De novo cardiomyocytes from within the activated adult heart after injury

Nicola Smart1,*, Sveva Bollini1,*, Karina N. Dubé1, Joaquim M. Vieira1, Bin Zhou2,3,4, Sean Davidson5, Derek Yellon5, Johannes Riegler6,7, Anthony N. Price8, Mark F. Lythgoe6, William T. Pu2,3 & Paul R. Riley1

A significant bottleneck in cardiovascular regenerative medicine is the identification of a viable source of stem/progenitor cells that could contribute new muscle after ischaemic heart disease and acute myocardial infarction1. A therapeutic ideal—relative to cell transplantation—would be to stimulate a resident source, thus avoiding the caveats of limited graft survival, restricted homing to the site of injury and host immune rejection. Here we demonstrate in mice that the adult heart contains a resident stem or progenitor cell population, which has the potential to contribute bona fide terminally differentiated cardiomyocytes after myocardial infarction. We reveal a novel genetic label of the activated adult progenitors via re-expression of a key embryonic epicardial gene, Wilm’s tumour 1 (Wt1), through priming by thymosin β4, a peptide previously shown to restore vascular potential to adult epicardium-derived progenitor cells2 with injury. Cumulative evidence indicates an epicardial origin of the progenitor population, and embryonic reprogramming results in the mobilization of this population and concomitant differentiation to give rise to de novo cardiomyocytes. Cell transplantation confirmed a progenitor source and chromosome painting of labelled donor cells revealed transdifferentiation to a myocyte fate in the absence of cell fusion. Derived cardiomyocytes are shown here to structurally and functionally integrate with resident muscle; as such, stimulation of this adult progenitor pool represents a significant step towards resident-cell-based therapy in human ischaemic heart disease.

Two previous studies have indicated a significant contribution of embryonic epicardial progenitor cells (EPDCs) to the cardiomyocyte lineage4–6. We investigated a basis for translating this myocardial potential in the adult heart. A significant problem in this regard is the lack of current adult epicardium-specific markers and authentic adult EPDC-Cre-expressing mouse strains for canonical lineage tracing. Previously reported genetic models to trace, or target, cells originating in the current adult epicardium-specific markers and authentic adult EPDC–Cre-expressing mouse strains for canonical lineage tracing. Previously reported genetic models to trace, or target, cells originating in the epicardium, such as Wt1CreERT2/+ and Tbx18Cre mice3,4, cannot be applied directly to their adult counterparts, as the epicardial markers are either restricted to embryonic stages (Supplementary Fig. 1) or additionally expressed in the myocardium, as in the case of the Tbx18 model3. Therefore, we sought to reactivate Wt1 expression in the adult heart by pre-treatment (‘priming’) with thymosin β4 (Tβ4), which we previously showed induces adult EPDCs to form vascular precursors for neovascularization3,6, followed by myocardial infarction (see schematic, Fig. 1a). Thus, we were able to establish both constitutive (GFP+) and pulse (YFP+) labelling of Wt1 progenitors to characterize the potential spatiotemporal distribution of primed adult epicardial progenitors.

We initially established epicardial explants from Tβ4-primed Wt1GFPCre/+ adult hearts (7 days of intraperitoneal injections without myocardial infarction; Supplementary Fig. 2a–d) as previously described7,8 and investigated Isl1 expression as a marker of postnatal cardioblasts along with Nkx2-5, an early marker of cardiomyocyte progenitors9–11. Isl1+/GFP+ cells and Nkx2-5+/GFP- cells were prevalent within the explant cultures with mean percentage incidences of 76.7 ± 6.3% and 72 ± 2.1%, respectively (mean percentage ± standard error of mean (s.e.m.); n = 12 explants), as were progenitor-like GFP+ cells, which expressed more mature markers of cardiomyocyte differentiation such as cardiac troponin T (CtnT, also known as Tnn2; 4.1 ± 1.6%; Supplementary Fig. 2i, j) sarcomeric α-actinin (SαA; 4.8 ± 2.4%; Supplementary Fig. 2i, m) and cardiac myosin binding protein C (MyBPC, also known as Mybpc2; 4.6 ± 0.8, mean percentage ± s.e.m.; Supplementary Fig. 2o, p). By day 14 in culture, cells adopted a more differentiated cardiac muscle phenotype; with evidence of sarcomeric structure in conjuction with compartmentalization of the GFP signal (Supplementary Fig. 2k–r).

Next we determined the extent of Wt1 re-expression in vivo along-side the quantity and distribution of GFP+ and YFP+ adult progenitors. With injury alone (no Tβ4 priming), expression of Wt1 and Tbx18 was significantly increased at day 7 after myocardial infarction, dependent on the severity of the injury (Supplementary Fig. 3a). Following Tβ4 priming, expression of both epicardial genes was precociously increased by day 2 after myocardial infarction (Supplementary Fig. 3b), and this persisted in Tβ4-primed GFP+ cells isolated by fluorescence-activated cell sorting (FACS) at day 4 after injury (Supplementary Fig. 4a). In situ hybridization, at an equivalent stage, revealed an upregulation in Wt1 expression in small round ‘progenitor-like’ cells within the epicardium, subepicardial region and underlying myocardium (Supplementary Fig. 4b–d).

Tβ4 priming resulted in significantly more sorted GFP+ and YFP+ cells from whole hearts taken at day 7 after myocardial infarction when compared to treatment with vehicle (Fig. 1b–e and Supplementary Fig. 5a–d; GFP, 6.12% ± 3.38% versus 0.74% ± 0.36% PBS; YFP, 0.74% ± 0.36% Tβ4 versus 0.36% ± 0.36% PBS). This was confirmed in situ by anti-GFP immunostainig on serial heart sections at the equivalent stage after injury (Supplementary Fig. 5e). Further flow cytometry characterization of a progenitor phenotype revealed that labelled cells, at day 4, were not c-Kit+/Sca-1− (Supplementary Fig. 6a–e). Instead, approximately 80% were positive for stem cell antigen factor 1 (Sca-1+; Supplementary Fig. 6f–j), consistent with the notion that the adult epicardium is a heterogeneous lineage2.

Two-photon molecular excitation laser scanning microscopy revealed pulse-labelled YFP+ cells in the epicardium and subepicardial region at day 7 after myocardial infarction (Fig. 1f, g), distributed in diminishing numbers towards the underlying myocardium (Fig. 1h). Proliferative Ki67+ progenitors were observed in epicardial and subepicardial regions (Supplementary Fig. 7a, b) along-side YFP+ cells positive for phospho-histone H3 (Supplementary Fig. 7c–f), which
**Figure 1** | Activated Wt1+ cells give rise to cardiac progenitors in the injured adult heart. a. Schematic of constitutive or pulse-chase labelling of Wt1+ cells. b-e. FACS analyses of whole hearts at day 7 (d7) after myocardial infarction (MI) revealed a significant increase in GFP+ (b, c) and YFP+ (d, e) cells following priming with Tj4, as compared to PBS-treated controls (Co); x-axes represent either GFP (b, c) or YFP (d, e) fluorescent wavelengths on a logarithmic scale and y-axes represent total cell numbers isolated by FACs (b-e). f, g. Multi-photon imaging at day 7 after myocardial infarction revealed YFP+ cells within the epicardium and subepicardial region migrating towards underlying myocardium. Scale bars in f, 20 μm; g, 10 μm. h, Three-dimensional Imaris reconstruction of migrating YFP+ cells (green) amidst non-labelled cells (red). ep, epicardium; my, myocardium. i-l. YFP+ cells that co-stained for Isl1 (highlighted by white arrowheads in k) resided in the epicardium proximal to areas of scarred myocardium 2 days (d2) after myocardial infarction. sc, scar region. Scale bar in l (also applies to i-l), 50 μm. m. Significant increase in Isl1 expression in primed hearts at days 2, 4 and 7 after myocardial infarction relative to sham-operated controls (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; MI group 1 and group 2 versus sham; myocardial infarction categories: purple, mild injury; cream, severe injury; n = 6 hearts per sham and MI groups. n. Significant increases in Isl1+/YFP+ cells at days 2 (*P ≤ 0.05), 4 (**P ≤ 0.01) and 7 (***P ≤ 0.001) after myocardial infarction and Nkx2-5+/YFP+ cells by day 7 (****P ≤ 0.001) alongside phospho-histone H3+ (P-HH3+) proliferating YFP+ progenitors at day 7 (****P ≤ 0.001), compared to sham-operated controls. P values were calculated by Student’s t-test (m) and paired ANOVA (n). Error bars represent mean ± s.e.m. N values are numbers of hearts analysed for each group: N = 3 (m); N = 4 (d2 and d4) and N = 7 (d7) (n).

To assess functional integration with resident myocardium, we recorded cellular calcium transients [Ca2+]i, between YFP+ and YFP− cells in situ, as previously described13 (Fig. 2m–s and Supplementary Fig. 10a–f). Two-photon imaging confirmed migration of YFP+ cells from the outer epicardial layer into the underlying myocardium (Supplementary Fig. 7m). At day 14 after myocardial infarction, evoked [Ca2+]i transients in YFP+ cardiomyocytes were synchronous, with kinetics indistinguishable from those of neighbouring YFP− cardiomyocytes (Fig. 2q–s). Apparent differences in resolution between resident YFP+ and de novo YFP+ cardiomyocyte transients (compare Fig. 2q with r) were observed, reflecting the newly acquired function of the YFP+ population.

To rule out the possibility that we traced resident cardiomyocytes that were labelled by virtue of ectopic activation of the fluorophore from the Wt1 knock-in alleles, we carried out a series of experiments transplanting FACs-isolated donor GFP+ cells into non-transgenic host hearts. Extensive analyses indicated that the prospective donor cells were progenitors of epicardial origin (Fig. 3). Wt1+ cells were restricted to the epicardium and subepicardial region throughout the heart, as confirmed by co-staining with an antibody against podoplanin, a transmembrane glycoprotein14 that specifically marked the...
epicardial and myocardial boundaries (Fig. 3a, b). Immunostaining for anti-GFP revealed GFP⁺ cells residing in the expanded epicardium but excluded from the myocardium throughout the heart (Fig. 3c, e).

Real-time quantitative polymerase chain reaction analyses on FACS-isolated donor cells at day 4 revealed no expression of the canonical cardiomyocyte markers cTnT, MyBPC and Actn2 (Fig. 3d). We also analysed hearts isolated from Tβ4-primed/injured MLC2vCre⁺;R26R²YFP⁺ mice (MLC2v-YFP; Fig. 3f), which lineage-traced ventricular cardiomyocytes as YFP⁺ from early developmental stages to adulthood, and confirmed an absence of Wt1 in both healthy and scarred myocardium respectively (Fig. 3g, h).
hearts and ruled out the possibility of the GFP⁺ progenitor population arising from existing vasculature (Fig. 3i,j). The FACS-isolated cells at day 4 revealed no expression of the canonical vascular markers Pecam and Tie2 (Fig. 3k), and cytospin with comparative immunostaining of the sorted GFP⁺ and GFP⁻ populations (Fig. 3l) revealed that the GFP⁺ cells were negative for both vascular markers Pecam and SMA and myocardial markers SβA and cTnT (Fig. 3m), whereas the GFP⁻ population contained cardiovascular cells (Fig. 3n).

After donor cell transplantation (Fig. 4a), GFP⁺ cells within the epicardial region of the host were restricted to the site of injection after 24 h (Fig. 4b). By day 7, GFP⁺/Nkx2-5⁺ donor cells, indicative of myocardial progenitor commitment, were located proximal to the subepicardium (Fig. 4c), in conjunction with morphologically immature, cTnT⁺ cardiomyocyte-like cells (Fig. 4d). Collectively, the presence of these staged donor derivatives suggested progressive differentiation towards a mature cardiomyocyte fate. More definitive donor GFP⁺ cardiomyocytes with myofibrillar structure and that co-expressed cTnT were observed residing within the host myocardium at day 14 (Fig. 4e,f). We subsequently traced donor GFP⁺ cardiomyocytes for fluorescent in situ hybridization (FISH) with X- and Y-chromosome paints to assess karyotype (Fig. 4a). Single XY GFP⁺ cardiomyocytes were detected within the XX host, indicating that transdifferentiation had occurred in the absence of cell fusion (predicted XXX fusion karyotype; Fig. 4g–j). To exclude the possibility of reductive divisions of fusion hybrids accounting for the XY diploid karyotype, reciprocal transplantation experiments injecting female XX GFP⁺ donor cells into male XY wild-type hosts (Fig. 4k) resulted in XX GFP⁺/SβA⁺ cardiomyocytes (Fig. 4l,m) in XY host myocardium (Fig. 4n,o). Quantitative assessment of FISH on GFP⁺ cardiomyocytes (total n = 22) excluded the presence of a host Y chromosome in each case. Finally, we reanalysed non-transplanted YFP⁺ cells in female hearts (see Fig. 2) and detected examples of both YFP⁺/cTnT⁺ (Fig. 4p) and YFP⁺/SβA⁺ (Fig. 4r) cardiomyocytes with a single XX karyotype (Fig. 4q,s).

To investigate the outcome of Tβ4 priming on cardiac function and myocardial regeneration, we carried out serial magnetic resonance imaging (MRI) 7, 14 and 28 days after myocardial infarction (Supplementary Fig. 11a–e and Supplementary Table 1). Significant improvement in functional parameters, including ejection fraction and end diastolic/systolic volumes, alongside beneficial changes in infarct/scar volume with increased left ventricular mass over time (Supplementary Table 1) were recorded with Tβ4 treatment, as a surrogate indicator of replenished myocardium (Supplementary Fig. 11f,g).

Collectively, these data indicate that the adult heart can respond to injury with a modest increase in Wt1⁺ progenitors but without initiating a cardiogenic program. Tβ4 enhances this response, via a precocious and significant reactivation of Wt1 expression ultimately resulting in cardiomyocyte restitution. Although we cannot unequivocally exclude the possibility that Wt1⁺ progenitors arise from a nonepicardial source, support for an adult EPDC myocardial contribution comes from the tight regulation of Wt1⁺ labelling in the epicardium and subepicardial region, and transplantation of donor GFP⁺ progenitors residing both within and immediately proximal to the epicardium.

Reactivation of Wt1 by injury and Tβ4 represents a robust means to faithfully tag the progression of adult cardiac progenitors to differentiated myocytes, and provides mechanistic insight into a molecular function of Tβ4 and downstream cellular events. Previously, Tβ4 was shown to upregulate ILK and Akt activity in the heart, enhancing early myocyte survival after ischaemic injury.²⁷ In addition, Tβ4 can induce the adult epicardium, in the same setting, to contribute coronary endothelial and smooth muscle cells and initiate vascular repair.²⁸ We now propose a further contribution, underpinning the initiation and migration of resident cardiovascular progenitors towards a cardiomyocyte fate. Although in the current study it is not possible to discriminate the relative contribution of each of these proposed functions, several effects of Tβ4 seem to be delineated with time post-myocardial

Figure 4 | Transplanted donor Wt1⁺ progenitors differentiate into cardiomyocytes within host myocardium in the absence of cell fusion. a, Schematic of cell transplantation regimen. b, After 24 h post-transplantation GFP⁺ cells within the epicardium and subepicardial region at the injection site were absent in remote regions. ep, epicardium; my, myocardium. c, Transplanted GFP⁺ cells expressed Nkx2-5, indicative of a myocardial progenitor phenotype (white arrowhead highlights a GFP⁺/Nkx2-5⁺ progenitor; white arrows highlight GFP⁺/Nkx2-5⁺ progenitors and asterisks highlight epicardium cells negative for both GFP and Nkx2-5). d–f, Donor GFP⁺ cells with an intermediate differentiated phenotype (highlighted by white arrowhead, d), alongside those with evidence of sarcomeric banding that co-expressed cTnT were observed within host myocardium (e, f). sc, scar. g–j, GFP⁺/cTnT⁺ cardiomyocytes (g–i) with sarcomeric banding (highlighted by white arrowheads in h) were traced for FISH analyses to reveal a single XY karyotype (j). X, X chromosome; Y, Y chromosome. k–o, Reciprocal transplantation (XX into XY) followed by confocal microscopy (k) revealed GFP⁺ cardiomyocytes (l) that had the donor XX karyotype (m) relative to the XX karyotype of host GFP⁺ cardiomyocytes (highlighted by white arrowhead; n, o). p–s, In female Wt1CreERT2/+;R26R/Cre/+ mice, previously tracked YFP⁺ cTnT⁺ (p) and SβA⁺ cardiomyocytes (r, s) were absent in the host myocardium (q, r). Scale bars: c, 25μm; d–f, 20 μm; g (applies to g–j), k (applies to k–o), p (applies to p–q), r (applies to r–s), 10 μm.
infarction. The pro-survival activity of Tβ4 is an early injury response to maintain the status quo of surviving myocardium, whereas the neovascularization and de novo cardiogenesis are longer-term regenerative functions potentially acting through the common target of adult EPDCs.

The identification of a bona fide source of myocardial progenitors is a significant step towards resident-cell-based therapy for acute myocardial infarction in human patients. The induced differentiation of the progenitor pool described into cardiomyocytes by Tβ4 is at present an inefficient process relative to the activated progenitor population as a whole. Consequently, the search is on via chemical and genetic screens to identify efficacious small molecules and other trophic factors to underpin optimal progenitor activation and replacement of destroyed myocardium.

METHODS SUMMARY

MLC2v–YFP and inducible Wt1CreERT2/+;R26R-EYFP/+ mice were generated by crossing Rosa26R–EYFP with MLC2vCre/+ (ref. 15) and Wt1CreERT2/+ (ref. 4) mice. Tβ4 expression in adult epicardium was achieved through daily intraperitoneal Tβ4 injections (RegenerEX; 12 mg kg⁻¹, 7 days; Fig. 1a). Wt1CreERT2/+;R26R-EYFP/+ mice received 2 mg tamoxifen. EPDC cultures from Tbx1-GFPcre/+ were differentiated for 14 days in IMDM plus 20% FBS and fixed in 4% paraformaldehyde. Permanent left anterior descending artery (LAD) ligation was performed in adult Wt1GFPCre/-. Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 16 March 2010; accepted 13 May 2011.

Published online 8 June 2011.


Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This work was funded by the British Heart Foundation. We are grateful to F. Costantini and S. Srinivas for providing the R26R–EYFP mouse strain, to B. Verryn for assistance with confocal microscopy and A. Edaoudi, P. Chana and A. Angelula for assistance in flow cytometry. We thank A. Taylor and V. Muthurangu for functional interpretation of MRI data and RegenerEX Biopharmaceuticals for provision of clinical grade Tβ4.

Author Contributions N.S. carried out the in vivo histological assessments of cardiomyocytes and FISH experiments. S.B. carried out the explant and FACS studies and jointly with K.N.D. established the myocardial infarction model and the cell transplantation. J.M.V. carried out the qRT–PCR analyses and assisted with cell transplantation. B.Z. generated the Wt1GFPCre and Wt1CreERT2 mice. S.D. and D.Y. performed the two-photon microscopy and Ca²+ transient recordings. J.R., A.N.P. and M.F.L. carried out the MRI functional analyses. W.T.P. provided the Wt1GFPCre and Wt1CreERT2 mice. P.R.R. established the hypotheses and experimental design, co-analysed data and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to P.R.R. (p.riley@ich.ucl.ac.uk).
**METHODS**

Generation of epicardial trace mice. Wt1*GFP*Cre*+/+* mice have been previously described,
inducible Wt1*CreERT2*+/−, R26*βFP*+ mice were generated by crossing the Wt1*CreERT2*+ strain with Rosa26*βFP* reporter mice and genotyping as previously described. Adult mice were primed with intraperitoneal (i.p.) injection of TGF at 4 or vehicle (PBS) into either Wt1*GFP*Cre*+ or Wt1*CreERT2*/−, R26*βFP*+ (plus tamoxifen) strains. Primed mice were subsequently given a myocardial infarction by coronary artery ligation (n = 85, Wt1*GFP*Cre*+ and n = 31, Wt1*CreERT2*/−, R26*βFP*+). Half of each myocardial infarction group were pre-treated with TGF and half were vehicle treated (n = 6 sham-operated controls were included per experiment). Hearts were subsequently assessed using a combination of FACS, cytospin, immunofluorescence and real-time qRT–PCR analyses for GFP/YFP expression and myocardial markers after 2, 4, 14 days (see schematic in Fig. 1a). With respect to monitoring progenitor-derived cardiomyocytes the focus was on the inducible Wt1*CreERT2*/−, R26*βFP*+ model to ensure specific temporal labelling of YFP+ derivatives and rule out ectopic activation of the Wt1 gene at the targeted allele. Mice that were PBS treated with tamoxifen and TGF primed in the absence of tamoxifen were used as controls and importantly we never observed YFP+ cardiomyocyte-like cells in these hearts.

**Generation of MLC2vCre*+/+*; R26*βFP*+ mice.** MLC2vCre*+/+*; R26*βFP*+ mice have been described previously and were crossed with the R26*βFP*+ strain to generate MLC2v*−*; R26*βFP*+ mice for myocardial infarction and to exclude Wt1 upregulation in YFP+ cardiomyocytes.

**Adult epicardial explant cultures.** Adult EPDCs were prepared, as previously described, from 8–10-week-old Wt1*GFP*Cre*+/+* mice that had received daily i.p. injections of TGF (RegenesRx, 12 mg/kg−1 in PBS) or vehicle (PBS) for 7 days. Cells were allowed to differentiate for up to 14 days in Isco’s modified Dulbecco’s medium (IMDM) containing 20% FBS, before fixing in 4% paraformaldehyde for immunostaining analysis. At the outset GFP+ cells were recorded emerging from TGF-treated explants (66±4; mean percentage of GFP+ cells relative to total number of cells in outgrowth ± s.e.m.; n=12 explants). Outgrowing cells, up to 6 days in culture, were documented as immature and phenotypically similar to Nck2.5+ progenitors previously isolated from embryonic hearts (Supplementary Fig. 2a–d). Throughout the ex vivo studies, PBS (vehicle)-treated explants were used as controls and revealed limited outgrowth or emergence of a significantly reduced number of GFP+ progenitor-like cells. The percentage incidence of GFP+ progenitors in the PBS treated/control explant cultures was recorded at 14±1.9% (mean ± s.e.m.; n = 12), significantly lower (P = 0.001) than those primed by TGF. Importantly,vehicle-treated cells failed to adopt a myocardial fate and only isolated fibroblast-derivatives were observed in control cultures (not shown).

Despite evidence of sarcomeric marker expression (cTnT, Sα and MyBPC) described, from 8–10-week-old Wt1GFPCre+/+ mice have been previously described and were crossed with the R26βFP+ strain with Rosa26REYFP mice to generate Wt1GFPCre+/+; R26βFP+ mice for myocardial infarction and to exclude Wt1 upregulation in YFP+ cardiomyocytes.

**TGF administration.** The injection regimen of TGF at 4 for priming Wt1*GFP*Cre*+/+* and Wt1*CreERT2*+/−, R26*βFP*+ mice, including induction with tamoxifen is outlined in Fig. 1a. In separate experiments, MLC2vCre; R26*βFP*+ and wild-type C57BL/6J mice were subject to the same injection regimen for epicardial priming. Adult mice received i.p. injection of TGF (12 mg kg−1) or vehicle (PBS) daily for 7 days. On the eighth day Wt1*CreERT2*+/−, R26*βFP*+ mice were injected with tamoxifen (2 mg suspended in peanut oil; i.p.) to induce CreERT2/Cre expression. Further injections of TGF/vehicle were given on day 9 and tamoxifen on day 10. Myocardial infarction was performed 3 days after this regimen.

**Myocardial infarction.** Mice were housed and maintained in a controlled environment. All surgical and pharmacological procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986, (Home Office, UK). For all experiments adult male Wt1GFPCre+/+ and Wt1CreERT2+/−, R26βFP+/+ mice, with fold-change expression of cardiac specific genes as described previously, were used. For each experiment (n = 8), both groups were divided into 3. Each group received either endogenous control (sham) treatment or were given tamoxifen to induce CreERT2/Cre expression. Hearts were harvested at 2, 4, 7 and 14 days after ligation and bisected transversely midway through the scar: the apex was snap frozen for RNA isolation and subsequent real-time qRT–PCR studies while the remaining tissue was fixed in 4% PFA for cryosectioning and immunostaining analyses.

**GFP+ YFP+ cell isolation and characterization.** Hearts from Wt1*GFP*Cre*+/+* and Wt1*CreERT2*+/−, R26βFP+/+ mice treated with TGF or vehicle were harvested 7 days after ligation and processed by enzymatic digestion using a 0.1% collagenase II–PBS solution (Worthington Biochemicals) to achieve a single-cell suspension. GFP+ or YFP+ cells were isolated from the total cardiac cell population using a Beckman Coulter MoFlo XDP cell sorter. All experiments were performed in the 530/40 nm channel and a 355 nm laser beam was used to excite GFP (collected in the 585/30 nm channel) and YFP (collected in the 530/40 nm channel).

To characterize GFP䟪cells, hearts from Wt1*GFP*Cre*+/+* mice treated with TGF were harvested 4 days after ligation and processed, as described earlier, to obtain a single-cell suspension. As a control, hearts from uninjured Wt1*GFP*Cre*+/+* mice treated either with TGF or with vehicle were also analysed after 7 days. Cells were incubated with the following primary and secondary antibodies: c-Ki (goat IgG, R&D System), Sca-1 (rat IgG2a, BD Pharmigen), Alexa Fluor 647 anti-goat or Alexa Fluor 647 anti-rat (Invitrogen) and analysed using a Beckman Coulter CyAn ADP analyser equipped with a 488 nm laser and 633 nm red diode and run by Summit Software. Data were analysed using the FlowJo Software.

Cell cytopsins were collected using a Shandon Cytospin 3 centrifuge. Cytopsin cells were then processed for immunostaining for epicardial and cardiovascular markers as described earlier.

YFP+ cardiomyocytes were assessed by cell counts through serial sections. The mean percentage of YFP+ progenitors that became cardiomyocytes was estimated across n = 7 hearts ± s.e.m. The incidence of YFP+ de novo cardiomyocytes relative to pre-existing myocardium was expressed as the mean ratio of YFP+ cells divided by YFP+ cells across serial sections per heart ± s.e.m. (n = 6 hearts analysed). Cell counts were also assessed after two-photon imaging in Langendorff-perfused hearts and expressed as the mean number of YFP+ cardiomyocytes per heart ± s.e.m. (n = 4 hearts analysed).

Immunofluorescence was performed on adult epicardial explant cultures, on cytopsin FACS-sorted cells and on cryosections of post-myocardial infarction hearts using standard protocols with the following antibodies: GFP (Clontech and Abcam, which also detect EYFP), Sα (Sigma), cardiac MyBPC (a gift from E. Ehler and M. Gautel), cTnT, cTNI, BrdU, Isl1 and Ki67 (Dako). To rule out the possibility of auto-fluorescence accounting for the detection of either GFP or YFP protein expression, sections through the left ventricle were stained with a polyclonal anti-GFP antibody (which detects both fluorescent proteins). The specificity of the anti-GFP antibody was ascertained by immunofluorescence on non-primed, intact hearts, which detected neither labelled cells in the epicardial region, nor their derivatives (no signal; not shown).

To detect BrdU-positive nuclei, sections were treated with 2 N HCl for 30 min at room temperature (22 °C) to denature the DNA, and neutralized in 0.1 M sodium borate pH 8.5 for 12 min before incubation with the anti-BrdU antibody. Owing to the destruction of cellular antigens resulting from acid treatment, these steps were performed after the incubation with antibodies to GFP and Sα. Images were acquired using either a Zeiss AxioImager with ApoTome or a Zeiss LSM 710 confocal microscope equipped with argon and helium neon lasers using ×20, ×40 and ×63/1.4 (oil immersion) objectives.

**RNA in situ hybridization.** RNA in situ hybridization on adult heart cryosections was performed as previously described, using a digoxigenin-labelled antisense riboprobe specific for Wt1 (ref. 4), alongside a sense control.

**RNA isolation and gene expression profiling.** Total RNA was isolated from the apex of collected hearts using the Trizol reagent (Invitrogen), according to the manufacturer’s instructions and reverse-transcribed using Superscript III RT (Invitrogen). Real-time qRT–PCR analysis was performed on an ABI 7900 Sequence Detector (Applied Biosystems) using SYBR Green (QuantitectTM SYBR Green PCR Kit, Qiagen). Data were normalized to Hprt expression (endogenous control). Gene expression was determined by the 2−ΔΔCt method and are presented relative to levels in non-myocardial infarction (sham) hearts. Complementary DNA PCR primer sequences were obtained from Primer Bank (http://pga.mgh.harvard.edu/primerbank/) and details are available on request.

To characterize GFP+ progenitors, total RNA was obtained from FACS-sorted GFP+ cells isolated after enzymatic digestion of the hearts of the TGF-treated Wt1*GFP*Cre*+/+* mice at 4 and 14 days following LAD ligation using a Beckman Coulter MoFlo XDP cell sorter. Total RNA was isolated using the RNeasy Micro Kit (Qiagen), according to the manufacturer’s instructions, and processed as above.

**Multiphoton imaging.** Multiphoton imaging was performed in Langendorff-perfused hearts loaded with Rhod-2/AM (Invitrogen) as described previously, except that 50 µM blebbistatin was used to inhibit myosin crossbridge cycling and prevent movement. Spontaneous calcium transients were visualized without

**LETTER RESEARCH**

©2011 Macmillan Publishers Limited. All rights reserved.
correction: Emitted light was collected using a bandpass 500–550 nm and 575–640 nm. Calcium transients were calculated by averaging along a line 40 pixels wide using ImageJ (http://rsbweb.nih.gov/ij) software. The ImageJ fourier filter was used to remove noise of less than 3 pixels and three-dimensional images were constructed using Imaris (Bitplane).

**MRI analysis.** Wt1CoxERT2+/R26R::EYFP/+ and wild-type C57BL/6J mice treated with Tjβ4 or vehicle, were subjected to MRI assessment at 7 days after LAD ligation. Where infarct size was within the range of 15–40%, follow-up MRI analysis was performed on the same mice at 14 and 28 days post-myocardial infarction, to determine temporal changes in infarct size and cardiac function. Mice were anaesthetized with isoflurane (4%), placed onto an animal cradle and maintained at 37 ± 0.5 °C with oxygen and anaesthetics (1–2% isoflurane), supplied via a nose cone (1 L/min). Cardiorespiratory monitoring and gating were performed using an MR-compatible system (SA Instruments) with needle electrodes inserted into the front limbs and a respiratory pillow placed on the chest. Imaging was performed using a 9.4T VNMRS horizontal bore scanner (Varian) with a shielded gradient system (1,000 mT m⁻¹) using a 39 mm diameter volume coil (Rapid Biomedical GmbH). An electrocardiogram and respiratory gated spoiled gradient echo sequence was used to acquire cine cardiac images with the following parameters for standard cine acquisitions: Time to echo (TE), 1.18 ms; time to repetition (TR), 4.5 ms; flip angle, 20°; slice thickness, 1 mm; no slice separation; field of view (FOV), 25.6 × 25.6 mm²; matrix size, 128 × 128; number of signal averages (NSA), 2. Twenty cine frames were recorded to cover the cardiac cycle. Infarct size was assessed using late gadolinium enhancement (LGE), as previously described21. Briefly, 0.6 mmol kg⁻¹ Gd-DTPA was administered i.p. followed by a Look-Locker acquisition with multiple time inversion (TI) to determine the optimum TI. This was followed by a multi-slice inversion recovery (IR) acquisition with flip angle (FA) = 90° using the following imaging parameters: TE, 1.58 ms; TR, ~500–600 ms; FA, 90°; slice thickness, 0.5 mm; 0.5 mm slice gap; 7–8 slices; FOV, 25.6 × 25.6 mm²; matrix size, 192 × 192; NSA, 2. A second stack of short-axis images offset by 0.5 mm was acquired to generate a continuous data set.

**MR image analysis.** Randomized and anonymized images were analysed using the cardiac analysis software Segment (http://segment.heidelberg.se). To estimate the infarct size, endocardial and epicardial borders were segmented on LGE images automatically with manual adjustments followed by automatic delineation of infarct tissue using a built-in fraction of segment. Manual corrections were performed when necessary. Infarct size, expressed as percentage of left ventricular mass, was calculated as infarct volume/left ventricular volume (from cine data). Results are shown as mean ± s.e.m. Comparisons between groups were performed using a repeated measures one-way ANOVA. All statistical analysis was performed using R software version 2.8.1.

**Cell transplantation.** Adult male (n = 14) and female (n = 14) Wt1GFPCreERT2 mice were primed with Tjβ4 and myocardial infarction was induced as described earlier. Pooled GFP⁺ progenitors were isolated (from eight surviving donors) using a Beckman Coulter MoFlo XDP cell sorter after enzymatic digestion of the hearts of Tjβ4-treated WT1GFPCreERT2 mice 4 days after ligation. Female (n = 3) and male (n = 3) non-transgenic mice were treated with Tjβ4 before surgery and myocardial infarction was induced as described above. 3–6 × 10⁶ FACS-sorted male GFP⁺ cells, resuspended in 10 μl of DMEM, were injected into the subepicardial space of the female host hearts (n = 3) immediately after LAD ligation. On recovery, animals received i.p. injection of Tjβ4 (12 mg kg⁻¹). Further injections were given every second day and hearts were harvested 14 days post-myocardial infarction and processed for immunofluorescence analysis as described earlier. In the second set of experiments, 5 × 10⁶ FACS-sorted female GFP⁺ cells, resuspended in 10 μl of DMEM, were injected into the subepicardial space of the male host hearts (n = 3) immediately after LAD ligation. On recovery, animals received i.p. injection of Tjβ4 (12 mg kg⁻¹). Further injections were given every second day and hearts were harvested at 24 h, 7 days and 14 days post-myocardial infarction and processed for immunofluorescence analysis.

**FISH.** After immunofluorescence analysis of EPDC-derived cardiomyocytes in adult heart cryosections, as described earlier, images were acquired before FISH. FISH was performed using mouse StarFISH probes (Cambio), essentially according to the manufacturer’s instructions, with the following modifications: muscle FISH was performed using mouse StarFISH probes (Cambio), essentially according to the manufacturer’s instructions, with the following modifications: muscle Cryochem. Cytochem. 49, 1–8 (2001).


