Inducible re-expression of HEXIM1 causes physiological cardiac hypertrophy in the adult mouse

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Aims	The transcription factor hexamethylene-bis-acetamide-inducible protein 1 (HEXIM1) regulates myocardial vascularization and growth during cardiogenesis. Our aim was to determine whether HEXIM1 also has a beneficial role in modulating vascularization, myocardial growth, and function within the adult heart.
Methods and results	To achieve our objective, we created and investigated a mouse line wherein HEXIM1 was re-expressed in adult cardiomyocytes to levels found in the foetal heart. Our findings support a beneficial role for HEXIM1 through increased vascularization, myocardial growth, and increased ejection fraction within the adult heart. HEXIM1 re-expression induces angiogenesis, that is, essential for physiological hypertrophy and maintenance of cardiac function. The ability of HEXIM1 to co-ordinate processes associated with physiological hypertrophy may be attributed to HEXIM1 regulation of other transcription factors (HIF-1-α, c-Myc, GATA4, and PPAR-α) that, in turn, control many genes involved in myocardial vascularization, growth, and metabolism. Moreover, the mechanism for HEXIM1-induced physiological hypertrophy appears to be distinct from that involving the PI3K/AKT pathway.
Conclusion	HEXIM1 re-expression results in the induction of angiogenesis that allows for the co-ordination of tissue growth and angiogenesis during physiological hypertrophy.
Keywords	HEXIM1 • Hypertrophy • Angiogenesis

1. Introduction

The adult heart can adapt to environmental stress by hypertrophy and vascular growth/remodelling.[1] Physiological or adaptive responses, as in the case of the exercised trained athletic heart, are characterized by balanced changes in both the cardiomyocytes and the vasculature. Negative consequences arise when these responses are not co-ordinated, as in the case of prolonged hypertension, and can lead to heart failure, arrhythmia, and death. The physiological and pathological responses overlap in some respects, especially, in the early stages of adaptation. However, there are distinct characteristics to the physiological and pathological responses that have been delineated in humans and rodents.[1] These differences lie in the degree of vascularization of the myocardium, functional parameters, gene and protein expression, and the response to ischaemic stress. An important goal in preventing heart disease is to understand how to induce physiological responses and suppress pathological responses.

We and others found that hexamethylene-bis-acetamide-inducible protein 1 (HEXIM1) is a tumour suppressor and cyclin-dependent kinase inhibitor, and that these functions are dependent on its C-terminal region.[2] We provided evidence that the HEXIM1 C-terminal region is critical for cardiovascular development. HEXIM1 protein was detected in the heart during cardiac growth and chamber maturation.[3] We created mice carrying an insertional mutation in the HEXIM1 gene that disrupted its C-terminal region and resulted in prenatal lethality. Heart defects in HEXIM1$_{1-312}$ mice included abnormal coronary

patterning, reduction of coronary vascularization within the myocardium and thin ventricular walls. The expression of vascular endothelial growth factor-A (VEGF), known to affect angioblast invasion and myocardial proliferation and survival, was decreased in HEXIM1$_{1-312}$ mice compared with control littermates. These results suggest that HEXIM1 is critical for coronary vessel development and myocardial growth. Here, we report that HEXIM1 induction in adult cardiomyocytes results in morphology, physiology, and gene expression that resembles those of a physiological rather than a pathological response of the heart.

2. Methods

2.1 Generation of HEXIM1 transgenic mice

All animal work has been approved by the CWRU Institutional Animal Care and Use Committee and conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). Mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. The Mhc-reverse tetracycline-controlled transactivator protein (rtTA) transactivator mouse strain (obtained from the Mutant Mouse Regional Resource Center) expresses the rtTA under the regulatory control of the rat alpha myosin heavy chain promoter that directs the expression of rtTA in cardiac myocytes.[4] pTET-HEXIM1 mice were generated as previously described[5] using a pTET-HEXIM1 transgenic construct containing the HEXIM1 coding sequence under the control of the tetracycline-dependent minimal promoter. Mating of Mhc-rtTA and pTET-HEXIM1 mice resulted in the creation of Mhc-HEXIM1 mice. These mice are on the Friend virus B-type (FVB) background strain. Genotype analysis is described in the Supplementary material online, Methods.

2.2 Treadmill

This test was modified from a previously protocol described.[6] Details are provided in the Supplementary material online, Methods.

2.3 Magnetic resonance imaging

In vivo magnetic resonance imaging (MRI) experiments were performed on a 9.4T Bruker system equipped with a gradient insert and a volume receiver coil as previously published.[7] Animals were anaesthetized via inhalation anaesthesia with 1.5% isoflurane and monitored by the evaluation of the toe pinch reflex and breathing rate. Other details are in the Supplementary material online, Methods.

2.4 Echocardiography

LV function was evaluated with a Sequoia C256 system (Siemens Medical) with a 15 MHz linear array transducer as previously described.[8] Mice were anaesthetized using 1.5−2.0% isoflurane, monitored by evaluation of toe pinch reflex and breathing rate, and situated supine on a warming pad with electrocardiogram (ECG) limb electrodes. Other details are in the Supplementary material online, Methods.

2.5 Telemetry

In preparation for implantation of transmitters, mice were anaesthesized with an ip injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) and monitored by evaluation of toe pinch reflex and breathing rate. We used a radio-telemetry system (ETA-F10, Data Sciences International, St. Paul, MN, USA) to monitor heart rate (HR) in conscious, unrestrained mice, as described previously.[9] Details are in the Supplementary material online, Methods.

2.6 Immunostaining

Frozen adult mouse hearts were processed for immunohistology and stained as previously described.[3] Details are in the Supplementary material online, Methods.

2.7 Quantitation of immunostaining results

Immunostaining results were analysed using a modified protocol.[10] Details are in the Supplementary material online, Methods.

2.8 Cell culture and transfections

H9C2 cells (originally derived from embryonic rat heart tissue[11]) were maintained and transfected as previously described.[3] Details are in the Supplementary material online, Methods.

2.9 Northern blot analyses

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and analysed using northern blotting as previously described.[12]
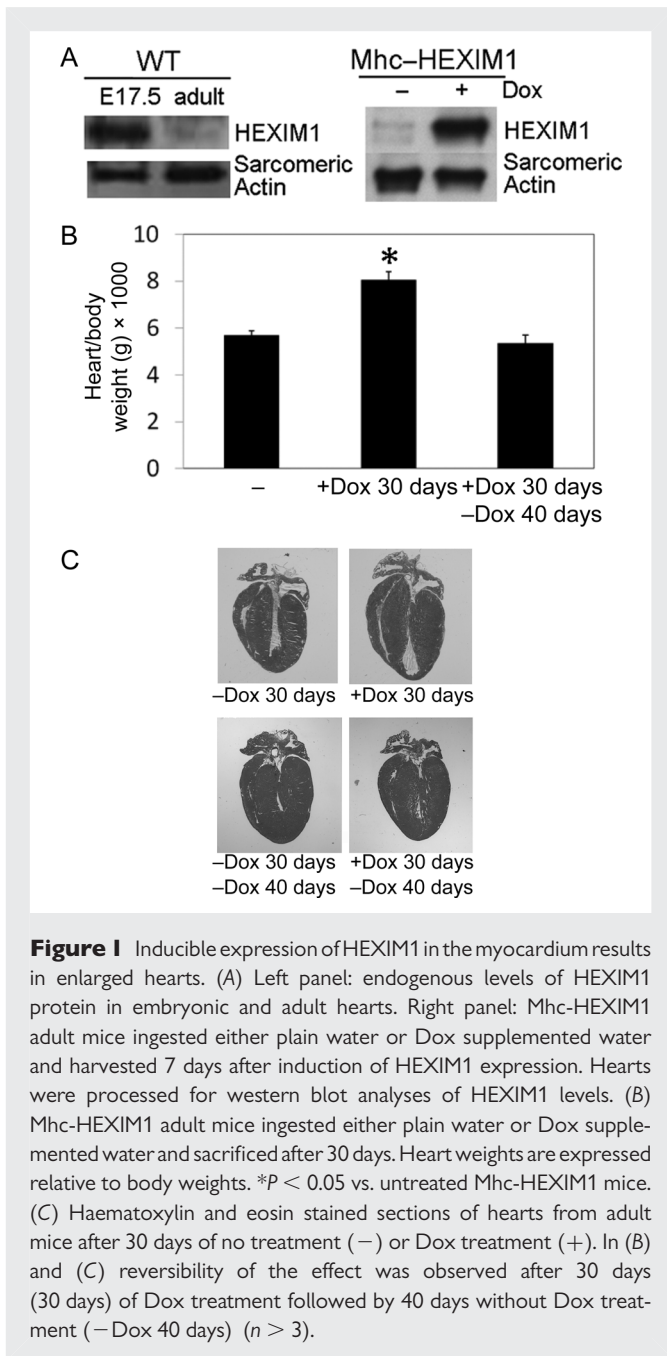
2.10 Western blot analyses

Western blot experiments were performed as previously described.[13] Details are in the Supplementary material online, Methods.

3. Results

3.1 Consequences of cardiomyocyte-specific expression of HEXIM1 to the phenotype of the adult heart

HEXIM1 is expressed in embryonic and foetal hearts, with decreased expression in adult hearts (*Figure 1A*). Because of the critical roles of HEXIM1 in coronary vessel development and myocardial growth in the developing heart, we investigated whether HEXIM1 re-expression would have beneficial effects on cardiovascular function in the adult heart. A tetracycline-responsive binary α-Mhc transgene system described in Section 2 was used to allow temporally regulated expression of HEXIM1 in cardiomyocytes. HEXIM1 expression was induced by doxycycline (Dox) in the hearts of Mhc-HEXIM1 mice as early as 7 days after the start of Dox treatment to a level observed in foetal mice (*Figure 1A*). HEXIM1 immunostaining showed little or no staining in the heart sections of the untreated mice but distinct nuclear staining was evident in the cardiomyocytes of the Dox-treated mice (Supplementary material online, *Figure S1A*). The induction of HEXIM1 is heart-specific (Supplementary material online, *Figure S1B*) and Dox dose-dependent (Supplementary material online, *Figure S1C*).

Heart sections stained with Masson's trichrome, Oil Red O, or TUNEL revealed no obvious qualitative differences in staining patterns between Dox-treated and untreated mice indicating HEXIM1 induction did not promote fibrosis, deposition of fat, nor apoptosis, respectively (Supplementary material online, *Figure S2*). Dox-treated mice had heavier hearts, higher heart weight to body weight ratios (*Figure 1B*), and larger hearts (*Figure 1C*). Dox treatment of Mhc-rtTA mice did not induce a change in heart weight to body weight ratios (Supplementary material online, *Figure S3A*). The effects of HEXIM1 re-expression were reversible; the hearts returned to normal weight and size after 40 days withdrawal of Dox. To determine the basis for heavier and enlarged hearts in Dox-treated Mhc-HEXIM1 mice, we assessed cardiomyocyte proliferation and vascularization of the myocardium. Hearts from Mhc-HEXIM1 adult mice show increased levels of phosphorylated histone H3 (PH3, marker of proliferation) by western blot analysis (*Figure 2A*). Analyses of immunostained histological sections show PH3
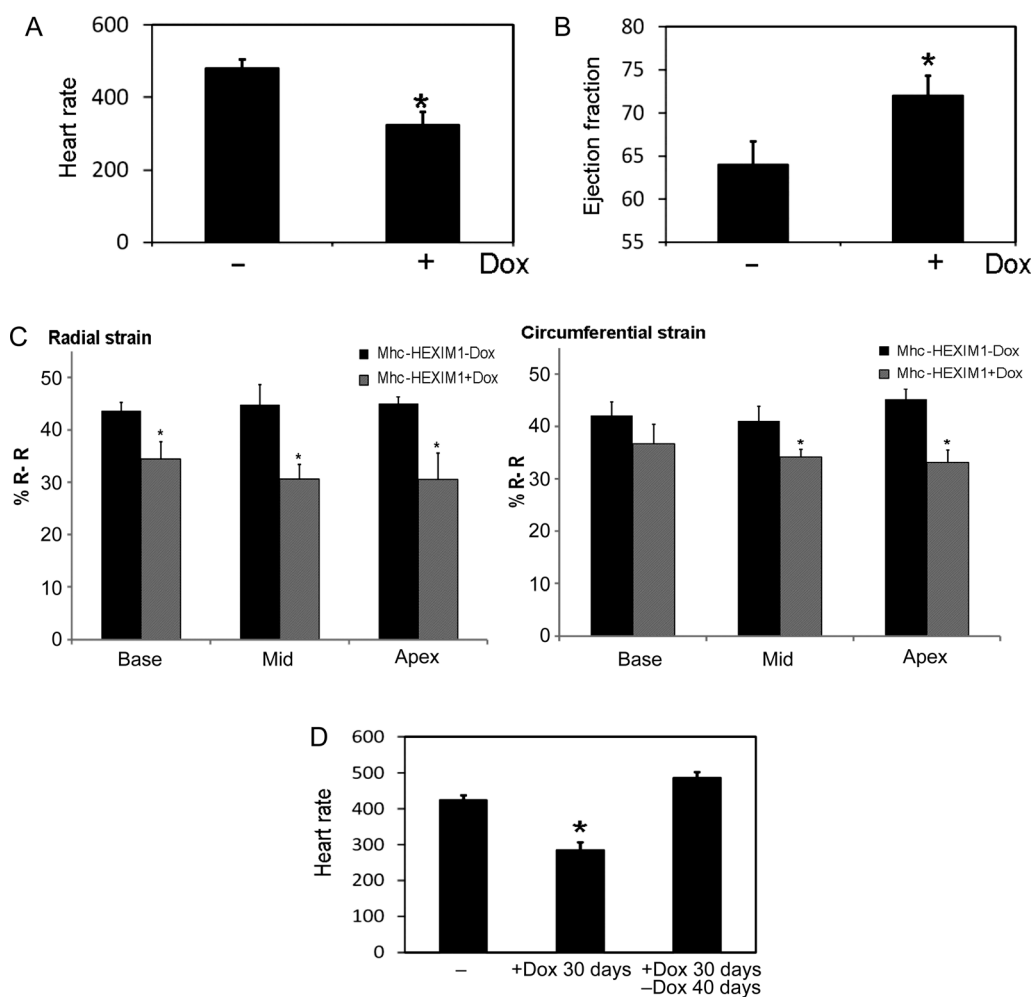
Figure 1 Inducible expression of HEXIM1 in the myocardium results in enlarged hearts. (*A*) Left panel: endogenous levels of HEXIM1 protein in embryonic and adult hearts. Right panel: Mhc-HEXIM1 adult mice ingested either plain water or Dox supplemented water and harvested 7 days after induction of HEXIM1 expression. Hearts were processed for western blot analyses of HEXIM1 levels. (*B*) Mhc-HEXIM1 adult mice ingested either plain water or Dox supplemented water and sacrificed after 30 days. Heart weights are expressed relative to body weights. *$P < 0.05$ vs. untreated Mhc-HEXIM1 mice. (*C*) Haematoxylin and eosin stained sections of hearts from adult mice after 30 days of no treatment (−) or Dox treatment (+). In (*B*) and (*C*) reversibility of the effect was observed after 30 days (30 days) of Dox treatment followed by 40 days without Dox treatment (−Dox 40 days) (*n* > 3).

Figure 2 Inducible expression of HEXIM1 in the myocardium results in increased proliferation and vascularization. Mhc-HEXIM1 adult mice ingested either plain water (−) or Dox supplemented water (+) and sacrificed 30 days after induction of HEXIM1 expression. Hearts were processed for (*A*) western blot analyses of phospho-histone 3 and (*B*) quantification of immunostaining for PECAM-1+ areas. Results were presented as fold change in expression in Mhc-HEXIM1 mice (+Dox) relative to control (−Dox). *$P < 0.05$ vs. untreated Mhc-HEXIM1 mice (*n* > 6). (*C*) Cells co-stained with PECAM1+ and PH3+ was quantified. Field size = 19 500 square micron. *$P < 0.05$ vs. untreated Mhc-HEXIM1 mice (*n* > 6).

staining of cardiomyocytes and endothelial cells (Supplementary material online, *Figure S4*). Our analyses also revealed a statistically significant increase in PECAM1+ elements per field (*Figure 2B* and Supplementary material online, *Table S1*) in the interventricular septum (IVS) and LV, indicating an increase in vascularization of the myocardium. Increased number of cells costained with PECAM1+ and PH3+ in Dox-treated Mhc-HEXIM1 mice suggest an induction of endothelial cell proliferation by HEXIM1 (*Figure 2C*). There was no statistically significant difference between untreated and Dox-treated animals in the number of nuclei per field (all cell types), number of large cells (largely cardiomyocytes) per field except in the IVS, nor in the area of the large cells (largely cardiomyocytes) per field. Thus, the larger/heavier heart cannot be explained by an increase in cardiomyocyte diameter or an increase in the density of cardiomyocytes.
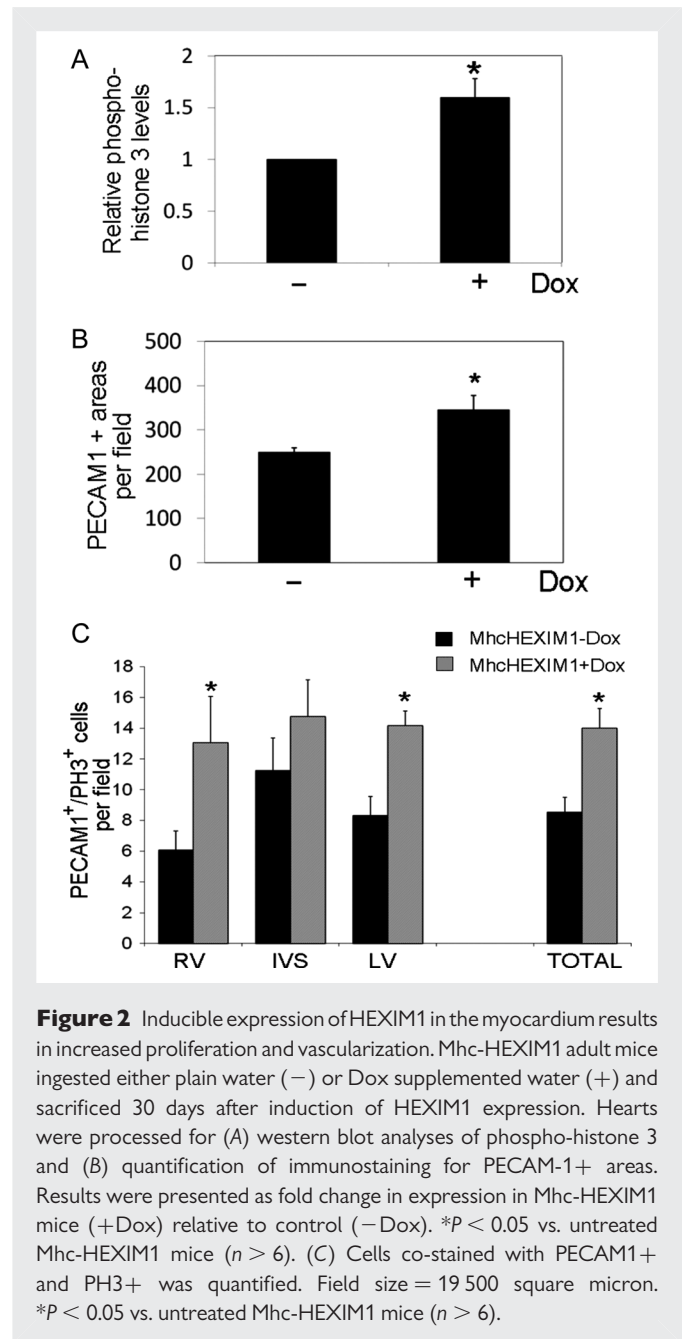
3.2 Cardiac performance assayed by *in vivo* MRI, echocardiography, and endurance testing by treadmill

We examined heart function by subjecting Mhc-HEXIM1 mice (± Dox treatment) to *in vivo* cardiac MRI and echocardiography. Data from anaesthetized mice revealed significantly lower heart rates in Dox-treated Mhc-HEXIM1 mice compared with controls (*Figure 3A*). Dox treatment of Mhc-rtTA mice did not induce changes in heart rates (Supplementary material online, *Figure S3B*). In addition, significantly higher ejection fractions were documented in Dox-treated Mhc-HEXIM1 mice (*Figure 3B*). Analysis of the ventricular volume at three levels showed a tendency towards being larger in Dox-treated Mhc-HEXIM1 mice, consistent with the higher heart−body weight ratio (36% higher than the control) and histological analysis. The MRI data also indicated

Figure 3 Inducible expression of HEXIM1 results in decreased heart rate, increased ejection fraction, and decreased time to peak strains. Mhc-HEXIM1 adult mice ingested either plain water (Mhc-HEXIM1−Dox) or Dox supplemented water (Mhc-HEXIM1+Dox) and heart function was analysed by *in vivo* MRI 30 days after induction of HEXIM1 expression for (*A*) heart rate and (*B*) ejection fraction. (*C*) Time to peak radial (left) and circumferential strains (right) in Mhc-HEXIM1−Dox and Mhc-HEXIM1+Dox mice expressed as a per cent of one cardiac cycle. (*D*) Heart rate was monitored by echocardiography in anaesthesized Mhc-HEXIM1 mice given plain water (−), or Dox supplemented water for 30 days (+Dox 30 days), or Dox supplemented water for 30 days followed by plain water for 40 days (+Dox 30 days, −Dox 40 days). Data are means ± SE. *$P < 0.05$ vs. untreated Mhc-HEXIM1 mice ($n > 5$).

that the time to peak radial and circumferential strains were significantly shorter in Dox-treated Mhc-HEXIM1 mice (*Figure 3C*). All other parameters of mechanical strain and torsion (Supplementary material online, *Figure S5*) were similar to that of the control untreated Mhc-HEXIM1 mice. We addressed the issue of mouse background differences and potential effects of the Mhc-HEXIM1 transgene itself with this assay. The Mhc-HEXIM1 mice are on the FVB background. The only significant difference between the non-transgenic SV/129 and FVB and the untreated Mhc-HEXIM1 mice was in the circular strain at the base (Supplementary material online, *Figure S6*). Heart rate and time to peak strain of the two background control animals with no transgene (FVB and SV/129) and the untreated Mhc-HEXIM1 mice were not different from each other. We also tested reversibility of the effects of HEXIM1 re-expression. After 40 days of Dox withdrawal, HR returned to levels not statistically different from untreated Mhc-HEXIM1 mice (*Figure 3D*).

Echocardiography data revealed that posterior wall thickness was significantly greater in Dox-treated Mhc-HEXIM1 mice consistent with the increase in the heart−body weight ratio (Supplementary material online, *Table S2*). Given the significant decrease in HR with no significant compensatory increase in stroke volume, cardiac index (CI) was significantly lower in the long-axis view of the Dox-treated Mhc-HEXIM1 group. There were no significant differences in other baseline structural or functional echocardiographic parameters between the treatment and control HEXIM1 groups. The increase in ejection fraction detected using MRI did not reach significance based on echocardiographic data. However, MRI analyses have been demonstrated to be more accurate and reproducible compared with 2D echocardiography and has increased sensitivity in detecting LV mass changes.[14] The functional responses (e.g. HR, CI, ejection fraction, and fractional shortening) to dobutamine stress were not different between Dox treated and untreated Mhc-HEXIM1 groups, suggesting that HEXIM1 re-expression did not alter the adrenergic response (Supplementary material online, *Table S3*).

Because restraint and anaesthesia can have an artefactual influence on physiological parameters, we used radiotelemetry to measure heart rates of conscious, untethered animals. Consistent with MRI and
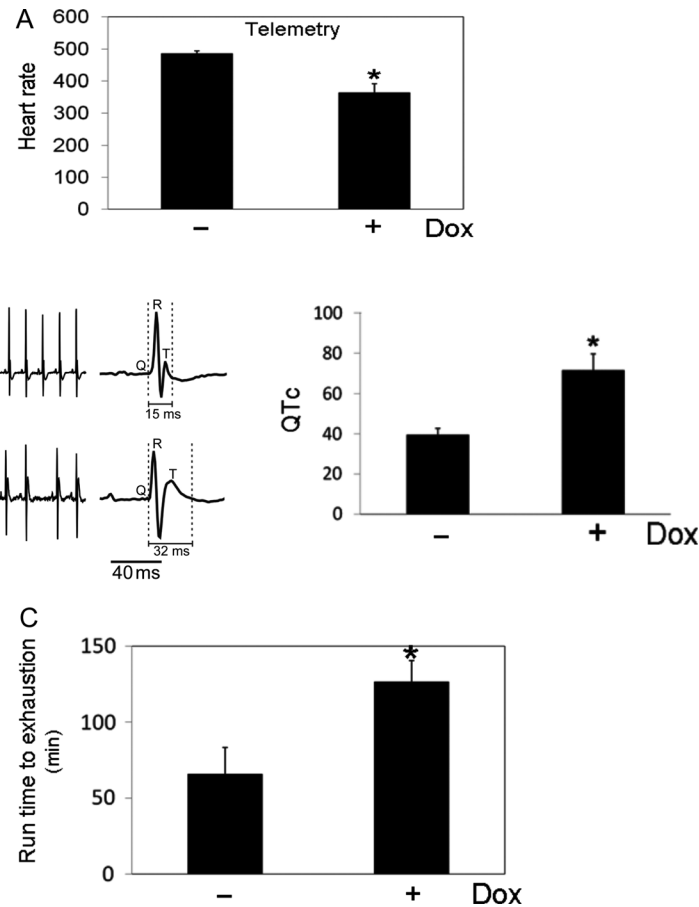
Figure 4 Physiology and exercise performance of Mhc-HEXIM1 mice with no anaesthesia. Mhc-HEXIM1 adult mice ingested either plain water (−) or Dox supplemented water (+) and analysed 30 days after induction of HEXIM1 expression. (*A*) Telemetry analyses of heart rate and (*B*) ECG (left) and cycle-triggered average (CTA, right) from conscious Mhc-HEXIM1 mice ± Dox. Representative 2-s data segments and CTA of ECG acquired using the telemetry device. *$P < 0.05$ vs. untreated Mhc-HEXIM1 mice (*n* > 6). (*C*) Performance in the treadmill exercise. Shown are times to exhaustion during submaximal endurance treadmill running (at 17 m/min, no incline). *$P < 0.05$ vs. untreated Mhc-HEXIM1 mice (*n* > 6).

echocardiography data, the heart rate of Dox-treated Mhc-HEXIM1 mice was lower than that of control mice (*Figure 4A*). In addition, ECG recordings from telemetry data revealed that Dox-treated Mhc-HEXIM1 mice had prolongation of the Q−Tc interval compared with control mice (*Figure 4B*). No sustained arrhythmias were noted during the 6 h of continuous recordings. While prolongation of the Q−T interval does not necessarily indicate a susceptibility to arrhythmias and sudden death,[15] it will be important to investigate any changes in the inducibility of arrhythmias and other electrophysiological parameters in these mice.

We examined endurance exercise capacity of control and Dox-treated Mhc-HEXIM1 mice using a run to exhaustion treadmill test without prior training. Time to exhaustion was significantly longer in the Mhc-HEXIM1 mice compared with controls (*Figure. 4C*).

3.3 HEXIM1 up-regulation of pro-angiogenic and growth regulatory factors

We examined the molecular basis for the phenotypic and functional changes in Mhc-HEXIM1 mice. As expected from our studies in the developing heart expressing mutant HEXIM1,[3] VEGF levels increased in HEXIM1-induced hearts (*Figure. 5A*). This may be attributable to increased levels of HIF-1-α in the hearts of Dox-treated compared with untreated Mhc-HEXIM1 mice (*Figure 5A*).

The transcription factor GATA4 has important growth stimulatory and pro-angiogenic roles. GATA4 levels were significantly increased after HEXIM1 re-expression (*Figure 5A*). Dox-treated Mhc-HEXIM1 heart sections immunostained for phosphorylated GATA4, the activated form of GATA4, had more positively stained and intensely stained nuclei than did heart sections from untreated mice (Supplementary material online, *Figure S7*).

FGF9 expression was also increased with increased HEXIM1 expression (*Figure 5A*). FGF9 promotes myocardial vascularization and hypertrophy in adult hearts[16] and likely contributes to the phenotype of Dox-treated Mhc-HEXIM1 mice.

3.4 Mechanism of regulation of HIF-1-α protein levels by HEXIM1

We tested the possibility that HIF-1-α is a direct target of HEXIM1 and thus likely to function as a critical mediator of HEXIM1 action in the adult heart. Thus, we examined if HEXIM1 can interact with HIF-1-α and in
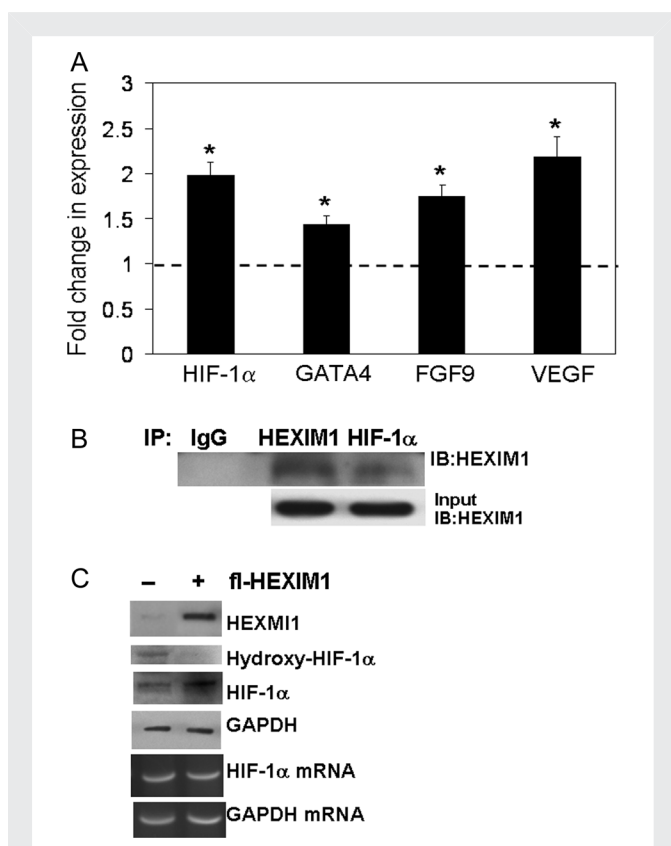
Figure 5 Inducible expression of HEXIM1 results in an increased expression of pro-angiogenic factors and HIF-1-α target genes involving an interaction between HEXIM1 and HIF-1-α. (A) Mhc-HEXIM1 adult mice ingested either plain water or Dox supplemented water and sacrificed 30 days after induction of HEXIM1 expression. Western blot analyses of HIF-1-α, VEGF, FGF9, and GATA4 expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control in western blots. Results were presented as fold change in the expression of indicated marker in Mhc-HEXIM1 mice (+Dox) relative to control (−Dox). *$P < 0.05$ vs. untreated Mhc-HEXIM1 mice ($n > 5$). (B) H9C2 cells were subjected to hypoxia treatment (1% O_2 for 18 h). Lysates were immunoprecipitated using antibodies against or HEXIM1 or HIF-1-α analysed for co-immunoprecipitating proteins by western blotting (IB) using HEXIM1 antibody. Normal rabbit immunoglobulin was used as a specificity control. (C) H9C2 cells were transfected with expression vector for fl-HEXIM1 or control vector. The expression of HEXIM1, HIF-1-α, hydroxylated HIF-1-α, and total HIF-1-α were analysed by western blot or northern blot analyses. GAPDH was used as a normalization control. Images represent at least three experiments.

doing so regulate HIF-1-α protein stability. Regulation of HIF-1-α stability is mediated by the oxygen-dependent degradation domain through various post-translational modifications.[17] HIF-1-α is hydroxylated at proline residues 402 and 564 by a family of HIF prolyl hydroxylase domain proteins, which require O_2.[18] Hydroxylated HIF-1-α subsequently interacts with the tumour-suppressor von Hippel−Lindau protein, which targets it for proteasomal degradation.[19] We observed that HEXIM1 interacts with HIF-1-α in H9C2 cardiomyocytes using endogenous co-immunoprecipitation experiments (*Figure 5B*). We also observed increased HIF-1-α protein, but not mRNA levels after induction of HEXIM1 expression (*Figure 5C*) consistent with post-translational
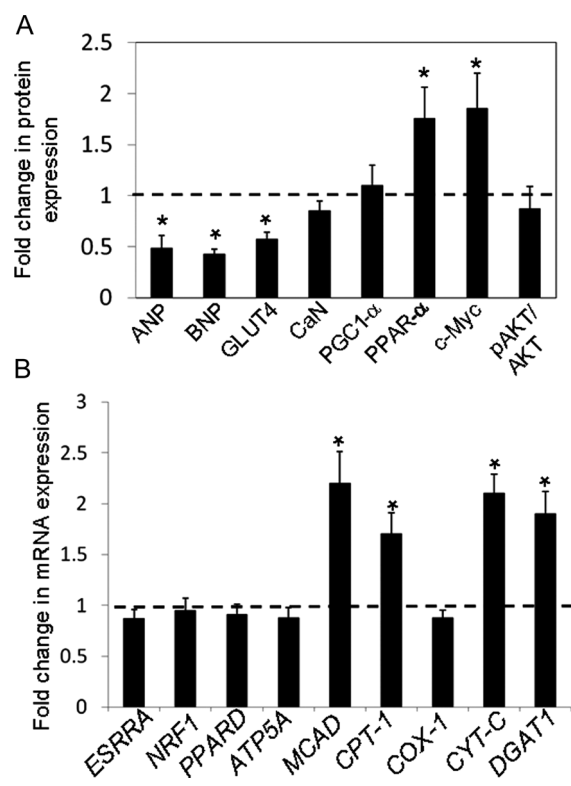
Figure 6 Expression of markers and regulators of physiological and pathological hypertrophy and genes involved in FA oxidation in control and Mhc-HEXIM1 adult mice. Mhc-HEXIM1 adult mice ingested either plain water or Dox supplemented water and sacrificed 15 days after induction of HEXIM1 expression. (A) Hearts were collected for western blot analyses of indicated cardiac markers. GAPDH was used as a loading control. Results were presented as fold change in the expression of an indicated marker in Mhc-HEXIM1 mice (+Dox) relative to control (no Dox). *$P < 0.05$ vs. untreated Mhc-HEXIM1 mice ($n > 5$). (B) Hearts were collected for RT−PCR analyses of FA metabolism genes normalized to GAPDH. Results were presented as fold change in the expression of an indicated marker in Mhc-HEXIM1 mice (+Dox) relative to control (no Dox). *$P < 0.05$ vs. untreated Mhc-HEXIM1 mice ($n > 4$).

regulation. In addition, the levels of the hydroxylated HIF-1-α protein were decreased in cells transfected with expression vector for flag-tagged HEXIM1 (fl-HEXIM1; *Figure 5C*).

3.5 Mhc-HEXIM1 mice express markers and regulators of physiological hypertrophy

To define other mechanisms for the phenotypic and functional changes in Mhc-HEXIM1 mice, we assessed levels of markers typically used to distinguish physiological from pathological hypertrophy. We did not observe increases in the expression of foetal cardiac genes (ANP and BNP) associated with pathological hypertrophy[20] in Mhc-HEXIM1 hearts (*Figure 6A*). Calcineurin (CaN) plays an important role in the development of pathological hypertrophy through dephosphorylation of the nuclear factor of activated T-cells-3 (NFAT3), inducing NFAT3 translocation to the nucleus and activation of its target genes (reviewed in Liu *et al.*[21]). Induction of CaN activity during pathological hypertrophy is associated with an increase in CaN protein.[22] HEXIM1 re-expression was not associated with changes in CaN expression (*Figure 6A*).

We also analysed expression levels of metabolic control genes associated with physiological and pathological hypertrophy[23] by western blot analyses. Relative to control hearts, Dox-treated Mhc-HEXIM1 mice exhibited decreased expression of GLUT4, that is, associated with pathological hypertrophy and increased expression of PPAR-α (*Figure 6A*), a critical regulator of fatty acid (FA) oxidation that is associated with physiological hypertrophy. c-Myc expression was also increased in Dox-treated Mhc-HEXIM1 mice (*Figure 6A*). c-Myc-regulated metabolic processes associated with preserved cardiac function and improved recovery from ischaemia.[24] c-Myc has been reported to regulate mitochondrial biogenesis in cardiomyocytes and is an important regulator of energy metabolism in the heart in response to pathological stress.[24] However, there are conflicting reports on the effect of c-Myc on mitochondrial biogenesis and heart function.[25] Pharmacological activation of PPAR-β/δ resulted in the induction of angiogenesis and cardiac growth, accompanied by the up-regulation of CaN and CaN target genes such as HIF1-α.[26] However, we did not observe changes in PPAR-β/δ levels upon up-regulation of HEXIM1 (*Figure 6B*).

We examined HEXIM1 regulation of genes critical in glucose and FA metabolism, partly to validate the involvement of PPAR-α in HEXIM1 action in the heart. HEXIM1 re-expression resulted in an increased expression of fatty oxidation (FAO) genes medium chain acyl-CoA dehydrogenase (*MCAD*), carnitine palmitoyltransferase 1 (*CPT-1*) and cytochrome *c* (*CYT-C*), and acyl-CoA:diacylglycerol acyltransferase (*DGAT1*), but no change in the expression of other transcriptional regulators of FAO, oestrogen-related receptor (*ESRRA*), and nuclear respiratory factor 1 (*NRF-1*), and other FAO genes, ATP synthase subunit alpha (*ATP5A*), cytochrome *c* oxidase complex IV, subunit I (*COX-I*) (*Figure 6B*).

Exercise-induced cardiac growth is reported to be regulated in large part by the growth hormone/IGF axis via signalling through the PI3K/AKT pathway.[27] However, we did not observe a significant difference in levels of phosphorylated AKT relative to total AKT (*Figure 6A*) in control and Dox-treated Mhc-HEXIM1 mice. This finding suggests that HEXIM1 regulates a distinct pathway from the prototypical growth factor-regulated pathway associated with physiological hypertrophy.

4. Discussion

The significance of our studies is that the transcription factor HEXIM1 may be a regulator that encourages an adaptive rather than a pathological response to cardiac stress. We uncovered novel molecular mechanisms of HEXIM1 action in the control of cardiac vasculogenesis and, potentially, metabolism. First, HEXIM1 up-regulates HIF-1-α expression, the hypoxia-sensitive component of HIF-1 that is known to regulate a host of genes including those involved in angiogenesis and metabolism.[28] Secondly, HEXIM1 up-regulates another key transcription factor GATA4 that is known to regulate growth regulatory and angiogenic genes.[29] Thirdly, our studies suggest new aspects of HEXIM1 action that involved regulation of gene expression that are expected to decrease glucose uptake and up-regulate FA metabolism associated with physiological hypertrophy. Together our studies provide insight into the molecular basis for hypertrophic effects of HEXIM1. Increased HEXIM1 expression may have therapeutic advantages by simultaneously regulating more than one pathway involved in physiological hypertrophy. Along this line, polymer-mediated delivery of Hexamethylene-bis-acetamide to mammary tissues resulted in increased HEXIM1 expression, without thrombocytopenia, the dose-limiting toxicity associated with HMBA in clinical trials.[30] A similar approach can be used to induce HEXIM1 expression in the heart.

Most of the literature on HEXIM1 has focused on its inhibition of positive transcriptional elongation factor b (P-TEFb) in defining HEXIM1 mechanism of action. The HEXIM1 knockout mouse exhibits the physical and molecular hallmarks of pathological cardiac hypertrophy and dies during the late foetal development.[31] Elevated P-TEFb activity, through overexpression of cyclin T1, was observed in cardiac hypertrophy *in vitro* and *in vivo*.[32] P-TEFb activity is also elevated in human heart failure.[33] Cyclin T1 transgenic mice that are heterozygous for HEXIM1 exhibited exacerbated hypertrophic response. The role of HEXIM1 in the mechanism governing compensatory hypertrophy in cardiomyocytes is supported by a report that HEXIM1 expression is decreased in a CaN model of cardiac hypertrophy, which coincides with an increase in P-TEFb activity.[34] Conversely, an increased expression of the full-length HEXIM1 would be expected to inhibit P-TEFb activity. Thus, some of the beneficial effects of HEXIM1 in cardiac function may be due to its ability to inhibit P-TEFb. A recent report on the prevention of right ventricular hypertrophy in hypoxia-induced pulmonary hypertension by cardiomyocyte-specific expression of HEXIM1 can also be attributed to HEXIM1 inhibition of P-TEFb activity.[35] It should be noted, however, that HEXIM1 overexpression in cardiomyocytes in that model was initiated during the embryonic stage and some of the effects on the adult heart in this model can be attributed to remodelling in the developing heart that can have effects on the function of the adult heart.

HEXIM1 regulation of VEGF gene transcription and vascularization is independent of its ability to inhibit the activity of the transcription elongation factor, P-TEFb.[3] Also, HEXIM1 regulation of HIF-1-α appears to be direct, through an interaction that may regulate the stability of HIF-1-α. HIF-1-α-mediated cardioprotection has been observed in cardiac-specific HIF-1-α transgenic mice after myocardial infarction.[36] The cause of cardioprotection likely involves many factors due to the activation of several HIF-1 target genes and the subsequent modulation of pathways involved in β-catenin signalling,[37] the purinergic signalling pathways,[38] glucose metabolism,[37] and lipid metabolism.[39]

Under some conditions such as during exercise and in certain pathologic states such as hypertrophy, the heart becomes increasingly dependent upon glucose to meet its metabolic demands.[40] Changes in GLUT transporters are an initial response to the hypertrophic stimulus.[41] High glucose conditions stimulate the production of angiotensin II, a known pathological modulator of cardiac remodelling.[42] Pathological cardiac hypertrophy is associated with reduced myocardial FA utilization that correlates with mitochondrial dysfunction, particularly during the transition to heart failure.[43] Dox-treated Mhc-HEXIM1 mice exhibited decreased expression of GLUT4 that is associated with pathological hypertrophy and increased expression of PPAR-α that is associated with physiological hypertrophy. PPAR-α regulates the expression of genes involved in FA oxidation.

A striking finding was that the HEXIM1 re-expression in adult cardiomyocytes resulted in bradycardia without compromise of other cardiomechanical parameters. One explanation for the bradycardia is that, as in physiological hypertrophy in humans,[44] the low resting heart rates are due to higher vagal tone as a result of more efficient cardiac function in the Dox-treated mice. Another possibility is that increased HEXIM1 expression in sinus node cardiomyocytes alters intrinsic pacemaker function of these cells. Prolonged QT as we have found in the Mhc-HEXIM1 mice has also been reported for physiological hypertrophy in humans (reviewed in Rowland[45]) and suggested to be due to

electrophysiological remodelling. These possibilities are currently being tested.

It has been previously reported that bradycardia induced by the administration of ivabradine or beta-blockers resulted in increased angiogenesis within the myocardium and increased cardiomyocyte survival after infarction in rodent models.[46] The clinical use of heart rate reduction therapy by pharmacological intervention reduces morbidity and mortality due to coronary artery disease and other cardiac pathologies.[47] However, the effect of reducing HR on the myocardial vasculature in these clinical studies is not known.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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