, deletion of PI3K without affecting systolic function under resting conditions (16). Our data suggest there are fundamental phenotypic differences between these distinct transgenic models (constitutive transgenic expression vs. temporally controlled expression).

Two major distinct but related branches of cellular effect can be induced by the class I PI3K: one on cell growth and one on cell cycle (review see Ref. 4). One possible mechanism underlying the different phenotypes between the temporal and constitutively active overexpression of cardiac PI3K is that constitutively active activation may push the pathway toward favoring cell growth effect, leading eventually to cardiac hypertrophy. Hence the increase in contractility presents an early phenotype following the temporal activation of cardiac PI3K.

Ca²⁺ turnover is a well-recognized mechanism for the regulation of contractility, and the dysregulation of Ca²⁺ turnover often leads to heart failure (review Refs. 6 and 15). The regulation of Ca²⁺ turnover has been speculated as a possible mechanism underlying the change in contractility seen in transgenic mice with the overexpression of constitutively ac-

effectively eliminating the chance of developing hypertrophy. We have noted no sign of hypertrophy in our transgenic mice if the induction periods were less than 2 mo. Hence the new conditional transgenic model can provide a unique window of opportunity to study the immediate phenotypic changes in vivo following the activation of cardiac PI3K but before hypertrophy is present.

Temporal overexpression of cardiac-specific PI3Kα increases contractility. It has been shown that the constitutive overexpression of cardiac PI3Kα results in hypertrophy without affecting systolic function under resting conditions (16). In contrast, deletion of PI3Kα results in hypercontractility (4). To examine whether the temporal overexpression of cardiac PI3Kα induces alterations in ventricular function, control and PI3Kα (after 2 wk transgene induction) mice were subjected to analysis on a Langendorff apparatus. We found that the temporal overexpression of cardiac-specific PI3Kα induced significant increases in LVDP, LVDp/dtmax, and LVDp/dtmin without altering heart rate and coronary flow (Table 1). Because heart rates were similar between the groups, these results suggest that there is enhanced contractility in the animals with temporal cardiac-specific overexpression of PI3Kα. This observation is reminiscent to that seen in transgenic mice overexpressing a constitutively active Akt (1). In contrast, the prolonged constitutive transgenically induced activation of cardiac PI3Kα is not associated with changes in LV dimensions or systolic function as assessed by M-mode echocardiography (16).

![Image](https://example.com/image.png)

**Fig. 3.** Absence of cardiomyopathy and hypertrophy following temporal (2 wk) overexpression of cardiac-specific PI3Kα. A: histological analysis of heart sections from 3-mo-old male PI3Kα and control litterate. A, top: hematoxylin and eosin (H & E) staining. A, bottom: Masson trichrome staining. Bars represent 10 μm. B: heart weight-to-body weight ratio in PI3Kα and control littersmates. Mice were 2 (n = 8), 4 (n = 10), or 6 (n = 8) mo old before initiation of cardiac PI3Kα overexpression.

| Table 1. Basal myocardial function assessed by Langendorff perfusion system |
|---------------------------------------------|---------------|-----------------|
|                                             | Control       | PI3Kα           | P Value |
| LV developed pressure, mmHg                | 125.4±4.5     | 148.2±6.5*      | 0.022   |
| LVDp/dtmax                                 | 3.066±125     | 3.619±108*      | 0.008   |
| LVDp/dtmin                                 | 3.000±142     | 3.500±105*      | 0.018   |
| Heart rate, beats/min                      | 335±25        | 323±20          | 0.716   |
| Coronary flow rate, ml/min                 | 3.72±0.15     | 3.27±0.30       | 0.234   |

Values are means ± SE; n, no. of littersmates/group. Male double transgenic littersmates (3 to 4 mo old) were maintained with doxycycline (control) or regular water [phosphatidylinositol 3-kinase (PI3Kα)] for 2 wk before measurement. *P < 0.05. LVDp/dtmax and LVDp/dtmin, the maximal and minimal positive values of the first derivative of left ventricular (LV) pressure, respectively.

![Image](https://example.com/image.png)

**Fig. 4.** Temporal overexpression of cardiac-specific PI3Kα induces upregulation of Ca²⁺-regulating proteins. Three-month-old male double transgenic littersmates were maintained with Dox water (control; n = 4) or regular drinking water (PI3Kα; n = 3) for 2 wk. Western blotting was performed using antibodies against L-type voltage-gated Ca²⁺ channel (Ca,v,1.2), ryanodine receptor (RyR) type 2, and sarco/endo/plasmatic reticulum Ca²⁺-ATPase 2a (SERCA2a; top). Actin levels were used as the loading control. Bottom: results of densitometric measurement of each antibody from 3 separate experiments. *P < 0.05 vs. control.
tive Akt (nonnuclear targeted), but this has not been confirmed (1). To explore the mechanism(s) underlying the increase in contractility following the temporally controlled overexpression of PI3Kα, we examined the expression of several Ca\(^{2+}\)-regulating proteins that are critical for Ca\(^{2+}\) homeostasis and myocardial function/adaptation (6, 15). We found that there were significant increases in the levels of RyR, Ca\(^{1,2}\), and SERCA2α, following the temporally controlled overexpression of cardiac-specific PI3Kα (Fig. 4). In cardiomyocytes, Ca\(^{1,2}\) is critical for Ca\(^{2+}\) influx and, along with RyR, is responsible for Ca\(^{2+}\)-induced Ca\(^{2+}\) release during the systolic period. Ca\(^{2+}\) ions are actively transported back into the sarcoplasmic reticulum by SERCA2α or pumped out by the Na\(^{+}\)-Ca\(^{2+}\) exchanger (NCX) during the diastolic (6, 15). The upregulation of these proteins should favor improved contraction and relaxation in cardiomyocytes and may represent a mechanism underlying the increase in contractility following the temporal activation of cardiac PI3Kα. These data set the tone for the future study of other Ca\(^{2+}\)-regulating proteins, e.g., NCX, phospholamban (PLB), calsequestrin (CSQ), and calreticulin. It is also crucial to examine the phosphorylation status of some of these proteins including CSQ, RyR, and PLB. To our knowledge, nevertheless, our data represent the first demonstration of the PI3Kα activation-induced induction of Ca\(^{2+}\)-regulating proteins in cardiomyocytes. It will be interesting to investigate the downstream factors involved in the transduction of the PI3Kα cascade.

In summary, we have engineered a new conditional transgenic mouse line that allows the temporally-controlled overexpression of cardiac-specific PI3Kα. The temporal activation of cardiac PI3Kα in these animals does not cause cardiomyopathy or hypertrophy but induces an increase in contractility and the upregulation of Ca\(^{2+}\)-regulating proteins. These changes may represent the early phenotypic characteristics following the activation of cardiac PI3Kα.

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