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Akt Promotes Increased Cardiomyocyte Cycling and Expansion of the Cardiomyocyte Progenitor Cell Population

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Abstract—Activation of Akt is associated with enhanced cell cycling and cellular proliferation in nonmyocytes, but this effect of nuclear Akt accumulation has not been explored in the context of the myocardium. Cardiac-specific expression of nuclear-targeted Akt (Akt/nuc) in transgensics prolongs postnatal cell cycling as evidenced by increased numbers of Ki67+ cardiomyocytes at 2 to 3 weeks after birth. Similarly, nuclear-targeting of Akt promotes expansion of the presumptive cardiac progenitor cell population as assessed by immunolabeling for c-kit in combination with myocyte-specific markers Nkx 2.5 or MEF 2C. Increases in pro-proliferative cytokines, including tumor-necrosis superfamily 8, interleukin-17c, and hepatocyte growth factor, were found in nuclear-targeted Akt myocardial samples. Concurrent signaling mediated by paracrine factors downstream of Akt/nuc expression may be responsible for phenotypic effects of nuclear-targeted Akt in the myocardium, including enhanced cell proliferation and expansion of the stem cell population. (Circ Res. 2006;99:381-388.)

Key Words: PKB/Akt cardiac stem cell proliferation cytokines postnatal growth

Postnatal growth of the myocardium results from combined actions of cellular proliferation and hypertrophy. Both processes are downregulated as the myocardium achieves structural and functional characteristics of the adult heart. Mechanisms determining the ultimate size and cellular density of the normal adult heart remain obscure, but it is clear that signaling to regulate growth and proliferation influences the outcome. Specifically, overexpression of insulin-like growth factor 1 (IGF-1) in the postnatal heart by cardiac-specific transgenesis results in hyperplastic growth without myocyte hypertrophy.1 In contrast, postnatal activation of various hypertrophic signaling cascades provokes cellular hypertrophy and substantial increases in cardiac mass.2,3

A nodal signaling kinase linked to both hyperplasia and hypertrophy of cardiomyocytes is Akt, a serine/threonine kinase with potent antiapoptotic action in vitro and in vivo.4 Akt is activated by multiple cardioprotective stimuli under physiological conditions, but Akt activation in the myocardium of genetically engineered mice has predominantly been associated with cardiomyopathic hypertrophy and transition to heart failure.5–7 These apparently paradoxical findings were reconciled by our recent reports that nuclear-targeting of Akt kinase promotes expansion of the presumptive cardiac progenitor cell population13,14 as assessed by immunolabeling for c-kit in combination with myocyte-specific markers Nkx 2.5 or MEF 2C. Increases in pro-proliferative cytokines, including tumor-necrosis superfamily 8, interleukin-17c, and hepatocyte growth factor, were found in nuclear-targeted Akt myocardial samples. Concurrent signaling mediated by paracrine factors downstream of Akt/nuc expression may be responsible for phenotypic effects of nuclear-targeted Akt in the myocardium, including enhanced cell proliferation and expansion of the stem cell population. (Circ Res. 2006;99:381-388.)

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Akt is associated with the promotion of cellular proliferation in many noncardiac cell types including oncogenic transformation.10–12 The commonality of cardiomyocyte hyperplasia without hypertrophy in transgenic lines expressing IGF-1 or nuclear-targeted Akt suggests that Akt kinase is responsible for the promotion of cellular proliferation in the postnatal heart. Mitotic replication of mature myocytes within the adult myocardium remains controversial, but the recent demonstration of progenitor cells within the myocardium13,14 raises the possibility that potentiation of this resident cardiac-committed stem cell pool could contribute to myocardial cellular proliferation driven by Akt.

The role of nuclear Akt kinase accumulation in the promotion of cellular proliferation at the myocytes and progenitor cell level was determined by comparing normal nontransgenic mice to those created with cardiac-restricted expression of nuclear-targeted Akt. Our results demonstrate that nuclear accumulation of Akt kinase expands the population of cycling myocytes in the postnatal heart as well as the number of progenitor cells expressing markers of myocyte lineage commitment in the adult heart. These distinctive effects raise the possibility of Akt kinase acting as a facilitator of cellular proliferation for cardiac progenitor cells and young committed myocytes in the heart.

Materials and Methods

Construction of Nuclear-Targeted Akt Transgensics

The creation and characterization of the nuclear-targeted Akt line has been previously described.8 Mice used in this study were homozygous for alleles carrying the transgene.

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Immunohistochemistry and Confocal Microscopy

Formalin-fixed, paraffin-embedded hearts were sectioned at 3 μm, dried, then deparaffinized in xylene and rehydrated through a series of graded alcohols into distilled water. Antigen retrieval was performed in 10 mmol/L citrate, pH 6.0, for 15 minutes at 50% power using an 1100-W microwave oven. Samples were cooled to room temperature, washed in several changes of purified water, and blocked for endogenous peroxidase activity using 3% H2O2 in 1× TN buffer (NaCl 150 mmol/L, Tris 100 mmol/L, pH 7.5) for 20 minutes at room temperature. Slides were washed in several changes of water and equilibrated in 1× TN buffer. Confocal micrographs were acquired using either a Bio-Rad Radiance or Molecular Dynamics 2010 confocal laser scanning microscope with ImageSpace software.

Antibodies and Immunolabeling

Immunostaining and morphometric analyses were based on protocols as previously described.13 Detailed information is provided in the online supplemental section, available at http://circres.ahajournals.org.

Real-Time PCR Gene Array

Total RNA was isolated from 6-week-old male FVB/N and Akt/nuc mouse hearts using Trizol (Invitrogen, Carlsbad, Calif). Detailed information is provided in the online supplemental section.

Data Analyses

All data are expressed as mean± SEM. Differences in quantitative variables were examined by the Student t test or 1-way ANOVA followed by Tukey post-hoc test for multiple comparisons. P<0.05 was considered significant. All statistical analyses were performed using Microsoft Excel software.

Results

c-Kit+ Cells Are Enriched in the Neonatal Myocardium

Analyses of postnatal c-kit expression demonstrate increased protein levels in neonatal myocardium that declines rapidly within the first week after birth. Immunoblot analyses of lysates prepared from the hearts of mice at various ages after birth show a rapid decline in the level of c-kit protein occurring within the first week after birth (Figure 1A). Quantitation of immunoblot results shows a significant 3-fold decrease in c-kit protein level between postnatal day 2 and 30 (P<0.05; Figure 1B). The presence of c-kit+ cells in hearts from mice at 2 days after birth can be readily observed by confocal immunofluorescence microscopy, but comparably labeled cells are comparatively rare at 6 weeks after birth (Figure 1C, green in overlay).

Nuclear-Targeted Akt Increases Ki67+ Myocyte Number in the Heart

Nuclear-targeting of Akt produces myocardium with more numerous, but smaller myocytes.10 The enrichment of myocytes in Akt/nuc hearts correlates with an increase in the number of cells possessing both Ki67 (a marker of cells typically undergoing mitotic replication) as well as GATA4 (a myocyte-specific transcription factor). Sections of ventricular myocardium were triple-labeled for Ki67 and GATA4 as well as either nuclei or desmin (to reveal sarcomeric structure) for evaluation by confocal microscopy (Figure 2A through 2F). Consistent with the slowing of cellular proliferation in postnatal development, the number of Ki67+ nuclei decreased by 2.3-fold from 2 days to 2 weeks after birth and then dropped another 3-fold to represent only 3.6% of the nuclei at 3 weeks after birth (Figure 2C).

Although Ki67+ nuclear frequency in the myocardium of Akt/nuc mice was comparable to nontransgenics at 2 days and 2 weeks, a significantly higher proportion of Ki67+ nuclei persist in Akt/nuc mice at age 3 weeks. The Akt/nuc samples at age 3 weeks possess 2.4-fold more Ki67+ nuclei than age-matched nontransgenic samples, representing 8.6% of the nuclei. In comparison, the percentage of nuclei committed to the myocyte lineage as evidenced by expression of GATA4 was comparable between nontransgenic and Akt/nuc mice (Figure 2D). Although Akt/nuc transgenic hearts are hypercellular and possess a greater
number of myocytes, both nontransgenic and Akt/nuc nuclei possess a similar percentage of GATA4 nuclei (Figure 2D). This suggests a concomitant increase in the number of nonmyocytes in the Akt/nuc heart to maintain a constant percentage of myocytes in the myocardium.

Akt/nuc promoted the accumulation of cells expressing Ki67 (Figure 2C) that should be associated with the myocyte-committed cell population since expression of the Akt/nuc transgene is driven by the α-myosin heavy chain promoter. Consistent with this postulate, nuclei possessing both Ki67 and GATA4 are more frequent in Akt/nuc myocardium (Figure 2E).

Moreover, the subpopulation of myocyte-committed cells expressing Ki67 was calculated by comparing nuclei possessing both Ki67/GATA4 relative to the number of GATA4 nuclei (Figure 2F). In this population, Ki67/GATA4 nuclei in the myocardium of normal mice decreased by 3.8-fold from 2 days after birth and then dropped another 2-fold by the following week to 2% of the population. Comparing the frequency of Ki67/GATA4 nuclei between normal and Akt/nuc mice shows that, although similar levels were present at 2 days after birth, a significant 2.2-fold increase in the number of these Ki67/GATA4 nuclei was evident in Akt/nuc samples relative to the nontransgenic heart by 2 weeks. This differential enrichment of Ki67/GATA4 nuclei was maintained in the Akt/nuc myocardium at 3 weeks after birth. The levels of Ki67/GATA4 nuclei in nontransgenic and Akt/nuc samples remained unchanged from values observed at 3 weeks after birth through early adulthood at 6 weeks after birth (data not shown).

Figure 2. Differences in Ki67+ and GATA4+ cell populations during postnatal development of normal and Akt/nuc hearts. Representative immunolabeling from postnatal day 2 Akt/nuc myocardium sections (A, B) and quantitation (C-F) of Ki67 and GATA4+ nuclei in normal vs Akt/nuc myocardium as a function of postnatal development. Confocal scans show nuclear labeling for Ki67 (A1, B1), GATA4 (A2, B2), total nuclei (A3), sarcomeric desmin (B3), and merged images (A4, B4). Indicated nuclei show coincident labeling for both Ki67 and GATA4 (at arrows) in A and B. Merged images show labeling for Ki67 (green) and GATA4 (blue) with nuclei (A, red) or sarcomeres (B, red). Scale bar as indicated in bottom left for each micrograph represents 10 μm. C–F. Quantitation of cells in the ventricular myocardium for Ki67/total nuclei (C), GATA4/total nuclei (D), Ki67+ and GATA4+/total nuclei (E), and Ki67+ and GATA4÷GATA4 nuclei (F). At least 3 views of ~100 cells per view were analyzed in at least 3 animals per group, totaling 900 to 1000 cells per sample group. Statistical significance values as indicated above bars, NS indicates not significant.

Nuclear-Targeted Akt Increases the Number of Myocyte-Committed Progenitor Cells in Heart

In addition to expansion of the cycling cell population (Figure 2), the Akt/nuc myocardium also exhibited increased frequencies of cells expressing the stem cell marker c-kit as well as cells expressing c-kit in combination with Nkx 2.5 or MEF2C. Expression of transcription factors MEF2C or Nkx-2.5 identifies cells committed to the myocyte lineage. The number of c-kit+ cells in Akt/nuc samples remained unchanged from values observed at 3 weeks after birth through early adulthood at 6 weeks after birth (data not shown).
denced by coincident Ki67+ labeling (Figure 3B), with these cycling stem cells more prevalent in the apex than the base or mid-region of the myocardium. Statistically significant increases in cycling stem cells were found in all 3 regions of the myocardium examined for Akt/nuc hearts relative to nontransgenic control samples (Figure 3B). Significant increases of 1.9- or 4.1-fold were also found for c-kit+/H11001/Nkx2.5+ cells in the ventricular midwall or atria (respectively) for Akt/nuc hearts relative to nontransgenic samples (Figure 3C). These values correlate with demonstration of a significant 2.9-fold increase in c-kit+/H11001/MEF2C+ cells in the midwall region of Akt/nuc samples relative to nontransgenic controls (Figure 3D and 3E).

Nuclear Akt Is Expressed at an Early Stage of Progenitor Cell Commitment

The coincident presence of c-kit and the Akt/nuc transgene was assessed in myocardium sections by confocal microscopy. Transgene expression was identified by immunolabeling for the expression of the myc-tag on the Akt/nuc construct. Examples of c-kit+/H11001/myc+ cells were sparsely detected throughout the myocardium in both the ventricular wall and atria and occasionally occurred in small clusters (Figure 4). Cells expressing the myc-tag are absent from nontransgenic samples, indicating specificity for the Akt/nuc transgene (data not shown). Mature myocytes express the myc-tag of Akt/nuc in their nuclei (Figure 4, arrowheads), together with rare c-kit+/myc-tag+ cells that comprise 2.3±2.1% of the total c-kit+ population (out of a total of 1783 c-kit+ cells examined in 10 separate mouse hearts, with the large standard deviation in the sampling reflecting variability between the 256 histological sections in the analysis). C-kit+ cells show the presence of myc+ as well as myc− nuclei, indicating that some progenitor cells express the α-mysin-heavy chain driven Akt/nuc transgene at an early stage of commitment to the myocyte lineage (Figure 4A). Supportive evidence for commitment of these cells to the myocyte lineage can be seen in occasional coincident labeling for the myc-tag as well as infrequently observed sarcomeric actin (Figure 4C).

Microarray Analysis Reveals Similarities in Cytokine mRNA Transcript Levels Between Akt/nuc and Neonatal Hearts

Recent studies implicate paracrine cytokine factors in the beneficial effects promoted by Akt activity in vivo. A comparison of cytokine mRNA transcript expression between Akt/nuc transgenic heart samples versus age and gender-matched nontransgenic controls (Figure 5, solid bars) reveals significant increases of 2-fold or more in transcript families encoding genes related to tumor necrosis factor (TNF), interleukins (ILs), and bone morphogenetic proteins. The
persistence of progenitor cells and proliferating myocyte precursors (Figures 2 and 3) in the Akt/nuc samples reminiscent of postnatal myocardium (Figures 1 and 2) prompted comparison between the cytokine expression profile of Akt/nuc transgenic hearts versus neonatal myocardium at postnatal day 3. Quantitative real-time PCR array analyses of myocardial samples show striking similarities in the transcriptional profile between the Akt/nuc and neonatal heart mRNA changes relative to adult nontransgenic control samples (Figure 5A, solid versus cross-hatched bars). Across the panel of 87 cytokine mRNAs screened in the array, 43 were induced or repressed 2-fold or more in heart samples prepared from postnatal day 3 compared with 6-week-old adults. Of these 43 mRNAs altered 2-fold or more in neonatal heart samples, 17 were similarly induced or repressed in Akt/nuc myocardium. This high level concordance, equivalent to 39.5% shared changes between neonatal versus Akt/nuc samples represents 19.5% of all the genes represented in the array. In contrast, concordance was absent from comparisons between the Akt/nuc transcriptional profile and that of another transgenic line created with cardiac-specific overexpression of a different serine/threonine kinase, Pim-1 (data not shown). Thus, Akt/nuc influences the transcriptional reprogramming of cytokine expression in the myocardium, which shares similarities with that found in the neonatal heart. Two genes that show marked increases in expression in both Akt/nuc and neonatal hearts are TNF superfamily 8 (Tnfsf8) and IL-17e. Increased cytokine protein expression for Tnfsf8, IL-17e, and hepatocyte growth factor (HGF) was demonstrated in myocardial lysates from adult Akt/nuc versus nontransgenic control mice (Figure 5B). Significantly higher protein levels for Tnfsf8 (6.9±0.5, *P<0.001) and IL-17e
(3.8±1.1, \(P=0.03\)) in Akt/nuc samples relative to nontransgenic controls corroborate array results (Figure 5A). In addition, the expression of HGF implicated in enhanced stem cell activity in vivo\(^9\) was increased (3.5±0.88, \(P=0.02\)) in myocardial lysates obtained from Akt/nuc relative to nontransgenic controls (Figure 5B, bottom).

**Discussion**

Findings of this study extend the multifaceted actions of Akt in the myocardium to include enhancement of cellular proliferation and expansion of the cardiac progenitor population in postnatal development. These novel observations build on a myriad of published observations regarding cardiac-related Akt actions including participation in IGF-mediated stimulation,\(^20–23\) with connections to hypertrophic,\(^24\) adrenergic,\(^24–26\) and antiapoptotic,\(^27–28\) signaling as well as effects on contractility,\(^29\) transcriptional reprogramming,\(^30\) and ultimately remodeling.\(^31,32\) Multiple similarities in cardiac phenotype between transgenic mice expressing either cardiac specific IGF-1\(^20,22\) or nuclear-targeted Akt\(^8,9\) indicate that focusing kinase activity within the nuclear compartment is essential to achieve cardioprotection without promoting maladaptive remodeling typical of other mutant Akt constructs. These beneficial effects are likely attributable to potentiation of cardiomyocyte precursor cell proliferation and survival.

Postnatal growth of the murine heart is characterized by a rapid proliferative phase through 4 days after birth that subsequently transitions into predominantly hypertrophic growth.\(^33\) This temporal shift is consistent with diminution of expression throughout the first week of postnatal life for c-kit (Figure 1) as well as Ki67.\(^34\) The coincidence of Ki67 and GATA transcription factor (Figure 2) indicates that early postnatal life is characterized by increased myocyte proliferation that declines with aging and that nuclear accumulation of Akt kinase promotes and extends the proliferative capacity of myocyte-lineage cells in the postnatal as well as adult myocardium. The increased number of smaller myocytes in the nuclear-targeted Akt transgenic heart is reminiscent of a young postnatal myocardium, opening the possibility that aging processes in the myocardium typified by the loss of Akt and telomerase and an increasing prevalence of cellular senescence could be antagonized by manipulation of Akt kinase activity.\(^35\)

Proliferative aspects of Akt action in the myocardium have been largely ignored because of a legacy of prevailing dogma that cardiomyocytes do not divide, although recent discoveries in adult myocytes\(^36\) and progenitor cells\(^13\) are changing this paradigm. As might be expected for a protein known to be a central player in oncogenic transformation, Akt impacts directly on cell cycle regulation by alteration of nuclear versus cytoplasmic distribution of proteins linked to cell cycle regulation, including FOXO family members, Mdm2, and p27\(^{Kip1}\).\(^37\) Among the plethora of inductive stimuli for Akt there are upstream connections to stem cell factor and c-kit receptor activation\(^38–40\) relevant for promoting stem cell proliferation. Involvement of Akt signaling in stem cell growth has already been documented in neural tissue,\(^31\) embryonic germ cells,\(^42\) endothelium,\(^43\) and erythroid progenitors.\(^43,44\) It is reasonable to speculate that expression of the nuclear-targeted Akt transgene in cardiac progenitor cells (Figure 4) contributes to expansion of the progenitor cell pool and myocyte number by promoting the proliferation of early committed cells expressing the \(\alpha\)-myosin heavy chain driven transgene. In addition, Akt has consistently been identified at the hub of protective signaling to prevent cardiomyocyte death, and nuclear targeting of Akt in the myocardium provides a powerful inhibition of apoptotic cell death without promoting maladaptive remodeling that is typical of other mutant Akt constructs.\(^4\) Thus, another mechanism for nuclear-targeted Akt to expand the cardiac progenitor pool and increase myocyte cell numbers is the inhibition of apoptotic cell death that normally occurs during cardiac growth and development. Interestingly, results of preliminary analyses show that the number of cycling myocytes (c-kit\(^+/\)/GATA4\(^+\)) in Akt-null mice does not differ significantly from nontransgenic counterparts at 2 weeks postnatal age. On the contrary, there is a trend (statistically insignificant) toward increased overall cell cycling (Ki67/total nuclei), progenitor cycling (Ki67/c-kit\(^+\)), and in the cycling myocyte (Ki67/GATA4\(^+\)) populations (data not shown). These results suggest that a loss of Akt activation in the myocardium pathways may lead to a change in the cycling cells population, although not a decrease as might be predicted from our Akt/nuc transgenic studies. Instead, a compensatory mechanism appears to exist that we cannot address within the scope of our present study. We suspect a maturational delay in the Akt-null animals, as was described in the original article describing this line.\(^45\) Because Akt-null animals were noticeably smaller than their heterozygous and wild-type littermates, the number of cycling cells may be developmentally shifted to an earlier postnatal phenotype.

![Figure 6](image-url)
Joining recent observations of cardiac progenitor cell biology with Akt kinase signaling opens novel possibilities for manipulation of myocardial cell survival, regeneration, and aging. Akt has already proven to be valuable as a genetic manipulation to enhance cardioprotective effects of stem cells after adoptive transfer, prompting renewed possibilities for therapeutic applications, although the predominant effect in these appears to be paracrine factor-mediated. Indeed, Akt/nuc prompts significant changes in cytokine expression in the hearts of transgenics reminiscent of neonatal transcription profiles for factors known to mediate cell survival, proliferation, and differentiation, such as TNF, interleukins, and bone morphogenetic proteins. Collectively, our results suggest a hypothetical model wherein nuclear Akt accumulation expands the cardiomyocyte progenitor population by a combination of proliferative and survival signaling mediated by intracellular signaling and paracrine factors (Figure 6). Thus, goals of ongoing studies are to (1) define specific molecular mechanisms responsible for the beneficial effects of nuclear-targeted Akt, (2) expand the potential application of nuclear-targeted Akt by genetic modification of cardiac progenitor cells and myocytes, and (3) promote recovery from cardiomyopathic injury and antagonize the progressive deterioration of hemodynamic performance in cardiomyopathies as well as normal aging.

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Disclosures

None.

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Antibodies and immunolabeling. Primary antibodies and dilutions used were: c-kit 1:100 (R&D Systems #AF1356), Ki-67 1:100 (Dako #M7249), GATA4 1:50 (Santa Cruz #sc-9053), desmin 1:40 (Santa Cruz #sc-7559), tropomyosin 1:50 (Sigma #T9283), MEF2C (Santa Cruz sc-13268 or 13266), α-sarcomeric actin Sigma #5C5), and myc-tag (Upstate Biotechnology #06-594). Primary antibodies were applied at 4°C overnight. Secondary conjugates and dilutions used were: biotin 1:2000-4000, FITC 1:200, Cy3, Cy5, and Texas Red 1:40-1:100, (all from Jackson Laboratories). Sarcomeric (tropomyosin) and cytoskeletal (desmin) markers were detected using standard indirect immunofluorescence. Ki67, c-kit and GATA-4 signals were amplified using the NEN TSA Fluorescence Systems kit (catalog #NEL701 for tyramide-fluorescein or NEL702 for tyramide-tetramethyl-rhodamine) following manufacturer recommendations at room temperature. All sections were blocked for 30 minutes in TNB blocking buffer (Blocking Buffer supplied with kit in 1X TN) without Tween-20. Biotinylated secondary antibodies were applied at the dilutions indicated for 1.5 hours at room temperature. After washing, sections were incubated in streptavidin-HRP (horseradish peroxidase) diluted 1:100 for 30 minutes, washed, and then developed with the tyramide substrate diluted 1:50 in amplification buffer for 10 minutes. For samples in which only a single amplification was necessary, slides were washed, stained with TOPRO-3 iodide, and coverslipped using VectaShield mounting medium. For samples requiring amplification of two signals in the same slide, slides were quenched in 3% H₂O₂ TN buffer for 20 minutes, washed and incubated with HRP-conjugated anti-FITC diluted 1:300 for 1 hour.
at room temperature or 1:500 overnight at 4C. After washing, slides were developed with tyramide-FITC substrate as described above. Nuclei were visualized using TOPRO-3 Iodide (Molecular Probes) diluted 1/10,000 in TN buffer for 20 minutes, washed briefly and coverslipped using VectaShield mounting medium. For immunoblotting studies, antibodies to detect cytokines include HGF (#AF2204), CD30L (#AF732; human equivalent of Tnfsf8), and IL-17e (#AF1399) (all from R & D Systems). Antibody bound to blots was detected with fluorescent-conjugated secondaries and scanned using a Molecular Dynamics Typhoon 9600 with quantitation by Imagequant software (GE Healthcare systems).

**Real-time PCR Gene Array.** Total RNA was cleaned using the Array Grade™ Total RNA Isolation Kit (SuperArray, Frederick, MD) and cDNA synthesis was performed in accordance with the protocol given for the RT2 Profiler™ PCR Array for mouse cytokines using 500ng of total RNA (SuperArray, Frederick, MD). Real-time PCR was then performed on each sample using the RT2 Profiler™ PCR Array for Mouse Common Cytokines (SuperArray, Frederick, MD) in an Opticon™ DNA Engine (Bio-Rad, Waltham, MA) with the following cycling conditions: an initial denaturation at 95°C 15 minutes, and 40 cycles of 95°C 15 seconds, 55°C 40 seconds, 72°C 30 seconds, with a final infinite 4°C hold. The C(t) for each gene from the different samples was standardized to the sample GAPDH value (ΔC(t)=Gene C(t)- GAPDH C(t)), and this value was then compared between NTG and Akt-nuc samples using the ΔΔC(t) method (ΔΔC(t)= [Akt Gene ΔC(t)] – [NTG AverageGene ΔC(t)]) which was then used to
determine the fold change (fold change= \[2^{\Delta\Delta C(t)}\]). The significance value for the fold change in each gene compared to fold change in GAPDH standard was determined using ANOVA. Standard error bars are representative of the SEM in fold change for each gene.