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Compensatory Hypertrophy Induced by Ventricular Cardiomyocyte Specific COX-2 Expression in Mice

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Abstract

Cyclooxygenase-2 (COX-2) is an important mediator of inflammation in stress and disease states. Recent attention has focused on the role of COX-2 in human heart failure and diseases, due to the finding that highly specific COX-2 inhibitors (i.e. Vioxx) increased the risk of myocardial infarction and stroke in chronic users. However, the specific impact of COX-2 expression in the intact heart remains to be determined. We report here the development of a transgenic mouse model, using a loxP-Cre approach, that displays robust COX-2 overexpression and subsequent prostaglandin synthesis specifically in ventricular myocytes. Histological, functional and molecular analyses showed that ventricular myocyte specific COX-2 overexpression led to cardiac hypertrophy and fetal gene marker activation, but with preserved cardiac function. Therefore, specific induction of COX-2 and prostaglandin *in vivo* is sufficient to induce compensated hypertrophy and molecular remodeling.

Keywords

cyclooxygenase; COX-2; transgenic; hypertrophy; compensatory

Introduction

The role of Cyclooxygenase-2 (COX-2) in inflammation and disease has long been appreciated due to the anti-inflammatory effects of mixed or preferential COX-2 inhibitors such as rofecoxib (Vioxx)[1]. The importance of COX-2 in human heart disease was brought to public attention by the relatively recent finding that chronic use of the COX-2 selective inhibitor Vioxx increased the risk of myocardial infarction and stroke[2]. While these findings

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definitively link COX-2 to heart disease, they reveal nothing about the underlying mechanisms of this pathology.

Conflicting results have been reported for the role of COX-2 in heart disease models of myocarditis. COX-2 promotes inflammation and disease in models of autoimmune myocarditis [3] and inflammatory lipid induced myocarditis[4]. On the other hand, COX-2 is protective in the disease states of virally induced myocarditis[5], as well as other disease models. Similarly, COX-2 is both detrimental [6] and protective [7] in experimental models of cardiac ischemia. These conflicting reports in the literature have prevented a clear understanding of the role of COX-2 in heart pathology, making it difficult to understand and manage the risks of COX-2 specific inhibitors in human health.

Genetic models of COX-2 deletion and overexpression can be important tools to determine the functional impact of COX-2 activity in heart. Several transgenic mouse models of COX-2 overexpression have been developed, including one driven by a non-tissue specific CMV promoter [8], and several tissue specific transgenic mice with targeted COX-2 expression in liver[9], brain[10], and keratinocytes [11]. Recently, a cardiomyocyte specific COX-2 transgenic mouse model was developed by Inserte *et al.*[12]. This model displayed no basal phenotype differing from control mice; however, the hearts were protected against ischemia-reperfusion injury. In this report, we also developed a heart specific transgenic (Tg) mouse over-expressing COX-2 based on an established COX-2 over-expressing mouse line, in which a universal promoter is placed in front of a floxed EGFP and STOP cassette, followed by the COX-2 coding region and a non-invasive reporter gene (*renilla* luciferase). The Cre-loxP mediated recombination allows tissue specific transgene expression in a variety of tissues [13]. In the report, we bred this mouse with a heart specific Cre mouse strain (MLC-2V-Cre [14]) allowed us to develop a model of heart specific COX-2 overexpression. These mice, in contrast to the model reported by Inserte *et al.* ([12]), displayed a significant basal phenotype characterized by a mild cardiomyocyte hypertrophy but preserved systolic function and altered expression of cardiac genes without any external stimuli. Our findings indicate a functional impact of COX-2 expression in cardiac remodeling and should help to provide additional insight to the role of COX-2 in heart physiology and pathology. The genetic model developed in this study should also provide a useful tool for future investigations.

Methods

Generation of Transgenic Animals

All animal handling and procedures were carried out in compliance with institutional guidelines and IACUC approved protocols. The mice from COX-2 COE (Tg) line established as described [13] were bred with mice from MLC-2v-Cre line (Cre) as described [14] with illustration in Figure 1. All pair breeding was carried out with Tg+/Cre- and Tg-/Cre+. Littermate controls (WT or one of either allele) were thus generated and compared to the double transgenic mice (Tg+/Cre+).

In Vivo Imaging

Imaging of the *Renilla* luciferase transgene was monitored as described in [13]. Briefly, the mice were anesthetized and secured in a light-tight chamber attached to a charge-coupled device camera (Xenogen, Alameda, CA). The *renilla* luciferase (rLuc) substrate coelenterazine (17µg/mouse) was injected via tail vein, and signal collected over a 60 second integration period. The data were analyzed and prepared by LIVING IMAGE (Xenogen). Individual organs and tissues were also removed and exposed to coelenterazine or UV light in order to obtain rLuc or EGFP signal respectively and collected with the same system.

mRNA and Protein Analysis

Hearts were removed, sectioned by chamber and snap frozen in liquid nitrogen prior to extraction of RNA or protein. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Left ventricle tissues were homogenized in TRIzol, using a glass-glass homogenizer. 0.5µg of the resultant total RNA was reverse transcribed into cDNA using the SuperScript II RT system (Invitrogen) with Oligo dT primers, according to the manufacturer's instructions.

mRNA levels of selected genes were determined by quantitative PCR from the generated cDNA. 50µl reactions were used with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 250 nM of each primer and 1µl of cDNA. The reactions were run in a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) and the data was collected using the Bio-Rad iQ5 software (Bio-Rad). The cycler program used was an initial denaturation at 95°C for 5min, 40 cycles of 45 seconds each of 95°C, 60°C, and 72°C, another denaturation at 95°C for 5min, and a final product melting curve. Data was normalized to the GAPDH mRNA levels which were run for each sample with every reaction. The threshold cycle count for each data point was subtracted from the GAPDH cycle count, and then used as in the formula $x=1/2^{\Delta\text{cycle}}$ in order to facilitate data analysis. Primers were also designed to flank intronic sequences, which would reveal the presence of genomic DNA contamination after the reaction had completed. The products were run on agarose gels to check for this contamination for every cDNA sample. The primer sequences used were as follows:

GAPDH F 5'- TCC TGC ACC ACC AAC TGC TTA G -3';

GAPDH R 5'- GAT GAC CTT GCC CAC AGC CTT G -3';

COX-2 F 5'- CCA GAT GCT ATC TTT GGG GA -3';

COX-2 R 5'- CGC CTT TTG ATT AGT ACT GTA G -3';

ANF F 5'- CTG ATG GAT TTC AAG AAC CTG CT -3';

ANF R 5'- CTC TGG GCT CCA ATC CTG TC -3';

βMHC F 5'- CTC AAC TGG GAA GAG CAT CCA -3';

βMHC R 5'- CCT TCA GCA AAC TCT GGA GGC -3';

TNFα F 5'- CTC TTC AAG GGA CAA GGC TG -3';

TNFα R 5'- TGG AAG ACT CCT CCC AGG TA -3';

CD45 F 5'- GGG TTG TTC TGT GCC TTG TT -3';

CD45 R 5'- GTT AGC ATC CTG CTT GCC TC -3';

IL-6 F 5'- TCC AGT TGC CTT CTT GGG AC -3';

IL-6 R 5'- GTG TAA TTA AGC CTC CGA CTT G -3'.

Total soluble protein was extracted from left ventricle tissue homogenized with a glass-glass homogenizer in a protein lysis buffer containing 20mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton-X100, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM sodium orthovanadate, 1mM PMSF, 10mM NaF and a complete mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The lysates were also spun down in order to separate the insoluble components. Protein concentration was determined by BCA Protein Assay (Thermo Scientific, Rockford, IL). 30µg of protein was separated onto 12% bis-tris polyacrylamide gels (Invitrogen) and transferred to Hybond-ECL membrane (Amersham Biosciences, Piscataway, NJ). COX-2 protein levels were determined by a COX-2

affinity purified primary antibody (Cayman Chemical, Ann Arbor, MI) and an anti-rabbit-HRP secondary antibody (Cell Signaling, Danvers, MA) with ECL detection (Thermo Scientific).

Prostaglandin ELISA

Total heart tissue prostaglandins were extracted by homogenizing frozen left ventricle pieces in 100% ethanol. The lysates were then diluted in 50mM citrate buffer, pH 3.5, so that the ethanol concentration dropped to 15% (85% citrate buffer). The lysates were spun down, and the pellets saved for protein concentration determination for the purposes of normalizing the final ELISA data. The supernatants were passed through C18 minicolumns (Amersham Biosciences) according to the manufacturer's instructions. The final eluate was dried by SpeedVac and stored at -80°C .

A Prostaglandin E_2 Biotrak Enzymeimmunoassay System kit (Amersham Biosciences) was used to measure PGE_2 levels in the heart tissue. A Thromboxane B_2 Biotrak Assay (Amersham Biosciences) was used to measure Thromboxane A_2 (TXA_2) levels by proxy in heart tissue (TXB_2 is the stable metabolite of TXA_2). The dried prostaglandin eluates were reconstituted in assay buffer, and the standard EIA protocol from the product literature was followed. The standard curve data was fit with an exponential function using Excel (Microsoft, Redmond, WA) and then used to calculate the sample PGE_2 and TXB_2 values.

Echocardiography

Mice were anesthetized and maintained with 2% isoflurane in 95% oxygen. A Vevo 770 (VisualSonics, Toronto, Canada) echocardiography system with a 30MHz scanhead was used to acquire the data. A parasternal short axis view was used to obtain M-mode images for analysis of fractional shortening, while a parasternal long axis view was used to obtain power doppler traces of mitral valve filling for the calculation of the E/A ratio. The heart rate was calculated directly from the short axis view of heart contraction.

Pressure Catheterization

Mice were anesthetized with 2g/kg urethane and maintained on a ventilator with 95% O_2 , 5% CO_2 . The chest cavity was opened, and an SPR-1000 micro-tip catheter pressure transducer (Millar Instruments, Houston, TX) was introduced through the apex into the center of the left ventricle. An MPCU-2000 pressure/volume conduction system (Millar Instruments) was used to control the catheter, and the data was acquired using Chart 5 (AD Instruments, Colorado Springs, CO). The resultant data was analyzed and functional parameters obtained using MPress (Millar Instruments).

Histology

The mouse hearts were perfused and fixed with 10% formalin prior to embedding in paraffin. All hearts were embedded in a cross section orientation, and all slices were cross sections of the heart. All slices were taken from the midpoint of the ventricle. $4\mu\text{m}$ slices were deparaffinized and rehydrated prior to staining with Masson's Trichrome stain[15]. Whole mount and trichrome stained images ($40\times$ objective) were collected using a SPOT digital camera system (Diagnostic Instruments, Sterling Heights, MI). For the purpose of calculating myocyte cross sectional area (CSA), 10 images were acquired from each heart from the epicardial region. In each image, 10 cells were quantified for their CSA using the SPOT Advanced software (Diagnostic Instruments). The resultant areas of 100 cells were all averaged to give the CSA of each heart, which counted as a sample size of 1 for statistical analysis.

Statistical Analysis

Comparisons between more than two groups were accomplished using a factorial analysis of variance (ANOVA) with Fisher's Protected Least Significant Difference post-hoc test. Comparisons between two groups were accomplished using an unpaired two-tailed T-test. Analysis was carried out using the StatView program (Abacus Concepts, Berkeley, CA). In all cases a significant result was defined as $p < 0.05$. The errors on all numerical figures were reported as standard error.

Results

Generation of a Heart Specific Transgenic COX-2 Overexpression Model

The COX-2/COE mouse is an established line [13] with a ubiquitous CAG promoter (CMV/chicken β -actin hybrid)[16] driving the expression of a floxed EGFP and a STOP cassette preceding the COX-2 protein coding sequence and an rLuc protein coding sequence linked by an internal ribosome entry site (IRES) (Figure 1A). These mice were bred with another established MLC-2V-cre line [14]. Cre mediated recombination of the loxP sites flanking the EGFP cassette results in its removal which permits COX-2 and rLuc transcription specifically in ventricular myocytes. The resultant double transgenic mice (Tg+/Cre +) and their age matched control littermates (Tg-/Cre -; Tg+/Cre -; Tg -/Cre +) were used in this study.

In vivo bioluminescence -imaging of the Tg +/Cre + transgenic mice revealed a discrete rLuc signal in the region of the heart after administration of the rLuc substrate (Figure 1B). In contrast, no signal was visible in a Tg+ control mouse with an inactive form of Cre recombinase (the inactive Cre control mouse was only used for this imaging experiment). To more closely examine the transgene expression, a number of different organs and tissues were removed from the Tg +/Cre + mouse, exposed to coelenterazine, and analyzed for rLuc-dependent bioluminescence (Figure 1C). These tissues were also exposed to UV light, to examine the expression of EGFP. The results showed specific luciferase signal only in the heart of the Tg +/Cre + mouse. In contrast, EGFP was widely expressed (Figure 1C).

The double transgenic hearts were then examined for the presence of COX-2 mRNA and protein. qPCR analysis showed robust COX-2 mRNA expression in the double transgenic hearts, with no expression in the Tg +/Cre - mouse (Figure 1D). Western blot analysis showed that this mRNA expression is accompanied by robust COX-2 protein induction, again with no detectable expression in the Tg +/Cre - mice (Figure 1E). Thus, we have generated a heart specific, robustly overexpressed COX-2 transgenic mouse.

Cardiac Specific COX-2 Overexpression Induces Mild Hypertrophy With No Signs of Heart Failure

To determine if the overexpressed COX-2 protein is functional, the hearts of control and Tg +/Cre + mice were tested for prostaglandin production, using an ELISA system to assay for PGE₂ and TXA₂ (via the stable metabolite TXB₂). PGE₂ and TXB₂ levels in the hearts of Tg +/Cre + mice were significantly induced compared to the hearts of WT littermate controls (Figure 2A). Thus the transgene COX-2 is functional, resulting in the elevated levels of multiple downstream prostaglandin production in the heart.

We then examined the hearts of double transgenic mice for signs of hypertrophy, as PGE₂ is a known inducer of hypertrophy in cardiomyocytes[17]. We found a mild (17%) but significant hypertrophy as measured by left ventricle weight normalized to the body weight in the Tg +/Cre + mice compared to their control littermates (Figure 2B). Hypertrophy was not accompanied by obvious signs of heart failure, such as edema or increased lung weight

normalized to the body weight (Figure 2B). Thus, functional COX-2 overexpression appears to cause mild hypertrophy without failure.

Cardiac Specific COX-2 Overexpression Does Not Induce Functional Changes in the Heart

While not accompanied by signs of overt failure, the hypertrophy we observed (Figure 2B) nonetheless has the potential to alter the function of the transgenic hearts. We thus examined functional parameters and performance of the heart by echocardiography and invasive pressure catheterization. Echocardiography revealed no differences in baseline heart rate (Figure 3A). There was also no difference in the fractional shortening, a basic measure of ventricular contraction, between the double transgenic mice and the WT and Tg +/Cre – mice (Figure 3A). There was an anomalous modest increase in fractional shortening in Tg –/Cre + mice. However, this increase is likely due to random variability since there were no other consistent differences in these mice showing an increase in function (data not shown). Lastly, there were no differences in mitral Doppler E/A ratio, a marker of diastolic dysfunction and wall stiffness, between any of the mouse genotypes (Figure 3A).

We also measured the function of these hearts by the more direct method of pressure catheterization. Again, the hearts of the double transgenic and WT control littermates had identical heart rates (Figure 3B). The maximum systolic pressure (Pmax), an indicator of systolic function, was unchanged between WT and Tg +/Cre + mice (Figure 3B). The dP/dT max, the maximum rate of change of the systolic pressure wave, another marker of systolic function, was also unchanged ($p > 0.05$) between the transgenic and control mice (Figure 3B). No other parameters measured (such as dP/dT min, Tau, etc.) indicated a difference between the control and transgenic mice (data not shown).

Cardiac Specific COX-2 Overexpression Induces Cardiomyocyte Hypertrophy Without Pathological Remodeling

The hearts of the Tg +/Cre + mice and their WT littermates were examined by histological methods, to search for signs of pathological remodeling. The whole hearts of the Tg +/Cre + mice were grossly normal, with no signs of enlargement, atrial remodeling, calcium deposits or other abnormalities (Figure 4A). Fixed hearts were sectioned and stained for fibrosis with Masson's trichrome stain. There were no signs of abnormal fibrosis, edema, or myocyte loss (Figure 4B). Indeed, small, infrequent patches of replacement fibrosis were observed in all WT hearts examined; however, these patches were not present in the hearts of Tg +/Cre + mice (Figure 4B). This observation raises the possibility that COX-2 overexpression prevents cardiac fibrosis. However, qPCR for Collagen I revealed no differences between the groups (data not shown), leaving this possibility unconfirmed.

Lastly, the cross sectional area (CSA) of the transgenic hearts was determined from an average of 100 cells from each heart examined. We found a significant increase in the CSA of the Tg +/Cre + hearts, supporting that the increase in heart size seen in Figure 2B is a *bona fide* myocyte hypertrophy. The gross heart size increase results – at least in part – from an enlargement of the cardiomyocytes themselves, as opposed to edema or other possible factors which could increase heart weight.

Cardiac Specific COX-2 Overexpression Induces Stress Gene Activation Without Inflammation

The hearts of the Tg +/Cre + mice were examined for pathological changes in inflammatory and stress gene transcription. TNF α and IL-6 are both pro-inflammatory cytokines, while CD45 is a pan-leukocyte marker which would reveal the infiltration of immune cells into the hearts. qPCR analysis of extracted RNA from the Tg +/Cre + and littermate control hearts showed no

differences in the inflammatory markers of TNF α , IL-6 or CD45, indicating no inflammatory induction in these hearts (Figure 5A).

The hearts were also examined for stress gene induction, specifically for members of the cardiac fetal gene program, a specific pattern of transcriptional changes seen in pathological remodeling and heart failure. By qPCR, we found that the levels of ANF and β MHC were both induced in the Tg +/Cre + hearts when compared to control littermates (Figure 5B). These data indicate that the remodeling seen in these hearts, while mild, nonetheless is associated with pathological stress gene induction. Interestingly, we also found a marked reduction of β MHC mRNA in both single transgenic control groups. This specific reduction of β MHC is not matched by reductions in ANF, or consistent with any of the other functional parameters measured.

Discussion

We report here that we have generated a robust, heart specific transgenic COX-2 overexpression model (Figure 1). The overexpressed COX-2 is functional, resulting in substantially elevated prostaglandin production in the transgenic heart tissues (Figure 2). The COX-2 transgenic hearts have no gross functional (Figure 3) or histological abnormalities (Figure 4), but do show *bona fide* cardiomyocyte hypertrophy (Figure 2 and 4C). Lastly, the hearts show no signs of inflammation (Figure 5A), but do displayed a pattern of pathological stress gene activation (Figure 5B).

Intriguingly, this model of COX-2 over-expression and resultant high level of prostaglandins (Figure 1 and 2A) only develops a mild hypertrophic phenotype, although PGE₂ alone is known to induce hypertrophy[17]. Furthermore, stress gene activation is observed in these hearts without signs of inflammation (Figure 5). The expression of stress genes, associated with pathological hypertrophy and heart failure[18], argues that the phenotype seen here, while mild, is still fundamentally pathological. Supporting this hypothesis, we found elevated levels of TXA₂ (via an assay for the stable metabolite TXB₂) in Tg +/Cre + hearts (Figure 2). Transgenic activation of the thromboxane receptor has been shown to induce pathological heart failure [19], and TXA₂ has been associated with multiple pathological conditions in heart [20,21]. Based on our observations that pro-inflammatory cytokines (TNF α and IL-6) or inflammatory marker gene (CD45) are not induced in COX-2 transgenic hearts, we can speculate that COX2 mediated prostaglandins in diseased heart can potentially contribute to cardiac hypertrophy and pathological changes in gene expression, but not sufficient to induce inflammatory response and overt heart failure.

Recently, Inserte *et al.* [12] also reported a cardiomyocyte specific COX-2 transgenic model with no differences in baseline phenotype from wild type mice, but exhibiting enhanced cardioprotection in response to ischemia/reperfusion injury. As in our model, these mice had no functional defects, however, our model showed significant hypertrophy and signs of pathological gene activation. The basis for the differences in their basal phenotypes between our model and the model by Inserte *et al.* is unclear to us. The model developed by Inserte *et al.* also had enhanced prostaglandin production. The lack of strong phenotype at baseline in these two models suggests that COX-2 activity at baseline can be restrained by other factors to prevent gross pathological responses. This may even be due to the balance of prostaglandins produced at baseline[20]. The finding that COX-2 also promotes cardioprotection in Inserte's report model was anticipated in the literature[20]. Thus, the nature of the stress employed may be critical in determining whether COX-2 is harmful, as some studies have shown [22], or beneficial, as in the study by Inserte *et al.*

One potential complicating factor in interpreting COX-2 overexpression is that alternative prostaglandins produced downstream of COX-2 can have widely varying effects on the heart. For instance, PGF_{2A} is associated with myocyte hypertrophy[23] while Thromboxane A₂ is associated with a variety of inflammatory and other pathological effects[21]. In contrast, PGI₂ has protective roles in blocking oxidative damage and preventing ischemic injury[24]. Thus, the balance of prostaglandin products may dictate the overall impact on cardiac pathology and protection. More investigation will be required to determine which prostaglandins are directly responsible for exacerbating or alleviating protective or detrimental effects downstream of COX2. Finally, although this is a plausible hypothesis, we cannot conclude definitively that cell-autonomous induction of the prostaglandins in COX-2 expressing myocytes is solely responsible for the observed phenotype, and exclude the possible contribution from other unknown cytokines or chemokines induced secondary to COX-2 expression.

The mild phenotype observed in COX2 transgenic hearts is also somewhat at odds with the findings in humans that chronic COX-2 inhibition is responsible for an increased heart disease risk[2]. Taken simply, the human data would suggest that COX-2 activity might be beneficial. However, COX-2 inhibition could be acting in other tissues, particularly kidney, to induce cardiovascular stress. Also, stress and inflammation related genes do not lend themselves well to such simple descriptions. For instance, both TNF- α overexpression[25] and knockout[26] have been associated with heart pathology. It is clear that stress and inflammatory genes have a role in both compensatory processes and pathological changes under prolonged or severe stresses. Lastly, since COX-2 overexpression is induced early in cardiac development in this model, compensatory mechanisms may have developed which complicate the analysis of COX-2 overexpression in the heart. Further investigation of the exact consequences of COX-2 induction or inhibition, particularly the prostaglandins production, using conditional transgenesis will be required in order to better understand its function.

Clearly, the biology of COX-2 action in the heart is complicated, and depends on the location and timing of induction and the exact balance of downstream products produced. This explains in part the controversy in the literature regarding this pathway. Our model should be useful in determining the role of COX-2 at the baseline and during stress, and which prostaglandins are produced by COX-2 in the heart, and how these prostaglandins impact heart function and pathology.

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References

- [1]. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* 2000;69:145–82. [PubMed: 10966456]
- [2]. Bombardier C, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, et al. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med* Nov 23;2000 343(21):1520–8. 2 p following 8. [PubMed: 11087881]
- [3]. Chang H, Hanawa H, Liu H, Yoshida T, Hayashi M, Watanabe R, et al. Hydrodynamic-based delivery of an interleukin-22-Ig fusion gene ameliorates experimental autoimmune myocarditis in rats. *J Immunol* Sep 15;2006 177(6):3635–43. [PubMed: 16951323]
- [4]. Grandel U, Hopf M, Buerke M, Hattar K, Heep M, Fink L, et al. Mechanisms of cardiac depression caused by lipoteichoic acids from *Staphylococcus aureus* in isolated rat hearts. *Circulation* Aug 2;2005 112(5):691–8. [PubMed: 16043646]

- [5]. Takahashi T, Zhu SJ, Sumino H, Saegusa S, Nakahashi T, Iwai K, et al. Inhibition of cyclooxygenase-2 enhances myocardial damage in a mouse model of viral myocarditis. *Life Sci* Nov 26;2005 78(2):195–204. [PubMed: 16107267]
- [6]. Abbate A, Salloum FN, Ockaili RA, Fowler AA 3rd, Biondi-Zoccai GG, Straino S, et al. Improvement of cardiac function with parecoxib, a cyclo-oxygenase-2 inhibitor, in a rat model of ischemic heart failure. *J Cardiovasc Pharmacol* 2007;416–8. [PubMed: 17577107]
- [7]. Timmers L, Sluijter JP, Verlaan CW, Steendijk P, Cramer MJ, Emons M, et al. Cyclooxygenase-2 inhibition increases mortality, enhances left ventricular remodeling, and impairs systolic function after myocardial infarction in the pig. *Circulation* Jan 23;2007 115(3):326–32. [PubMed: 17210840]
- [8]. Leung WK, Wu KC, Wong CY, Cheng AS, Ching AK, Chan AW, et al. Transgenic cyclooxygenase-2 expression and high salt enhanced susceptibility to chemical-induced gastric cancer development in mice. *Carcinogenesis* Aug;2008 29(8):1648–54. [PubMed: 18611916]
- [9]. Mayoral R, Molla B, Flores JM, Bosca L, Casado M, Martin Sanz P. Constitutive expression of cyclooxygenase 2 transgene in hepatocytes protects against liver injury. *Biochem J.* Aug 1;2008
- [10]. Xiang Z, Thomas S, Pasinetti G. Increased neuronal injury in transgenic mice with neuronal overexpression of human cyclooxygenase-2 is reversed by hypothermia and rofecoxib treatment. *Curr Neurovasc Res* Nov;2007 4(4):274–9. [PubMed: 18045153]
- [11]. Wang X, Colby JK, Rengel RC, Fischer SM, Clinton SK, Klein RD. Overexpression of cyclooxygenase-2 (COX-2) in the mouse urinary bladder induces the expression of immune- and cell proliferation-related genes. *Mol Carcinog.* Apr 28;2008
- [12]. Inserte J, Molla B, Aguilar R, Traves PG, Barba I, Martin-Sanz P, et al. Constitutive COX-2 activity in cardiomyocytes confers permanent cardioprotection Constitutive COX-2 expression and cardioprotection. *J Mol Cell Cardiol* Feb;2009 46(2):160–8. [PubMed: 19084534]
- [13]. Kamei K, Ishikawa TO, Herschman HR. Transgenic mouse for conditional, tissue-specific Cox-2 overexpression. *Genesis* Apr;2006 44(4):177–82. [PubMed: 16604526]
- [14]. Chen J, Kubalak SW, Minamisawa S, Price RL, Becker KD, Hickey R, et al. Selective requirement of myosin light chain 2v in embryonic heart function. *J Biol Chem* Jan 9;1998 273(2):1252–6. [PubMed: 9422794]
- [15]. Garvey W. Modified elastic tissue-Masson trichrome stain. *Stain Technol* Jul;1984 59(4):213–6. [PubMed: 6208643]
- [16]. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* Dec 15;1991 108(2):193–9. [PubMed: 1660837]
- [17]. Mendez M, LaPointe MC. PGE2-induced hypertrophy of cardiac myocytes involves EP4 receptor-dependent activation of p42/44 MAPK and EGFR transactivation. *Am J Physiol Heart Circ Physiol* May;2005 288(5):H2111–7. [PubMed: 15626689]
- [18]. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: a strong hypothesis. *Heart Fail Rev* Dec;2007 12(3–4):331–43. [PubMed: 17516164]
- [19]. Zhang Z, Vezza R, Plappert T, McNamara P, Lawson JA, Austin S, et al. COX-2-dependent cardiac failure in Gh/tTG transgenic mice. *Circ Res* May 30;2003 92(10):1153–61. [PubMed: 12702643]
- [20]. Streicher JM, Wang Y. The role of COX-2 in heart pathology. *Cardiovasc Hematol Agents Med Chem* Jan;2008 6(1):69–79. [PubMed: 18220723]
- [21]. Wacker MJ, Best SR, Kosloski LM, Stachura CJ, Smoot RL, Porter CB, et al. Thromboxane A2-induced arrhythmias in the anesthetized rabbit. *Am J Physiol Heart Circ Physiol* Apr;2006 290(4):H1353–61. [PubMed: 16339832]
- [22]. Suzuki J, Ogawa M, Futamatsu H, Kosuge H, Tanaka H, Isobe M. A cyclooxygenase-2 inhibitor alters Th1/Th2 cytokine balance and suppresses autoimmune myocarditis in rats. *J Mol Cell Cardiol* May;2006 40(5):688–95. [PubMed: 16499924]
- [23]. Adams JW, Migita DS, Yu MK, Young R, Hellickson MS, Castro-Vargas FE, et al. Prostaglandin F2 alpha stimulates hypertrophic growth of cultured neonatal rat ventricular myocytes. *J Biol Chem* Jan 12;1996 271(2):1179–86. [PubMed: 8557648]
- [24]. Shinmura K, Tamaki K, Sato T, Ishida H, Bolli R. Prostacyclin attenuates oxidative damage of myocytes by opening mitochondrial ATP-sensitive K⁺ channels via the EP3 receptor. *Am J Physiol Heart Circ Physiol* May;2005 288(5):H2093–101. [PubMed: 15604124]

- [25]. Kubota T, McTiernan CF, Frye CS, Slawson SE, Lemster BH, Koretsky AP, et al. Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor- α . *Circ Res* Oct;1997 81(4):627–35. [PubMed: 9314845]
- [26]. Lien YC, Lin SM, Nithipongvanitch R, Oberley TD, Noel T, Zhao Q, et al. Tumor necrosis factor receptor deficiency exacerbated Adriamycin-induced cardiomyocytes apoptosis: an insight into the Fas connection. *Mol Cancer Ther* Feb;2006 5(2):261–9. [PubMed: 16505099]

Figure 1. Heart Specific COX-2 Overexpression in Tg +/Cre + Transgenic Mice

A) Diagram of the COX-2 COE transgene. A promiscuous CMV promoter drives expression of a floxed EGFP cassette and STOP sequence which, together, prevent translation of the COX-2 and *Renilla* luciferase “downstream” coding regions. The COX-2 and rLuc coding regions, connected by an internal ribosomal entry sequence (IRES), form a bicistronic message. Cre directed recombination of the EGFP-STOP cassette permits downstream COX-2 and rLuc expression. **B)** Live mice were anesthetized, injected with coelenterazine, an rLuc substrate, and imaged using a CCD camera. Signal in the region of the heart is visible in the Tg +/Cre + double transgenic mice, and not in the Tg +/inactive Cre control mouse (the inactive Cre mouse was only used for this imaging experiment). **C)** Various organs and tissues were imaged after exposure either to rLuc substrate (to monitor Cre-specific transgene expression) or to UV light to image EGFP. rLuc signal is only visible in heart, indicating specific cardiac recombination. Fluorescence is visible in a wide range of tissues, indicating widespread expression of the unrecombined EGFP transgene. **D)** Hearts of the Tg +/Cre + mice and their control littermates were removed, total RNA extracted, and COX-2 mRNA measured by qPCR. Substantially elevated COX-2 mRNA is present in the double transgenic mice, when compared to controls. *= $p < 0.05$ vs. WT. **E)** Hearts of the Tg +/Cre+ mice and control littermates were removed, protein extracted, and the levels of COX-2 and EGFP measured by Western blot. Robust COX-2 protein expression is visible in the double transgenic samples. Evidence of loss of EGFP expression due to recombination is also visible.

Figure 2. Cardiac Specific COX-2 Overexpression Results in Elevated Prostaglandins and Mild Hypertrophy

A) The hearts of Tg +/Cre + mice and their WT littermates were removed and PGE₂ and TXB₂ (the stable metabolite of TXA₂) levels were determined by ELISA. *= $p < 0.05$ vs. WT.
B) The left ventricles and lungs of Tg +/Cre + mice and their littermate controls were removed and weighed. The data are normalized to mouse body weights. *= $p < 0.05$ vs. WT.

Figure 3. Cardiac Specific COX-2 Overexpression Does Not Change Heart Function

A) Functional parameters of the Tg +/Cre + and control littermate hearts were measured by echocardiography. The heart rates were unchanged among groups, which is important due to the effects of heart rate on contractility. The fractional shortening was measured as a percent change between the left ventricular inner diameter at systole and diastole. The Doppler E/A ratio was measured from the peaks of the E and A waves during diastole measured from the mitral valve. *= $p < 0.05$ vs. WT. **B)** Functional parameters of the Tg +/Cre + and WT littermate hearts measured by pressure catheterization. The heart rates were again unchanged among groups. The Pmax was measured at the peak of systole, and the dP/dT max was found from the maximum rate of change of the systolic pressure wave. Both were unchanged among groups.

Figure 4. Cardiac Specific COX-2 Overexpression Induces Cardiomyocyte Hypertrophy Without Gross Histological Remodeling

A) Hearts from the WT and Tg +/Cre + mice were removed and imaged with a digital camera. A size marker in millimeters is provided. No gross differences are visible. **B)** WT and Tg +/Cre + hearts were fixed, sectioned, and stained by Masson trichrome. Collagen fibers stain blue, myocytes red. No gross differences are visible between the genotypes in histological features. The worst example of replacement fibrosis from each of two hearts from each genotype is displayed. Replacement fibrosis appears to be decreased in the Tg +/Cre + transgenic mice. **C)** The average of 100 cells from each heart for three hearts per genotype was measured for myocyte cross sectional area (CSA). The Tg +/Cre + hearts have a significant increase in CSA relative to WT hearts, indicating cardiomyocyte hypertrophy. *= $p < 0.05$ vs. WT.

Figure 5. Cardiac Specific COX-2 Overexpression Induces Stress Gene Upregulation Without Inflammatory Gene Activation

A) The hearts of Tg +/Cre + mice were removed, RNA extracted, and the mRNA levels of TNFα, IL-6 and CD45 measured by qPCR. No differences were found among any of the groups for any inflammatory marker examined. **B)** The hearts from **A** were also examined for stress gene activation by qPCR. The levels of both ANF and βMHC were elevated in Tg +/Cre + mice. *= $p < 0.05$ vs. WT. #= $p < 0.05$ vs. single transgenic controls.