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Human epicardial cell-conditioned medium contains HGF/IgG complexes that phosphorylate RYK and protect against vascular injury

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Aims The aim of this study was to evaluate the paracrine activity of human epicardial-derived cells (hEPDCs) to screen for secreted vasoprotective factors and develop therapeutics to treat vascular reperfusion injury.

Methods and results Epicardial cells support cardiac development, repair, and remodelling after injury in part, through paracrine activity. We hypothesized that secreted ligands from hEPDCs would protect vascular integrity after myocardial infarction (MI) with reperfusion. During simulated ischaemia in culture (24–48 h), concentrated hEPDC-conditioned medium (EPI CdM) increased survival of primary cardiac endothelial cells. In a rat MI model, EPI CdM treatment reduced vascular injury *in vivo* after reperfusion. By phospho-receptor tyrosine kinase (RTK) arrays, ELISA, and neutralizing antibody screens, we identified hepatocyte growth factor (HGF) as a key vasoprotective factor in EPI CdM. Unexpectedly, we observed that some of the HGF in EPI CdM formed complexes with polyclonal IgG. Following reperfusion, preparations of HGF/IgG complexes provided greater vascular protection than free HGF with IgG. HGF/IgG complexes localized to blood vessels *in vivo* and increased HGF retention time after administration. In subsequent screens, we found that 'related to tyrosine kinase' (RYK) receptor was phosphorylated after exposure of cardiac endothelial cells to HGF/IgG complexes, but not to free HGF with IgG. The enhanced protection conferred by HGF/IgG complexes was lost after antibody blockade of RYK. Notably, the HGF/IgG complex is the first 'ligand' shown to promote phosphorylation of RYK.

Conclusion Early treatment with HGF/IgG complexes after myocardial ischaemia with reperfusion may rescue tissue through vasoprotection conferred by c-Met and RYK signalling.

Keywords Myocardial infarction • Reperfusion • Epicardial • Progenitor cell • Precursor cell • EPDC • HGF

1. Introduction

The epicardium, a specialized epithelial cell layer that covers the heart, is essential to cardiac development, structure, and function.[1,2] During development, a subset of epicardial cells undergo epithelial-to-mesenchymal transformation (EMT) into epicardial-derived cells (EPDCs), precursor cells that invade the subepicardium and myocardium, and contribute to the subepicardial and coronary vasculature and the pool of interstitial fibroblasts.[3–5] EPDCs interact with neighbouring cells such as cardiomyocytes and Purkinje fibres and affect their proliferation and function,[3,6,7] in part, through paracrine activity.[8] In adults, epicardial cells play important roles in repair and remodelling after cardiac injury. Ventricular regeneration in zebrafish requires epicardial cell proliferation, EMT, invasion, and subsequent neovascularization of myocardium.[9] Factors secreted by EPDCs of adult mice were reported to increase angiogenesis after myocardial infarction (MI); these paracrine effects correlated with decreased infarct size and improved cardiac function.[10]

Historically, the field of cardiac regenerative medicine has focused on transplantation of stem/progenitor cells to reduce infarct size and improve cardiac function through direct replacement of damaged myocytes and vascular cells.[11,12] However, numerous reports now indicate that paracrine effects were responsible for much of the observed benefits of cell therapy. Stimulation of angiogenesis is frequently reported

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due to higher numbers of blood vessels observed in tissues/organs treated with stem/progenitor cells,[13,14] but few studies have considered rescue and/or repair of pre-existing blood vessels as opposed to angiogenesis. Importantly, secreted factors that protect microvascular endothelial cells early after reperfusion may reduce vascular leak and infarct expansion after MI. To better understand the role of vasoprotection (rescue) in the paracrine benefits of cell transplantation, we isolated human EPDCs (hEPDCs) and investigated the ability of hEPDC-conditioned medium (EPI CdM) to protect the vasculature early after cardiac ischaemia with reperfusion. Through screens of EPI CdM, we identified novel HGF/IgG protein complexes that enhance vasoprotection, in part, by activating a Wnt co-receptor called 'related to tyrosine kinase' (RYK). Our results suggest that treatment with HGF/IgG complexes may reduce reperfusion injury and infarct expansion in patients with MI.

2. Methods

A detailed description of methods is available in Supplementary materials online.

2.1 Human epicardial-derived cell-conditioned medium

Conforming with the principles outlined in the Declaration of Helsinki, and with patient's informed consent, right atrial appendages were obtained during cardiac bypass surgery. The right atrial biopsies were used to derive primary cultures of hEPDCs. After EMT, passage 2 hEPDCs were seeded and grown in 150 cm^2 dishes (Nunc) in Claycomb base medium supplemented with 10% FCS (lot selected for rapid growth of human multipotent stromal cells, Atlanta Biologicals, Lawrenceville, GA, USA), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM L-glutamine (Mediatech, Inc., Hendron, VA, USA). When the cells reached 80–90% confluence, the plates were washed twice with PBS and serum-free MEM (MEM) was placed on the cells (20 mL per plate). After 48 h of incubation, the EPI CdM was collected, filtered (0.2 μm PES membrane, Nalgene MF75, Rochester, NY, USA), and concentrated to 10- or 30-fold with a Labscale^{TM} TFF diafiltration system with a 5 kDa cut-off filter (Millipore, Bedford, MA, USA) or with Amicon Centricon filters (Millipore) with a 3 kDa cut-off. One millilitre vials of EPI CdM were frozen and stored at −80°C. Some 1× unconcentrated EPI CdM was aliquoted and reserved for ELISAs and cell protection assays with primary human cardiac endothelial cells. ELISAs for human growth factors were performed with commercially available kits (R&D systems).

2.2 Myocardial ischaemia–reperfusion surgery and treatment with EPI CdM

All animal procedures conformed to the Guide of the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 2011) and to the IACUC guidelines for animal care approved by the University of Vermont. Fischer 488 rats (males, 7 weeks of age) were weighed, shaved, anaesthetized under 4% isoflurane, and endotracheally intubated. Rats were ventilated at a respiration rate of 65 bpm under a peak inspiration pressure of 15 cmH_2O (Kent Scientific). Body temperature was maintained at 37°C with a heated pad (Gaymar). Through a dermal incision, a blunt dissection of the fascia was performed and the intercostal muscles were separated. The heart was exposed by retraction of the pericardium to expose the left anterior descending coronary artery (LAD). The LAD was then encircled with a 6-0 nylon suture. To facilitate release of suture at the time of reperfusion, a 0.4 cm length of PE10 tubing was placed over the LAD. The LAD was then occluded; this was confirmed by blanching of the anterior free wall of the left ventricle (LV). Animals were allowed to recover off the ventilator. After 2 h, the rats were reintubated under anaesthesia, ventilated, and the chest wall was re-opened. Hearts were exposed to reveal the suture, which was released, and reperfusion was visually confirmed by blood flow through the LAD and 're-pinking' of the blanched area. For each rat, we injected 1 mL of MEM, EPI CdM, or other treatments steadily over a period of 1.5 min through LV wall into the ventricle lumen (intra-arterial, a 30.5-gauge needle). After the injection, the chest wall was closed and the rats recovered in their cages for 24 h prior to euthanization and tissue harvest. Under anaesthesia, the chest of sham-operated animals was opened to visualize the intact pericardium (twice), corresponding to the times of ischaemia and reperfusion surgeries. No further manipulations or treatments were performed on the sham animals.

2.3 Measurement of vascular permeability *in vivo*

At 22 h post-ischaemia, animals were injected with 0.5 mL of FITC-albumin (tail vein, 5 mg/mL). After 2 h, rats were anaesthetized, perfused with 50 mL of sterile PBS to wash out circulating FITC-albumin from blood vessels, and whole hearts were excised into sterile PBS. To isolate the left ventricles with septum, the adventitial tissue, major vessels, and left and right atria were separated. Hearts were then homogenized in PBS (2 mL/g wet weight of tissue) using a Polytron dispersing tool (Euro Turrax T206 IKA Labortechnik; Dispersing Tool S25N-10G, outer diameter 10 mm; IKA Works, Inc., Wilmington, NC, USA) and centrifuged at 13 000 × *g* for 20 min. The soluble fraction was separated and 100 μL volumes were aliquoted to determine the amount of FITC extravasated into myocardial tissue. Fluorescence readings were measured in duplicate at 480 nm excitation and 520 nm emission wavelengths on an HT Synergy plate reader (BioTek Instruments, Winooski, VT, USA).

2.4 Preparation of HGF/IgG complexes

Recombinant human HGF was diluted to a working concentration of 10 μg/mL in sterile PBS. Mixed polyclonal IgG (non-specific) from human serum was diluted to 14 μg/mL. The IgG was mixed with a diluted HGF in a total volume of 10 mL (1 : 1 molar ratio; HGF : IgG). The mixture was then concentrated 40-fold (from 10 mL to 250 μL) using a Centricon device (Centricon Plus-70 Centrifugal Filter, Ultracel-PL Membrane, 3 kDa, Millipore). This concentrated mixture was diluted in PBS to give final HGF : IgG doses of 1× or 10×. Different concentrations of HGF were then used for treatment studies either in the free 'uncomplexed' form or in the HGF/IgG complexes.

2.5 Statistical analysis

Comparisons of data from individual control and treatment groups were made by two-tailed Student's *t*-test. For studies with comparison of multiple groups, we performed one-way ANOVA with Bonferroni *post hoc* testing. Values of $P \leq 0.05$ were considered statistically significant.

3. Results

3.1 Isolation of adult human epicardial progenitor cells and EMT into precursor cells

The epicardium of healthy adult human atrial tissue had a single layer of epithelial cells that stained positive for the intermediate filament protein keratin (a marker of epithelial cells) (*Figure 1A*), and negative for vimentin (mesenchymal cell marker, *Figure 1A*). Human epicardial cells were isolated from right atrial appendages commonly removed to place a cardiopulmonary bypass cannula as part of cardiac surgery. The cells were successfully isolated from 12 of 13 donors (see Supplementary material online, *Table S1*). We developed methods to isolate proliferating epicardial progenitor cells from right atrial appendages (for details,

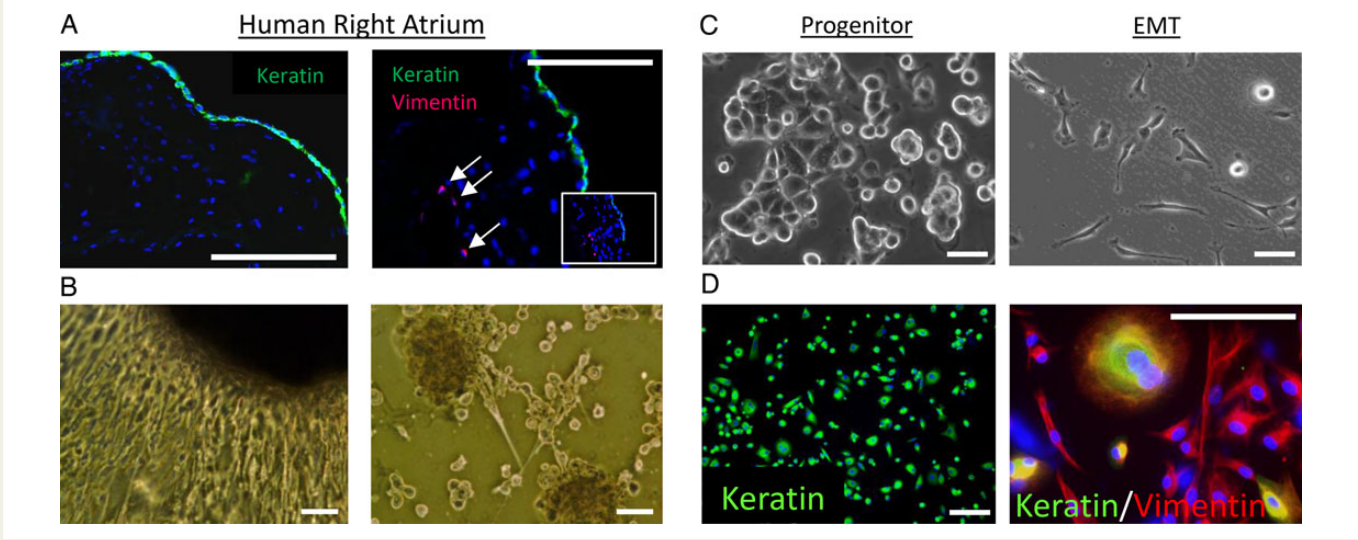

Figure 1 Isolation of adult human epicardial progenitor-like cells and EMT into precursor cells. (*A*) Right, immunohistochemistry of a section of right atrial appendage from a human donor showing the epicardium. Left, epicardial cells are keratin-positive (epithelial cell marker) and vimentin-negative (mesenchymal cell marker). Arrows point to vimentin-positive cells. Inset: note absence of vimentin staining in the epicardial layer ($n = 4$ donors). (*B*) Left, explant culture of right atrial appendage to generate feeder layer. Right, formation of floating spheroids and bunches of cells following switch to medium that favors the growth of stem/progenitor cells. (*C*) Left, maintenance of epicardial progenitor-like cells with epithelial phenotype on uncoated dishes. Right, EMT of epicardial progenitor-like cells 3 days after incubation in medium with 10% FBS. (*D*) Left, epicardial progenitor-like cells express keratins (green). Right, precursors derived by EMT express vimentin (red) but not keratin (green) ($n = 5–7$ donors). Scale bar = 100 μM.

see Supplementary Materials and Methods). Explant cultures were generated from minced right atrial appendage (*Figure 1B*). Once fibroblast outgrowth reached ~70% confluence, the medium was switched to a low serum stem/progenitor cell growth medium containing leukemia inhibitory factor, epidermal growth factor, and basic FGF. Under these conditions, floating spheroids and bunches of cells formed and were collected, placed into new culture dishes, and expanded (*Figure 1C*). The epicardial progenitor cells had an epithelial morphology and expressed keratins (*Figure 1D*). Differentiation (EMT) of the epicardial progenitor cells into a mixture of transitory-amplifying precursor cells (hEPDCs) was induced by culture in medium containing 10% fetal calf serum (*Figure 1D*). During EMT into precursor cells, the progenitor cells down-regulated their expression of keratins and expressed vimentin (*Figure 1D*). By RT-PCR, we detected several mRNAs for markers of EMT: Snail, Slug, Twist, and Smad1 (see Supplementary material online, *Figure S2A*). In addition, the human precursor cells expressed transcription factors reported to be expressed by murine epicardial cells: WT1 (cardiac development),[15,16] GATA4 (myocardial differentiation and function),[17] TCF21 (epicardial EMT and cardiac fibroblast specification),[18] and RALDH2 (enzyme for retionoic acid synthesis[19]; *Figure 2A*). Expression of mRNA for several epicardial-related transcription factors decreased as the cells differentiated (see GATA5, WT1, Isl-1, and TBX18; *Figure 2B*).[20] In contrast, expression of mRNA for smooth muscle actin (SMA) and Von Willebrand Factor (vWF) increased (*Figure 2B*), indicating that some hEPDCs might be differentiating into vascular smooth muscle cells or myofibroblasts and others into endothelial cells, respectively. At 2 weeks after EMT, by immunocytochemistry, we detected a few cells that were positive for smooth muscle myosin or vWF, confirming that some of the cells were precursors of smooth muscle cells and endothelial cells, respectively. The majority of epicardial-derived cells were positive for prolyl-4-hydroxylase, an enzyme involved in collagen biosynthesis and a marker of fibroblasts (see Supplementary material online, *Figure S2B*). Cell surface phenotyping demonstrated that the hEPDCs were negative for haematopoietic and endothelial cell surface markers such as CD31, CD34, CD45, and c-kit, and also the vascular pericyte marker NG2. Multiple cell surface antigens typical of bone marrow multipotent stromal cells such as CD105, CD90, CD73, CD54, CD49d, CD44, and CD29 were expressed by hEPDCs after EMT (see Supplementary material online, *Figure S3* and data not shown).

For the second isolation method, epicardial tissue was carefully dissected from the surfaces of human right atrial appendages and cultured directly (see Supplementary material online, *Figure S1* and Materials and Methods). Comparison by immunocytochemistry, cell surface phenotyping, and ELISA data indicated that the precursor cells derived from EMT did not differ whether the cells were rapidly induced to undergo EMT or were maintained for several weeks as epithelial progenitor cells and then induced to undergo EMT.

3.2 EPI CdM treatment promotes vascular integrity *in vivo*

To determine whether human EPI CdM could provide vascular protection *in vivo*, adult male Fischer rats underwent 2 h of transient ischaemia followed by 24 h of reperfusion. They were then treated with either MEM (vehicle control) or EPI CdM at the time of reperfusion. Twenty-four hours after ischaemia/reperfusion, we quantified the amount of FITC-albumin extravasation in each treated animal by normalizing it to the level of extravasation in sham-operated animals. On tissue sections, we observed a greater level of FITC-albumin extravasation from damaged vessels in the infarcted region from the left ventricle (LV) compared with no extravasation outside the region of infarction,

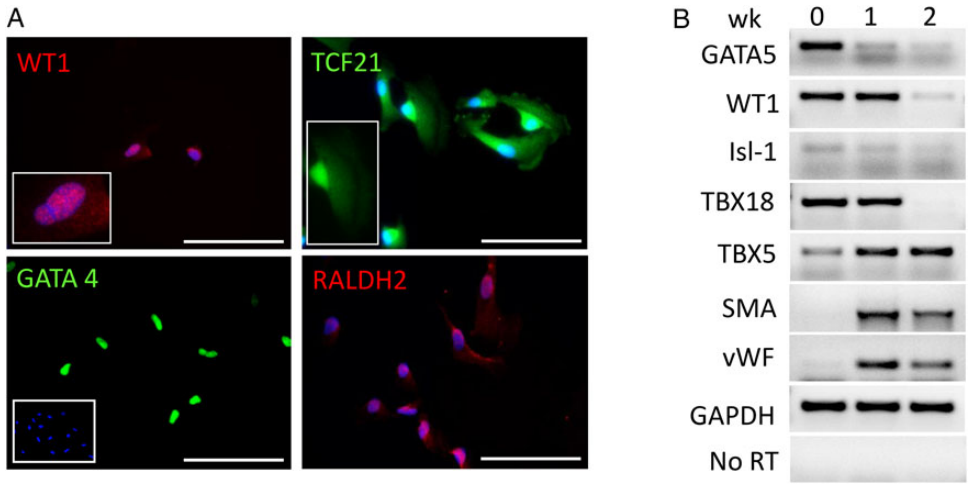

Figure 2 Characterization of isolated human cells. (*A*) Immunocytochemistry for epicardial transcription factors: WT1 (Top, Left); TCF21 (Top, Right); GATA4 (Bottom, Left); and RALDH2 (Bottom, Right) ($n = 5–7$ donors). (*B*) By RT-PCR, several transcription factors associated with cardiac development are downregulated in epicardial progenitor-like cells during EMT into precursor cells. The mRNAs for other proteins associated with differentiation such as smooth muscle actin (SMA) and vWF are upregulated. Left lane shows amplification of RNA isolated from cells prior to EMT. Middle and right lanes show amplification of RNA isolated from cells after EMT and 1 or 2 weeks of culture ($n = 3$ donors). Note: The lower fuzzy bands in the gel data for GATA5, Isl-1, and TBX5 are primer dimers. Scale bar = 100 μM.

including the right ventricle (RV) (*Figure 3A*). The amount of extravasated FITC-albumin in the MEM-treated group was 527.4 ± 109.33% of sham, whereas in the EPI CdM-treated group it was 359.7 ± 78.82% of sham ($P \leq 0.05$, $n = 5$ per group; *Figure 3B*).

To investigate vascular effects of EPI CdM treatment, we performed immunoblotting on LV homogenates. VE-Cadherin is a key junctional protein involved in maintenance of endothelial barrier integrity, and the level of phosphorylated VE-Cadherin (pVE-Cadherin) is a useful indicator of increased vascular permeability.[21] At 24 h after MI, reperfusion, and treatment, the level of pVE-Cadherin (pY^{658}) was significantly higher in the MEM-treated group of animals than in the EPI CdM-treated group ($P \leq 0.01$, $n = 3$; *Figure 3C*). To examine the relative effects of EPI CdM treatment on vascular endothelial cells and smooth muscle cells, we next compared the levels of CD31 (PECAM1, endothelial marker) and smooth muscle alpha actin (α-SMA) in LV homogenates from the MEM and EPI CdM treatment groups. Consistent with enhanced endothelial cell survival, we observed significantly higher levels of CD31 in animals treated with EPI CdM, compared with MEM-treated controls ($P \leq 0.05$, $n = 3$; *Figure 3C*). In contrast to the CD31 results, we observed equal amounts of α-SMA for the MEM and EPI CdM treatment groups (*Figure 3C*), indicating that EPI CdM protected endothelial cells.

3.3 EPI CdM protects human cardiac endothelial cells during simulated ischaemia

To investigate EPI CdM-mediated protection of cardiac endothelial cells, human cardiac endothelial cells were purchased from Lonza (Catalog # CC-2585 and CC-7030, Passage 1). Cell protection assays were performed under conditions of simulated ischaemia (low glucose medium, 1% oxygen, for 24 or 48 h). We first performed an MTS assay (Promega), which measures cell metabolism, a measure of cell viability (*Figure 4A*). Compared with incubation in MEM (vehicle control), EPI CdM generated from hEPDC of donors from 52 to 80 years of age all protected primary human cardiac endothelial cells from simulated ischaemic injury for 24 h (*Figure 4A*). To determine the effects of EPI CdM on cell survival, we quantified cell number (nuclei) by dye-binding of the nucleic acids (CyQuant assay, Molecular Probes, Invitrogen). The CyQuant assay confirmed cell protection by EPI CdM (*Figure 4B*).

3.4 HGF is a key vascular protective component of EPI CdM

To identify signalling pathways stimulated in cardiac endothelial cells following exposure to EPI CdM, we performed phospho-receptor tyrosine kinase (RTK) arrays with lysates from human coronary artery endothelial cells previously exposed to MEM or 1× EPI CdM. We observed that c-Met, vascular endothelial growth factor receptor (VEGFR)1, VEGFR2, and Tie2 (angiopoietin-1 receptor), but not FGFR1, were phosphorylated after EPI CdM exposure based on array signal relative to that of MEM (*Figure 4C*). By ELISA, unconcentrated (1×) EPI CdM from several different human donors contained HGF (~3 ng/mL; *Figure 4D*), angiopoietin-1 (ANG1, between 446 pg/mL and 1.1 ng/mL), VEGFA (between 500 pg/mL and 2.5 ng/mL), and SDF-1 alpha (<150 pg/mL; see Supplementary material online, *Figure S4*).

To examine the relative role of HGF and other angiogenic factors in protection conferred by EPI CdM, we performed pull-down assays with growth factor-specific antisera. Compared with non-specific pull-down (PD), HGF PD significantly reduced the protective effects of EPI CdM (*Figure 4E* and see Supplementary material online, *Figure S5*). Importantly, pull-down of the other angiogenic factors from EPI CdM did not decrease its ability to protect endothelial cells under hypoxic conditions (*Figure 4E*).

3.5 Protein complexes containing HGF and IgG are present in concentrated EPI CdM

In experiments designed to neutralize HGF in 30× EPI CdM using protein A Sepharose, we observed an unexpected decrease in the HGF

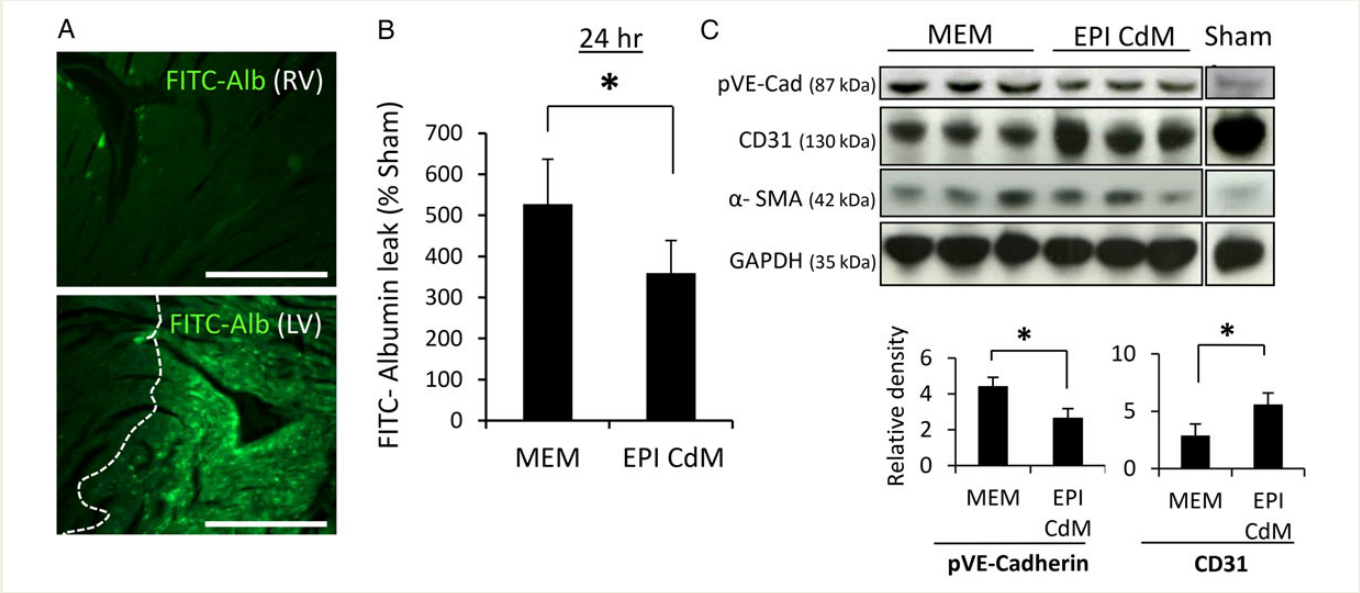

Figure 3 EPI CdM treatment improves vascular integrity after myocardial ischemia with reperfusion in rats. (*A*) Representative immunofluoresence images showing the extent of FITC-albumin extravasation from an uninjured blood vessel in the right ventricle (RV, top) and from an injured LV vessel (bottom). Dashed white line indicates infarct border at 24 h after reperfusion. We confirmed a similar staining pattern in hearts of several different animals ($n = 3$). (*B*) Treatment with 30× EPI CdM significantly decreased extravasation of FITC-albumin in the LV wall of rats at 24 h after reperfusion ($n = 5$ animals per group). In each group, treatment animals are calculated as % of sham. (*C*) Top, western blot image of 3 representative animals per group showing lower levels of phospho-VE-Cadherin (pY^{658}) and higher levels of CD31 in heart homogenates from EPI CdM-treated rats compared with MEM-treated rats. Note: α-SMA levels were similar in both treatment groups. Bottom, quantification of western blot bands. The levels of phospho-VE-Cadherin were higher in MEM (control) vs. EPI CdM ($n = 3$) and CD31 levels were higher in heart homogenates of EPI CdM-treated rats compared with MEM-treated rats ($n = 3$). GM, Growth Medium; Con CdM, Control (EPI) CdM; PD, Pull down; Con IgG, Control IgG. Student's *t*-Test: $^*p \leq 0.05$. Scale bar = 100 μM.

concentration of EPI CdM when it was incubated with non-specific polyclonal IgG alone (typically used as a control for specific IgG). We have previously described the presence of left-over serum carrier proteins in CdM preparations, due to dynamic recycling of extracellular proteins by cellular vesicles.[22] We performed experiments to determine whether HGF interacted with IgG from serum in EPI CdM to form protein complexes as a consequence of concentrating it *ex vivo*. To test this idea, we first concentrated EPI CdM to varying degrees (1× to 40×). To determine whether IgG was interacting with HGF, we added a standard amount of IgG (non-specific polyclonal IgG, 2 μg/mL) to the series of EPI CdM concentrations to use as bait for HGF and performed pull-down assays with protein A Sepharose. As expected, we detected increasing amounts of HGF by ELISA when unmodified EPI CdM was concentrated from 1× to 40×. In the instance that IgG and HGF were interacting, we would observe a reduction in HGF level and detect HGF on the protein A beads. To test our hypothesis, we performed pull-down of HGF from increasing concentrations of EPI CdM using a non-specific IgG, and compared the levels of HGF with before and after pull-down at the various concentrations (*Figure 5A*). We then tested the co-precipitation of HGF with IgG from EPI CdM, by washing the beads thoroughly after pull-down and then incubating them in sodium deoxycholate. We performed ELISA on the deoxycholate-soluble fraction to determine whether we could recover HGF from the beads. We were able to detect increasing levels of HGF after pull-down with IgG, corresponding to increasing EPI CdM concentration. We detected ~1.5 ng/mL HGF from beads with 30× CdM and a significantly greater amount from beads with 40× CdM (~2.8 ng/mL, *Figure 5B*; $n = 3$). Although some interaction may have been present at EPI CdM concentrations lower than 30×, we could not detect HGF from beads by ELISA. To visualize the direct interaction between HGF and IgG in solution, electrophoretic mobilities of human IgG (Sigma) and human HGF were compared in free and complexed states by native agarose (1.5%) gel electrophoresis using MES buffer (50 mM, pH 6.7). By staining with Coomasie Brilliant Blue dye, we were able to observe a band-shift for the HGF when loaded as a complex with IgG compared with free HGF (*Figure 5C*).

3.6 HGF/IgG complexes provide enhanced vascular protection by activating RYK

As the above protein interactions were observed *in vitro*, we were interested to learn if they had any physiological benefit(s). To investigate whether HGF/IgG complexes might protect vascular endothelial cells differently during tissue injury than would free HGF, we performed *ex vivo* cell protection assays under conditions designed to simulate ischaemia (1% oxygen combined with nutrient deprivation). To ensure reproducibility across experiments, we first produced and purified several mg of soluble, human HGF from stable clones of HEK293 cells that were grown in 5% serum (*Figure 6A*). Importantly, the recombinant HGF produced by this method was 100% processed by factors contained in serum (e.g. HGF-activating factor) as indicated by complete cleavage of the 90 kDa HGF protein into its subunits under reducing conditions (*Figure 6A*). Activity of HGF was further confirmed by performing an ELISA to measure activated c-Met receptor after exposure of human cells (see Supplementary material online, *Figure S6*). For protection assays, 100 ng/mL of HGF was added to cells as HGF/IgG

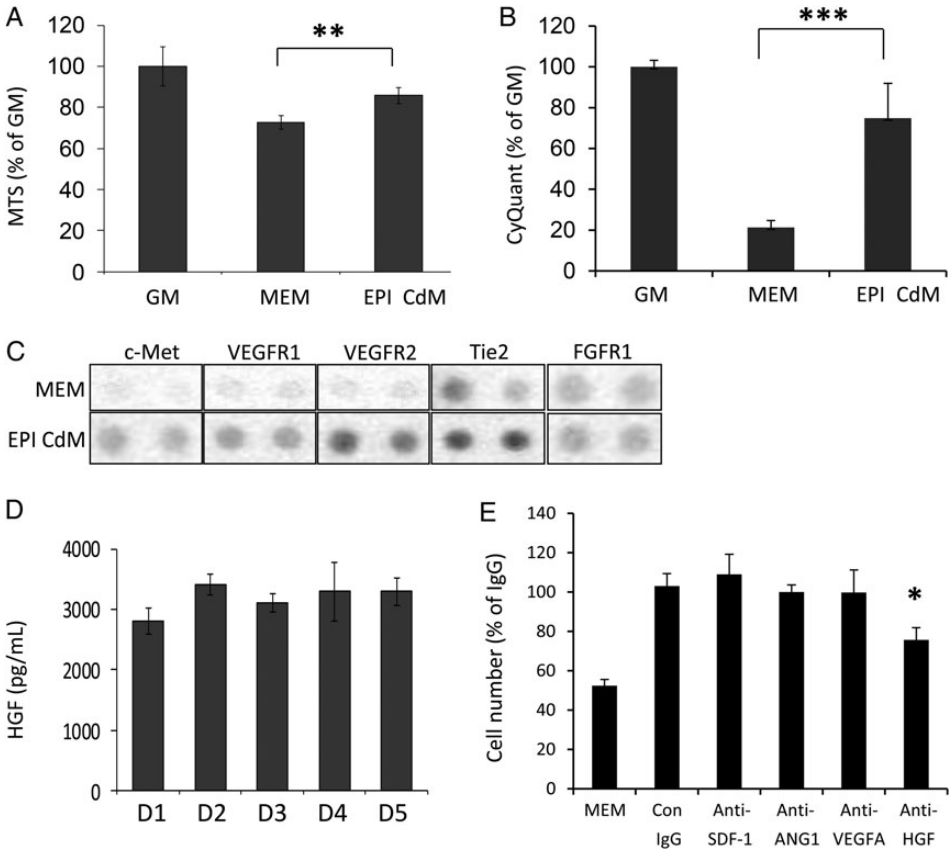

Figure 4 HGF is a key vaso-protective factor in EPI CdM. (*A*) EPI CdM protected cultured primary human coronary artery endothelial cells for 48 h under simulated ischemia, as measured by MTS assay ($n = 5$ donors). Data are mean $\pm$ S.D. (*B*) EPI CdM protected cultured human microvascular endothelial cells for 48 h under simulated ischemia, as measured by CyQuant assay ($n = 3$ donors). Data are mean $\pm$ S.D. In both experiments, survival of cells in growth medium (GM) was considered as 100%. (*C*) Receptor tyrosine kinase (RTK) array demonstrates 30 min exposure to 1× EPI CdM induces phosphorylation of multiple growth factor receptors of coronary artery endothelial cells compared with MEM treatment ($n = 2$). (*D*) HGF ELISA data are for 5 different EPI CdM donors (D1–D5). Measurements are in duplicate. (*E*) Pull down (PD) of HGF from EPI CdM with an HGF-specific antibody (2 μg/ml) decreased EPI CdM-mediated protection of microvascular endothelial cells during simulated ischemia (48 h) compared to PD with a non-specific IgG antibody (*$P \leq 0.05$ Control IgG vs. Anti-HGF). In contrast, PD of SDF-1, ANG1 or VEGFA from EPI CdM did not alter its protection of microvascular endothelial cells (all antibodies used at 2 μg/ml) ($n = 3$). Cell numbers were determined by CyQuant assay (dye binding of nucleic acids). Data are mean $\pm$ S.D. One way ANOVA for A,B, and E: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. Scale bar = 100 μM.

complexes (see detailed description in methods) or as free HGF combined with a concentration of IgG that matched that of the HGF/IgG complexes. We observed a significant increase in protection conferred by HGF/IgG complexes compared with uncomplexed HGF and IgG at individual, matched protein concentrations ($P \leq 0.01$, $n = 4$; *Figure 6B*). Notably, uncomplexed HGF with IgG provided similar level of protection in culture when compared with free HGF alone. We therefore chose uncomplexed HGF and IgG mixture as appropriate control for HGF/IgG complexes in subsequent experiments.

To determine whether HGF/IgG complexes mediated their protective effects via a receptor (or receptors) that differed from c-Met, we performed a second phospho-RTK array with lysates of human coronary artery endothelial cells that were incubated for 30 min with HGF/IgG complexes or with a matched concentration of HGF and IgG. On the array, the level of c-Met phosphorylation was comparable in both the treatment groups, suggesting an additional mechanism for protection mediated by HGF/IgG complexes (*Figure 6C*). Of special interest, we observed phosphorylation of RYK after treatment with HGF/IgG complexes, but not after treatment with free HGF and IgG (*Figure 6C*). RYK is an 'orphan' receptor tyrosine kinase with no ligand yet identified that promotes its phosphorylation.[23,24] We next performed a cell protection assay under conditions of simulated ischaemia in the presence or absence of RYK neutralizing antibodies. Blocking RYK on coronary artery endothelial cells significantly decreased the protective effect of the HGF/IgG complexes (complexed HGF with anti-RYK 155.46 $\pm$ 14.62% of MEM, complexed HGF with non-specific control IgG, 223.46 $\pm$ 10.307% of MEM, $P \leq 0.01$; *Figure 6D*, $n = 4$), but not the protection conferred by uncomplexed HGF with IgG ($P = 0.09$; *Figure 6D*, $n = 4$).

3.7 HGF/IgG complexes localize to blood vessels and promote vascular protection after MI

To test whether treatment by HGF/IgG complexes was advantageous over uncomplexed HGF with IgG *in vivo*, we compared the relative

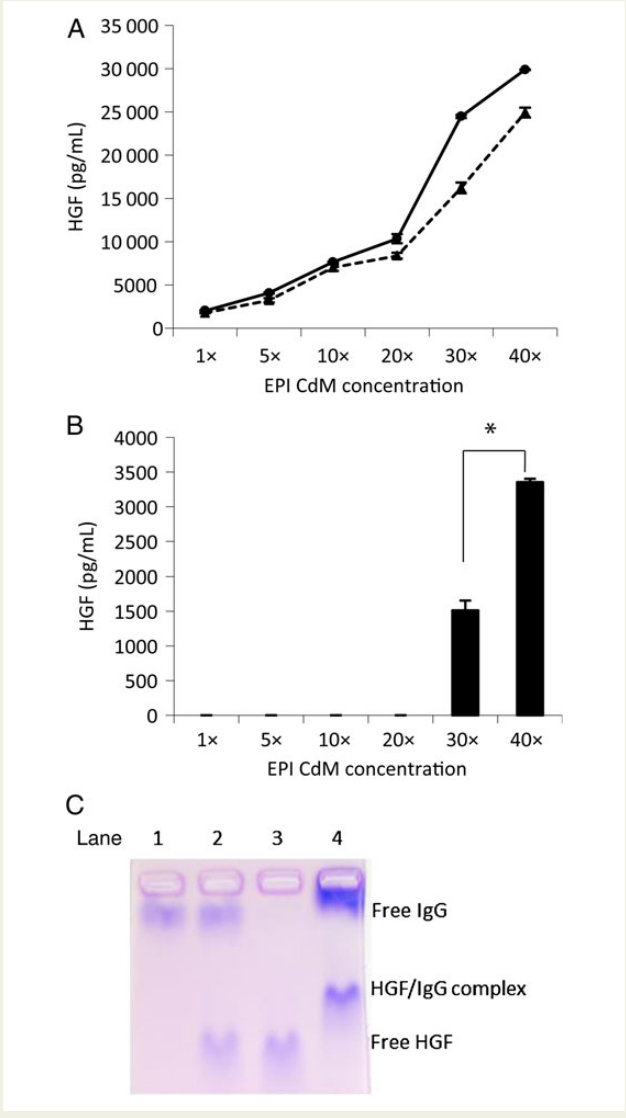

Figure 5 Concentrated EPI CdM contains HGF/IgG protein complexes. (*A*) With increasing concentration of EPI CdM, we observed greater formation of HGF/IgG complexes as detected by protein A pull-down and ELISA. Solid line= EPI CdM, dashed line= EPI CdM + non-specific polyclonal IgG (3 EPI CdM donors, $n = 3$ each). (*B*) Quantification of HGF after dissociation from protein A Sepharose beads by incubation in sodium deoxycholate (3 EPI CdM donors, $n = 3$ each). (*C*) Native 1% agarose gel demonstrating change in mobility for HGF when complexed with IgG. Human IgG (lane 1) migrates towards the anode at a much slower rate at pH 6.7 compared with human HGF (lane 3). A simple mixture of the 2 proteins (free) did not affect their individual mobilities (lane 2). However, HGF and IgG complexes formed by concentration resulted in altered HGF mobility (i.e. band shift; lane 4) ($n = 3$). Student's *t*-test: $^*P \leq 0.05$.

level of vascular integrity after MI with reperfusion. We mixed HGF (10 μg/mL) with non-specific polyclonal rat IgG (carrier) either in a complexed form or in an uncomplexed form and compared effects with MEM infusion. We then administered HGF/IgG complexes or uncomplexed HGF with IgG (control) to two groups of rats at the time of reperfusion, 2 h after LAD occlusion (steady injection of 1 mL treatment with a 30-gauge needle into the LV lumen at the apical end of heart). By FITC-albumin assay, treatment with HGF/IgG complexes significantly improved vascular integrity at 24 h after ischaemia–reperfusion when compared with treatment by free HGF and IgG or MEM (MEM, 544.1 ± 94.18% of sham, $n = 4$; complex, 370.2 ± 102.86% of sham, $n = 11$; free HGF, 516.5 ± 53.93% of sham, $n = 8$; complex vs. MEM, $P \leq 0.001$; MEM vs. free HGF, n.s.; complex vs. free HGF, $P \leq 0.01$; *Figure 6E*).

Immunohistochemistry using an antibody targeting the N-terminal His-tag of our recombinant HGF demonstrated a perivascular localization of His (HGF) when administered as HGF/IgG complexes, but we were unable to detect His (HGF) in tissue sections of control animals that received free His (HGF) and IgG (*Figure 6F*). For rats treated with HGF/IgG complexes, His (HGF) was readily detectable in larger vessels (*Figure 6F* and *F'*) and the microvasculature and the remaining capillary bed within the infarcted region (*Figure 6F'*).

4. Discussion

Based on their multipotent capacity for differentiation and paracrine activity, hEPDCs are a promising cell type for cardiac regenerative medicine.[10,15] However, safe and effective clinical use of hEPDC will require a more complete understanding of cytokines and growth factors secreted by hEPDC and their roles under homeostatic conditions and after cardiac injury. Here, we identified HGF as a factor produced by hEPDCs that protected cardiac microvascular endothelial cells during hypoxia/ischaemia. When administered upon reperfusion after MI, concentrated human EPI CdM prevented vascular leak and promoted microvascular cell survival. Previous reports demonstrated that HGF increased myocyte survival after cardiac injury (ischaemia/reperfusion)[25] and provided vascular protection after lung or brain injury.[26,27]

We found that EPI CdM contained unique HGF/IgG complexes that promoted endothelial cell survival in a manner superior to 'free' HGF. Furthermore, we determined that recombinant human HGF and IgG interact in a predictable manner *in vitro*, and could be reproducibly prepared as complexes for *in vivo* administration. Compared with free HGF, HGF/IgG complexes increased the preservation of vascular integrity after myocardial ischaemia with reperfusion. Immunohistochemical data suggested that HGF/IgG complexes localized to blood vessels in a different fashion than did free HGF, both spatially and temporally; this may increase local concentration and/or retention time, thereby enhancing its protective effect(s). Exposure of vascular endothelial cells to either HGF/IgG complexes or free HGF promoted the phosphorylation of c-Met to the same extent, but only HGF/IgG complexes were able to induce phosphorylation of RYK. Furthermore, protection of endothelial cells by HGF/IgG complexes was substantially diminished when RYK was blocked by antisera.

At present, the intracellular signalling cascade downstream of RYK phosphorylation has yet to be determined, but several reports describe RYK as a modulator of Wnt ligands and Wnt signalling.[28–31] RYK has also been shown to associate with Ephrins, which have known roles in vascular permeability during developmental angiogenesis and after injury.[32,33] In the case of reperfusion injury and endothelial cell protection, RYK signalling could promote intracellular signal transduction that directly increases cell survival. Notably, HGF/IgG appears to be the first 'ligand' reported to induce RYK phosphorylation and may therefore provide a tool to help delineate signalling downstream of p-RYK.

In the clinic, patients with MI commonly undergo percutaneous coronary intervention or thrombolytic treatment (e.g. tPA)[34] to restore blood flow to occluded vessels. Unfortunately, re-canalization of

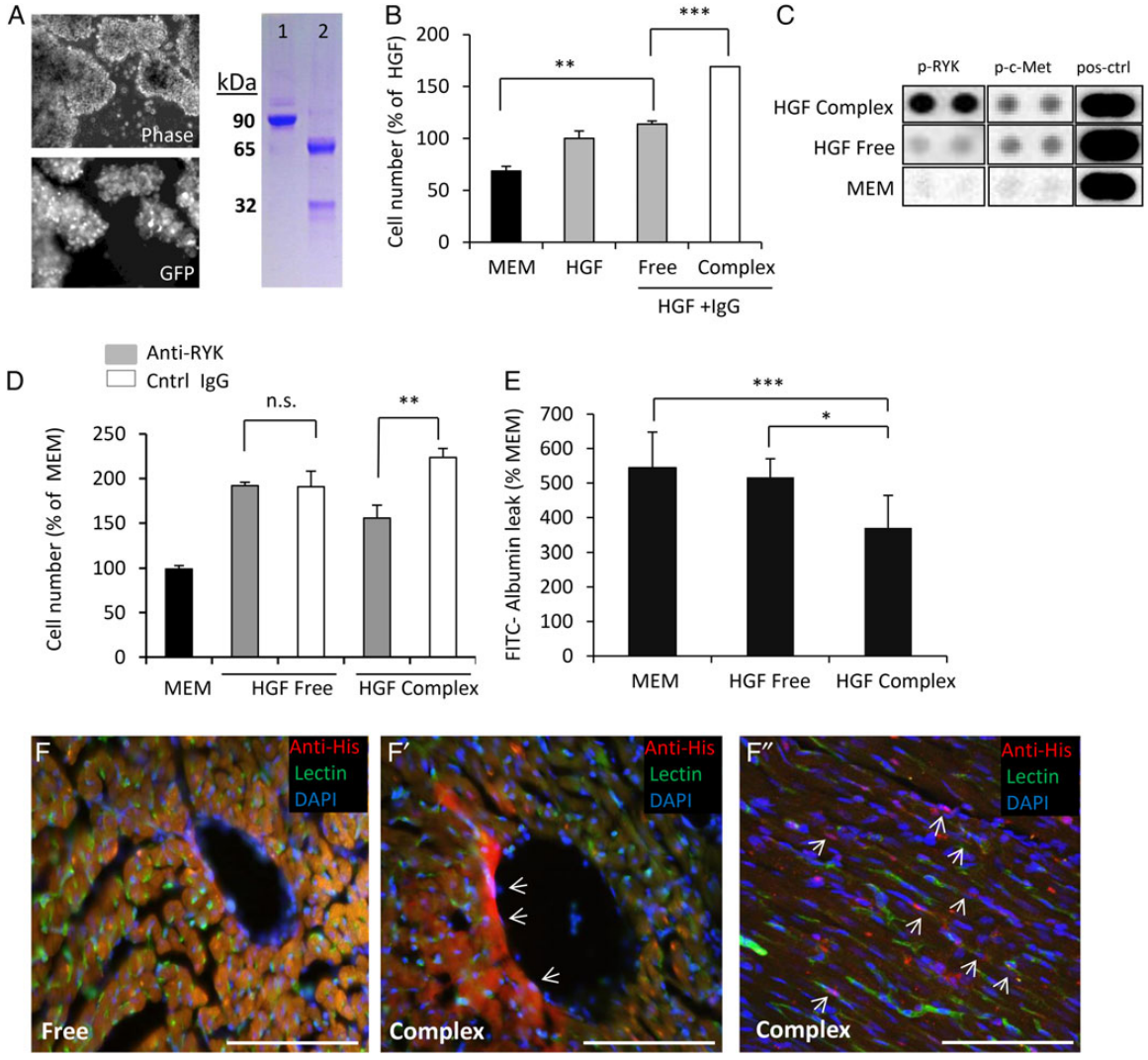

Figure 6 HGF/IgG complexes enhance vascular protection by interacting with RYK. (*A*) Stable producer cell lines and purification of bioactive recombinant human HGF. (Left, top) Phase contrast images of HEK 293 cells with genomic integration of pIRES-puro vector expressing human HGF-His tag-F2A-GFP. (Left, bottom) Epifluorescence image showing GFP expression (FITC channel). (Right) Gel electrophoresis illustrating purification of HGF. Lane 1, electrophoresis of eluted material under non-reducing conditions demonstrates purity and molecular weight of single chain pro-HGF (90 kDa). Gel stained with Coomassie Brilliant Blue. Lane 2, purified protein run under reducing conditions (beta-mercaptoethanol) demonstrates the active heterodimer of human HGF (see bands at 65 kDa and 32 kDa). (*B*) CyQuant assay demonstrates enhanced protective effects of HGF/IgG complexes when compared with free HGF (alone) or free HGF with IgG (Both free, unconcentrated factors). For greater detail, please refer to Methods. Cell number was assayed after 48 h of simulated ischemia (MEM, 69 $\pm$ 24.7% of HGF; free HGF alone, 100 $\pm$ 7.3%; free HGF with IgG [Free], 114 $\pm$ 3.1% of HGF; HGF with IgG [Complex], 169 $\pm$ 4.0% of HGF [$n = 4$ for all]). Free HGF with IgG (Free) conferred protection ($P \leq 0.01$, vs. MEM). However, HGF/IgG complexes (Complex) protected better than did free HGF with IgG (Free) ($P \leq 0.001$). (*C*) Treatment of coronary endothelial cells with HGF/IgG complexes (Complex, top), free HGF and IgG (Free, middle) or MEM (bottom) affects the level of phosphorylation for RYK, but not c-Met. Positive control signal (pos-ctrl) has been included for each membrane to compare for normalization ($n = 2$). (*D*) Blocking RYK reduced protection conferred by HGF/IgG complexes ($P \leq 0.01$, $n = 4$) but not protection by free HGF with IgG ($P = 0.09$, $n = 4$). Note: coronary artery endothelial cells were incubated under simulated ischemia for 24 h. (*E*) Intra-arterial treatment with HGF/IgG complexes significantly decreased FITC-albumin extravasation in the LV wall of rats at 24 hr after reperfusion (MEM, $n = 4$; free HGF with IgG [Free], $n = 8$; HGF/IgG complexes [Complex], $n = 11$; $P \leq 0.01$). (*F* and *F′*) Epifluorescent image showing localization of His-HGF outside of blood vessels (by anti-His antibody) in animals treated with free HGF (*F*) and complex HGF (*F′*). HGF/IgG complexes were localized to large blood vessels (*F* and *F′*) and the capillary network at the borders of infarcted regions (*F″*). We confirmed a similar staining pattern in hearts of 3 different animals. Note: the presence of HGF not localized to capillaries is likely indicative of leakage from damaged or dead blood vessels in the capillary bed. Data are mean $\pm$ S.D. One way ANOVA for B,D, and E: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Scale bar = 100 μM.

macroscopic vessels does not lead to improved microvascular perfusion in ~30% of cardiac patients.[35] This phenomenon is described as 'no re-flow' or 'low re-flow', and the extent of no re-flow is a major determinant of infarct expansion after MI.[36,37] No re-flow may result from destruction of microscopic vessels, which we have termed 'vascular rhexis', or from other factors such as microemboli, inflammation, release of toxic cellular metabolites, or oxidative stress that cause endothelial cell dysfunction and induce microvascular leaks.[38,39] Our results indicate that HGF/IgG treatment protects against reperfusion injury after MI as opposed to stimulation of angiogenesis, which is a process that requires several days. Further administration of HGF/IgG complexes may provide additional protection or induce angiogenesis, but this remains to be tested.

By simultaneously signalling through c-Met and RYK, HGF/IgG complexes have potential as a new therapeutic to improve vascular integrity. Infusion of HGF/IgG complexes may also reduce vascular rhexis and the incidence of no/low reflow after MI. In addition to cardiac ischaemia/reperfusion injury, many other forms of tissue injury or disease that involve damage to vascular endothelium, such as stroke and peripheral artery disease, may also benefit from treatment with HGF/IgG complexes.

Authors' contributions

K.S.R. and J.L.S. designed research; K.S.R., A.A., K.L.M.-Y., B.B., and J.L.S. performed research; K.S.R. and J.L.S. analysed data; P.V.B. provided critical reagents and biological samples; K.S.R., B.E.S., and J.L.S wrote the paper.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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