Journal of the American College of Cardiology
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Published by Elsevier Inc.

Vol. 58, No. 9, 2011
ISSN 0735-1097/$36.00
doi:10.1016/j.jacc.2011.05.013

Endogenous Cardiac Stem Cell Activation by Insulin-Like Growth Factor-1/Hepatocyte Growth Factor Intracoronary Injection Fosters Survival and Regeneration of the Infarcted Pig Heart

Georgina M. Ellison, PHD,*† Daniele Torella, MD, PHD,*† Santo Dellegrottaglie, MD, PHD,§ Claudia Perez-Martinez, DVM, PHD,‖ Armando Perez de Prado, MD,‖ Carla Vicinanza, PHD,*† Saranya Purushothaman, BSC,* Valentina Galuppo, MD,† Claudio Iaconetti, PHD,† Cheryl D. Waring, PHD,* Andrew Smith, PHD,* Michele Torella, MD, PHD,# Carlos Cuellas Ramon, MD,‖ Jose Manuel Gonzalo-Orden, DVM, PHD,‖ Valter Agosti, MD, PHD,‡ Ciro Indolfi, MD,† Manuel Galiñanes, MD, PHD,¶ Felipe Fernandez-Vazquez, MD, PHD,‖ Bernardo Nadal-Ginard, MD, PHD*

Liverpool, United Kingdom; Catanzaro and Naples, Italy; and León and Barcelona, Spain

Objectives The purpose of this study was to test the ability of insulin-like growth factor (IGF)-1/hepatocyte growth factor (HGF) to activate resident endogenous porcine cardiac stem/progenitor cells (epCSCs) and to promote myocardial repair through a clinically applicable intracoronary injection protocol in a pig model of myocardial infarction (MI) relevant to human disease.

Background In rodents, cardiac stem/progenitor cell (CSC) transplantation as well as in situ activation through intramyocardial injection of specific growth factors has been shown to result in myocardial regeneration after acute myocardial infarction (AMI).

Methods Acute MI was induced in pigs by a 60-min percutaneous transluminal coronary angiography left anterior descending artery occlusion. The IGF-1 and HGF were co-administered through the infarct-related artery in a single dose (ranging from 0.5 to 2 μg HGF and 2 to 8 μg IGF-1) 30 min after coronary reperfusion. Pigs were sacrificed 21 days later for dose-response relationship evaluation by immunohistopathology or 2 months later for cardiac function evaluation by cardiac magnetic resonance imaging.

Results The IGF-1/HGF activated c-kit positive–CD45 negative epCSCs and increased their myogenic differentiation in vitro. The IGF-1/HGF, in a dose-dependent manner, improved cardiomyocyte survival, and reduced fibrosis and cardiomyocyte reactive hypertrophy. It significantly increased c-kit positive–CD45 negative epCSC number and fostered the generation of new myocardium (myocytes and microvasculature) in infarcted and peri-infarct/border regions at 21 and 60 days after AMI. The IGF-1/HGF reduced infarct size and improved left ventricular function at 2 months after AMI.

Conclusions In an animal model of AMI relevant to the human disease, intracoronary administration of IGF-1/HGF is a practical and effective strategy to reduce pathological cardiac remodeling, induce myocardial regeneration, and improve ventricular function. (J Am Coll Cardiol 2011;58:977–86) © 2011 by the American College of Cardiology Foundation

From *The Stem Cell and Regenerative Biology Unit (BioStem), RISES, Liverpool John Moores University, Liverpool, United Kingdom; †Molecular and Cellular Cardiology, Department of Medicine, Magna Graecia University, Catanzaro, Italy; ‡Molecular Oncology, Department of Medicine, Magna Graecia University, Catanzaro, Italy; §Division of Cardiology, Federico II University, Naples, Italy; ‖IBIOMED y Fundación Investigación Sanitaria en León, Universidad y Hospital Universitario de León, León, Spain; ¶Department of Cardiac Surgery, Reparative Therapy of the Heart, Area del Cor and Research Institute, University Hospital Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain; and the #Department of Cardio-Thoracic and Respiratory Sciences, Second University of Naples, Naples, Italy. This work was in part supported by grants from the British Heart Foundation (PG/06/045; PG 08/085), Marie Curie FP7 (PIRG02-GA-2007-224853), Cardio Repair European Multidisciplinary Initiative FP7 (FP7-HEALTH-2009-242038), FIRB-Futuro-in-Ricerca (RBFR081CCS), Associazione Italiana per la Ricerca sul Cancro (MFAG-2008), Italian Ministry of Health (GR-2008-1142673), and Spanish Ministry of Science and Innovation (MoSI, Factostem DEX-580000-2008-0043). The Instituto Carlos III through the "Red de Terapia Celular and the Junta de Castilla y Leon" supported cardiac magnetic resonance imaging studies. Iberhospitex

Abbreviations and Acronyms

AMI = acute myocardial infarction
BrdU = bromodeoxyuridine
CD45^{neg} = CD45 negative
c-kit^{pos} = c-kit positive
cMRI = cardiac magnetic resonance imaging
CSC = cardiac stem/progenitor cell
CTRL = control
eCSC = endogenous resident cardiac stem/progenitor cells
epCSC = endogenous porcine cardiac stem/progenitor cell
HGF = hepatocyte growth factor
IGF = insulin-like growth factor
LV = left ventricular
MI = myocardial infarction

The presence of endogenous resident cardiac stem and progenitor cells (eCSCs) in the adult mammalian heart of different species, including human (1,2), supports the notion that the adult myocardium has an intrinsic regenerative capacity, which could be exploited to produce meaningful myocardial regeneration (3–6).

Data from mice and dogs show that the regenerative response of eCSCs to a ischemic insult can be enhanced in situ by administration of growth factors (7,8). Nevertheless, before such protocols can be clinically tested, they need to be validated in a large-animal model that closely mimics the human heart using a growth factor dose and method of administration compatible with clinical application. Swine, because of similarities to ours in organ size, coronary anatomy, immunology, and physiology, stands up as the most attractive model for pre-clinical protocols of eCSC activation for myocardial regeneration.

Here, we show that the adult pig myocardium harbors c-kit positive (c-kit^{pos}) CD45 negative (CD45^{neg}) eCSCs, which show rapid activation and robust cardiomyogenic differentiation when stimulated by insulin-like growth factor (IGF)-1 and/or hepatocyte growth factor (HGF) in vitro. Small amounts of IGF-1 and HGF administered to the damaged porcine myocardium through the coronary circulation after acute myocardial infarction (AMI), in a linear dose/effect relationship, salvages a significant portion of the myocardium at risk, produces a robust activation of the resident eCSC pool and regenerates most myocytes and microvasculature lost by the ischemic insult. At 2 months after myocardial infarction (MI), this treatment results in improved left ventricular (LV) function and reduced infarct size.

S. L., Spain, provided catheters, stents, and other technical supplies. Dr. Nadal-Ginard is a cofounder of and worked as Scientific Director of Coretherapix, SRL, Madrid, Spain, a biotechnology start-up company. Coretherapix was not involved in any aspect of the experiments described in this paper apart from participating in the Factostem, a multicenter cooperative grant awarded to Coretherapix as the coordinator and three other Institutions among which is the Fundación Investigación Sanitaria of Leon (FIS). The Factostem grant funded a portion of the animal experiments with cMRI performed at FIS. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose. Drs. G. Ellison and D. Torella contributed equally to this work. Drs. Fernandez-Vazquez and Nadal-Ginard shared the senior responsibility for the project.

Manuscript received April 20, 2011; revised manuscript received May 20, 2011, accepted May 24, 2011.

Methods

See the Online Appendix for a complete description of Methods.

Results

Porcine resident c-kit^{pos}CD45^{neg} cardiac cells are multipotent stem/progenitor cells. Small cells positive for c-kit (c-kit^{pos}) and negative for blood-cell lineage markers (i.e., CD45), Lin^{neg}, are ubiquitous in the adult porcine myocardium, with higher density in the atria and the ventricular apex (Online Fig. 1).

The c-kit^{pos} cardiac cells constituted 9 ± 2% and 4 ± 2% of the starting myocyte-depleted cardiac small cell population from atria and LV, respectively (Fig. 1A). Moreover, 75 ± 12% and 50 ± 8% of these c-kit^{pos} cells from the atria and the left ventricle, respectively, also expressed CD45 (Figs. 1B and 1C), which, when co-expressed with c-kit, identifies resident cardiac mast cells (9). Thus, using negative (CD45) and positive (c-kit) sorting by magnetic activated cell sorting technology, we obtained >95% enriched c-kit^{pos} cardiac cells, depleted of tryptase^{pos} and CD45^{pos} mast cells (Figs. 1D and 1E), and endothelial/hematopoietic CD34^{pos} progenitors (Online Fig. 2). A fraction of c-kit^{pos}CD45^{neg} cardiac cells expressed the mesenchymal marker, CD90 (38 ± 6%) and adhesion molecule/cardiac progenitor marker, CD166 (74 ± 11%) (Fig. 1E). Porcine c-kit^{pos}CD45^{neg} cardiac cells were negative for a panel of CD markers specific for other hematopoietic, mesenchymal, and endothelial cell lineages (Online Fig. 2). Freshly isolated c-kit^{pos}CD45^{neg} cells expressed Oct3/4 (3 ± 1%) (Fig. 1F to 1H), SSEA-4 (11 ± 2%) (Fig. 1F), SSEA-3 (10 ± 3%), Nanog (9 ± 3%), Telomerase (58 ± 8%), Bmi-1 (59 ± 11%), Flk-1 (46 ± 5%), Gata-4 (49 ± 7%), Nkx2.5 (29 ± 10%), and Isl-1 (1 ± 1%).

The c-kit^{pos}CD45^{neg} cells from atria, ventricles, and apex exhibited ~20% clonal efficiency, independently of their chamber of origin (Online Fig. 3). Three randomly picked clones each from atria-, ventricle-, and apex-derived cells were further expanded. These clones had a ~22 h doubling time. They have been propagated for >65 passages and serially subcloned every 10 passages without reaching growth arrest, senescence, or showing any detectable chromosomal alterations (Online Fig. 4). Cloned cells showed positivity for c-kit (90 ± 8%), Oct3/4 (62 ± 11%), SSEA-3 (65 ± 10%), SSEA-4 (59 ± 14%), Nanog (46 ± 5%), telomerase (81 ± 10%), Bmi-1 (70 ± 14%), Flk-1 (86 ± 9%), Gata-4 (60 ± 11%), Nkx2.5 (52 ± 8%) and Isl-1 (8 ± 6%) (Figs. 1G and 1H, Online Figs. 3 and 5), indicating higher clonal efficiency of cells expressing multipotency genes.

Cloned c-kit^{pos}CD45^{neg} cardiac cells grew in suspension and generated cardiospheres (1,2). Cardiospheres placed in differentiation medium attached, and cells spread out from the sphere (Online Fig. 3), differentiating into myocytes (27 ± 4%), endothelial (10 ± 6%), and smooth muscle cells (34 ± 5%) (Online Fig. 3). These results show that porcine resident c-kit^{pos}CD45^{neg} cardiac cells (hereafter identified

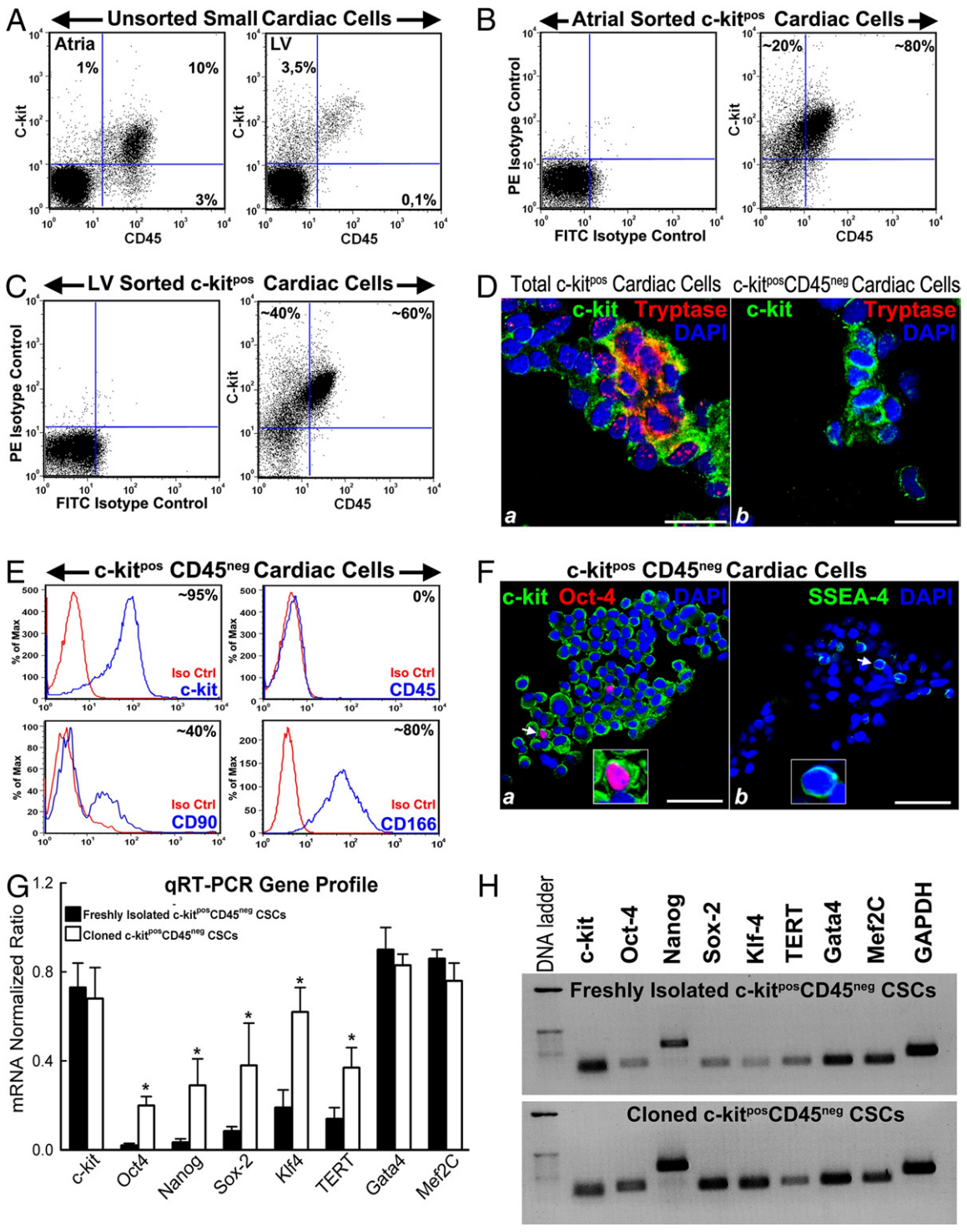

Figure 1 **Phenotype of Porcine c-kit^{pos}CD45^{neg} Cardiac Stem/Progenitor Cells**

(A) Florescence activated cell sorting (FACS) analysis of c-kit^{pos} CD45^{pos} cells within the myocyte-depleted cardiac small cells from the atria and left ventricle (LV). **(B, C)** FACS analysis of c-kit^{pos} magnetic activated cell sorting (MACS)-sorted cells from right atria and LV distinguishes CD45 positive and negative fractions. **(D)** A significant fraction of freshly isolated porcine c-kit^{pos} **(green)** cardiac cells express tryptase **(red; a)** but when depleted of CD45^{pos}, cells are negative for tryptase **(b)**. Bar = 20 μm. **(E)** Phenotype of c-kit^{pos}CD45^{neg} cells obtained using MACS sorting. **(F)** Oct-4 expression **(red; a)** and SSEA-4 expression **(green; b)** in freshly isolated ckit^{pos}CD45^{neg} cardiac cells. Nuclei stained by 4',6-diamidino-2-phenylindole (DAPI) in **blue**. Bar = 50 μm. **(G, H)** Bar graph and representative gel showing stemness and cardiac gene transcripts in freshly isolated and cloned endogenous porcine cardiac stem/progenitor cell (epCSCs). *$p < 0.05$ versus freshly isolated epCSCs. DNA = deoxyribonucleic acid; FITC = fluorescein isothiocyanate; mRNA = messenger ribonucleic acid; PE = phycoerythrin; qRT-PCR = quantitative reverse transcription-polymerase chain reaction.

as endogenous porcine CSCs [epCSCs]) have true stem/progenitor cell characteristics (2).

The epCSCs express intact IGF-1/HGF signaling pathways that modulate their activation and differentiation in vitro. The epCSCs express IGF-1 and HGF (known as c-met) receptors (Online Fig. 3). When grown in culture, freshly isolated and cloned c-kit^{pos}CD45^{neg} epCSCs respond to stimulation with human recombinant IGF-1 and human recombinant HGF with proliferation, migration, and activation of specific downstream effector pathways (Online Fig. 6).

When IGF-1/HGF were separately added to epCSCs, IGF-1 had no effect on myogenic differentiation, as measured by the number of Nkx2.5 and cardiac troponin I positive cells (Figs. 2A to 2D). In contrast, HGF increased significantly the number of these cells (Figs. 2A to 2D). The combination of the 2 factors did not increase myogenic differentiation over HGF alone (Figs. 2A to 2D). Yet, neither factor alone or in combination was able to induce epCSC differentiation into beating myocytes. However, co-culture with adult rat ventricular myocytes induced epCSCs (tagged with green fluorescent protein lentiviral construct [GFP^{pos}]) to synchronized beating (Online Video 1). The HGF was a stronger inducer of functional cardiomyogenic differentiation than IGF-1, while the combination of both growth factors significantly improved the effects of each alone (Figs. 2E and 2F). These data suggest that HGF is able to prompt epCSC cardiomyogenic commitment per se without inducing cell contraction. Together, IGF-1 and HGF stimulate a paracrine response in adult myocytes that induces functional cardiomyogenic maturation of differentiating epCSCs in vitro.

Intracoronary IGF-1/HGF injection after AMI preserves the organization of the infarcted tissue and improves cardiomyocyte survival. The IGF-1/HGF were administered to female juvenile White pigs (22 ± 3 kg) after AMI produced by 1 h of total balloon occlusion of the left anterior descending coronary artery below the origin of the first diagonal branch, followed by 30 min reperfusion (10). Different doses of IGF-1/HGF, identified as 1×, 2×, and 4× dose (0.5/2 μg, 1/4 μg, and 2/8 μg, respectively) (see Online Fig. 7) were administered in 15 ml of phosphate-buffered saline over 15 min at a rate of 1 ml/min with 1 min reperfusion every 5 min. The control group pigs (CTRL) were submitted to the identical protocol but received saline alone. To track myocardial cell regeneration, animals were administered bromodeoxyuridine (BrdU) through an implanted osmotic pump for 14 days.

There was no statistical difference in the infarcted area at the time of sacrifice, between the IGF-1/HGF-treated and the CTRL group (23 ± 2%, 21 ± 3%, 20 ± 3% in IGF-1/HGF 1×, 2×, and 4×, respectively, vs. 21 ± 3% in CTRL) 21 days after AMI. However, histological analysis revealed islands of survived myocardial tissue distributed among the fibrotic infarcted zone that were more abundant in the IGF-1/HGF treated than in the CTRL infarcted myocardium (Figs. 2G and 2H). These islands consisted of large BrdU-negative cardiomyocytes, a phenotype that, together with their mature, even hypertrophic nature confirmed their survival as pre-infarct myocytes (Fig. 2I). Compared with CTRL, the IGF-1/HGF-treated hearts, in a dose-dependent manner, had significantly less fibrotic tissue in the infarct region (Figs. 2J to 2L). Additionally, IGF-1/HGF reduced myocyte apoptosis and hypertrophy in the peri-infarct/border zone in a dose-dependent manner (Figs. 2M to 2O). These findings indicate that early IGF-1/HGF administration after AMI rescues myocytes at risk and improves myocardial remodeling in pigs.

Intracoronary IGF-1/HGF administration after AMI activates epCSCs. In healthy and post-AMI hearts, ~90% of c-kit^{pos} epCSCs express IGF-1 and c-met (HGF) receptors (Figs. 3A and 3B). Accordingly, 21 days after AMI, IGF-1/HGF-treated infarcted hearts showed a significant increase of c-kit^{pos}CD45^{neg} epCSCs in the border region and even higher in the infarcted area (Figs. 3C and 3D). These cells are CD45^{neg} and tryptase^{neg} and were clearly distinguishable from resident c-kit^{pos}CD45^{pos} cardiac mast cells (Fig. 1D).

That the increase in c-kit^{pos}CD45^{neg} epCSCs is the result of IGF-1/HGF administration was confirmed by its direct correlation to the IGF-1/HGF-dose administered (Fig. 3D). At the highest dose, the number of epCSCs in the infarcted area is more than 6-fold greater than in the CTRL hearts (Fig. 3D, Table 1). Most epCSCs were BrdU positive, a fixture that documents their birth after AMI (Fig. 3E). Many epCSCs expressed either transcription factor Nkx-2.5, Ets-1, or Gata6, indicative of their commitment to the myocyte, endothelial, and smooth muscle lineage, respectively (Figs. 3F to 3I). The number of committed myogenic progenitors (c-kit^{pos}Nkx2.5^{pos} cells) significantly increased in the infarct and border regions of the treated hearts in a dose-dependent manner (Fig. 3G, Table 1).

IGF-1/HGF administration produces robust myocardial cell regeneration after AMI. As expected from the increase in epCSCs and committed myocyte progenitors, IGF-1/HGF-treated hearts harbored a large population of very small, newly formed BrdU^{pos} and still-proliferating (Ki67^{pos}) myocytes in the infarct and border regions (Figs. 4A to 4F). Furthermore, some were in mitosis and cytokinesis, confirming their immature nature (Fig. 4G). Newly formed BrdU^{pos} myocytes were also present in the border region of the CTRL pigs. However, their number was less than one-fifth of those in IGF-1/HGF 1× treated hearts, and they were practically absent in the infarct zone (Figs. 4H and 4I, Online Fig. 8, Table 1).

There was a direct correlation between the number of small BrdU^{pos}/Ki67^{pos} newly formed myocytes and IGF-1/HGF-dose (Fig. 4H and 4I, Table 1). The BrdU^{pos} myocytes were organized as clusters of regenerating bands in the infarct zone, which were more organized and compacted with increasing IGF-1/HGF dose (Fig. 4A and 4B).

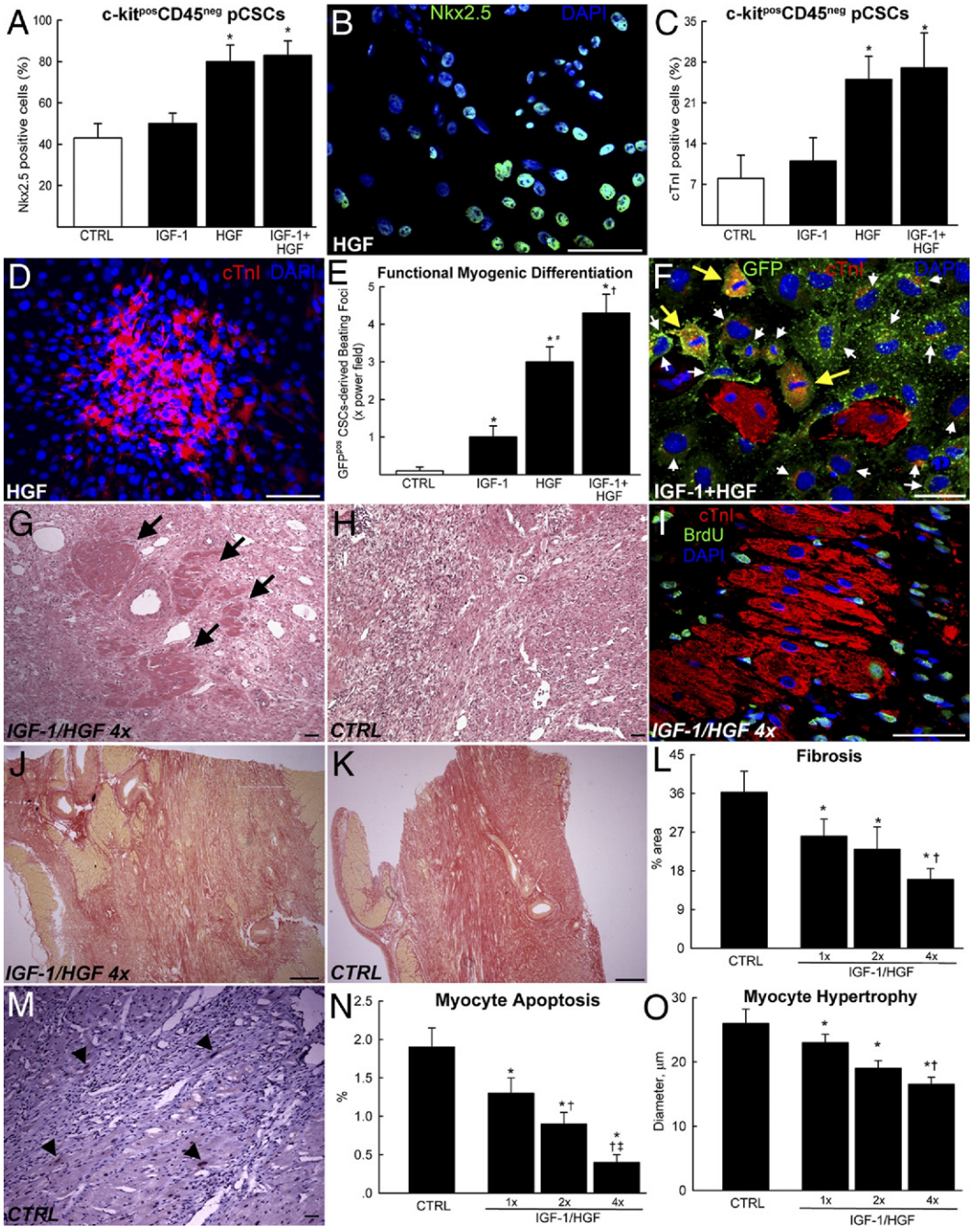

Figure 2 **Effects of IGF-1/HGF on epCSC Myogenic Differentiation In Vitro and on Myocardial Cell Remodeling After AMI In Vivo**

(A, B) Number and representative staining of Nkx-2.5 **(green)** positive myocyte progenitor endogenous porcine cardiac stem/progenitor cells (epCSCs) upon insulin-like growth factor (IGF)-1/hepatocyte growth factor (HGF) treatment (*p < 0.05 vs. control [CTRL] and IGF-1). Bar = 50 μm. **(C, D)** Number and representative staining of cardiac troponin I (cTnI) positive epCSCs (cTnI; **red**) upon IGF-1/HGF treatment (*p < 0.05 vs. CTRL and IGF-1). Bar = 50 μm. **(E, F)** Number and representative staining of GFP^{pos} **(green)** epCSC-derived beating cardiomyocytes (cTnI; **red**), when co-cultured with adult rat ventricular myocytes upon IGF-1/HGF treatment (*p < 0.05 vs. CTRL; #p < 0.05 vs. IGF-1; †p < 0.05 vs. all). **Yellow arrows** point to newly formed contractile GFP^{pos} epCSC-derived myocytes, while **small white arrows** point to very immature GFP-^{pos} epCSC-derived small myocyte precursors. Bar = 20 μm. **(G, H)** Islands of survived myocardial tissue in the infarct zone of 4× IGF-1/HGF-treated pigs **(G; arrows)**, which were infrequent and less defined in structure in the CTRL (H). Bar = 50 μm. **(I)** Surviving, mature, bromodeoxyuridine (BrdU) negative cardiomyocytes (cTnI; **red**) constituted these survived myocardial islands. Bar = 50 μm. **(J, K)** Representative fibrotic tissue (sirius red staining) in the infarct zone, in 4× IGF-1/HGF-treated **(J)** and CTRL **(K)** pig hearts. Bar = 500 μm. **(L)** Percentage area fraction of fibrosis in the infarct zone. *p < 0.05 versus CTRL; †p < 0.05 versus IGF-1/HGF 1×. **(M)** Apoptotic myocytes (casapse-3, **brown**; **arrowheads**) in the peri-infarct/border zone of a CTRL pig heart. Bar = 50 μm. **(N)** Cumulative data on myocyte apoptosis in the peri-infarct/border zone. *p < 0.05 versus CTRL; †p < 0.05 versus IGF-1/HGF 1×; ‡p < 0.05 versus IGF-1/HGF 2×. **(O)** Cumulative data on myocyte hypertrophy. *p < 0.05 versus CTRL; †p < 0.05 versus IGF-1/HGF 1×. All data are mean ± SD, n = 5, 4, 5, and 4 for CTRL, IGF-1/HGF 1×, 2×, and 4×, respectively. AMI = acute myocardial infarction. See Online Video 1.

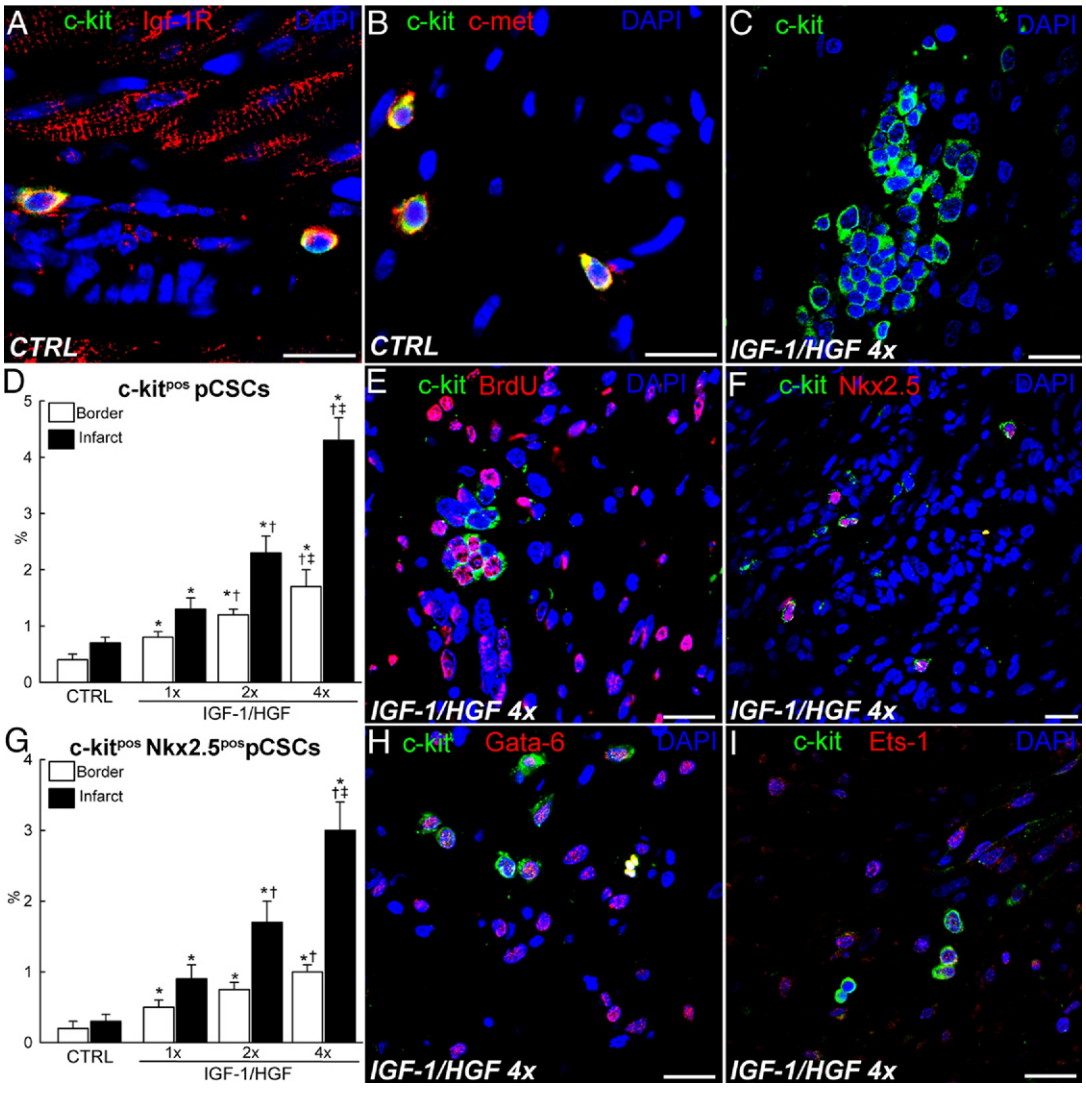

Figure 3 **IGF-1/HGF Intracoronary Administration After AMI Activates epCSCs Driving Their Cardiac Commitment**

(A, B) The c-kit^{pos} endogenous porcine cardiac stem/progenitor cells (epCSCs) **(green)** express **(A)** insulin-like growth factor (IGF)-1 **(red)** and **(B)** c-met **(red)** receptors in vivo. **(C)** A large cluster of c-kit^{pos} **(green)** epCSCs in the infarct zone of an IGF-1/hepatocyte growth factor (HGF) 4× treated pig heart. **(D)** Number of c-kit^{pos} epCSCs in the border **(open bars)** and infarcted **(solid bars)** regions of IGF-1/HGF-treated and control (CTRL) pigs. **(E)** Newly generated c-kit^{pos}, bromodeoxyuridine (BrdU) **(red)** positive epCSCs in the IGF-1/HGF 4× treated pig heart. **(F)** The c-kit^{pos} epCSCs expressed the cardiac transcription factor, Nkx2.5 **(red)**, representing myocyte progenitor cells. **(G)** Number of c-kit^{pos}Nkx2.5^{pos} myocyte progenitor cells. **(H, I)** Some c-kit^{pos} epCSCs expressed the transcription factors, **(H)** Gata-6 **(red)** and **(I)** Ets-1 **(red)**, indicative of smooth muscle and endothelial cell differentiation, respectively. Nuclei are stained by 4',6-diamidino-2-phenylindole (DAPI). All data are mean ± SD; n = 5, 4, 5, and 4 for CTRL, IGF-1/HGF 1×, 2×, and 4×, respectively. *p < 0.05 versus CTRL, †p < 0.05 versus IGF-1/HGF 1×; ǂp < 0.05 versus IGF-1/HGF 2×. All scale bars = 20 μm. AMI = acute myocardial infarction.

In association with the appearance of epCSCs committed to the vascular lineage in response to IGF-1/HGF (Figs. 3H and 3I), newly formed BrdU-positive vascular structures were evident in the border and infarcted myocardium (Figs. 4J and 4K, Online Fig. 9). The IGF-1/HGF-treated hearts displayed increased number of capillaries in the infarct zone, compared with CTRL, and this response was dose dependent (Fig. 4L, Table 1, Online Fig. 9). Interestingly, new microvessels and BrdU^{pos} myocytes in regenerating bands were most evident around survived myocardium islands within the infarct (Online Fig. 9). This organization, together with the in vitro data (Figs. 2A to 2F), suggests that adult spared myocytes produce cardiopoietic factors (11) acting on the epCSCs.

It should be stressed that, at 21 days after MI, the regenerated myocytes in the infarct were still immature, as demonstrated by their size which, on average, was significantly smaller than normal adult porcine myocytes (Table 1). Furthermore, many of these regenerating myocytes were still cycling, as demonstrated by the expression of Ki67 (Fig. 4). In agreement with the suggested cardiopoietic factors secreted by

Table 1 Histological and Immunohistochemical Data 21 Days After MI

	CTRL (n = 5)	IGF-1/HGF 1× (n = 4)	IGF-1/HGF 2× (n = 5)	IGF-1/HGF 4× (n = 4)
Fibrosis, %	36.2 ± 5.9	26 ± 7.0†	23 ± 7.0†	16 ± 2.5†‡
Myocyte apoptosis, %	1.9 ± 0.25	1.3 ± 0.2†	0.9 ± 0.15†‡	0.4 ± 0.1†‡§
Myocyte hypertrophy, μm	26 ± 2.2	23 ± 1.2†	19 ± 1.3†	16.5 ± 1.1†‡
c-kit^{pos} CSCs (border zone), %	0.4 ± 0.1	0.8 ± 0.2†	1.2 ± 0.1†‡	1.7 ± 0.3†‡§
c-kit^{pos} CSCs (infarct area), %	0.7 ± 0.1	1.3 ± 0.2†	2.3 ± 0.3†‡	4.3 ± 0.4†‡§
c-kit^{pos}Nkx2.5^{pos} CSCs (border zone), %	0.2 ± 0.1	0.5 ± 0.0†	0.7 ± 0.1†‡	0.9 ± 0.05†‡§
c-kit^{pos}Nkx2.5^{pos} CSCs (infarct area), %	0.3 ± 0.1	0.9 ± 0.2†	1.7 ± 0.3†‡	3.0 ± 0.4†‡§
BrdU^{pos} myocytes (infarct zone), %	0.5 ± 0.1	7.1 ± 0.8†	12.7 ± 1.5†‡	19.3 ± 2.6†‡§
BrdU^{pos} myocytes (border area), %	1.2 ± 0.3	5.4 ± 0.6†	9.2 ± 1.3†‡	11.8 ± 1.0†‡§
Ki67^{pos} myocytes (border zone), %	0.7 ± 0.2	3.3 ± 0.3†	4.7 ± 0.4†‡	6.5 ± 0.9†‡§
Ki67^{pos} myocytes (infarct area), %	0.3 ± 0.0	3.9 ± 0.6†	8.2 ± 0.6†‡	10.4 ± 1.1†‡§
Number of capillaries, ×0.2 μm^2	5 ± 1	7 ± 1	9 ± 2†	11 ± 1†‡§
BrdU^{pos} myocyte diameter* (infarct zone), μm	6.4 ± 1.2	9.2 ± 1.3	10.4 ± 1.5†	12.3 ± 1.2†

*Diameter of normal BrdU^{neg} myocyte = 17 ± 1 μm; †$p < 0.05$ versus control (CTRL); ‡$p < 0.05$ versus IGF-1/HGF 1×; §$p < 0.05$ versus IGF-1/HGF 2×.

BrdU = bromodeoxyuridine; CSC = cardiac stem/progenitor cell; HGF = hepatocyte growth factor; IGF = insulin-like growth factor; MI = myocardial infarction.

mature myocytes, those newly formed myocytes in contact or close proximity with mature ones (i.e., in the border zone) were significantly larger than those in the middle of the scar with no proximity to spared myocytes (Fig. 4, Online Fig. 9). Also, IGF-1/HGF enhanced myocyte maturation, as shown by the increased average size of BrdU^{pos} myocytes with increasing dose (Table 1). However, despite this robust regeneration of myocyte number, the treated hearts still had a severe myocyte-mass deficit due to the significantly smaller size of the regenerated myocytes compared with those lost by the AMI.

IGF-1/HGF treatment improves cardiac function after AMI. To test the effects on cardiac function after AMI, IGF-1/HGF at a 4× dose or saline (CTRL) was given by intracoronary administration to additional pigs. Cardiac magnetic resonance imaging (cMRI) was performed before coronary occlusion, at 72 h, at 1 month and before sacrifice, and at 2 months after AMI (Online Fig. 7).

Intracoronary administration of a 4× dose of IGF-1/HGF (n = 5) maintained the favorable histological effects on myocardial repair also at 2 months after MI (Online Fig. 10). Remarkably, epCSC number, BrdU^{pos} myocytes and capillary density were significantly higher in IGF-1/HGF-treated pigs compared with CTRL (n = 4) (Fig. 5A, Online Fig. 10). Interestingly, at 2 months, BrdU^{pos} myocyte maturation in the infarct region of treated pigs (diameter = 14.3 ± 0.7 μm) was increased compared with 21 days (at 4× dose, 12.3 ± 1.2 μm, $p < 0.05$), but they were still smaller than the average adult porcine myocyte (17 ± 1 μm, $p < 0.05$). More importantly, infarct size, as measured by computed planimetric analysis at sacrifice, was significantly smaller in treated animals (16 ± 4% of the total left ventricular free wall) than in CTRL animals (24 ± 2%, $p < 0.05$).

The anatomical and histological improvement of cardiac repair after MI in IGF-1/HGF-treated pigs was associated with better LV function (Figs. 5B to 5F). Indeed, in the CTRL pigs, LV volumes and function progressively worsened from 72 h to 2 months after AMI, whereas IGF-1/HGF prevented LV cardiac dilation, which resulted in a better LV ejection fraction (45 ± 8% vs. 33 ± 6%, $p < 0.05$) at 2 months after AMI (Figs. 5B to 5F, Online Table 1).

Discussion

Since its birth, regenerative cardiology's holy grail has been the development of procedures to either replace lost myocytes with transplanted stem cells or to use stem cells to mediate functional repair through paracrine effects (2,4,5,12,13). Although the discovery of eCSCs together with definitive proof of adult mammalian myocyte renewal were initially met with enthusiasm (14), mounting skepticism has developed about the physiological significance and regenerative potential of the resident c-kit^{pos} eCSC population (3,15,16). A recent report claimed that the cardiogenic potential in vitro of the c-kit^{pos} cardiac ells is robust only during neonatal age, but it is severely limited or lost in the adult (17).

Undoubtedly, resident adult stem-progenitor cells are more abundant in early post-natal life and decline thereafter (18). These progenitor cells are highly active in the neonatal period because they are main participants in the myocardium hyperplastic growth and maturation to adulthood. In contrast, most eCSCs in the adult myocardium are quiescent (2). This difference might explain why eCSCs from the adult have a higher threshold than those from the neonate to activate their myogenic pathway in co-culture with fetal cardiomyocytes (17). Moreover, isolated c-kit^{pos} cardiac cells are a mixed population containing not only real eCSCs and progenitor/precursors but also cells committed to other lineages, including mast cells. Indeed, we show that the adult c-kit^{pos}CD45^{neg} fraction is enriched for cells with cardiac stem and progenitor cell properties, while the

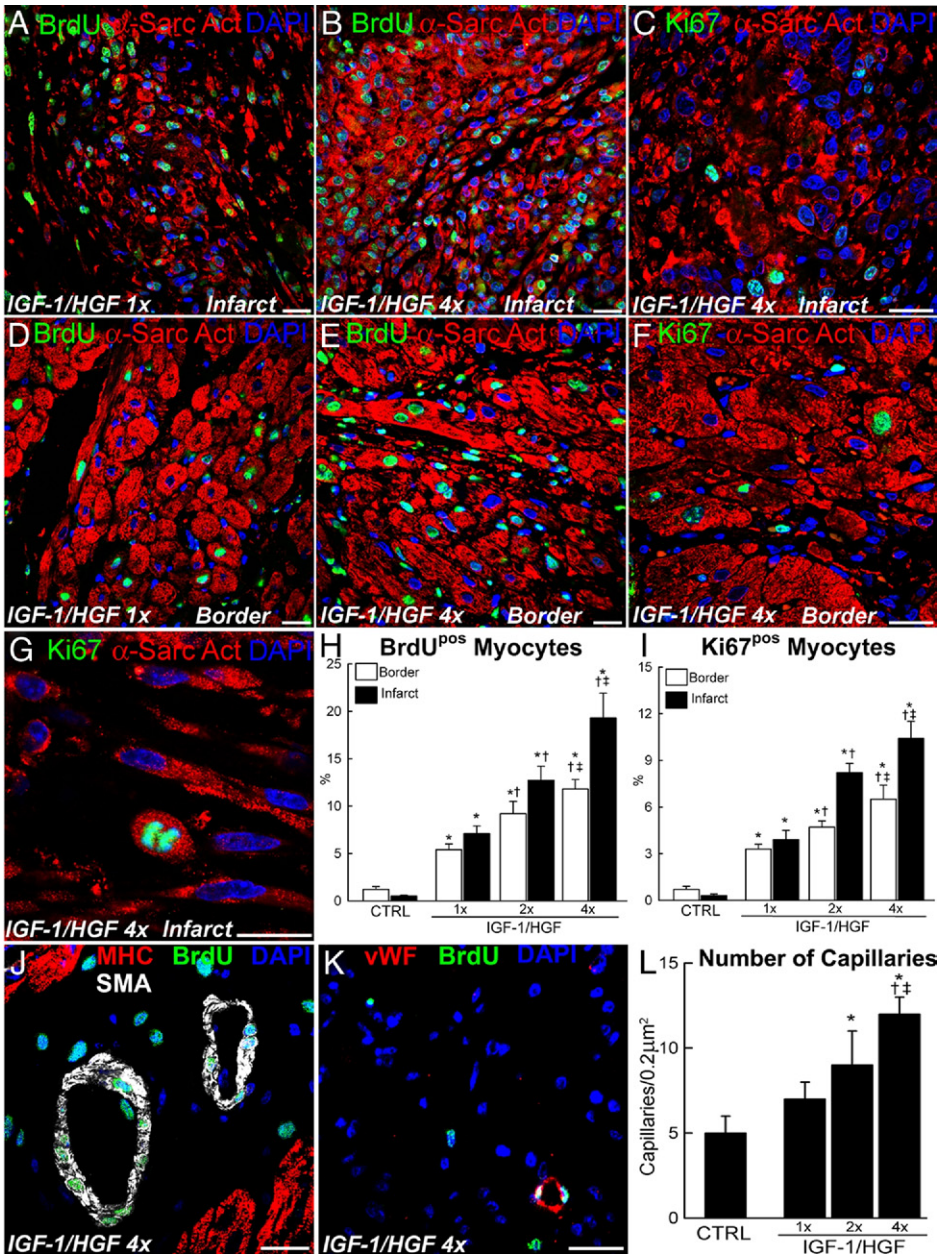

Figure 4 **IGF-1/HGF Intracoronary Administration Induces Myocardial Regeneration After AMI**

(A, B) Small, newly formed bromodeoxyuridine ($BrdU^{pos}$) **(green)** myocytes **(red)** (α-sarcomeric actin) in the infarct regions of insulin-like growth factor (IGF)-1/hepatocyte growth factor (HGF) of **(A)** 1× and **(B)** 4× treated pig hearts. **(C)** Within these regenerating bands were small $Ki67^{pos}$ **(green)** proliferating myocytes. **(D, E)** Newly formed small $BrdU^{pos}$ myocytes **(red)** (α-sarcomeric actin) in the border zone after IGF-1/HGF **(D)** 1× and **(E)** 4× doses. **(F)** Small $Ki67^{pos}$ myocytes were also present in the border zone after IGF-1/HGF-injection. **(G)** A small $Ki67^{pos}$ **(green)** mitotic myocyte precursor. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) in **blue**. All scale bars = 20 μm. **(H, I)** $BrdU^{pos}$ and $Ki67^{pos}$ myocyte number. **Open bars** indicate border region; **solid bars** indicate infarct region. **(J, K)** Newly formed arterial and capillary structures: BrdU = **green**; α-smooth muscle actin (SMA) = **white**; myosin heavy chain (MHC) = **red**; von Willebrand factor (vWF) = **red**; DAPI = **blue** in the infarcted region of IGF-1/HGF 4× treated pig hearts. Bar = 20 μm. **(L)** Number of capillaries in the infarct zone. All data are mean ± SD; n = 5, 4, 5, and 4 for CTRL, IGF-1/HGF 1×, 2×, and 4×, respectively. *p < 0.05 versus CTRL; †p < 0.05 versus IGF-1/HGF 1×; ‡p < 0.05 versus IGF-1/HGF 2×. AMI = acute myocardial infarction.

fraction expressing CD45 does not contain cardiomyogenic cells (data not shown). This is not different from bone marrow stem cells, whereby only a very small fraction of $c\text{-}kit^{pos}$ cells are actually stem/progenitor cells (18). Thus, it is not surprising that when the identity and number of true stem/progenitor cells is not known, as in Zaruba et al. (17), such undefined cell mixtures produce uninterpretable results in vitro and even more so in vivo. Furthermore, the failure

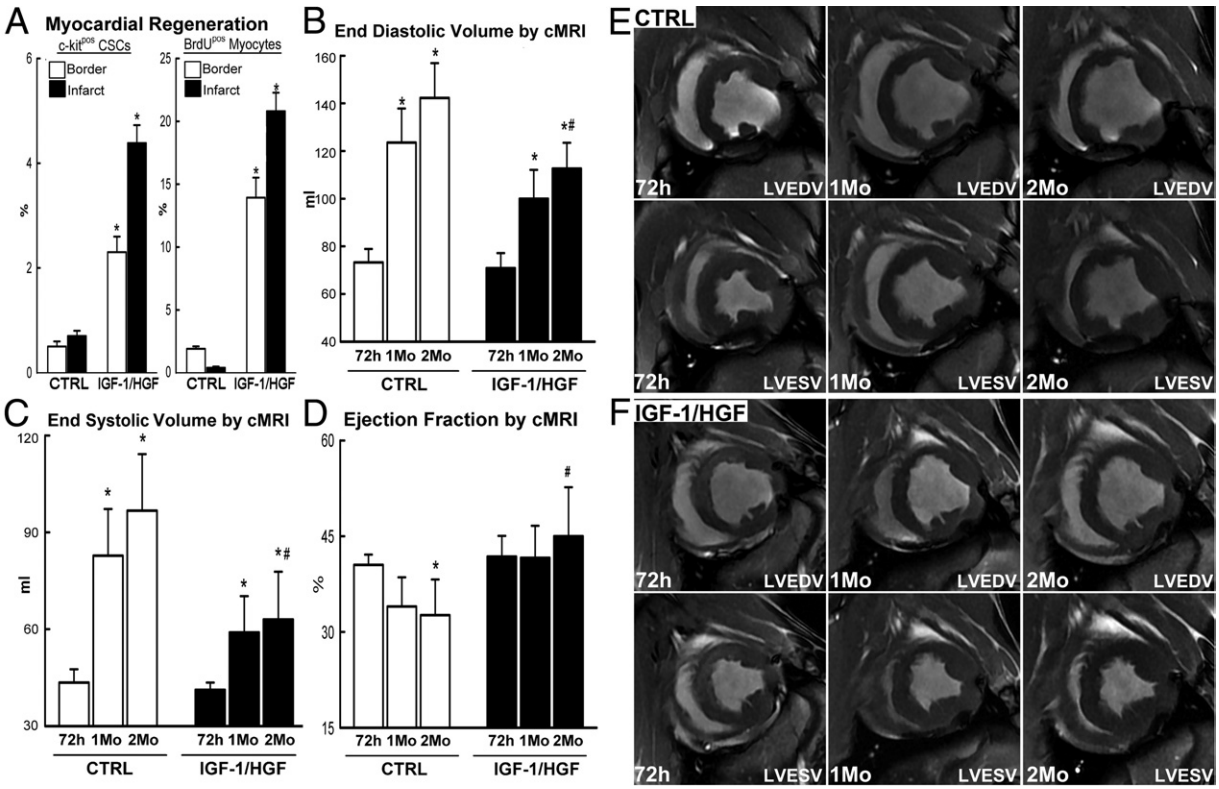

Figure 5 IGF-1/HGF Intracoronary Administration Improves Cardiac Function After AMI

(A) Number of c-kit^{pos} endogenous porcine cardiac stem/progenitor cells (epCSCs) and bromodeoxyuridine-positive (BrdU^{pos}) myocytes 2 months after acute myocardial infarction (AMI). *$p < 0.05$ versus control (CTRL). **(B to D)** Left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), and left ventricular ejection fraction (LVEF) in the 2 groups of pigs. *$p < 0.05$ versus 72 h; #$p < 0.05$ versus CTRL. **(E, F)** Cardiac magnetic resonance imaging (cMRI) images showing progressive cardiac enlargement in CTRL as opposed to insulin-like growth factor (IGF)-1/hepatocyte growth factor (HGF) treatment. All data are mean ± SD; n = 4 for CTRL and n = 5 for IGF-1/HGF 4×.

of adult c-kit^{pos} cells to differentiate in Zaruba et al. (17) unlikely arises from their lack of actual regenerative potential but rather from the lack of effective protocols to stimulate their differentiation. Accordingly, the data presented here on the effects of IGF-1/HGF on adult c-kit^{pos} epCSCs activation, differentiation and maturation in vivo and in vitro, further ascertains the role of these cells in myocardial cell homeostasis and regeneration.

There is little controversy that the best replacement for the lost myocardium after MI is functional autologous myocardial tissue. However, as presently practiced, the isolation and expansion of eCSCs for autologous cell transplantation is slow, expensive, and of uneven quality (2). For that reason, it is unlikely that autologous eCSCs transplantation will ever become widely available to treat acute and subacute MI (2,19). Even for late and chronic MI, autologous eCSCs can become available only to a very small fraction of patients in need of myocardial regeneration and at very high costs. Thus, there is need for strategies to specifically activate in situ the intrinsic cardiac regenerative potential represented by the resident eCSCs using combinations of growth factors, cytokines, and drugs, obviating the need for cell transplantation (2,19).

Recently, some growth factors/receptors axis—and activating small molecules—modulating CSCs fate have been described (20,21). Indeed, eCSCs possess IGF-1 and HGF signaling pathways that regulate their growth, survival, and migration (7,8,22). Also, HGF induces expression of cardiac-specific markers in embryonic stem cells (23).

Despite their value as proof of concept, the extrapolation of murine and dog data (7,8) to the human pathology is debatable. The 3 orders of magnitude difference in heart size between mice and humans and the abundance of collaterals in the dog coronary circulation make these experimental infarct models not comparable to that of the human. More importantly, in the mouse and canine studies, the growth factors have been injected transepicardially through open-chest surgery, which is not a suitable option in the treatment of AMI. In contrast, the protocol used here could easily be applied at the time of primary revascularization for AMI.

Six major conclusions emanate from this study: 1) the adult porcine myocardium harbors epCSCs—phenotypically distin-

guishable from cardiac mast cells—that differentiate into myocytes, a process specifically stimulated by HGF; 2) injection of small amounts of IGF-1/HGF in the coronary artery supplying the infarcted region improves cardiomyocyte survival and remodeling; it has significant protective effect on myocardial tissue organization and structure; 3) intracoronary IGF-1/HGF administration activates the resident epCSCs, which multiply and differentiate, fostering the regeneration of the myocytes and microvasculature lost by the AMI; 4) the intensity of epCSC activation, myocardial survival, and cell regeneration are directly correlated to the dose of IGF-1/HGF administered, strongly pointing to a direct cause-effect relationship; 5) the effects of a single administration of IGF-1/HGF is still measurable 2 months after its application, suggesting the existence of a feedback loop triggered by the external stimuli that activates the production of growth and survival factors by the targeted cells, which explains the persistence and long duration of the regenerative myocardial response; and 6) IGF-1/HGF treatment improves cardiac function by decreasing cell death, infarct size, and preventing LV cardiac dilation, resulting in better LV ejection fraction at 2 months after AMI, as measured by cMRI.

Taken together, these results provide the proof of concept needed to justify further experimental development and refinement of this approach, which ultimately could lead to an effective, simple, clinically applicable, and widely available protocol of myocardial regeneration.

Study limitations. By extrapolation from fetal myocytes (24), it is likely that, despite the large number and progressive maturation of the regenerated myocytes from 21 days to 2 months, these immature myocytes have limited force-generating capacity when compared with the adult surviving counterparts. Therefore, the documented beneficial effect of IGF-1/HGF on ventricular performance is unlikely due only to the direct force-generating contribution of the regenerated myocytes. Most probably, the effects on ventricular performance shown in Figure 5 are due to a combination of better preservation of myocardial architecture, decreased myocyte death, reduced remodeling, and regeneration of new myocardial cells including neomyogenesis as well neoangiogenesis. For further progress, the identification of the factors that drive full CSC-derived cardiomyocyte maturation is warranted.

Reprint requests and correspondence: Dr. Bernardo Nadal-Ginard, The Stem Cell and Regenerative Biology Unit (BioStem), RISES, Liverpool John Moores University, Tom Reilly Building, Room 1.41, Byrom Street, Liverpool L3 3AF, United Kingdom. E-mail: b.nadalginard@ljmu.ac.uk. OR Dr. Daniele Torella, Molecular and Cellular Cardiology, Department of Experimental and Clinical Medicine, Magna Graecia University, Campus S.Venuta, Viale Europa, 88100 Catanzaro, Italy. E-mail: dtorella@unicz.it.

REFERENCES

1. Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003;114:763–76.
2. Torella D, Ellison GM, Karakikes I, Nadal-Ginard B. Resident cardiac stem cells. Cell Mol Life Sci 2007;64:661–73.
3. Martin-Puig S, Wang Z, Chien KR. Lives of a heart cell: tracing the origins of cardiac progenitors. Cell Stem Cell 2008;2:320–31.
4. Ellison GM, Torella D, Karakikes I, Nadal-Ginard B. Myocyte death and renewal: modern concepts of cardiac cellular homeostasis. Nat Clin Pract Cardiovasc Med 2007;4 Suppl 1:52–9.
5. Janssens S. Stem cells in the treatment of heart disease. Annu Rev Med 2010;61:287–300.
6. Terzic A, Nelson TJ. Regenerative medicine advancing health care 2020. J Am Coll Cardiol 2010;55:2254–7.
7. Urbanek K, Rota M, Cascapera S, et al. Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. Circ Res 2005;97:663–73.
8. Linke A, Müller P, Nurzynska D, et al. Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. Proc Natl Acad Sci U S A 2005;102:8966–71.
9. Sperr WR, Bankl HC, Mundigler G, et al. The human cardiac mast cell: localization, isolation, phenotype, and functional characterization. Blood 1994;84:3876–84.
10. de Prado AP, Cuellas-Ramón C, Regueiro-Purriños M, et al. Closed-chest experimental porcine model of acute myocardial infarction-reperfusion. J Pharmacol Toxicol Meth 2009;60:301–6.
11. Behfar A, Perez-Terzic C, Faustino RS, et al. Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. J Exp Med 2007;204:405–20.
12. Kubal C, Sheth K, Nadal-Ginard B, Galiñanes M. Bone marrow cells have a potent anti-ischemic effect against myocardial cell death in man. J Thorac Cardiovasc Surg 2006;132:1112–8.
13. Lai VK, Linares-Palomino J, Nadal-Ginard B, Galiñanes M. Bone marrow cell-induced protection of the human myocardial: characterization and mechanism of action. J Thorac Cardiovasc Surg 2009;138:1400–8.
14. Leinwand LA. Hope for a broken heart? Cell 2003;114:658–9.
15. Passier R, van Laake LW, Mummery CL. Stem-cell-based therapy and lessons from the heart. Nature 2008;453:322–9.
16. Pouly J, Bruneval P, Mandet C, et al. Cardiac stem cells in the real world. J Thorac Cardiovasc Surg 2008;135:673–8.
17. Zaruba MM, Soonpaa M, Reuter S, Field LJ. Cardiomyogenic potential of C-kit(+)-expressing cells derived from neonatal and adult mouse hearts. Circulation 2010;121:1992–2000.
18. Rossi DJ, Jamieson CH, Weissman IL. Stems cells and the pathways to aging and cancer. Cell 2008:132;681–96.
19. Nadal-Ginard B, Torella D, Ellison G. Cardiovascular regenerative medicine at the crossroads. Clinical trials of cellular therapy must now be based on reliable experimental data from animals with characteristics similar to human's. Rev Esp Cardiol 2006:59;1175–89.
20. Sadek H, Hannack B, Choe E, et al. Cardiogenic small molecules that enhance myocardial repair by stem cells. Proc Natl Acad Sci U S A 2008;105:6063–8.
21. Engel FB, Hsieh PC, Lee RT, Keating MT. FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. Proc Natl Acad Sci U S A 2006;103:15546–51.
22. Torella D, Rota M, Nurzynska D, et al. Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res 2004;94:514–24.
23. Roggia C, Ukena C, Böhm M, Kilter H. Hepatocyte growth factor (HGF) enhances cardiac commitment of differentiating embryonic stem cells by activating PI3 kinase. Exp Cell Res 2007;313:921–30.
24. Siedner S, Krüger M, Schroeter M, et al. Developmental changes in contractility and sarcomeric proteins from the early embryonic to the adult stage in the mouse heart. J Physiol 2003;548:493–505.

Key Words: cardiac magnetic resonance imaging ■ cardiac stem cells ■ growth factors ■ myocardial infarction ■ myocardial regeneration.

APPENDIX

For a complete description of Methods, supplementary figures, a supplementary table, and a supplementary video, please see the online version of this article.