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ORIGINAL PAPER

A mouse model for spatial and temporal expression of HGF in the heart

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Abstract In order to study the effects of Hepatocyte Growth Factor (HGF) in the heart, two transgenic mice were developed, one carrying a bidirectional HGF-TetO-GFP responder construct and the other carrying a α-MHC-tTA transactivator construct. Crosses were carried out between heterozygotes, so that litters contained bitransgenic α-MHC-tTA/HGF-TetO-GFP+, thus expressing HGF and GFP exclusively in the heart and only in the absence of Doxycycline. Our data show that the expression of HGF was indeed restricted to the heart and that the expression was limited to the timeframe of the absence of Doxycycline. Surprisingly the expression was variable even between bitransgenic littermates. In the setting of a model of ischemia–reperfusion, the expression of HGF ameliorates cardiac functionality, enhances proliferation and diminishes the scarred area, proving that this is a good model to study the beneficial influences and functional roles of HGF in the heart.

Keywords HGF · Heart · Transgenic mice · Myocardial infarction

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Introduction

The Hepatocyte Growth Factor (HGF) is a mesenchymal-derived multifunctional cytokine that raises mitogenic and morphogenic activities in development, as well as in many patho-physiological processes (Birchmeier et al. 2003; Boccaccio and Comoglio 2006). The HGF receptor is the Met tyrosine kinase, the product of the Met proto-oncogene, which is expressed in different cell types, including epithelial, endothelial and mesenchymal cells. After binding to its ligand, Met undergoes autophosphorylation on several tyrosine residues and constitutes a multifunctional-docking site for adaptor proteins containing the SH2 domain. Recruitment of these molecules leads to the activation of downstream signalling cascades, such as Ras-Raf-MEK-ERK and PI3 K pathways. Concomitant

stimulation of these pathways induces cellular changes that collectively give rise to a complex morphogenetic program known as "invasive growth" (Boccaccio and Comoglio 2006; Moumen et al. 2007; Xiao et al. 2001). This program implies cell spreading, cell–cell dissociation (scattering), migration, invasion, proliferation and differentiation. Notably, during these changes, cells result protected from apoptosis (Moumen et al. 2007; Xiao et al. 2001).

There is evidence that HGF has beneficial effects in the setting of ischemic injury. Both HGF and Met mRNA are upregulated in adult cardiomyocytes following heart injury (Nakamura et al. 2000; Ueda et al. 2001). In rats subjected to myocardial infarction, HGF elicits anti-apoptotic and cardioprotective effects (Nakamura et al. 2000; Ueda et al. 1999). In addition, HGF has proangiogenic activity and antifibrotic action (Taniyama et al. 2002). Finally, it plays a role in cardiac regeneration after myocardial infarction (Urbanek et al. 2005). Despite the clear indications for HGF to effectively treat post-ischemic heart failure, the information on the role of the HGF/Met system in normal cardiac development is still limited. HGF/Met are co-expressed in cardiomyocytes during very early embryonic development (Rappolee et al. 1996; Song et al. 1999), whilst their expression and function in the heart during late prenatal or early postnatal life have not been investigated.

In this paper we present a new mouse model which expresses, in an inducible and cardiac tissue specific way, the HGF protein. Up to now, the effects of HGF have been studied extensively mainly delivering the recombinant protein from an exogenous source. Our sophisticated mouse model with conditional gene expression offers a wealth of possibilities for research. Development of successful models of HGF expression will provide extensive information of the role of this protein in development and injury repair.

Materials and methods

Animal models

All animal procedures were approved by the Ethical Commission of the University of Torino, Italy, and by the Italian Ministry of Health. Mice harbouring the α-MHC-tTA (cardiac-specific) promoter construct were kindly donated by Dr. G. Fishman (Yu et al. 1996) and crossed with FVB pure mice for ten generations. Then mice were subsequently kept in a FVB background.

α-MHC-tTA/HGF-TetO-GFP transgenic mice

The bidirectional HGF-TetO-GFP responder plasmid was built as described (Giacobini et al. 2007). The HGF-TetO-GFP construct was cut with *AseI* and a 6.1-kb gel-purified fragment (GenElute™ Gel Extraction Kit, Sigma) was microinjected into the fertilized eggs of FVB mice in the San Raffaele-Telethon Core Facility for Conditional Mutagenesis (Milan, Italy). Founder mice were identified by PCR analysis of genomic DNA prepared from tail biopsies and screened for expression as described (Crepaldi et al. 2007). A total of five transgenic founders were obtained upon two series of microinjections. Ear fibroblasts from founder #24 expressed GFP upon infection with LV-TA1 lentiviral vector carrying the tTA under the phosphoglycerate kinase promoter. A transgenic line was established from this founder, analyzed by Southern blot, bred to α-MHC-tTA transactivator mice and maintained in a FVB background. Crosses were carried out between heterozygotes, so that most litters contained bitransgenic α-MHC-tTA/HGF-TetO-GFP+ and internal controls of α-MHC-tTA+, HGF-TetO-GFP+ and the wild-type genotype. Transmission of both transgenes followed typical Mendelian inheritance patterns. Mice were genotyped by PCR analysis of tail genomic DNA of P7-P9 mice. The tTA transgene was identified with primers annealing to tTA cDNA, resulting in a 547-bp fragment. The HGF-TetO-GFP responder transgene was identified with a primer annealing to exon 16 of HGF and the other to the globin poly A sequence downstream to the HGF transgene, resulting in a 524-bp fragment (Supplemental Table 1 for primer sequences).

To induce HGF-TetO-GFP transgene, α-MHC-tTA/HGF-TetO-GFP+ mice were fed in the absence of Doxycycline (DOX, Sigma). To repress HGF-TetO-GFP transgene, DOX was diluted in 3% sucrose in water to a final concentration of 200 μg/ml and freshly supplied every 2–3 days to α-MHC-tTA/HGF-TetO-GFP+ mice as drinking water.

PCR, real-time PCR and semi-quantitative RT–PCR

For the extraction, hearts were first homogenized with Turrax©, then RNA was extracted using TRIzol protocol

(Invitrogen, Carlsbad, CA, USA). After quantification (NanoDrop® ND-1000, Spectrophotometer, NanoDrop Technologies), cDNA was made using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA, USA). For Real-time PCR, primers and Taqman probe specific for the transgene were designed using the File builder 3.1 program (Applied Biosystems, Foster city, CA, USA). Then real-time PCR was performed on a 7,300 Real-time PCR instrument (Applied Biosystem). All real-time PCR sample reactions were performed in triplicate and normalized to 18S mRNA expression. For semi-quantitative RT–PCR, control samples (no RT) were prepared without adding the RT enzyme to the RT–PCR reaction. Tubulin was used as endogenous control. The fragments were amplified using specific primers (Supplemental Table 1). The conditions for the reactions were: Real-time PCR: Step 1: 1 cycle, 50°C-2 min. Step 2: 1 cycle, 95°C-2 min. Step 3: 45 cycles, 90°C-15 s/60°C-30 s/72°C-1 s. Genotyping PCR: Step 1: 1 cycle, 94°C-2:30 min. Step 2: 35 cycles, 94°C-20 s/61°C-45 s/72°C-30 s. Step 3: 1 cycle, 72°C-3 min. Semi-quantitative RT–PCR: Step 1: 1 cycle, 95°C-5 min. Step 2: 32 cycles, 95°C-1 min/60°C-45 s/72°C-45 s. Step 3: 1 cycle: 72°C-10 min.

Western blot (WB)

Protein extracts from heart ventricles were prepared using RIPA buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid sodium salt, 5 mM EDTA) to which Protease Inhibitor Cocktail (Sigma) was added. Heart lysates were subsequently sonicated twice for 10'' in ice and centrifuged at 14,000 rpm for 25 min at 4°C.

Protein concentration was determined by Bio-Rad protein assay and equal amounts of total cell lysate (5 μg) were separated by 8 or 12% SDS–PAGE under denaturing conditions and transferred onto nitrocellulose Hybond-C-extra membrane (Amersham). Membranes were saturated with 10% BSA (Sigma) in TBS-T (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4 added with NaN2), incubated overnight at 4°C with primary antibodies and then incubated with appropriate secondary anti-rabbit, anti-mouse or anti-goat antibodies conjugated to horseradish peroxidase (Amersham). Proteins were then revealed by enhanced chemiluminescence SuperSignal detection reagents (Pierce) and quantified with a GS800 model (Bio-Rad).

Stereomicroscopy analysis

The hearts of α-MHC-tTA/HGF-TetO-GFP+ and control mice were removed and rinsed in ice-cold Tyrode solution. Hearts were then fixed in 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Sodium Phosphate dibasic, 2 mM Potassium Phosphate monobasic, pH of 7.4) for 4 h at 4°C. After being washed in PBS for 20 min (5 times), hearts were incubated in 30% sucrose in PBS overnight at 4°C to preserve GFP fluorescence. GFP fluorescence was monitored by Leica MZ12 stereomicroscope and imaged by Evolution VF colour cool camera and Image-Pro Plus software.

Immunofluorescence (IF) and confocal analysis

The hearts of mice were removed and rinsed in ice-cold Tyrode solution (154 mM NaCl, 4 mM KCl, 1 mM MgCl2, 5.5 mM glucose, 5 mM Hepes, 10 mM 2,3 butanedionemonoxime, 5 mM Taurine, pH 7.4 adjusted with NaOH). Hearts were then fixed in 4% paraformaldehyde in PBS for 4 h at 4°C. After being washed in PBS for 20 min (5 times), hearts were incubated in 30% sucrose in PBS overnight at 4°C. These tissues were embedded in OCT mounting medium (Biooptica), quickly frozen in dry isopentan, and stored at −80°C. Sections were cut with a cryotome (Leica) at a thickness of 35-μm and washed with PBS (three times for 10 min each). Sections were then incubated with buffer containing 10% normal goat serum (Calbiochem), 1% Triton X-100 (Sigma) and primary antibodies for 48 h at 4°C followed by a wash in PBS (three 10 min steps). They were then incubated for 1 h at room temperature with the secondary anti-rabbit or anti-mouse antibody Cy3 (C 2306, Sigma) or Alexa Fluor (Molecular probes) in concentration 1:500 in PBS and washed in PBS (three 10 min steps). Sections were then mounted. Confocal microscope imaging was performed with a Fluoview 200 scan head on a IX 70 (Olympus Europa, Germany) inverted microscope with a 100 × 1.3 NA objective and a Ar/Kr laser. The 488 nm laser line was used to acquire enhanced green florescence protein fluorescence and to excite Alexa fluorescence, while simultaneously the 568 nm line was used to excite Cy3 fluorescence. Optical slices (1,024 by 1,024 pixels) were acquired every 200 nm on the z axis and processed with

ImageJ (Rasband W. National Institute of Health, Bethesda, MD, 2004) on a Macbook Pro (Apple, CA, USA) computer.

Antibodies

Anti-Cx43 (1:5,000 for WB; 1:300 for IF, C6219), anti-α-actinin (1:1,000 for WB and IF, clone EA-53) were from Sigma. Anti-ERK2 (1:1,000, C14), anti-p140Met (1:1,000 for WB; 1:300 for IF, SP260, Santa Cruz Biotechnology), anti-HGF (1:500, AF294-NA, R&D), Griffonia (Bandeiraea) Simplicifolia lectin I (Vector), anti-Ki67 (1:300, NCL-Ki67p, Novacastra). Secondary antibodies were either from Molecular Probes (Alexa Fluor 488, Alexa Fluor 546) or Sigma (anti-mouse Cy3, C 2306).

Heart explants

2–3 months-old mice were killed by cervical dislocation; under sterile conditions, hearts were excised and rapidly washed in D-PBS to eliminate blood, then cut in 2 mm^2 pieces by using surgical scissors and seeded in 10 cm plates in a final volume of 15 ml. In the control sample (HGF Heart explant CM +DOX) Doxycycline was added at a final concentration of 1 mg/ml. At day 6, conditioned media were collected, gently centrifugated and kept frozen at −80°C. All conditioned media were serially diluted with DMEM 10% FBS for subsequent scatter assays.

MDCK scatter assay

The Madin Derby Canine Kidney epithelial (MDCK) cells were maintained in Dulbecco's Modified Medium (DMEM) supplemented with 10% FBS. For scatter experiments, MDCK were seeded at a density of 500 cells per well in 96 wells plates. At the second day of proliferation, MDCK were added of serial dilutions of purified HGF (Sigma), explant-conditioned medium or DMEM 10% FBS; after 24 h, cells were PBS-washed, fixed with 4% paraformaldehyde and stained with cresyl violet using standard protocol.

Surgical procedure for cardiac ischemia and reperfusion

All procedures were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies. Animals were anesthetized with isoflurane 1, 5% and ventilated with a mechanical ventilator (Ugo Basile 28026 mouse ventilator tidal volume 0.2 mL; 120 stokes/min) through an endotracheal cannula. The left anterior descending coronary artery was ligated with a 7-O silk (Ethicon) suture after exteriorization of the heart through a 15-mm opening at the fourth-intercostal space. An overhand knot was tied with two pieces of suture to arrest blood flow and then removed after 45 min at 37°C. Ischemia was confirmed by the appearance of ventricular ectopy and blanching of the myocardium. The chest was then closed under negative pressure and mice were weaned from mechanical ventilation. Post surgical analgesia was achieved by buprenorphine (0.1 mg/kg s.c. q12 h for 1 day).

Echocardiography

Before sacrifice at week 10 after infarction, conscious mice underwent transthoracic echocardiography (Aloka SSD-5500, Tokyo, Japan) with the use of a 13 MHz linear transducer at high frame rate imaging (102 Hz). Short and long-axis two dimensional (2D) views and M-mode at the level of infarction were analyzed in real time and recorded on magneto-optic disk for off-line analysis by a sonographer blind to study groups. Left ventricular (LV) internal dimensions were measured, as recommended by the American Society of Echocardiography. Shortening Fraction (SF) was calculated from the composite LV internal, diastolic (LVIDd) and systolic (LVIDs) dimensions as: $SF = ((LVIDd\text{-}LVIDs)/LVIDd) \times 100$ from M-mode short axis view. Transmitral LV inflow velocities were measured from 4 and 5 apical chamber views respectively by pulsed- wave Doppler with a sample volume length of 3.5–7.5 mm; the ultrasound beam was aligned as parallel as possible to Colour Doppler flow and to record the highest velocity.

Histology

Infarct size

Upon completion of echocardiographic measurements, the heart was arrested in diastole by i.v. injection of a 2.5-M solution of KCl, quickly

removed from the chest, blotted dry, weighted, and immediately frozen at −80°C in OCT for histological studies. The heart was serially sectioned (10 μm) in a cryotome, and one every ten sections was stained with hematoxilin and eosin in order to locate the infarct and to measure its area by image analysis of high density digitized cardiac sections (ImageTool UTHSCSA).

Capillary counts

To visualize capillaries of myocardium, endothelial cells were stained with Griffonia simplicifolia lectin I (GS-I). Briefly, sections were pretreated with trypsin (1 mg/ml) for 30 min at 37°C and incubated with goat serum 10% for 1 h. Subsequently they were incubated with rodaminated Griffonia (Bandeiraea) Simplicifolia lectin I (Vector) (10 μg/ml) overnight at room temperature. After rinsing with PBS, the slides were incubated with bisbenzimide (0.5×) to visualize nuclei. Vascular density was assessed by counting lectin-positive vessels at the margins of the infarcted area (4.5–9.8% of the total area of the ventricles) at 400× in 50 fields/heart in a Olympus fluorescence microscope.

Interstitial collagen

Interstitial collagen was assessed in myocardium by staining 10 micron sections with Sirius red and analyzing digitized images at 400× magnification on the IMAGE ANALYZER IBAS 2.0 (Kontron–Zeiss image analysis system). Collagen was expressed as percent area of the total area of tissue within the high power field.

Proliferation count

Ki67 positive nuclei were counted in at least 8 fields per mouse.

Statistics

Data are expressed as the mean ± SD. Differences between groups were determined by using the two-tailed independent *T* test. The level of significance was set at $P < 0.05$.

Results

Generation of α-MHC-tTA/HGF-TetO-GFP bitransgenic mice

To examine the influence of inducible HGF expression in the myocardium, we generated a novel transgenic mouse model with tetracycline–regulatable expression of HGF in the heart (HGF responder). The mouse HGF cDNA was subcloned into the bidirectional responder plasmid pBI-eGFP, in which the bidirectional minimal promoters, downstream to TetO sequences (TetO)7, control the expression of the transgene of interest and that of GFP gene. The GFP reporter gene was chosen as being a convenient tracer of transgene expression. Bitransgenic α-MHC-tTA/HGF-TetO-GFP mice (HGF On) were obtained by crossing the HGF responder with an activator mouse, in which tTA expression is driven by α-MHC promoter (α-MHC-tTA transactivator; Fig. 1a-b). The α-MHC promoter is a well known promoter with tissue specific expression in the heart (Yu et al. 1996). In rodents the α-MHC gene transcript is transiently expressed throughout the myocardium between E8 and E9, being subsequently downregulated in the left ventricular muscle (Lyons et al. 1990; Zammit et al. 2000). Before birth α-MHC levels increase progressively and completely replace β-MHC by 14 days after birth (Siedner et al. 2003). In offspring resulting from mating α-MHC-tTA+ and HGF-TetO-GFP+ heterozygotes ($0 > 100$), transmission of both transgenes followed typical Mendelian inheritance patterns, with no significant increase in mortality of animals at weaning, indicating that transgenic HGF expression was not lethal.

Histological and immunohistochemical characterization of α-MHC-tTA/HGF-TetO-GFP bitransgenic hearts

GFP inspection under fluorescent stereomicroscopy of sacrificed induced bitransgenic E 14.5 embryos showed specific, although still modest, expression of the transgene only in the heart and not in other organs (Fig. 2a). A normal morphological heart phenotype of bitransgenic α-MHC-tTA/HGF-TetO-GFP (HGF On) newborn mice was observed using histological and stereo microscopy analysis, respectively (Fig. 2b-d). Expression of GFP transgene in the young mice (post-natal day 20) was chimeric and over 50% of cells in the

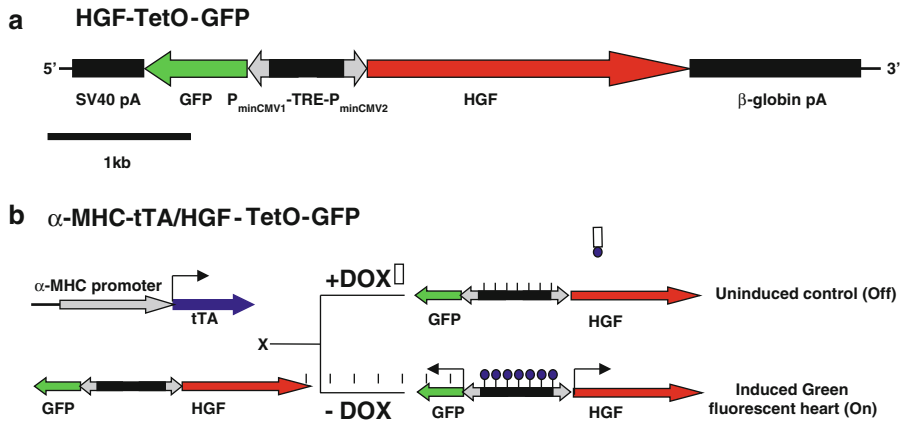

Fig. 1 Generation of α-MHC-tTA/HGF-TetO-GFP bitransgenic mice. **a** The HGF-TetO-GFP responder mouse was produced by microinjecting the construct containing the HGF and GFP (reporter gene) cDNA sequences under control of the TetO7 responsive element (TetO) fused to the bidirectional minimal promoters (PminCMV1, PminCMV2). The *β*-globin and late SV40 polyadenilation sites were downstream to HGF and GFP, respectively. **b** The responder mouse was crossed with the transactivator line containing the TetR/VP16 transactivator (tTA) under control of the α-MHC promoter. The HGF and GFP expression is induced in cardiac muscle by doxycycline (DOX) withdrawal conditions

left ventricle were positive for GFP (Fig. 2c). Tissue section analysis for transgenic GFP, Connexin43 and α-actinin demonstrated that the transgene was expressed specifically in cardiomyocytes (Fig. 3). Consistently, Met was found to be expressed in the heart of young adult mice, supporting the potentiality and effectiveness of stimulating the heart with HGF (Fig. 3).

Western blot analysis of HGF transgene and endogenous Met receptor

We validated the tissue specific and suppressible properties of α-MHC-tTA by expression analysis of transgenic HGF and GFP proteins. An increase in cleaved HGF protein over the basal level was observed in the absence of DOX (Fig. 4, first lane). Accordingly, no GFP protein was detected by western blot in uninduced bitransgenic mice (data not shown). Met expression in hearts was also confirmed (Fig. 4, middle lane). ERK2 was used as loading control.

Real time-PCR evaluation of transgenic HGF expression

We validated the tissue specific and suppressible properties of α-MHC-tTA/HGF-TetO-GFP by expression analysis of transgenic HGF mRNA (Fig. 5). Pregnant mothers and their progeny were left without Doxycycline (DOX) treatment to induce transgene expression (−DOX). In parallel matings, pregnant mothers were given DOX starting from conception, throughout pregnancy and breastfeeding period (+DOX). Quantitative RT–PCR experiments revealed that the transgenic specific HGF mRNA was detectable in bitransgenic hearts (1–4 months-old) in the absence of DOX (Fig. 5a). The set of primers and probe created to assess the expression of the transgenic HGF generated an aspecific signal in the WT mice reaction. This signal was constantly under the relative expression of 50. Therefore a cut-off expression of relative expression over 50 was settled for the positive mice, leaving everything that is less than 50 as a negative expression of the transgene. Surprisingly, we found that the expression of transgenic HGF was variable between littermate bitransgenic mice. GFP expression followed the same variability trend of transgenic HGF (Fig. 5a). Basing on an arbitrary evaluation of GFP fluorescence, we were able to divide the relative expression of HGF in bitransgenic mice in 4 groups (G+, G, G−, NG), accordingly with GFP levels (graph in Fig. 5a). Transgenic HGF expression was not found in DOX-treated bitransgenics (HGF Off), nor in littermate wild-type controls (both under relative expression of 50 as shown in Fig. 5b). This demonstrated efficiency of HGF probe and no detectable leakiness in transgenic expression.

To assess the reversibility of the Tet-Off system, DOX was administered until the age of 30 days

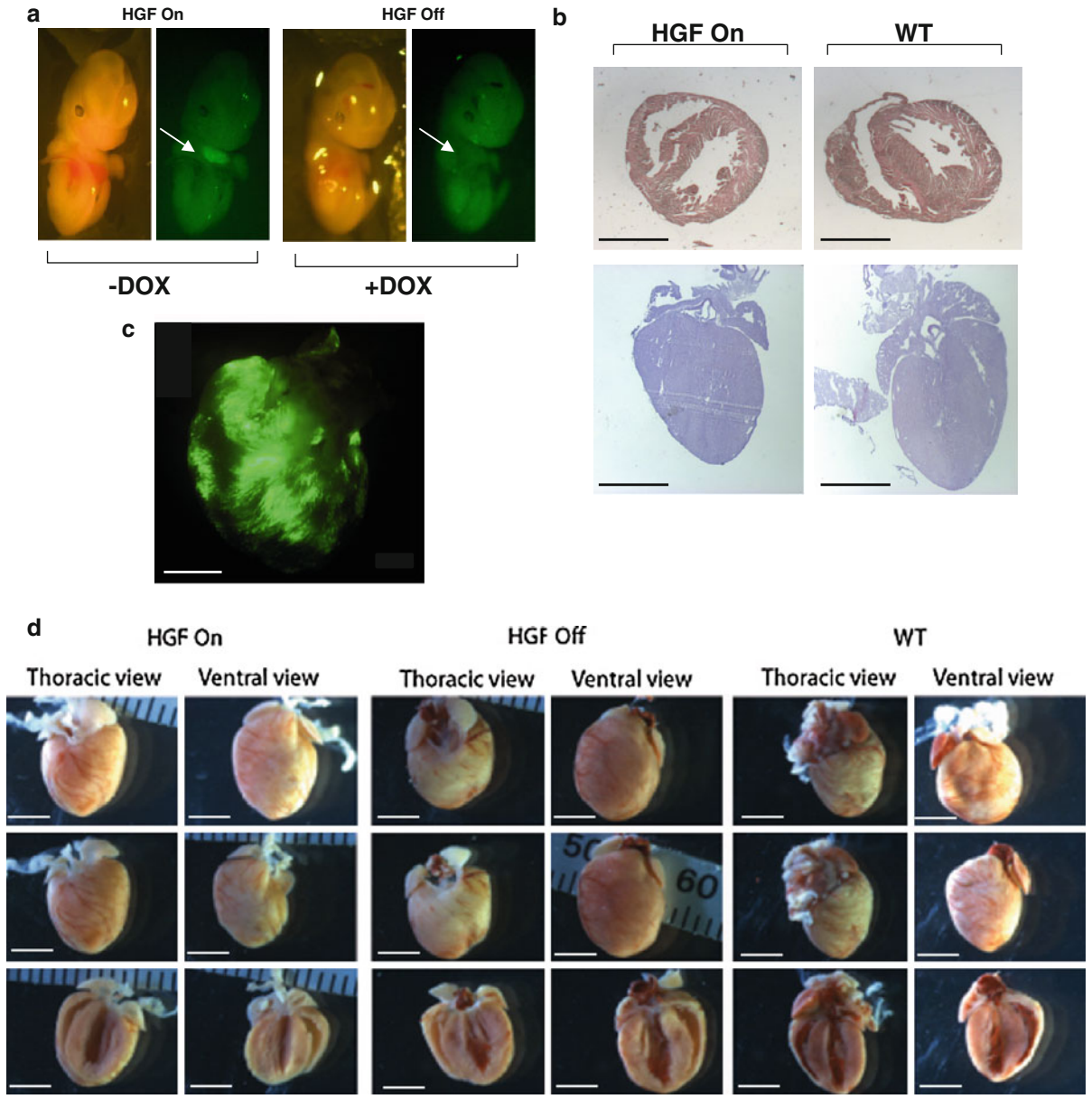

Fig. 2 Prenatal, postnatal and young HGF On (α-MHC-tTA/HGF-TetO-GFP bitransgenic mice kept without DOX) hearts show no morphological defects. **a** The α-MHC-tTA/HGF-TetO-GFP double transgenic embryos (E14.5) were raised in the absence (HGF On) or presence (HGF Off) of DOX during pregnancy starting from conception. The HGF On embryos showed strong *green fluorescence* localized in heart (*arrow*). **b** Haematoxylin-eosin staining of transversal (*upper panels*) and four-chamber cut (*lower panels*) sections of P7 HGF On (*left*) and control (WT, *right*) hearts. **c** Fluorescence of GFP (*strong green*) localized in young mice (P20 days) HGF On photographed under stereomicroscopy. **d** Visible stereomicroscopy of P7 HGF On (*left*), HGF Off (*middle*) and littermate wild-type (*WT*) control (*right*) hearts. Bars: b-d 2 mm

(+DOX), then expression was induced for 2 weeks (−DOX), until the age of 45 days when DOX was again administered for 1 week. Six points were chosen to assess the expression of transgenic HGF (scheme in lower left panel of Fig. 5c). After withdrawal of DOX, transgenic HGF mRNA was detected already after 1 week (points P3 and P4 in Fig. 5c). DOX treatment completely abrogated

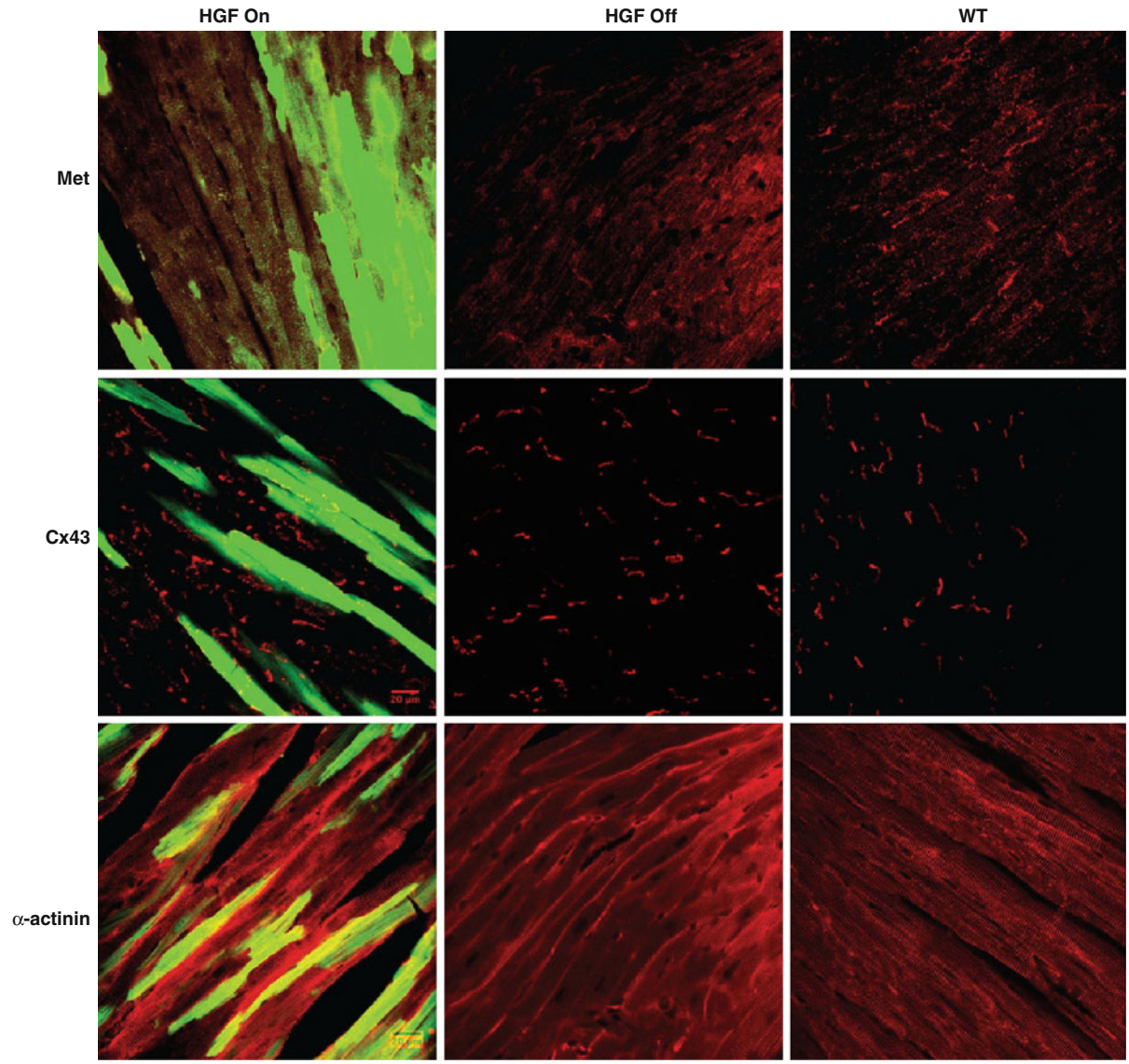

Fig. 3 Immunofluorescence of young mice hearts (P20 days). HGF On (*left line*), HGF Off (*middle line*) and littermate WT (*right line*) were stained for the transgenic expression of the GFP (*green* staining), the endogenous expression of Met, Cx43 and α-actinin (red stainings). Scale Bar: 20 μm

transgenic HGF mRNA within 12–24 h (points P5 and P6 in Fig. 5c). As shown before, also in this experiment the expression of the transgenic HGF was variable between mice.

Real time-PCR was performed also on other tissues to confirm the specificity of transgene expression (supplemental Figure 1). To better understand the reason of the variability of transgene expression, we specifically evaluated the expression of the target responder gene (HGF-TetO-GFP) and the expression of the α-MHC driven transactivator at different ages (supplemental Figures 2 and 3). In adults, the expression of the transgenic GFP and HGF was comparable; this was expected since both are expressed simultaneously (supplemental Figure 2). In contrast, the expression of the α-MHC-tTA didn't show any suppression in of the samples, whereas it was similar to that of the endogenous α-MHC gene

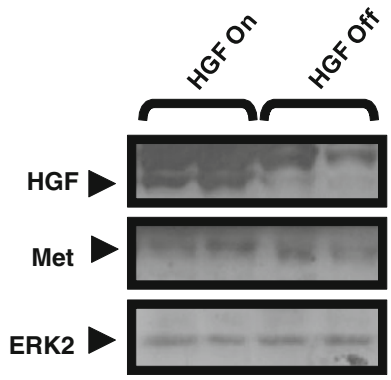

Fig. 4 Western blot of HGF expression in HGF On and HGF Off mice showing effective suppression of transgene expression by DOX. The expression of Met and ERK2, as loading control, is also shown

(supplemental Figure 2). In neonates the variability of GFP expression was less pronounced (supplemental Figure 3). At both ages, no correlation was seen between the expression of the α-MHC gene and the transgenic HGF. These results indicate that the variability between littermates is not caused by a defect in the transactivator promoter.

Scatter assay for HGF biological activity analysis

HGF protein was also detected by measuring the ability of heart explants to produce and release HGF in the culture medium. HGF, which is also known as Scatter Factor, has the unique property to induce cell–cell dissociation (scattering) in MDCK cells (Li et al. 1992). The production of the special conditioned medium (CM) is shown in Fig. 6a. The presence of HGF in conditioned medium (CM) was tested by using the typical scatter assay on MDCK epithelial cells. In the absence of HGF, these cells grow in compact colonies (Fig. 6b). The addition of 2 ng/ml purified HGF for 24 h to MDCK cells induced a typical change in morphology and a scatter response resulting in cell dispersion (Fig. 6c). CM from HGF On heart explants induced a strong cell-scatter response (Fig. 6d) comparable to that obtained by using purified HGF. CM from HGF On heart explants cultured in the presence of DOX for 5 days induced a scarce scatter response (Fig. 6e), comparable to that of WT CM (not shown). These data indicated that the HGF protein was released by heart explants in the culture medium and was turned-over after DOX treatment.

I/R model for evaluation of beneficial HGF effects in the heart

We checked if temporally limited expression of HGF specifically in the adulthood could influence heart functionality. Echocardiography assay was performed to show that there is no difference between induced, uninduced bitransgenic, single transgenic and wild-type mice (data not shown). A preliminary experiment was made to assess if the activation of HGF in the heart reduces ischemia/reperfusion induced injury. Ecocardiographic analysis showed significant differences between the induced bitransgenic group (HGF-ind) and wild-type (WT) (Fig. 7a). The anterior wall thickness, heart weight and heart-to-body weight ratio were significantly increased while the deceleration time (DT) was decreased. WT littermates were used instead of un-induced (+Dox) mice because it seems like Doxycycline has a beneficial effect on the infarct size in comparison with WT littermates (Supplemental Figure 3 and Cheung et al. 2000). Heart section analysis by histological staining revealed lower levels of collagen (Fig. 7b) and immunostaining of Ki67 positive cells showed a higher number of proliferating cells in bitransgenic hearts (Fig. 7c). Furthermore, immunofluorescence for Griffonia simplicifolia showed increased amounts of capillaries in the area next to the infarction (Fig. 7d) between induced (HGF-ind) and uninduced (HGF Off) bitransgenic mice, while in the septum there were no significant differences in capillary density (data not shown). These results were expected, since HGF is a molecule with known anti-fibrotic and pro-angiogenic properties.

Discussion

Cardiovascular diseases represent the leading cause for morbidity and mortality in the western world. However, myocardial infarction treatment remains poor and a great deal of research must still be focused on enhancing cardiac functionality and muscle regeneration. HGF has long been studied as a potential tool to enhance myocardial repair, avoid cell death and recruit stem cells. Our study proposes a new mouse model that enables the study of HGF in a temporally controlled window of activation and in a tissue specific manner. Using the α-MHC promoter,

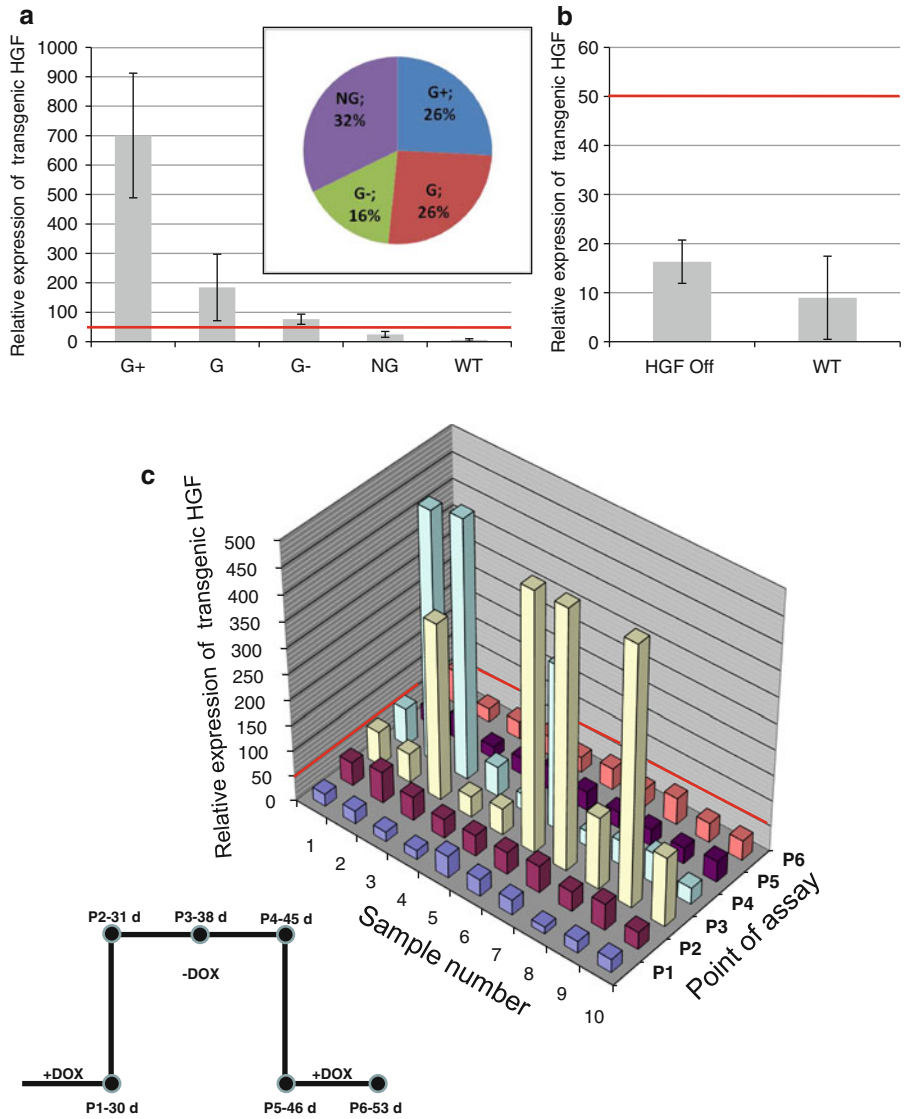

Fig. 5 Real time-PCR analysis. **a** Relative expression of the transgene (normalized to 18S mRNA) correlates with visual arbitrary evaluation of expression of the GFP reporter gene. GFP expression was divided in 4 groups: G+: very green ($n = 8$), G: green ($n = 8$), G−: less green ($n = 5$), NG: no green ($n = 10$). It is evident that the arbitrary evaluation of GFP levels through visual inspection of hearts perfectly matches with the respective quantitative measurement of transgenic HGF levels. **b** Relative expression of HGF in HGF Off ($n = 10$) mice is comparable to that of WT controls ($n = 6$). **c** Relative expression of transgenic HGF during the Curve of Induction (scheme on lower left side). Six points were chosen (P1-P6) to evaluate the expression of HGF, each point was composed by 10 double transgenic mice and 5 littermate WT controls (not shown in graph). As can be seen in points P1, P5 and P6 (HGF Off/+DOX) there is no expression of the transgene. In points P2, P3 and P4 (HGF-ind/−DOX) the expression is none in point P2 and variable within the mice in points P3 and P4. The cut-off (*red line*) of the expression in all the assays is 50. All real-time PCR sample reactions were performed in triplicate and normalized to 18S mRNA expression

we succeeded in expressing the transgenic protein HGF with a reporter Green Fluorescent Protein specifically in the heart. Thanks to the TetO-tTA Doxycycline inducible system, we were able to regulate the time frame of the transgene activation. Our results showed that both proteins are expressed in the heart when the transgenes transcription is activated. No adverse effects in gross morphology were

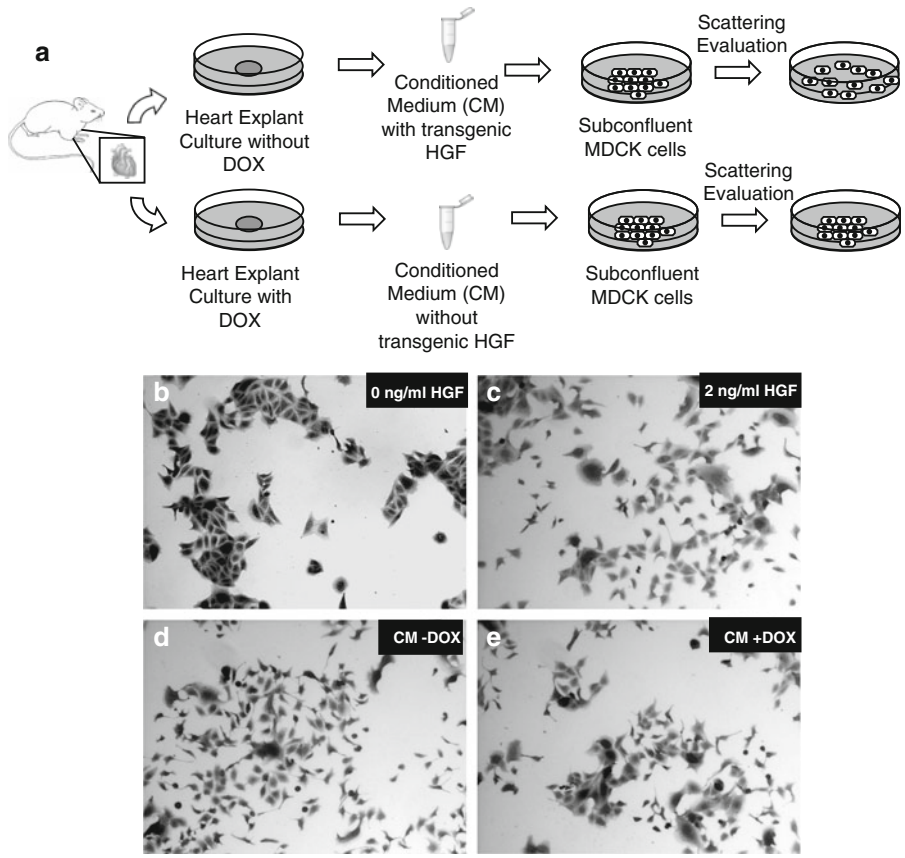

Fig. 6 HGF expression is reversible in α-MHC-tTA/HGF-TetO-GFP bitransgenic mice. **a** Scheme of production of conditioned medium (CM). **b**-**e**) MDCK epithelial cells form colonies in cell culture (b), while they "scatter" when exposed to purified HGF for 24 h (**c**). Heart explants from HGF On mice were grown in culture for 5 days in the absence or presence of DOX to create and collect the conditioned mediums CM -DOX (HGF positive) and CM +DOX (HGF negative), then they were tested on MDCK cells (**d**, **e**)

observed in neonatal or adult bitransgenic mice, when induced from conception. No leakiness in transgene expression could be detected in uninduced bitransgenic mice, thus supporting the robustness of our model. Effective activity of mature HGF protein released by induced bitransgenic hearts was confirmed. GFP transgene showed a chimeric pattern of expression; however, we evaluated that more than 50% of cardiomyocytes in the left ventricle showed GFP expression. These results are in accordance with previous data obtained with the Tet-Off binary system using the present α-MHC-tTA transactivator and may depend on position-related negative control (Fishman 1998; Redfern et al. 1999). Variability in expression levels of transgenic HGF has been also observed in littermate mice. Defects in the transactivator promoter have been excluded by Real-time PCR of the α-MHC-tTA gene, confirming the efficacy of the transactivator line. Furthermore, variability of HGF/GFP expression among littermates was higher in adults than in neonates, suggesting an age-dependent mechanism of downregulation. This issue will be further investigated in the future. We will also evaluate whether bringing the transgene into homozygosity will enable to solve this matter. Finally, we also observed differences between transgenic animals and the WT controls in terms of endogenous α-MHC expression. This suggests that the α-MHC-tTA construct used in our study may have influence on gene expression in the myocardium, as previously reported (McCloskey et al. 2005).

The expression of HGF in the adult heart revealed no negative effects, but rather was able to improve cardiac functionality, diminish scar area and increase

Echocardiographic and post-mortem available parameters in mice with ischemia and reperfusion (I/R)

a

	HGF-ind+I/R n=8	WT+I/R N=9	p
HR (bpm)	618±10*	604±16	NS
LVIDd (mm)	3.28±0.12	3.03±0.13	NS
AWTh (mm)	1.10±0.03	0.97±0.05	0.0465
SF (%)	45.1±3.62	52.21±3.88	NS
DT (ms)	24.25±2.51	35.33±1.91	0.0028
BW (g)	28.19±1.30*	27.34±1.35	NS
HW (mg)	134.75±5.38	114.44±5.49	0.0189
HW/BW (mg/g)	4.83±0.18	4.30±0.13	0.0262

*mean±SEM; *p*: Student t-test was used. HR: heart rate; LVIDd: left ventricular internal diastolic diameter; AWTh: anterior wall thickness; SF:shortening fraction; DT:deceleration time; BW:body weight; HW: left and right ventricular weight.

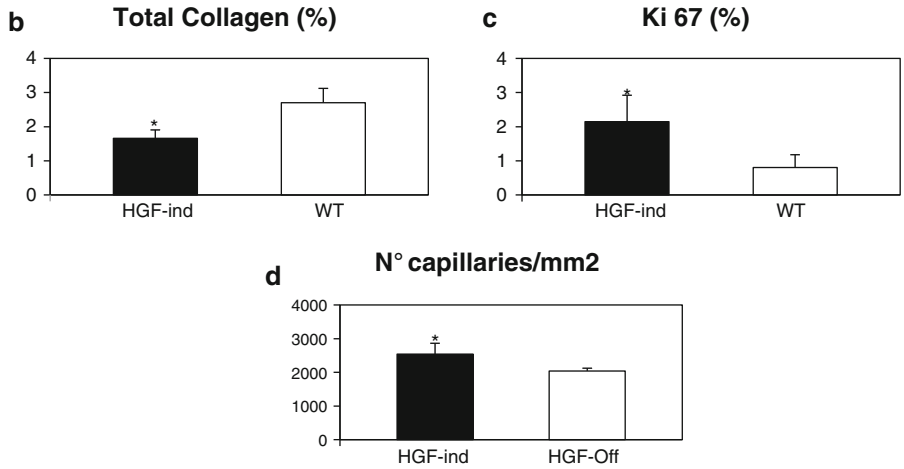

Fig. 7 **a** Echocardiographic analysis showing that the only significant differences between the two groups (HGF induced in the adult and WT) are: the anterior wall thickness, the deceleration time, the heart weight and the relation between body and heart weight. **b** The level of collagen was lower in HGF-ind mice. Results were normalized: % of collagen stain in total ventricular area. **c** The measurement of Ki67 positive cells showed a higher number of proliferating cells in HGF-ind vs WT mice. Results were normalized: number of Ki67 positive cells in total of 100 nuclei. $n = 3$ animals per group **d** The Griffonia stain showed a higher amount of capillary density in the area close to the infarction in the HGF-ind ($n = 6$) vs HGF-Offmice ($n = 4$)

proliferation in the heart of mice subjected to myocardial ischemia–reperfusion injury. Paracrine secretion of HGF protein resulted in a clearly detectable proangiogenic activity, indicating an important role of HGF in the regeneration of endothelial cells. This is consistent with the finding that Met was found in myocardial infarction, where it coexisted with CD31, CD34 and VWF-positive cells (Wang et al. 2004). In fact, HGF gene transfer has been shown to be effective in improving angiogenesis

in several ischemic models (Morishita et al. 1999; Aoki et al. 2000; Hayashi et al. 1999; Taniyama et al. 2001; Wang et al. 2006). Our results reinforce the concept that HGF can be a powerful tool to treat cardiovascular diseases and that our model is an innovative instrument to study these matters. As a word of caution, it should be noted that some results in the literature suggest a possible effect of the current α-MHC-tTA transactivator line in ischemia models (Turnbull et al. 2006). Thus, although a link between HGF and cardioprotection against I/R injury is suggested in this study, further work will be required to validate our HGF/GFP responder using other transactivators driven by newly developed cardiac-specific promoters (Sanbe et al. 2003; Breckenridge et al. 2009).

In this paper, we concentrated our attention on the characterization of our mouse model with HGF induction specifically in the adulthood. In future work, we intend to explore the effects of HGF on embryogenesis by expressing the protein during the developmental phases.

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