Cardiac phenotype induced by a dysfunctional \( \alpha_{1C} \) transgene
A general problem for the transgenic approach

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**Key words:** transgenic animals, MRI, change of phenotype

**Abbreviations:** BW, body weight; CO, cardiac output; ED, end-diastolic; EF, ejection fraction; ES, end-systolic; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HR, heart rate; HW, heart weight; ISO, isoproterenol; LV, left ventricle; ORF, open reading frame; SV, stroke volume; TG, transgenic; UTR, untranslated region; veh, vehicle; WT, wild-type

Based on stable integration of recombinant DNA into a host genome, transgenic technology has become an important genetic engineering methodology. An organism whose genetic characteristics have been altered by the insertion of foreign DNA is supposed to exhibit a new phenotype associated with the function of the transgene. However, successful insertion may not be sufficient to achieve specific modification of function. In this study we describe a strain of transgenic mouse, G7-882, generated by incorporation into the mouse genome of human \( \alpha_{1.2} \) \( \alpha_{1C} \) cDNA deprived of 3'-UTR to exclude transcription. We found that, in response to chronic infusion of isoproterenol, G7-882 develops dilated cardiomyopathy, a misleading “transgenic artifact” compatible with the expected function of the incorporated “correct” transgene. Specifically, using magnetic resonance imaging (MRI), we found that chronic \( \beta \)-adrenergic stimulation of G7-882 mice caused left ventricular hypertrophy and aggravated development of dilated cardiomyopathy, although no significant changes in the kinetics, density and voltage dependence of the calcium current were observed in G7-882 cardiomyocytes as compared to cells from wild type mice. This result illustrates the possibility that even when a functional transgene is expressed, an observed change in phenotype may be due to the artifact of “incidental incorporation” leading to misleading conclusions. To exclude this possibility and thus provide a robust tool for exploring biological function, the new transgenic phenotype must be replicated in several independently generated transgenic strains.

**Introduction**

Transgenic (TG) technology is based on stable integration of a recombinant DNA into a host genome with the aim of modifying the phenotype of the host to include new functions associated with the transgene. After three decades of exploration, this technology has become an indispensable research tool and an important genetic engineering methodology used in biotechnology and medicine. Conceptually, however, a transgene may produce its effect on the host’s phenotype either (A) as the result of its expression or (B) indirectly, by virtue of its incidental incorporation into the host’s genome. The problem is that A and B may occur simultaneously, when a functional transgene may get expressed, but the resulting modification of the phenotype may have nothing to do with its properties, being rather due to the incidental incorporation. In this case one can’t separate A and B based on the observation of the “expected” induction of a functional pattern associated with the transgene. Here, we report a previously never characterized example of this phenomenon in which the incorporation of a human calcium channel \( \alpha_{1C} \) transgene deprived of 3'-UTR caused, in the absence of any evidence of transgene expression, a cardiac effect that one would expect in response to calcium channel overexpression. Because the new cardiac phenotype in our experiment can only be ascribed to incidental incorporation, one can expect that this artifact would occur even with the “functional” \( \alpha_{1C} \) transgene not deprived of 3'-UTR. It appears that the vast majority of TG studies have this potential source of misinterpretation. Our study of the modified cardiac phenotype of the TG mouse strain carrying a dysfunctional human \( \alpha_{1C} \) transgene clearly defines the problem and offers a new essential requirement for TG studies: to provide an adequate tool for exploring biological function.
the new phenotype must be replicated simultaneously in several independently generated TG strains.

**Results**

**Generation of homozygous G7-882 TG mouse.** Voltage-gated Ca1.2 calcium channels play a central role in controlling cardiovascular function.1 The channel is composed of the pore-forming α1C and accessory β and δ subunits.4 The Ca1.2 α1C subunits are subject to alternative splicing that generates multiple splice variants.5 The transgenic approach can provide important insights into the physiological significance of the calcium channel transcripts.6,7 The aim of this study was to generate a transgenic (TG) mouse strain carrying dysfunctional α1C cDNA to be used as a control TG line for calcium channel studies. To generate this TG mouse strain, we used the fusion construct (GFP-α1C,β77) of N-terminal GFP with human α1C,β77 lacking 94.2% of the α1C 3'-UTR. By deleting a large portion of the 3'-UTR, we eliminated post-transcriptional control of the transgene α1C expression by the majority of potential poly(A) binding proteins.8 Using PCR genotyping, we identified six homozygous TG strains carrying CMV-GFP-HLCC77 cDNA lacking 3'-UTR, and strain α1C,β77 lacking 94.2% of the

| Target          | Nucleotide sequence                  | Product size (bp) |
|-----------------|--------------------------------------|-------------------|
| 18S RNA         | 5'-AGG GGA GAG GAG GGT AAG GA A-3'   | 240               |
|                 | 5'-GGA CAG GAC TAG GGG GAA CA-3'    |                   |
| GAPDH           | 5'-GGT GAA GGT GGG TGG GAA CG-3'    | 200               |
|                 | 5'-CTC GCT CCT GGA AGA TGG TG-3'    |                   |
| Mouse α1C (M1) | 5'-GCT CTC TTC ACC GTC TCC AC-3'    | 177               |
|                 | 5'-GAC GAA ACC CAC GAC GAT GT-3'   |                   |
| Mouse α1C (M2) | 5'-TGA ACA TGG CTC TGA ACA GC-3'    | 113               |
|                 | 5'-CCT CAT TGG CTT GCT CTA GG-3'    |                   |
| GFP-α1C,β77 (1)| 5'-ATG GTC CTG CTG GAG TTT GT-3'    | 178               |
|                 | 5'-GCA TGG GCA TCT AGT TG G-3'     |                   |
| GFP-α1C,β77 (2)| 5'-ACA TGG TCC TGG TGG AGT TT-3'   | 180               |
|                 | 5'-GCA TGG GCA TCT ATG TGG G-3'    |                   |

with nested sense and antisense primers M2 (Table 1). Using GAPDH as a reference, the difference between TG and WT mice was estimated to be 1.52 ± 0.50- and 2.10 ± 0.84-fold for the M1 and M2 primers (Table 1), respectively, while using 18S RNA as a reference, those values were 0.45 ± 0.16 (M1) and 0.63 ± 0.27 (M2), respectively (n = 4). To exclude the presence of the nuclear GFP-α1C transcripts, we isolated cytoplasmic and nuclear RNA fractions from the myocardium of G7-882 mice and subjected both fractions to RT-PCR as described above. Results of this analysis showed no evidence of the presence of the GFP-α1C transcripts in either fraction. Lack of GFP-α1C expression was also confirmed by the western blot analysis of the myocardial tissue isolated from G7-882 and control WT mice (Fig. 1).

Immunoprecipitation and western blot analysis with an anti-α1C antibody revealed only the WT (endogenous) α1C band distinctly different by mass from GFP-α1C. No GFP reactivity was observed in the WT and G7-882 myocardium samples with an anti-living color (anti-GFP) antibody.

Electrophysiological evaluation confirmed that the properties of the net Ca2+ current in cardiac myocytes were not significantly different in G7-882 as compared with the controls (Fig. 2). The current density (I_{Ca,park} = 7.7 ± 0.3 pA/pF, n = 8) and time constant of inactivation of the maximum calcium current in G7-882 cells (τ = 104 ± 4 ms, n = 42) were not significantly different from those observed in control WT cells (I_{Ca,park} = 7.3 ± 0.2 pA/pF, n = 11; τ = 107 ± 8 ms, n = 25) (Fig. 2A and B). The averaged characteristics of current-voltage relations measured in cardiac myocytes from WT and TG mice also were not significantly different (Fig. 2C). Taken together, these results show that G7-882 did not express GFP-α1C and retained its endogenous calcium channels intact.

**General characterization of G7-882 transgenic mice.** Because of the small size and rapid heart rate of mice, functional analysis of cardiac phenotype in transgenic mice remains challenging.3 Successful miniaturization of the well-characterized isolated perfused heart preparation has been demonstrated using either the working heart or Langendorff perfusion models.10–12 However, the isolated heart is unsuitable for the study of complex pathophysiology of the intact cardiovascular system due to the absence of neurohumoral regulation and blood circulation. Thus, in vivo studies are essential for a thorough investigation of the consequences of genetic alteration.

G7-882 mice did not show any distinct physiological abnormalities under normal physiological conditions. To uncover possible cardiac abnormalities, we applied sustained stimulation of endogenous β-adrenergic receptors (β-AR’s) which are assembled with Ca1.2 channels in signaling complexes.13 This is a relevant approach to test for cardiac abnormalities associated with calcium channels because β-AR-mediated signal transduction pathways involving adenyl cyclase and protein kinase A regulate Ca2+ influx through Ca1.2 channels.14 β-AR-stimulation is the dominant mechanism of positive chronotropy and inotropy in the heart.15 Activation of β-AR’s enhances cardiac L-type Ca2+ channel current most likely by phosphorylating specific sites on the channel protein.16,17 As with other adrenergic receptors, β-adrenergic receptor function is a dynamically regulated
component of normal physiological adaptation for maintaining homeostasis. During pathologic processes, such regulation can be compensatory or it can contribute to the pathophysiology of the condition. It has been reported that sustained adrenergic stimulation results in the development of pathological hypertrophy with progression to heart failure in animal models and humans. To investigate whether G7-882 transgenic mice may develop pathological cardiac conditions in response to sustained adrenergic stimulation, we compared the effects of chronic ISO-induced stress on the physiological and cardiovascular characteristics of G7-882 and WT mice.

**Mortality.** No elevated mortality of G7-882 or WT mice was observed during the continuous 7-day infusion of either vehicle or ISO different at a dose of 30 mg/kg/day (n = 6; Sup. Table 1). However, at a higher ISO dose of 45 mg/kg/day, the mortality was 41% in G7-882 (n = 17) as compared with 16% in WT mice (n = 6). The mortality was greater still on infusion of ISO at a dose 60 mg/kg/day, which resulted in 100% mortality in G7-882 as compared with 0% in WT mice (n = 6). Thus, G7-882 exhibited higher sensitivity to β-adrenergic stimulation under our chronic isoproterenol stress conditions.

**Body weight (BW).** Direct measurements showed that the BWs of the vehicle and ISO-treated mice were not significantly different at a dose of 30 mg/kg/d (Table 2). The heart weight (HW) and the HW/BW ratio of WT and G7-882 mice were markedly higher for both tested doses of ISO (30 and 45 mg/kg/d) as compared to the corresponding vehicle-treated mice. The HW/BW ratio was 32% (p < 0.001) and 50% (p < 0.0001) higher in G7-882 mice with infusion of ISO at a rate of 30 and 45 mg/kg/d, respectively, as compared to the corresponding vehicle-treated G7-882 mice.

**Heart rate.** No differences in the heart rate (HR) were observed between WT and G7-882 mice treated with vehicle (Sup. Table 2). In contrast, ISO-induced stress significantly increased the HR in both WT and G7-882 mice 24 h of infusion, but this increase in HR was not significantly different between WT and G7-882 mice. After seven days of chronic ISO-induced stress at a dose of 30 mg/kg/d, WT mice showed a significantly higher HR than G7-882 mice, but there was no significant difference in HR between WT mice infused at a rate of 30 versus 45 mg/kg/d.

**Hypertrophic response as detected by in vivo MRI.** Magnetic resonance imaging (MRI) has been shown to yield accurate and reliable quantification of cardiac function and morphology in studies of both animals and man. MRI is a three-dimensional tomographic technique which is uniquely suited for the assessment of volumetric and functional changes in hearts with abnormal morphology. In the present work, MRI was used to detect changes in myocardial morphology and systolic and diastolic left ventricular volumes before and after chronic isoproterenol stress. Our results showed that under ISO-induced stress, G7-882 transgenic mice developed an enhanced degree of LV hypertrophy, which resulted in a progression to dilated cardiomyopathy and severe contractile dysfunction.

A positive correlation has been reported between LV mass, as measured either by in vivo echocardiography or ex-vivo weighing, and LV functional changes during pharmacological...
ventricular mass due to partial volume effects, which can occur in areas with high curvature such as near the apex of the heart. However, to minimize this error, we carefully identified the long axis of the heart and carefully chose the orientation of the short axis images. We employed a ciné MRI imaging technique using the shortest possible echo time, which prevented image artifacts from turbulent blood flow. Although the motion of the heart may cause artifacts especially given the high heart rate in mice, we used ECG and respiratory gating with careful control of the triggering point to give images with high spatial resolution and negligible motion artifacts.

The short total scan times achieved using the ciné MRI imaging technique are particularly important in light of anesthesia, which must be used for immobilization during in vivo MRI in the mouse. Many general anesthetics cause respiration depression and negative chronotropic and inotropic effects. Inhalation anesthesia with isoflurane was chosen because it is easy to administer and control during scanning, facilitating stress. Moreover, the assessment of LV functional changes in a gene-targeted mouse model of heart failure has been addressed by echocardiography. However, echocardiography measures are based on geometric assumptions, which may no longer be valid when the ventricle undergoes asymmetrical shape changes during remodeling. In contrast, magnetic resonance imaging, as a three dimensional tomographic technique offers quantitative information without relying on geometric assumptions and provides high temporal and spatial resolution. We performed MRI at the high magnetic field strength of 7T, which offered excellent signal-to-noise ratio, allowing us to perform rapid imaging with high spatial resolution. Due to saturation of the nuclear spins within the myocardium by frequent radio frequency pulse application, good contrast between blood and myocardium was obtained. This contrast resulted in very sharp endocardial borders, permitting accurate measurements of LV mass and volume (Fig. 3) and a good agreement between ex-vivo and MRI-determined LV weight was achieved. It has been observed that MRI is subject to overestimation errors when measuring left ventricular mass due to partial volume effects, which can occur in areas with high curvature such as near the apex of the heart. However, to minimize this error, we carefully identified the long axis of the heart and carefully chose the orientation of the short axis images.

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### Table 2. Comparison of in-vivo MRI (i) (at end-diastole) and ex-vivo (e) assessment of total heart and LV weight in WT and G7-882 mice infused for seven days with vehicle or ISO at a dose of 30 or 45 mg/kg/d

| Genotype | Drug (mg/kg/d) | BW (g) | HW* (mg) | HW*/BW, (mg/g) | LV* (mg) | LV/BW (mg/g) | LV*/BW (mg/g) | N  |
|----------|----------------|--------|-----------|---------------|----------|--------------|---------------|----|
| WT       | veh (30)       | 30.6 ± 2.8 | 114.6 ± 9.1 | 3.8 ± 0.2     | 84.4 ± 11.2 | 87.3 ± 13.3  | 2.8 ± 0.3     | 5  |
| ISO (30) |                | 29.5 ± 1.2 | 123.2 ± 9.9 | 4.2 ± 0.4     | 101.8 ± 8.1* | 100.7 ± 8.3  | 3.5 ± 0.3*    | 6  |
| WT       | veh (30)       | 30.8 ± 0.9 | 117.9 ± 7.5 | 3.8 ± 0.2     | 95.7 ± 6.4  | 94.2 ± 10.2  | 3.1 ± 0.2     | 5  |
| ISO (30) |                | 29.8 ± 2.8 | 1479 ± 16.5** | 5.0 ± 0.2**   | 119.5 ± 9.1** | 116.0 ± 9.9** | 4.0 ± 0.3**   | 6  |
| WT       | veh (45)       | 34.2 ± 1.3 | 115.8 ± 10.0 | 3.4 ± 0.2     | 94.4 ± 6.9  | 92.3 ± 8.1   | 2.8 ± 0.2     | 5  |
| ISO (45) |                | 30.4 ± 0.9 | 128.6 ± 9.3 | 4.2 ± 0.3*    | 109.6 ± 7.8* | 106.7 ± 7.1* | 3.6 ± 0.2*    | 5  |
| WT       | veh (45)       | 34.8 ± 2.5 | 119.1 ± 4.3 | 3.4 ± 0.3     | 97.4 ± 4.6  | 95.3 ± 5.8   | 2.8 ± 0.2     | 7  |
| ISO (45) |                | 31.0 ± 1.0* | 157.1 ± 6.2** | 5.1 ± 0.2**   | 132.9 ± 6.8** | 129.7 ± 11.7** | 4.3 ± 0.2**   | 9  |

Mean ± SD. HW, total heart weight; BW, body weight; LV, left ventricle weight. *p < 0.05, ISO vs. veh; †p < 0.05, WT vs. TG with veh; ‡p < 0.05, WT vs. TG with ISO.

**Figure 3.** MR images of mouse hearts at the end-diastolic phase. Shown are representative MR images in the four-chamber (A) and mid-ventricular short axis views (B). Short axis MR images were acquired after seven days of vehicle (parts a, c, e and g) or ISO (30 mg/kg/d) administration (parts b, d, f and h) in WT (parts a, b, e and f) and G7-882 mice (parts c, d, g and h). Arrows point to the aorta (Aor), right atrium (RA), right ventricle (RV), septum (SEP), left atrium (LA), left ventricle (LV), right ventricle free wall (RVW), right ventricle cavity (RVC), left ventricle cavity (LVC), left ventricle free wall (LVW) and papillary muscle (PM).
the maintenance of stable cardiac function while inducing a relatively low degree of negative chronotropic effects. 39 In previous Kubota et al.34 observed a depressed mean heart rate of 218 bpm for intraperitoneal tribromoethanol (Avertin) administration.33 MRI studies in mice, a mean heart rate of 356 bpm was reported and ejection fraction values (demonstrating baseline LV end-diastolic volume, cardiac output isoflurane, MRI in mice could be performed closer to realistic assessed at end-diastole revealed no significant differences in LV volume and mass.

Table 3. Comparison of LV end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), ejection fraction (EF) and cardiac output (CO) in WT and G7-882 mice in response to vehicle or chronic (7 d) ISO stress at two doses

| Genotype | Drug (mg/kg/d) | EDV (μl) | ESV (μl) | SV (μl) | EF (%) | CO (ml/min) | n   |
|----------|----------------|----------|----------|---------|--------|-------------|-----|
| WT       | veh (30)       | 35.8 ± 4.8 | 9.4 ± 1.8 | 26.4 ± 5.6 | 73.2 ± 6.6 | 14.1 ± 2.6 | 5   |
|          | ISO (30)       | 53.2 ± 7.6* | 18.5 ± 7.2* | 34.7 ± 6.1* | 65.8 ± 10.8 | 19.0 ± 2.7* | 6   |
| G7-882   | veh (30)       | 37.0 ± 13.8 | 10.8 ± 4.1 | 26.2 ± 11.3 | 69.7 ± 11.4 | 13.0 ± 5.7 | 5   |
|          | ISO (30)       | 48.5 ± 9.2 | 25.8 ± 9.1* | 22.7 ± 8.0* | 46.9 ± 16.8* | 12.1 ± 4.0* | 6   |
| WT       | veh (45)       | 37.0 ± 2.2 | 11 ± 1.4  | 26.0 ± 2.1 | 70.3 ± 3.6 | 14.8 ± 1.1 | 5   |
|          | ISO (45)       | 50.4 ± 8.2* | 25.4 ± 11.1 | 25.4 ± 7.0 | 49.8 ± 8.7* | 12.1 ± 3.7 | 5   |
| G7-882   | veh (45)       | 42.3 ± 7.5 | 13 ± 2.6  | 29.3 ± 5.8 | 69.1 ± 4.8 | 13.9 ± 2.4 | 7   |
|          | ISO (45)       | 57.3 ± 6.9* | 35.6 ± 4.3* | 21.8 ± 6.1* | 37.5 ± 8.1* | 9.5 ± 3.0* | 9   |

Mean ± SD. *p < 0.05, ISO vs. veh; ‡p < 0.05, WT vs. G7-882 with ISO.

Effects of chronic β-adrenergic stimulation on cardiac function of transgenic G7-882 mice. MRI did not reveal a significant difference in end-diastolic LV volume between WT and TG mice infused with vehicle only. Chronic ISO infusion at 45 mg/kg/day resulted in significantly greater LV EDV in WT and G7-882 mice as compared to the corresponding vehicle-treated mice (Table 3). In response to chronic β-adrenergic stimulation, ESV was significantly greater in both WT and G7-882 mice as compared to the corresponding vehicle-treated mice. MRI revealed a larger LV cavity size at systole in chronically ISO-treated G7-882 as compared to WT mice under ISO stress (Fig. 4), indicating that G7-882 mice developed a severe left ventricular contractile dysfunction. At an ISO dose of 30 mg/kg/d, G7-882 mice exhibited a substantially smaller SV as compared to ISO-treated WT mice (Table 3). No differences were detected in ejection fraction in WT mice at an ISO dose of 30 mg/kg/day; however, at the higher ISO dose of 45 mg/kg/day, the EF was 29% lower (p < 0.05) as compared to the respective vehicle-treated WT mice (Table 3). Under conditions of sustained ISO stress, G7-882 mice showed a significantly reduced EF that was 33% (p < 0.05) and 46% (p < 0.01) lower in the 30 and 45 mg/kg/day ISO-treated groups, respectively, as compared to the respective vehicle-treated G7-882 mice (Table 3). Both tested doses of ISO (30 and 45 mg/kg/day) revealed that EF was significantly lower in TG mice as compared to that of WT mice, further indicating that TG mice developed severe contractile dysfunction. Due to their higher heart rate, WT mice treated with ISO at the lower dose (30 mg/kg/day) exhibited 35% greater cardiac output than vehicle treated WT mice. However, ISO-treated TG mice showed insignificantly lower cardiac output when compared to vehicle treated TG mice (Table 3). Finally, at the higher dose of ISO (45 mg/kg/day), TG mice exhibited significantly lower cardiac output than their respective vehicle-treated controls.

Measurement of LV geometry revealed that ED wall thickness in WT and G7-882 mice was significantly greater in ISO-treated mice as compared to corresponding vehicle-infused mice at a dosage of 30 mg/kg/d (Table 4). No other significant differences in ES wall thickness were found. The end-systolic epicardial diameter in ISO-stressed G7-882 mice was significantly greater by 10% (p<0.05) at both doses as compared to the corresponding groups of ISO-treated WT mice (Table 4). In addition,
pathology developed in WT and TG mice in response to chronic administration of vehicle (Fig. 5C and D, parts a and c). Focal areas of fibrosis, however, were observed in the left ventricles of WT and TG mice after seven days of chronic β-adrenergic stimulation (Fig. 5C and D, parts b and d). Dense fibrous tissue replaced most of the muscle fibers in this area while within some muscle fibers of G7-882 mice treated with ISO, clear vacuoles infiltrated between the muscle fibers. Masson’s trichrome staining for collagen (Fig. 5E and F) showed that no collagen accumulation (blue staining) was present in WT and TG mice infused with vehicle (Fig. 5E and F, parts a and c). Focal areas of fibrosis were observed within the left ventricles, septae and papillary muscles of WT and G7-882 mice subjected to ISO-induced stress (parts b and d). Multifocal lesions, increased interstitial fibrosis, enlarged myocytes, enlarged bizarre nuclei, sometimes with vacuoles, focal cytoplasmic vacuoles in myocytes and evidence of myocyte degeneration atrophy and cell loss were markedly more prevalent in the hearts of G7-882 mice chronically treated with the higher dose of ISO (45 mg/kg/d) as compared to wild type

d-end systolic endocardial diameter was significantly larger in WT mice infused with ISO at 30 and at 45 mg/kg/d, as compared to corresponding vehicle treated mice (Table 4). In summary, these results indicate that chronic ISO stress caused the loss of LV contraction during systole and that this effect was more pronounced in TG than in WT mice.

Histopathology. Consistent with the MRI data, gross morphology and histological sections showed a greater overall LV size in G7-882 and WT mice infused with ISO than in corresponding vehicle-treated animals (Fig. 5). Moreover, the development of cardiac hypertrophy in response to ISO-induced stress appeared to be more pronounced in G7-882 mice (Fig. 5A and B, part d) as compared to WT mice (Fig. 5A and B, part b). A more detailed histological examination demonstrated that mild to severe myocyte hypertrophy and fibrosis were present in the hearts of chronically ISO treated WT and TG mice, whereas pathological lesions were essentially absent in TG and WT mice subjected to vehicle infusion. Specifically, hematoxylin and eosin (H&E) staining showed that no left ventricular

Table 4. Effect of sustained (7 d) ISO-induced stress on LV wall thickness and thickening and LV diameter in WT and transgenic G7-882 mice

| Genotype | Drug (mg/kg/d) | Wall thickness | Epicardial diameter | Endocardial diameter |
|----------|----------------|----------------|---------------------|---------------------|
|          |                | ED (mm)  | ES (mm)  | Systolic thickening (mm) | ED (mm)  | ES (mm)  | ED (mm)  | ES (mm)  | n |
| WT       | veh (30)       | 0.89 ± 0.04 | 1.41 ± 0.08 | 0.51 ± 0.09 | 4.94 ± 0.43 | 4.53 ± 0.45 | 2.51 ± 0.38 | 0.94 ± 0.22 | 5 |
| ISO (30) | 1.09 ± 0.14*   | 1.44 ± 0.05 | 0.35 ± 0.14* | 5.38 ± 0.53 | 4.96 ± 0.32 | 3.05 ± 0.74 | 1.76 ± 0.51* | 6 |
|          | veh (30)       | 0.99 ± 0.07† | 1.38 ± 0.03 | 0.38 ± 0.05† | 5.39 ± 0.48 | 5.11 ± 0.42 | 2.59 ± 0.56 | 1.27 ± 0.29 | 5 |
| ISO (30) | 1.22 ± 0.2*    | 1.47 ± 0.12 | 0.25 ± 0.17 | 5.52 ± 0.48 | 5.46 ± 0.41| 3.18 ± 0.55 | 2.15 ± 0.61* | 6 |
| WT       | veh (45)       | 0.91 ± 0.06 | 1.45 ± 0.07 | 0.53 ± 0.11 | 4.89 ± 0.4 | 4.8 ± 0.55 | 2.56 ± 0.35 | 1.02 ± 0.13 | 5 |
| ISO (45) | 1.08 ± 0.17    | 1.47 ± 0.09 | 0.39 ± 0.17 | 5.12 ± 0.42 | 5.12 ± 0.22 | 3.17 ± 0.59 | 1.87 ± 0.53* | 5 |
|          | veh (45)       | 0.98 ± 0.12 | 1.33 ± 0.13 | 0.35 ± 0.17 | 5.08 ± 0.19 | 4.98 ± 0.43 | 2.43 ± 0.62 | 1.30 ± 0.28 | 7 |
| ISO (45) | 1.2 ± 0.12*    | 1.46 ± 0.11 | 0.32 ± 0.08 | 5.49 ± 0.29* | 5.65 ± 0.31* | 3.18 ± 0.21* | 2.21 ± 0.29* | 9 |

ED, end-diastolic; ES, end-systolic. Values are mean ± SD. *p < 0.05 for ISO treated vs. veh groups. †p < 0.05 for vehicle-treated WT vs. G7-882 groups. ‡p < 0.05 for WT vs. G7-882 groups after ISO-induced stress.
enhanced cardiac dysfunction and more severe histopathological abnormalities than WT mice receiving the same dose of ISO.

Chronic β-adrenergic stimulation at an ISO dose of 45 mg/kg/d resulted in significantly greater LV EDV and ESV in both WT and G7-882 mice when compared to mice infused with vehicle (Table 3). MRI revealed deformation of the left ventricular wall (Table 4), suggesting loss of relaxation and contraction and the development of both eccentric and concentric hypertrophy. The effect of chronic β-adrenergic stimulation was significantly greater in G7-882 mice than in WT mice. Furthermore, left ventricular SV, EF and CO were significantly lower in TG mice following chronic administration of ISO at a rate of 30 mg/kg/d as compared to WT mice under the same conditions (Table 3). Under chronic ISO-induced stress G7-882 mice appear to have developed decompensated hypertrophy evolving into cardiomyopathy while the corresponding ISO-treated WT mice had significantly higher heart rates, which resulted in higher CO. The latter suggests that the hearts of WT mice are still exhibiting compensated hypertrophy and this enhanced cardiac function may serve to improve myocardial performance as has been observed in models of dilated cardiomyopathy.19 The other indication that TG mice are more susceptible than WT mice to cardiac hypertrophy in response to β-adrenergic stimulation is the finding of a significantly larger ES epicardial diameter in G7-882 mice after chronic ISO-induced stress as compared to WT mice. Taken together, our data indicate that under β-adrenergic receptor stimulation, G7-882 mouse hearts showed impaired contractility and relaxation, which resulted in cardiac hypertrophy and accelerated development of cardiomyopathy. One can anticipate these changes as a result of overexpression of Ca\textsubscript{1.2}α\textsubscript{1C}.2

To our knowledge, this is the first study where a transgenic animal with a dysfunctional transgene was characterized at the molecular, cellular and phenotypic levels. Results of this study demonstrate that an inactive transgene, unable to produce a transcript, may induce a physiological phenotype. This effect is probably due to an incidental incorporation of the foreign DNA into the host’s genome (structural effect) and the mechanism of this effect must necessarily be very complicated and may involve multiple compensatory regulatory elements.42 Because this genetic modification was random and artificial, the investigation of its nature was beyond the scope of our investigation. Multiple naturally

mice infused with the same dose of ISO (Fig. 5F, parts b and d). Histopathological scores were significantly higher for G7-882 mice that received chronic β-adrenergic stimulation as compared to the corresponding WT mice (data not shown).

Discussion

Results of this study showed that the Ca\textsubscript{1.2}α\textsubscript{1C} subunit cDNA deprived of 3’-UTR caused accelerated development of cardiovascular pathology in a transgenic animal model despite the absence of expression of the transgene and lack of changes in the kinetics, density and voltage-dependent properties of the net calcium channel current in cardiomyocytes. Under normal conditions the transgenic G7-882 mouse hearts were obtained after administration of vehicle (a and c) or ISO (b and d) at a dose of 30 (A, C and E) and 45 mg/kg/day (B, D and F) for a total of seven days.

Figure 5. Hypertrophic response to isoproterenol. Representative images of hematoxylin/eosin (H&E)-stained frontal sections (A and B) and H&E (C and D) and Masson’s trichrome (E and F) stained histopathological sections (10x magnification) of the left ventricle of WT (a and b) and transgenic G7-882 (c and d) mouse hearts were obtained after administration of vehicle (a and c) or ISO (b and d) at a dose of 30 (A, C and E) and 45 mg/kg/day (B, D and F) for a total of seven days.
occurring mutations associated with cardiomyopathy are known in humans (reviewed in refs. 43 and 44). From our experiment we cannot conclude whether the mutation observed in G7-882 is a frequently occurring case or an extremely rare exception. It was not replicated in five other independent transgenic strains indicating that it is unrelated to the transgene, and therefore sporadic and unpredictable. Although the associated defect is retained in the G7-882 genome, it is not lethal. The most surprising observation is that the altered phenotype may correspond to the anticipated modified phenotype induced by an active transgene. To what extent the altered phenotype is due to the structural effect in the case of an active transgene is a crucial question for transgenic technology to be addressed in light of our findings. To exclude the possibility of misinterpretation due to the structural TG effect, we conclude that a new transgenic phenotype must be replicated in independently generated transgenic strains.

Methods

Generation of transgenic mice. The TG mice were generated at the NIA animal core facility by standard methods. The GFP-coding sequence (pGreen Lantern, Gibco) was incorporated frame upstream of the human \( \alpha_{c,77} \) DNA open reading frame (ORF) to ease genotyping and detection. The CMV-GFP-HLCC77 cDNA fragment containing the Kozaq sequence in front of the ORF and only 97 nt of the 3' UTR was cleaved from the S'-GFP-77pcDNA3 plasmid by SspI and NotI. The purified DNA fragment was microinjected into the pronuclei of day 1 embryos of C57BL/6J mice and the surviving zygotes were implanted into pseudopregnant foster mothers. Transgenic founder mice were identified by PCR genotyping of the chromosomal DNA isolated from tail snips from pre-weanling pups aged 14–21 days. PCR with the sense primer (5'-CCA CTA CCA GCA GAACAC C-3') specific for the nucleotide sequence of GFP and antisense primer (5'-GCC GCC TGC CAC GAC AG3') located at the nucleotide positions 1778–1794 of the HLCC77 ORF (z34815) yielded a 350 bp product from mice expressing the transgene. Both genotyping primers showed nonsignificant nucleotide identity with the mouse \( \alpha_{c} \) DNA sequence (<45%). The adopted PCR procedure was highly specific for GFP-HLCC77 and allowed for identification of a single copy of the transgene in the murine genomic background. Six homozygous TG strains were generated and strain G7-882 was used for the study. All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of the NIA and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985).

Chronic ISO infusion. Miniosmotic pumps (Alzet model 1007D) filled with ISO in vehicle (50 mM ascorbic acid in saline) or vehicle alone and set to deliver the drug at 30 or 45 mg/kg/d for seven days were subcutaneously implanted in mice after midscapular incision under isoflurane anesthesia. A total of 48 adult male mice were used in eight experimental groups. Four groups of 6-month-old mice were infused at a rate of 30 mg/kg/d for seven days: group 1, WT + veh (n = 5); group 2, WT + ISO (n = 6); group 3, G7-882 + veh (n = 5); group 4, G7-882 + ISO, (n = 6). To examine the maximum tolerance of TG mice, we exposed 12-month-old mice to an increased infusion rate of 45 mg/kg/day for 7 days: group 5, WT + veh (n = 5); group 6, WT + ISO (n = 5); group 7, G7-882 + veh (n = 7); group 8, G7-882 + ISO, (n = 9). For measurement of the heart rate (HR), both prior to pump implantation and after seven days of infusion, electrodes were attached to two front and one back paw, and HR was recorded under isoflurane anesthesia using the Power Lab ECG system (AD Instruments).

In vivo cine MRI. MRI was performed on a 7 Tesla/30 cm Bruker Biospec MRI Scanner (Bruker Biospin). The animal was positioned in a feet first and prone position on a surface coil used as a proton receiver (diameter 20 mm). Homogeneous radio frequency excitation was achieved using a proton resonator (diameter 72 mm). During MRI scanning, anesthesia was maintained with 1.5% v/v isoflurane:O\(_2\) delivered to the mouse via a mask. The general anesthesia regimen with isoflurane was shown to have no impact on mouse cardiac function. The body temperature was maintained at 37°C using a MRI-compatible heater system with a rectal thermometer feedback (SA Instruments, Stony Brook, NY). To acquire an ECG signal, surface electrodes were placed on both front paws and connected to a small animal MR ECG trigger unit while the respiration triggering signal was obtained using a pressure sensor placed on the back of the mouse (SA Instruments). Cardiac-respiratory triggering signals were used for synchronization of the image acquisition to physiological motion. A respiratory-gated, ECG-triggered, fast gradient echo (FLASH) sequence was used, with the following acquisition parameters: echo time (TE), 2.3 ms; repetition time (TR), 7 ms; flip angle, 40°; field of view, 30 x 30 mm; acquisition matrix, 128 x 128; slice thickness, 1 mm. A pilot coronal image of the heart was acquired and used to plan short axis images. Using these short axis images, the long axis of the heart was identified and four-chamber views were planned and acquired. Six to eight contiguous ventricular short axis slices were acquired to cover the entire heart, and the number of frames adjusted to cover the entire cardiac cycle. To increase the signal-to-noise ratio and obtain sufficient image quality, four signal averages were acquired per scan. In order to maintain the MRI signal in a steady state, only one ECG trigger per respiratory cycle was used. These conditions resulted in an acquisition time of approximately 4 min per cine sequence, depending on the respiration rate. After completion of MRI studies, the mouse was sacrificed and the heart was dissected for histological examination and to determine the HW and LV weight.

MRI data analysis. For LV mass and volume measurements, epicardial and endocardial borders were manually delineated at end-diastole and end-systole for each short axis slice and the area enclosed between these borders was tabulated. The LV end-diastolic volume (EDV) was calculated by summing the areas within the endocardial borders in each slice and multiplying by the slice thickness. A similar procedure was used to determine the LV end-systolic volume (ESV). Stroke volume (SV) was calculated as EDV minus ESV, the ejection fraction (EF%) was calculated as 100 times the ratio of SV to EDV, and cardiac output (CO) was calculated as SV multiplied by heart rate. Measurement of LV
ED and ES wall thickness, systolic wall thickening as well as ED and ES epicardial and endocardial diameter were obtained from the midventricular short axis slice. Left ventricular mass was calculated as the volume within the epicardial border at end-diastole minus the LV EDV, multiplied by a factor of 1.05 (assumming a myocardial specific gravity of 1.05 g/cm³). Papillary muscle was included in the left ventricular mass.

**Histopathological analysis.** For histopathological analysis, hearts were dissected and fixed in 10% neutral buffered formalin. The cardiac tissues were embedded in paraffin and 4–5 μm sections were cut on a Reichert-Jung RV-2035 microtome. Tissue sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) for histopathological evaluation. Additional sections of the heart were stained with Masson’s trichrome to visualize areas of fibrosis within the cardiac tissue. The degree of histopathological changes in cardiac tissue was evaluated by a pathologist blinded to the genotype of the donor and ISO dose. The tissues were viewed using a Nikon Eclipse E 400 microscope to identify myocardial fiber thinning, vacuolation and other degenerative and fibrotic changes of cardiac tissue. The severity of the histopathological changes in LV tissue was scored as follows: 0 (no effect), 1 (minimal), 2 (mild), 3 (moderate) and 4 (severe).

**Expression analysis.** The comparative threshold cycle method was used to determine expression of the endogenous Ca\(_{1.2}\) α\(_{1C}\) in WT and TG mouse hearts. Four WT C57BL/6J hearts and four TG mice were used. RNA was extracted from ~50 mg myocardium samples with the RNAeasy kit (Qiagen). The first strand cDNA was generated with Superscript III First-strand Synthesis Super Mix (Invitrogen) using either oligo(dT)\(_{20}\) or random hexamer primer. Reaction mixtures (25 μl) contained 12.5 μl of SYBR Green, cDNA and 40 pmol of sense and antisense primers (Table 1). The quality of cDNA was checked by regular PCR using mouse GAPDH primers (Table 1). Each PCR product showed a single band of expected size (113–240 bp) on agarose gel electrophoresis. Real time PCR was carried out in an Applied Biosystems 7300 Real-Time PCR System. PCR results with 18S RNA were consistent with those for GAPDH. Data were normalized against the values obtained for 18S RNA. The threshold cycle (C\(_T\)) value for 18S RNA was a general indicator of the transcript abundance and was thus subtracted from the C\(_T\) value obtained for Ca\(_{1.2}\) α\(_{1C}\) transcripts to give ΔC\(_T\). The ΔC\(_T\) values for the exogenous gene and endogenous gene were then compared by subtracting one from the other to give ∆∆C\(_T\). 2\(^{-ΔΔC_T}\) gave an estimate of the change in the target gene expression. The cytoplasmic and nuclear RNA fractions from cardiac tissue samples were isolated using a Cytoplasmic and Nuclear RNA purification kit (Norgen Biotech).

**Measurement of whole-cell calcium current.** Cardiac myocytes were isolated from left ventricles of 3–5-month-old WT and TG littermates using the enzymatic method of Zhou et al.\(^{46}\) Ca\(^{2+}\) currents were recorded at 20–22°C by the patch clamp technique in whole-cell configuration. Bath medium contained (in mM) NaCl, 137; CsCl, 10; MgCl\(_2\), 1.2; CaCl\(_2\), 2; glucose, 15; HEPES, 10; tetrodotoxin, 0.02 (pH 7.4 with CsOH). The pipette solution contained (in mM) CsCl, 120; NaCl, 10; tetaethylammonium, 10; MgATP, 5; HEPES, 20 (pH 7.2 with CsOH). Calcium currents were activated by a series of 200-ms depolarization pulses applied from the holding potential of -50 mV to a test potential ranging from -40 mV to +70 mV with 10-s intervals.

**Statistical analysis.** All results are given as mean ± SD except electrophysiology and RT-PCR (mean ± SEM). A value of p < 0.05 was considered significant. All data were compared between groups using an unpaired Student's t-test while the heart rate within each group was compared using one-way ANOVA. Mortality was analyzed using Richard's exact test.

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**Note**
Supplemental materials can be found at: [www.landesbioscience.com/journals/channels/article/14314](http://www.landesbioscience.com/journals/channels/article/14314)
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