Cardiomyocyte-Specific Deletion of the Vitamin D Receptor Gene Results in Cardiac Hypertrophy

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Background—A variety of studies carried out using either human subjects or laboratory animals suggest that vitamin D and its analogues possess important beneficial activity in the cardiovascular system. Using Cre-Lox technology we have selectively deleted the vitamin D receptor (VDR) gene in the cardiac myocyte in an effort to better understand the role of vitamin D in regulating myocyte structure and function.

Methods and Results—Targeted deletion of the exon 4 coding sequence in the VDR gene resulted in an increase in myocyte size and left ventricular weight/body weight versus controls both at baseline and following a 7-day infusion of isoproterenol. There was no increase in interstitial fibrosis. These knockout mice demonstrated a reduction in end-diastolic and end-systolic volume by echocardiography, activation of the fetal gene program (ie, increased atrial natriuretic peptide and alpha skeletal actin gene expression), and increased expression of modulatory calcineurin inhibitory protein 1 (MCIP1), a direct downstream target of calcineurin/nuclear factor of activated T cell signaling. Treatment of neonatal cardiomyocytes with 1,25-dihydroxyvitamin D partially reduced isoproterenol-induced MCIP1 mRNA and protein levels and MCIP1 gene promoter activity.

Conclusions—Collectively, these studies demonstrate that the vitamin D-VDR signaling system possesses direct, antihypertrophic activity in the heart. This appears to involve, at least in part, suppression of the prohypertrophic calcineurin/NFAT/MCIP1 pathway. These studies identify a potential mechanism to account for the reported beneficial effects of vitamin D in the cardiovascular system. (Circulation. 2011;124:1838-1847.)

Key Words: vitamin D receptor • gene deletion • isoproterenol • cardiac hypertrophy

Vitamin D is ingested in the diet or generated de novo through scission of cholesterol precursors in the skin by ultraviolet light.1 Once formed, vitamin D is activated through 2 sequential hydroxylation reactions. The first of these, a 25-hydroxylation, takes place largely in the liver to produce 25-hydroxyvitamin D. This molecule circulates bound to a plasma protein and is the form that is measured to assess the adequacy of vitamin D stores in the organism. The second hydroxylation, a 1α-hydroxylation, takes place in a variety of tissues, including the kidney, to generate 1,25-dihydroxyvitamin D [1,25(OH)2D], the most polar and biologically active of the vitamin D metabolites. 1,25(OH)2D functions as a ligand for the nuclear vitamin D receptor (VDR) which, when paired with its heterodimeric retinoid X receptor partner, binds to sequence-specific recognition elements on DNA and stimulates or represses transcription of contiguous genes.2

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The prevalence of vitamin D deficiency and insufficiency (ie, plasma levels of 25-hydroxyvitamin D <20 and <30 ng/mL, respectively) is estimated at greater than one billion people worldwide.1 In developed countries, it is seen more commonly in otherwise healthy individuals with dark skin pigmentation, older individuals, obese individuals, and individuals living at higher latitudes, in particular, during the winter months.1,3 Vitamin D insufficiency has been shown to cluster with a variety of cardiovascular and metabolic disorders of clinical importance, including hypertension,4–6 insulin resistance,7 metabolic syndrome,8 diabetes mellitus,9,10 peripheral vascular disease,11 and congestive heart failure,12,13 raising the intriguing possibility of a mechanistic link between vitamin D insufficiency and cardiovascular disease.

A limited number of interventional studies have linked vitamin D repletion to reduction in blood pressure14,15 in humans, and the use of 1,25(OH)2D and its bioactive analogues has led to reversion of cardiac hypertrophy in rats16 and in patients with end-stage renal failure on dialysis.17

We have shown previously that the liganded VDR displays antihypertrophic activity in neonatal rat ventricular myocytes in culture.18 Recent studies from Li and colleagues have...
shown that the VDR gene-deleted (VDR<sup>−/−</sup>) mouse displays both hyperreninemic hypertension<sup>19</sup> and ventricular hypertrophy.<sup>20</sup> The presence of hypertension and inferred increased ventricular afterload make it difficult to assign this antihypertrophic activity to VDR-signaled events within the myocyte itself. The presence of secondary hyperparathyroidism in these mice<sup>21</sup> and the postulated link between parathyroid hormone excess and cardiovascular dysfunction<sup>22</sup> add to the complexity of data interpretation in this model. In an effort to address this issue in a directed fashion, we have generated a mouse with selective deletion of the VDR gene in the myocytes of the heart, and have shown that these mice demonstrate increased cardiac hypertrophy both at baseline and after the administration of isoproterenol (ISO), a hypertrophic stimulus.

Methods

**Cardiomyocyte-Specific VDR Knockout Mice**

Generation and characterization of a floxed VDR-targeting construct, transfection into embryonic stem cells, and introduction of these cells into mice are described in the Methods section of the online-only Data Supplement. The recombinant VDR allele bearing the loxP-bordered exon 4 was maintained in the C57/BL6J background. The neomycin resistance gene cassette was excised through a cross with the Flpe mouse.<sup>23</sup> Mice harboring the recombinant allele (VDR<sup>loxP/loxP</sup> or VDR<sup>loxP−/−</sup>) were bred with MLC-Cre<sup>24</sup> or Sox2-Cre mice<sup>25</sup> to create cardiomyocyte-specific or global VDR knockout mice, respectively. All experiments were carried out in mice at ~6 months of age. All animal-related experimental protocols were approved by the Institutional Animal Care and Use Committee at University of California San Francisco.

**ISO Infusion and Blood Pressure Measurement**

Mice were anesthetized with 1.5% isoflurane and subcutaneous Alzet osmotic minipumps containing PBS or ISO, the latter calibrated to release the drug at the rate of 15 mg·kg<sup>−1</sup>·d<sup>−1</sup> for 7 days, were surgically implanted subcutaneously in the interscapular region of the mouse. Mouse blood pressure was measured using the tail-cuff method. Mice were trained on the Hatteras Instruments SC1000 Blood Pressure Analysis System, used according to the manufacturer’s instructions, for 4 days. Ten consecutive measurements on the last day were averaged to calculate systolic and diastolic blood pressure. Mean arterial blood pressure was calculated as 2/3 diastolic pressure plus 1/3 systolic blood pressure.

**Ventricular Myocyte Isolation and Cell Sorting**

Adult mouse ventricular myocytes were isolated with the use of a previously described retrograde perfusion technique,<sup>26</sup> with minor modifications. The detailed procedure for isolation of the myocytes is described in the Methods section of the online-only Data Supplement. To purify myocytes further, cells were sorted at a flow rate of 250 cells/s on a MoFlo cytometer (Beckman Coulter, Brea, CA) using a 100-μm tip and a 488-nm Argon laser for excitation. Rod-shaped cardiac myocytes were sorted with the use of the following sort parameters: autofluorescence (excitation at 488 nm; m tip and a 488-nm Argon laser for excitation). Rod-shaped cardiac myocytes were sorted with the use of the following sort parameters: autofluorescence (excitation at 488 nm; m tip and a 488-nm Argon laser for excitation).

**Echocardiography**

Mice were anesthetized with 1.5% isoflurane, and transthoracic echocardiography was performed using a Vevo 660 system (VisualSonics, Toronto, Canada) equipped with a 30-MHz transducer, according to the methods of Zhang et al.<sup>27</sup> Left ventricular ejection fraction, end-systolic volume, end-diastolic volume, and wall thickness were assessed at different time points accordingly. Diastolic function parameters, including peak early (E) and late (A) mitral inflow velocities, were measured. Three cycles were measured for each assessment, and the average values were obtained. Analyses of the echocardiographic images were performed in a blinded manner.

**Tissue Harvesting, Histology, and Morphometric Analyses**

Mice were weighed and euthanized. The atria and right ventricular free walls were dissected away from the left ventricle. The left ventricles, including septum, were weighed, quick frozen, and stored at −80°C for later preparation of DNA and RNA. Selected mice were deeply anesthetized, and the hearts were perfused with saline followed by Z-Fix (Anatech Ltd, Battle Creek, MI). Fixed left ventricles were embedded in paraffin, and cross sections were stained with hematoxylin and eosin or Masson’s trichrome to evaluate gross morphology and cardiac fibrosis. To quantify individual myocyte size, some sections were stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin, as described by Xu et al.<sup>28</sup> Myocyte area was assessed with ImageJ (National Institutes of Health) with the investigator blinded to the genotype. The mean cardiomyocyte area was evaluated by measurement of 400 cells per heart (4–5 hearts per genotype).

**Plasma Measurements**

Plasma was collected at the time of euthanasia and used to measure total calcium (IDEXX Laboratories, West Sacramento, CA). Plasma parathyroid hormone was measured with the use of a mouse intact PTH ELISA kit (Immunotops, San Clemente, CA) and plasma atrial natriuretic peptide (ANP) concentration was measured with a commercial radioimmunoassay kit (Phoenix Pharmaceutical, Mountain View, CA) according to the manufacturer’s instructions.

**Plasmid Construction**

An 852-bp segment of the MCIP1 promoter in the intron region upstream of exon 4 from mouse genome (Gene: Rcan1 (ENSMUSG00000022951) containing multiple nuclear factor of activated T cell [NFAT] binding sites)<sup>29</sup> was amplified and subcloned into the KpnI and XhoI restriction sites in pGL3 vector (Promega, Madison, WI). The structure was confirmed by DNA sequencing.

**Gene Array and Differential Expression Analysis**

DNase-digested total RNA was isolated from the cardiomyocytes of wild-type and myocyte-specific VDR knockout mice. Sample quality and quantity was determined by assaying samples in Bioanalyzer RNA Nano Assays (a RNA integrity number score >8.5 was recorded for all samples) and with a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). All samples were amplified with the use of NuGEN Appliance Plus kits (NuGEN Technologies Inc, San Carlos, CA), converted to sense strand DNA, and then fragmented and labeled according to the manufacturer’s instructions. Samples were hybridized to Affymetrix Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA), stained, and scanned according to the manufacturer’s protocols. A total of 26,581 known gene expression profiles were detected. Microarrays were normalized to control for array-specific effects with the use of the Affymetrix Power Tools software with the Robust Multi-Array normalization. The average signal intensity in our microarrays was 7.5 after normalization. For statistical analyses, all probe sets where none of the groups had an average log 2 intensity >3.0 were removed. This is a standard cutoff, under which the expression levels are indistinguishable from background. All log values were log 2. Linear models were fitted for each gene with the use of the Limma package (Smyth 2008) in R/Bioconductor. Moderated t statistics and the associated probability values were calculated.
Genotyping, Southern Blot Analysis

Neonatal cardiomyocyte preparation, luciferase assay, real-time polymerase chain reaction (PCR) and Western blot analysis are presented in the Methods section of the online-only Data Supplement.

Statistical Analysis

Data are expressed as mean±SD. Data were analyzed by the use of 2-way ANOVA. Statistical significance (P<0.05) was assessed by the use of the Newman-Keuls test.

Results

Using a strategy outlined in Figure 1A, we engineered loxP sites around the fourth exon of the murine VDR gene and placed it in a targeting construct containing a neomycin resistance cassette. The fourth exon was selected for deletion based on the phenotype of the whole animal knockout generated by Li and coworkers.21 The targeting construct was transfected into SV/129 embryonic stem cells (Figure 1B and 1C), which, in turn, were introduced into the blastocysts of C57/BL6J mice. Chimeric offspring were identified and screened for the recombinant allele. This allele was tracked in subsequent generations (at least 6 generations bred into the C57/BL6 background) using conventional PCR analysis of tail genomic DNA. Additional details on the generation of the VDRloxP/loxP mice are provided in the Methods section and Figure IA–ID in the online-only Data Supplement.

In an effort to document accurate positioning and accessibility of the loxP sites, we mated a Sox2-Cre transgenic mouse, which allows for ubiquitous expression of Cre in mice harboring this transgene, with mice homozygous for the recombinant floxed VDR allele (VDRloxP/loxP). Subsequent derivatives of this cross showed complete elimination of exon 4 at the VDR gene locus, effectively confirming their geno-
type as VDR−/− (Figure 1D). As shown in the online-only Data Supplement, VDR−/− mice demonstrated no immunoreactive VDR in extracts from tail tissue (versus robust signal in wild-type mice) (Figure IIA in the online-only Data Supplement). They also displayed alopecia and reduced body weight (Figure IIB in the online-only Data Supplement) as previously reported for mice homozygous for the VDR gene deletion.21 This confirms both correct positioning and accessibility of the loxP sites in the targeted VDR gene allele.

When VDRloxP/loxP mice were mated with mice bearing a Cre recombinase, as a single copy gene knock-in under control of the myosin light chain gene promoter (MLC-Cre),24 the observed efficiency of exon 4 deletion was <50% (Figure 1D), likely reflecting the fact that the majority of nuclear DNA in these preparations is derived from nonmyocyte cells (eg, interstitial fibroblasts), which would not be predicted to express the MLC-Cre gene. MLC-Cre-dependent excision was more obvious when we introduced 1 null allele into the VDR gene locus (M−/−) (Figure 1D, right lane). To be more precise about the level of exon 4 deletion in the MLC-Cre/VDRloxP/loxP mice, we isolated individual myocytes from these hearts, and those from animals bearing the uncut recombinant floxed allele (VDRloxPloxP), as well, and carried out the same PCR analysis. As shown in Figure 1E, there was ∼90% efficiency in the excision of exon 4 in myocytes of the MLC-Cre/VDRloxPloxP mice.

To maximize the efficiency of the proposed myocyte-specific VDR gene excision, we mated the VDRloxPloxP mouse with the heterozygous whole animal knockout (VDR+/−) to generate the VDRloxPloxP mouse (see Figure 1D). This mouse, like the VDR+/− reported previously,21 did not appear to have phenotypic characteristics that differ from the wild-type VDR−/−/− mouse (see below). Moving MLC-Cre into the VDRloxP−/− background was predicted to effect near-complete deletion of the VDR gene in the cardiac myocytes. This was documented by real-time PCR analysis of RNA collected from these hearts, as shown in Figure 2A. Although VDRloxP−/−, similar to VDR+/−, displayed ∼50% wild-type cardiac VDR transcript levels, the cardiac selective VDR gene deletion displayed levels that were ∼20% of wild-type levels. The residual presumably reflects VDR expression in cardiac fibroblasts heterozygous for the wild-type gene. Expression was greatly reduced (<5% of wild-type cardiac VDR mRNA levels) in the partially purified myocytes and undetectable in myocytes that were further purified by cell sorting. To confirm VDR deletion in mouse cardiomyocytes, we further characterized the VDR mRNA products from the wild-type and cardiac-specific VDR null myocytes. We reverse transcribed DNase-digested mRNA isolated from wild-type myocytes versus myocytes collected from the cardiomyocyte-specific VDR null hearts. VDR cDNA was amplified with the use of a specific primer pair positioned in exons 3 and 5. As shown in Figure IIIA in the online-only Data Supplement, a shorter VDR transcript was detected in cardiac-specific VDR-deleted myocytes, whereas the wild-type myocytes contained a transcript of the normal predicted size. Wild-type and exon 4-deleted VDR cDNAs were purified from the gel, and the cDNAs were sequenced. As expected, wild-type VDR cDNA sequences were identical to the published sequence (http://uswest.ensembl.org/index.html; Transcript: VDR-001 [ENSMUST00000023119]) (Figure IIB in the online-only Data Supplement). As shown in Figure IIIIC in the online-only Data Supplement, deletion of exon 4 led to direct, out-of-frame fusion of exon 3 with the exon 5 cDNA sequence. This fusion converts AGG (AG from exon 3 and G from exon 4) encoding arginine, to AGT (AG from exon 3 and T from exon 5) encoding serine. Furthermore, the fusion places a translation stop codon (TGA) in frame 4 amino acids further downstream (Figure IIIIC and IID in the online-only Data Supplement), implying that any protein translated through this fused carboxy-terminal segment would be very significantly foreshortened (lacking the second zinc finger in the DNA binding domain and the entire hormone binding domain). Identical sequence was obtained using the cDNA transcribed from total mRNA isolated from the cardiomyocytes of global VDR knockout mice (data not shown). These results confirm wild-type VDR gene expression in adult mouse cardiomyocytes and document the absence of VDR gene expression, assessed at the mRNA level, in cells harboring a cardiomyocyte-specific deletion of the VDR gene. Unlike the VDR−/− mice, the MLC-Cre/VDRloxPloxP mice were not hypocalcemic on a standard chow diet (Figure 2B), nor was hyperparathyroidism present (Figure 2C), supporting the cardiac specificity of the deletion.

Figure 2. Demonstration of normal plasma calcium and PTH levels in mice with deletion of VDR in cardiomyocytes. (A) RT-PCR analyses of VDR and GAPDH mRNA levels in isolated and sorted left ventricular myocytes from mice with different genotypes, as indicated. (B) Determination of the plasma calcium levels from VDRloxPloxP (C), VDR−/− (−/−), and MLC-Cre/VDRloxPloxP [M(−/−)] mice. (C) Measurement of plasma PTH levels from same mice. Bar graphs display mean and standard deviation from n = 8 to 10 per group. *P < 0.01 versus control. PTH indicates parathyroid hormone; VDR, vitamin D receptor; RT-PCR, real-time polymerase chain reaction.
We next compared the morphology of hearts from MLC-Cre/VDRloxP/H11002 mice with those of VDRloxP/H11002 mice at baseline and after 7 days of exposure to continuous ISO infusion, a maneuver that reliably generates myocardial hypertrophy in the mouse.30 As shown in Figure 3A and Table, at baseline, hearts from the cardiac-specific knockout animals were larger than those from the VDRloxP/H11002 controls. ISO infusion led to the expected increase in cardiac size in both the VDRloxP/H11002 and the MLC-Cre/VDRloxP/H11002 mice, but the increase in the latter was considerably more robust, approaching levels seen in the whole animal knockout (VDR−/−) (see Figure IVA in the online-only Data Supplement). The macroscopic changes in cardiac morphology were accompanied by hypertrophy of individual myocytes at the microscopic level. As shown in Figure 3B and 3C, myocytes from the MLC-Cre/VDRloxP/H11002 mice were significantly larger than those from the VDRloxP/H11002 animals, in the presence or absence of ISO. Interestingly, this was not accompanied by an increase in interstitial fibrosis, as assessed by Masson’s trichrome staining of myocardial tissue sections in Figure V in the online-only Data Supplement. Neither MLC-Cre/VDRloxP/H11002 mice nor VDRloxP/H11002 mice displayed >1% collagen fraction in the sections examined. Heart size was unchanged in the MLC-Cre, VDRloxPloxP, or VDRloxPloxP−/− mice versus the wild-type littermates (see Figure IVA in the online-only Data Supplement).

The morphological changes of cardiac hypertrophy were accompanied by increased expression of the fetal gene program that is typically associated with hypertrophy of neonatal and adult ventricular myocytes.31 As shown in Figure 4, expression of the ANP (Figure 4A) or alpha skeletal actin (Figure 4B), both representatives of the fetal gene program, was increased in the MLC-Cre/VDRloxPloxP−/− mice above those seen in the VDRloxPloxP−/−. Expression of these genes

### Table. Cardiomyocyte-Specific Knockout of the VDR Gene Results in Left Ventricular Hypertrophy

<table>
<thead>
<tr>
<th>Animal</th>
<th>BW (g)</th>
<th>LWV (mg)</th>
<th>LWV/BW (mg/g)</th>
<th>HR (bpm)</th>
<th>HR (bpm)</th>
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<tbody>
<tr>
<td>Control</td>
<td>39.1±7.0</td>
<td>105.7±8.1</td>
<td>2.69±0.21</td>
<td>679.7±147.0</td>
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</tr>
<tr>
<td>M(−/−)</td>
<td>38.5±3.5</td>
<td>121.8±12.3†</td>
<td>3.16±0.27†</td>
<td>676.7±91.7</td>
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</tr>
<tr>
<td>ISO</td>
<td>40.4±4.2</td>
<td>132.1±11.8†</td>
<td>3.27±0.20†</td>
<td>686.3±42.0</td>
<td></td>
</tr>
<tr>
<td>M(−/−)+ISO</td>
<td>40.3±2.8</td>
<td>150.3±9.1*</td>
<td>3.78±0.370*</td>
<td>737.0±56.0</td>
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</tbody>
</table>

*P<0.01 vs control; †P<0.05 vs M(−/−); *P<0.01 vs M(−/−)+ISO group.

**Figure 3.** Cardiac hypertrophy in cardiomyocyte-specific VDR knockout mice. (A) Left ventricular sections of hearts from mice with genotype indicated were stained by hematoxylin and eosin. Representative sections are shown. (B) Cardiac sections were stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin, which delineates cell dimensions by staining glycoprotein enveloping individual myocytes. Representative photomicrographs are shown. (C) Individual myocyte size was assessed using ImageJ (National Institutes of Health). The mean myocyte area was evaluated by measurement of 400 cells per heart (4–5 hearts per genotype). Bar graphs displaying mean and standard deviation from 8 to 12 mice per group are shown. *P<0.05; **P<0.01 versus control; #P<0.05 versus M(−/−)+ISO. M(−/−) indicates MLC-Cre/VDRloxPloxP−/−; ISO, isoproterenol.
at baseline in MLC-Cre/VDRloxP/− mice approached those levels achieved with ISO infusion in the VDRloxP/− mice. The combination of ISO in the MLC-Cre/VDRloxP/− mice led to at least an additive increase in fetal gene expression. Expression of ANP (Figure IVB in the online-only Data Supplement) and alpha skeletal actin (Figure IVC in the online-only Data Supplement) in MLC-Cre, VDRloxP/loxP, or VDRloxP/− mice was not different from the wild-type littermates.

The increment in heart size was confirmed by echocardiography in living mice (Figure 5A and 5B). The MLC-Cre/VDRloxP/− mice displayed increased thickness of the interventricular septum and posterior left ventricular free wall relative to the VDRloxP/− controls, in the presence or absence of the ISO infusion, paralleling the gravimetric findings described above. There was a reduction in end-diastolic volume and end-systolic volume in the MLC-Cre/VDRloxP/− mice (in the presence or absence of ISO), but no significant change in left ventricular systolic or diastolic function in the MLC-Cre/VDRloxP/− versus the VDRloxP/− mice (Table I in the online-only Data Supplement).

As mentioned above, although the whole animal VDR gene knockouts (VDR−/−) display cardiac hypertrophy,20 interpretation of this finding is complicated by the presence of hypertension in these animals. To provide assurance that blood pressure and, inferentially, ventricular afterload were not contributing to the development of left ventricular hypertrophy in the MLC-Cre/VDRloxP/− mice, we measured blood pressure in previously acclimated mice by the use of the tail-cuff technique.32 As shown in Figure 6A, the MLC-Cre/VDRloxP/− mice had lower diastolic and mean arterial blood pressure relative to the controls. Although systolic blood pressure tended to be lower in these mice versus controls, this difference did not reach statistical significance. The mechanism underlying the reduction in blood pressure remains unclear; however, increased ANP gene expression (Figure 4A) and modestly increased ANP secretion (Figure 6B) from the hearts of the MLC-Cre/VDRloxP/− mice might account for at least an element of the reduction in blood pressure. A similar mechanism has been suggested to account for the reduction in blood pressure noted following the myocyte-specific deletion of the type A natriuretic peptide receptor.33

Global VDR-null mice20 display an increase in renin gene expression in the heart. Renin expression was extremely low at baseline in the adult murine left ventricle. As shown in Figure VI in the online-only Data Supplement, we did not detect increased renin expression in the left ventricles of cardiomyocyte-specific VDR knockout mice. These results suggest that local renin expression is unlikely to play an important role in the cardiac hypertrophy seen in the myocyte-specific, VDR-deficient mice. Consistent with the findings of Li et al,19 we did find increased renin expression in the kidneys of our VDR−/− mice (data not shown).

To explore the mechanism underlying myocyte hypertrophy in the cardiomyocyte-specific VDR gene-deleted mice, we performed gene array analysis using conventional hybridization array methodology. In comparing wild-type with VDR-deleted cardiac myocytes (n=5 samples/group), we found that a limited number of genes (n=15) were upregulated >2-fold in the latter mice (Figure 7A and Figure VII in the online-only Data Supplement). One of these was the modulatory calcineurin inhibitory protein 1 (MClP1 or Rcan1), a gene product that functions as a downstream effector for calcineurin-dependent activity.34,35 Activation of calcineurin/ the NFAT cascade has been linked to the development of pathological myocyte hypertrophy in several experimental models,34–41 and some forms of hypertrophy are abrogated in the MClP1−/− mouse.34,35 Using real-time PCR analysis, we confirmed that MClP1 expression was selectively increased in whole hearts from VDR−/− and myocyte-specific VDR-deleted mice (Figure 7B and 7C). Using cultured neonatal rat ventricular myocytes, we showed that the hypertrophic agonist ISO increased MClP1 mRNA levels, MClP1 protein levels, and MClP1 promoter activity, whereas pretreatment with 1,25(OH)2D resulted in partial inhibition of this induction (Figure 8). These results imply that MClP1 is an important target of the liganded VDR in cardiomyocytes and suggest that antagonism of the calcineurin/NFAT/MClP1 signaling pathway plays a major role in defining the antihypertrophic activity of the liganded VDR.
Discussion

The studies reported here describe, for the first time, a direct role for the VDR in the antagonism of hypertrophic growth of the cardiac myocytes in vivo. This antihypertrophic activity may account for some fraction of the beneficial effects that the hormonal ligand for the VDR, 1,25(OH)2D, is reported to have in the cardiovascular system.42

Ventricular hypertrophy has been reported in the whole animal VDR gene knockout mouse (VDR−/−),20 but this is in the setting of 10 to 15 mm Hg elevations in systolic and diastolic blood pressure,19 systemic hyperreninemia, and the potential for dysregulated mineral homeostasis (eg, lower calcium and phosphate and elevated parathyroid hormone levels) in these mice in comparison with their wild-type counterparts. Hyperparathyroidism has been linked to elevations in blood pressure43 and cardiac hypertrophy,44 and in the studies of Park et al,17 vitamin D-dependent reversion of cardiac hypertrophy correlated with reduction in parathyroid hormone levels. The hypertrophy seen in the cardiomyocyte-specific VDR knockout (Figure 3) is somewhat less than reported in the VDR−/− mouse20; however, given the differences in arterial blood pressure (ie, estimated ∼20 mm Hg difference in BP in the cardiac-specific knockout versus VDR−/−), and the fact that the reduction in blood pressure-dependent afterload would be predicted to reduce the magnitude of the hypertrophic response, we are probably underestimating the true antihypertrophic effect of liganded VDR signaling in mice with the cardiomyocyte-specific VDR deletion.

The antihypertrophic activity described here is likely to be one of a number of beneficial effects that vitamin D and its derivatives exert in the cardiovascular and renal systems. These agents have been shown to suppress proliferation of vascular smooth muscle cells in culture45 and inhibit chole-

Figure 5. Echocardiography of mice with cardiomyocyte-specific VDR knockout in presence or absence of ISO infusion. (A) Representative M-mode images are demonstrated. (B) Thickness of interventricular septum (IVS) and posterior wall (PW) was measured, and bar graphs displaying mean and standard deviation from n=6 to 8 per group are shown. *P<0.05; **P<0.01 versus control; #P<0.05; ###P<0.01 versus M(−/−) + ISO. M(−/−), MLC-Cre/VDRlox/lox; ISO, isoproterenol.

Figure 6. Reduction of blood pressure and increase in plasma ANP level in cardiomyocyte-specific VDR gene knockout mice. (A) Mouse blood pressure was measured using tail-cuff technique described in Methods. The histogram shows the pooled data from 6 to 8 animals in each group. (B) Plasma ANP level (n=10–12 each group) was determined by ANP radioimmunoassay kit. **P<0.01 versus control. VDR indicates vitamin D receptor; ANP, atrial natriuretic peptide; C, control; M(−/−), cardiomyocyte-specific VDR knockout; −/−, whole animal knockout; MAP, mean arterial pressure; BP, blood pressure.
terol sequestration in macrophages collected from patients with diabetes mellitus, both of which would be predicted to mitigate progression of vascular lesions in diseases like atherosclerosis. The liganded VDR also reduces renin production, which could lead to beneficial effects on blood pressure and vascular structural integrity.

The liganded VDR also suppresses proliferative and synthetic activity in the interstitial compartment (ie, fibroblasts and other mesenchymal elements) of the heart. It is noteworthy that we found little evidence of interstitial fibrosis in our cardiac-specific VDR knockout, whereas fibrosis is an important feature in the whole animal knockout, implying that VDR exerts effects in the fibroblast population that are largely independent of the observed direct effects in the myocyte population.

A variety of studies support a central role for calcineurin signaling in the development of pathological cardiac hypertrophy. Calcineurin dephosphorylates and promotes the translocation of NFAT to the nucleus. This sequence of events has been shown to be both necessary and sufficient for the induction of cardiac hypertrophy. MCIP1 gene expression is tightly controlled by an alternative promoter containing multiple NFAT binding sites in the region upstream of exon 4. Several in vivo studies have demonstrated that MCIP1 expression levels correlate closely with activation of calcineurin/NFAT signaling in the heart. Indeed, MCIP1 knockout mice display a significant reduction of cardiac hypertrophy induced by pressure overload, neuroendocrine stimulation, or exercise, suggesting that MCIP1 mediates calcineurin/NFAT-induced cardiac hypertrophy. We found increased MCIP1 gene expression in hearts of cardiomyocyte-specific VDR gene-deleted mice and global VDR knockout mice, both of which displayed cardiac hypertrophy.
Treatment of rat cardiomyocytes with 1,25(OH)2D following induction with ISO, led to reduction of MCIP1 mRNA, protein, and promoter activity. Collectively, these results suggest that VDR-dependent antihypertrophic activity in the myocyte is linked to suppression of the calcineurin/NFAT/MCIP1 pathway(s).

These findings add a new and important piece of evidence to incorporate into a growing body of epidemiological,1–13 clinical,14,17 in vitro,18 and whole animal in vivo studies19 that support the need for adequate vitamin D repletion in the general population to promote cardiovascular health. This takes on particular importance given the high degree of vitamin D insufficiency that exists worldwide.1 These findings also suggest that targeted use of 1,25(OH)2D or its active analogues in clinical situations characterized by aberrant or undesirable hypertrophy of the myocardium may be useful in preventing or slowing the progression of cardiovascular diseases where this is a dominant feature.

Acknowledgments

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Disclosures

None.

References

Clinically, vitamin D has usually been linked to disorders of the skeletal system where vitamin D deficiency is associated with an increase in fracture risk. More recently, it has become clear that vitamin D has important actions in nonclassic target tissues like the immune system, heart, and vasculature. This study provides the first demonstration for a direct effect of the liganded vitamin D receptor (VDR) in the heart. Using a specially engineered mouse model, we have produced selective deletion of the VDR in the cardiac myocyte. This deletion leads to an increase in heart weight-to-body weight ratio, an increase in myocyte size without interstitial fibrosis, and activation of a gene expression program that is typically associated with hypertrophy and fibrosis. Of note, deletion of the VDR leads to activation of a signaling molecule called the modulatory calcineurin-interacting protein 1 (MCIP1) that is known to play a role in the prohypertrophic, calcineurin-dependent signaling cascade. Treatment of myocytes in culture with a potent vitamin D metabolite leads to a reduction in MCIP1 gene expression. These findings present the first definitive support for a direct beneficial effect of vitamin D and its metabolites in the atrial natriuretic peptide receptor guanylyl cyclase-A. J Clin Invest. 2003;111:1399–1407.


Cardiomyocyte-Specific Deletion of the Vitamin D Receptor Gene Results in Cardiac Hypertrophy
Songcang Chen, Christopher S. Law, Christopher L. Grigsby, Keith Olsen, Ting-Ting Hong, Yan Zhang, Yerem Yeghiazarians and David G. Gardner

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CARDIOMYOCYTE-SPECIFIC DELETION OF THE VITAMIN D RECEPTOR GENE
RESULTS IN CARDIAC HYPERTROPHY

Chen et al. Cardiomyocyte-specific VDR knockout

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Online-Only Supplement Material
SUPPLEMENTARY METHODS

Generation of cardiomyocyte-specific VDR knockout mice

To generate a floxed exon 4 in the murine VDR gene, approximately 4 kilobases (kb) of mouse genomic DNA containing introns 3-4 and exon 4 from the murine VDR gene were amplified by PCR from the genomic DNA of SV/129 stem cells. The 4 kb fragment was inserted into the Not1 site of the core targeting vector (pBS KS+) containing a loxP-FRT-NeoR-FRT cassette. To flank exon 4 with loxP sites, a short fragment consisting of a loxP site and an Ssp1 restriction site was incorporated into the BbvCI site upstream from exon 4. To complete the construct, intron 4 from the murine VDR (~4 kb) was amplified and inserted into the Kpn1 site downstream from the loxP-FRT-NeoR-FRT cassette in the targeting vector. The completed targeting vector (Fig. 1A) was confirmed structurally by PCR amplification and DNA sequencing across critical junctions of the construct. The targeting vector DNA was linearized using BcgI, purified and dissolved in sterile TE buffer. SV/129 embryonic stem (ES) cells were electroporated with linearized targeting vector. Transfected cells were plated and subjected to G418 selection. 286 clones were picked and replica-plated in 96-well format. Once confluent, one replicate plate containing cells was frozen; the second was used to prepare DNA for PCR screening using primer pair A (sense 5’ GGAAGCTGAGAAATGGGGAAC and antisense 5’ GAGAACCTGCGTGCAATCCATC). This primer was designed to detect the recombinant allele (i.e. one primer in the VDR gene sequence not contained in the targeting vector and one in the NeoR gene; see Fig. 1A). Two positive clones (Fig. 1B) were identified using primer pair A. The same positive clones from the replicate plate were thawed and expanded. DNA from these cells was prepared and subjected to PCR and Southern blot analysis (see below) to confirm structure.
(Fig. 1C). When this was completed, ES cells from the positive clones were microinjected into 50 blastocysts in the UCSF Diabetes Center Genomic Core. Injected blastocysts were then transferred into pseudopregnant C57/BL6J females. Mouse pups, representing the products of these pregnancies, were weaned and tail clippings were obtained to screen for chimeric mice (mixture of cells possessing two wild type alleles and cells possessing one wild type and one loxP-bordered [recombinant allele]). Germline transmission of the recombinant allele in F1 mice was identified by PCR using primer pair A (Supplemental Fig. 1A). Positive males were bred with C57/BL6J females (and continued to be bred into this line in subsequent generations) to establish and maintain heterozygotes. Offspring positive for the recombinant allele (i.e. flox’d VDR) were bred with the FLPe mouse. FLPe mice constitutively express the Flp gene product, a recombinase which cuts and religates DNA at FRT sites in DNA. Cross of the VDR<sup>loxP/+</sup> mouse with the FLPe mouse resulted in excision of the NeoR cassette. PCR using primer pair D (sense 5’-GATGGATTTGCACGCAGGTTCTC and antisense 5’-GTCAAGAAGGCGATAGAAGGCG) was carried out to confirm deletion of NeoR in the offspring (Supplemental Fig. 1B). Heterozygous VDR<sup>loxP/+</sup> mice were crossbred to develop homozygotes (i.e. VDR<sup>loxP/loxP</sup>) identified by PCR using primer pair B (sense 5’ CCTGGCTGTCCTGGAACTCAG and antisense 5’ GTACACCTAGTGCAGGGTG) (Supplemental Fig. 1C). Male VDR<sup>loxP/loxP</sup> mice were then mated with female Sox2-Cre mice which express the Cre recombinase in the germline to generate Sox2-Cre/VDR<sup>+/-</sup>, which, in turn, were backcrossed to the VDR<sup>loxP/loxP</sup> mice to generate VDR<sup>-/-</sup> mice, identified by PCR using primer pair C (sense 5’-GCTGACTGGGGGCTTGAGT and antisense 5’-GGTCTGAGGACAGATGCTGA) (Supplemental Fig. 1D).
**Southern blot and genotyping**

The DNA from ES cells was prepared, digested with Ssp1, fractionated on an agarose gel, and transferred to nylon membranes. The membranes were then hybridized with a $^{32}$P-labeled 0.58-kb probe outside the targeting construct region (Fig. 1A), generated through PCR amplification with primers (sense: 5’ GGCTCAATCAAGTCCAAGGG and antisense 5’ GTGTTCTGGACTCTCACCC). Genomic DNA was isolated from tail clippings with the UltraClean® Tissue & Cells DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). Genotypes were assessed by PCR with 40 nanograms of DNA in each reaction. VDR genotyping employed primer pair C, which generated a 1.2 kb band corresponding to the wild type allele, a 1.5 kb band corresponding to the recombinant allele (floxed exon 4), or a 1 kb band corresponding to the deletion allele (excision of exon 4). Amplification of VDR bands began with denaturation at 95°C for 2 min followed by 31 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 1 min and concluded with a cleanup at 72°C for 5 min. MLC-Cre genotyping of positive mice produced an amplicon roughly 550 bases in length with primers (sense 5’ CATGTTCAAGGATCGCCAGG and antisense 5’ TGCGGTGCTAACCAGCGTTTT). Amplification of the Cre band began with denaturation at 95°C for 2 min followed by 32 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 40 s and concluded with a cleanup at 72°C for 5 min.

**Isolation of adult mouse ventricular cardiomyocytes**

Adult mouse was anesthetized with 3% isoflurane and 100% O$_2$ (0.5 L/min) in an anesthesia chamber and injected with 0.5 ml heparin (100 IU/ml, i.p.) to prevent coagulation. After cervical dislocation, the heart was removed rapidly and immersed in ice-cold calcium-free perfusion buffer containing (in mmol/L) NaCl 120.4, KCl 14.7, KH$_2$PO$_4$ 0.6, Na$_2$HPO$_4$ 0.6,
MgSO₄·7H₂O 1.2, Na-HEPES 10, NaHCO₃ 4.6, taurine 30, butanedione monoxime (BDM) 10, glucose 5.5. Extraneous tissue was removed, and the aorta was cannulated under a dissection scope. After cannulation, the heart was retrograde perfused through the aorta using a Langendorff perfusion apparatus with calcium-free perfusion buffer (3ml/min) for 4 min, then switched to calcium-free digestion buffer (perfusion buffer containing collagenase II [2mg/ml] from Worthington Biochemical Co., Lakewood, NJ) for 10 min. This was followed by perfusion with digestion buffer containing 100 μmol/L CaCl₂ for another 8-10 min. After digestion, the heart was removed from the perfusion apparatus and placed in a 10 cm Petri dish containing 2 ml digestion buffer and 3 ml of stop buffer (perfusion buffer supplemented with 10% fetal bovine serum). The atria were removed and the ventricles were pulled into 10-12 equally sized pieces. The tissue was then gently dispersed into cell suspension using plastic transfer pipettes. The cell suspension was collected in a 15 ml falcon tube, brought to 10 ml with stop buffer and centrifuged at 40 g for 3 min to pellet the myocytes. Damaged myocytes and non-myocytes were removed by a series of washes in 10 ml stop buffer containing, sequentially, 100, 400, or 900 μmol/L CaCl₂. Reintroduction of calcium removes calcium intolerant damaged cardiomyocytes. Healthy rod-shape cardiomyocytes were pelleted by centrifugation at 40 g for 3 min after each wash. After the final wash, cardiomyocytes were resuspended in 10 ml cardiomyocyte culture medium (Catalog #6201, ScienCell Research Lab., Carlsbad, CA) for cell sorting or preparation of DNA or RNA.

**Total RNA isolation, cDNA sequencing and real-time PCR (RT-PCR)**

Total RNA was isolated from left ventricles or cardiomyocytes with Trizol Reagent (Invitrogen, Carlsbad, CA) and the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse
transcribed into cDNA with SuperScript III (Invitrogen). VDR cDNA was used as a template to generate an amplicon encompassing exon 3 through 5 sequences with primers (sense 5’ GGCTTCCACTTCAACGCTAT and antisense 5’ TTACGTCTGCACGAATTGGA).

Amplicons were gel purified with the QIAquick Gel Extraction kit (Qiagen) and sequenced (Sequetech, Mountain View, CA) with the primer 5’ ACGAATTGGAGGCCGGAAGT.

Real-time PCR was performed with mouse ANP (Mm01255748_g1), α skeletal actin (αSA) (Mm00808218_g1) and GAPDH (4352932E) Taqman primers (Applied Biosystems, Foster City, CA) and VDR (sense 5’ AGGACAAACCGCGACACT and antisense: 5’ CTTACGCTGCACCTCCTCAT), MCIP 1 (sense 5’ AGCTCCCTGATTGCTTGTGT and antisense 5’ GCAGATAAGGGTTGCTGAA), renin (sense 5’-GAGGCCTTCCTTGACCAATC-3’ and antisense 5’-TGTGAATCCCACAAGCAAGG-3’), and GAPDH (sense 5’ GACGTGCCGCCTGGAGAAAC and antisense 5’ AGCCCAAGATGCCCTTCAGT) SYBR Green primers; or rat MCIP 1 (sense 5’ AGCTCCCTGATTGCTTGTGT and antisense 5’ TTTGGCCCTGGTCTCACTTT) or rat GAPDH (sense 5’ GACATGCGCGCTGGAGAAAC and antisense 5’ AGCCCAAGATGCCCTTCAGT) SYBR Green primers. ANP, VDR, αSA, and MCIP1 mRNA levels were normalized to GAPDH mRNA expression. Real-time PCR was performed on the ABI Prism 7900HT (Applied Biosystems).

**Western blot analysis**

A small segment of tail from mice was immediately homogenized or cardiomyocytes were directly lysed in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 10% glycerol, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium
fluoride, 1 mmol/L glycerophosphate) containing protease inhibitors (1 Complete tablet/50 ml, Roche Diagnostics, Indianapolis, IN). Forty micrograms of total protein was denatured at 100°C for 5 minutes, subjected to 10% SDS-PAGE, and transferred onto ImmunBlot PVDF membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat milk in TBST (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween-20) and probed with a rabbit polyclonal antibody directed against VDR (sc-1008; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or with rat polyclonal antibody directed against MCIP 1 (a gift kindly provided by Dr. Timothy A. McKinsey, University of Colorado Denver, CO). An HRP-conjugated secondary antibody was used to detect immunoreactive bands with SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL). The same membranes were rebotted with anti-GAPDH antibody (sc-32233; Santa Cruz Biotechnology Inc.). VDR signals were quantified using a Kodak Scientific Imaging system and normalized to the GAPDH signal.

Neonatal cardiomyocyte preparation and luciferase assay
Neonatal ventricular myocytes were prepared from one day-old Sprague-Dawley rats (Charles River, Wilmington, MA) as described previously 1. Cells were maintained in Dulbecco’s modified Eagle’s medium H-21 supplemented with 10% enriched calf serum (Gemini Bioproducts, West Sacramento, CA) for 36 hours. A portion of the cells were transfected with MCIP 1-Luc and Renilla-Luc using lipofectin reagent (Invitrogen) as reported previously 2. Twenty-four hours after transfection, cells were incubated with different doses of 1,25 (OH)2 D for 48 hours with or without 10-6 mol/L isoproterenol (ISO) for the final 24 hours. At that time point, the cells were collected and lysed. Luciferase activity was measured using the Dual-Luciferase kit (Promega). MCIP 1-Luc activity was normalized for Renilla luciferase activity.
The remainder of the myocytes were changed from media containing 10% enriched calf serum to media containing 10% serum substitute for 24 hours. At that point, fresh media containing different concentrations of 1,25 (OH)₂ D was added. Cells were cultured for another 48 hours, and 10⁻⁶ mol/L ISO or vehicle was added, where indicated, in the final 24 hours. Total protein and mRNA were prepared for Western blot and RT-PCR analysis, respectively, as described above.

**Supplemental Figure Legends**

**Supplemental Fig. 1.** Generation of floxed VDR mice. **A:** Germline transmission of recombinant allele was confirmed in F1 mice by PCR using primer pair A, identified in Fig 1A, and genomic tail DNA as template. **B:** Neo gene-deleted F2 mice were obtained by mating F1 mice with Flpe mice. PCR using primer pair D for Neo gene and primer B pair for the inserted loxP site, as shown in Fig. 1A, was used to confirm genotype. **C:** F3 mice with VDRloxp/loxP genotype were obtained by mating VDRloxp/+ with VDRloxp/+ mice; offspring were screened using primer pair B. **D:** VDR deletion (VDR⁻/⁻) was obtained by mating VDRloxp/loxP mice with Sox2-Cre mice. VDR allele screen was carried out using primer pair C.

**Supplemental Fig. 2.** Development of the global VDR knockout mice by deleting recombinant VDR alleles in germline. **A:** Western blot showed no VDR protein expression in VDR⁻/⁻ mice. **B:** VDR⁻/⁻ mice displayed alopecia and reduced body weight.
Supplemental Fig. 3. The measurement of the quality of templates which guide VDR protein synthesis in cardiac-specific VDR-null cardiomyocytes. A: Amplification of VDR cDNA by PCR from a cDNA library generated from total mRNA of wild type and cardiac-specific VDR-null cardiomyocytes. B&C: Sequencing wild type and VDR- deleted cDNAs. D&E: Comparison of the cDNA and predicted amino acid sequences in wild type vs. cardiac-specific VDR-null cardiomyocytes. Stop codons: TGA (*), TAA are in bold font. Dashed line indicates deleted nucleotide sequence.

Supplemental Fig. 4. M(-/-) and VDR^loxP/loxP mice are phenotypically indistinguishable from VDR^+/+ mice. A: Ratio of left ventricular weight:body weight from mice with the indicated genotypes. B: RT-PCR analysis of left ventricular ANP/GAPDH mRNA levels in the groups indicated. C: RT-PCR analysis of left ventricular αSA/GAPDH mRNA levels in the groups indicated. Bar graphs display mean and standard deviation from 8 animals in each group. **P<0.01 vs. control.

Supplemental Fig. 5. Cardiomyocyte-specific VDR deletion does not lead to cardiac fibrosis. Left ventricular sections were stained with Masson’s Trichrome stain. Representative photomicrographs are shown (n=4-5 per group).

Supplemental Fig. 6. M(-/-) mice show no increase in ventricular renin gene expression. Total RNA was extracted from left ventricular tissue and RT-PCR was carried out to measure renin
and GAPDH mRNA levels. Renin expression was normalized to GAPDH levels. Bar graph showing mean and standard deviation is derived from 4 samples per group.

**Supplemental Fig. 7.** A heatmap displays the expression profile of the genes shown in Fig 7. based on the normalized intensities in the isolated ventricular myocytes of control (n=5) and cardiomyocyte-specific VDR knockout (n=5) mice. C: Control, M: Cardiomyocyte-specific VDR knockout.

**Supplemental Table 1.** Echocardiographic parameters of control vs cardiomyocyte-selective VDR knockout mice in presence and absence of isoproterenol infusion.

**References:**


Suppl. Fig. 1.

A

B

C

D

Recombinant/Floxed allele
Wild type allele
Neo Cassette

Floxed allele
Wild type allele
Deletion allele
Suppl. Fig. 3.

A

C  M(-/-)

380 bp  250 bp

B

Exon 3-4 border

C

Exon 3-5 border
Suppl. Fig. 3.

D

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Suppl. Fig. 4.

A

LVW/BW (mg/kg)

**

+/

MLC-Cre

Loxp/Loxp

Loxp/-

-/

B

ANP/GAPDH mRNA

(FOILD INDUCTION)

**

+/+

MLC-Cre

Loxp/Loxp

Loxp/-

-/

C

α.SA/GAPDH mRNA

(FOILD INDUCTION)

**

+/+

MLC-Cre

Loxp/Loxp

Loxp/-

-/-
Suppl. Fig. 5.

C  M(-/-)  ISO  M (-/-) + ISO
Suppl. Fig. 6.

Renin/GAPDH mRNA (FOLD INDUCTION)

C  M (-/-)
Suppl. Fig. 7.

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**Expression (log2 scale):**

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*P<0.05, **P<0.01 vs. Control, †P<0.05 vs. M(-/-) alone; #P<0.05, ##P<0.01 vs. M(-/-) + ISO group