Myc overexpression enhances epicardial contribution to the developing heart and promotes extensive expansion of the cardiomyocyte population

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Myc is an essential regulator of cell growth and proliferation. Myc overexpression promotes the homeostatic expansion of cardiomyocyte populations by cell competition, however whether this applies to other cardiac lineages remains unknown. The epicardium contributes signals and cells to the developing and adult injured heart and exploring strategies for modulating its activity is of great interest. Using inducible genetic mosaics, we overexpressed Myc in the epicardium and determined the differential expansion of Myc-overexpressing cells with respect to their wild type counterparts. Myc-overexpressing cells overcolonized all epicardial-derived lineages and showed increased ability to invade the myocardium and populate the vasculature. We also found massive colonization of the myocardium by Wt1Cre-derived Myc-overexpressing cells, with preservation of cardiac development. Detailed analyses showed that this contribution is unlikely to derive from Cre activity in early cardiomyocytes but does not either derive from established epicardial cells, suggesting that early precursors expressing Wt1Cre originate the recombined cardiomyocytes. Myc overexpression does not modify the initial distribution of Wt1Cre-recombined cardiomyocytes, indicating that it does not stimulate the incorporation of early expressing Wt1Cre lineages to the myocardium, but differentially expands this initial population. We propose that strategies using epicardial lineages for heart repair may benefit from promoting cell competitive ability.

The epicardium is the mesothelium-derived outer layer of the heart. Epicardial cells derive from the proepicardium -a group of cells that bulges from the dorsal pericardial wall-, and are transferred to the heart surface at midgestation (E9.5–10.5 in the mouse)1-2. Initially epicardial cells conform a squamous single-layered epithelium completely covering the cardiac surface of the heart. From E11, epicardial cells undergo epithelial-mesenchymal transition (EMT) invading and colonizing the subepicardial space and the myocardium3-5. Epicardial-derived cells (EPDCs) contribute extensively to the myocardial connective tissues, extensively to smooth muscle and mesenchyme of the coronary vasculature and less so to the endothelium6-10. In the mouse, but not in other vertebrates, the epicardium has also been reported to contribute to the cardiomyocyte lineage, however controversy remains as to whether these findings derive from undesired recombination of the epicardial Cre lines or represent true contributions8,10-12.

The Wilms’ tumor gene Wt1 is dynamically expressed in the coelomic epithelium as well as in coelomic epithelium-derived cells in many organs, including the epicardium, therefore several studies have used Wt1 as a lineage marker and tracer for the coelomic and coelomic-derived cells10,13-18. Wt1 expression has also been reported in adult19 and embryonic endothelial and endocardial cells12,20. Wt1 codes for a zinc-finger transcription factor that is involved in cell proliferation and the control of cell cycle progression, and has been implicated in several developmental processes such as nephron formation, gonadogenesis, and cardiovascular development21-23. The role of Wt1 in cardiac development has been extensively studied in mouse models of congenital heart defects24,25. Wt1 expression is essential for the development of the cardiac outflow tract and the formation of the atrioventricular canal26,27. Wt1 expression is also required for the development of the cardiac conduction system28,29. Wt1 expression is strongly induced in response to hypoxia, which is a key signaling pathway regulating cardiac development24,26,28,29.

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factor which has been involved in many normal and pathological processes\textsuperscript{21,22}. The postnatal epicardium is normally quiescent, however it shows cellular and signaling activation upon injury in the fish and the mouse, contributing cells and signals that could be relevant in cardiac repair processes\textsuperscript{21–25}.

Cell competition is a tissue homeostasis mechanism by which low-anabolizing -but otherwise viable- cells are eliminated from tissues due to confrontation with higher-anabolizing cells\textsuperscript{26–28}. Increasing anabolism by moderate Myc overexpression in a mosaic fashion leads to cardiomyocyte competition during cardiac development and adult myocardium homeostasis\textsuperscript{29}. Cell competition leads to the homeostatic replacement of wild type cardiomyocytes by the Myc-enhanced cardiomyocytes without producing any cardiac anatomical or functional alteration\textsuperscript{29}.

Here we studied whether cell competition modifies the myocardial colonization pattern of EPDCs, determining the preferential expansion of Myc-enhanced epicardial cells in the niches usually colonized by EPDCs and in the cardiomyocyte lineage.

**Results**

**Myc-overexpression in the WT1-Cre lineage promotes the extensive colonization of the myocardium during cardiac development.** To study whether increased Myc levels modify the behavior and contribution of the epicardial cell lineages, we used the Wt1Cre driver\textsuperscript{26} to induce recombination of the iMOS\textsuperscript{WT} and iMOS\textsuperscript{T1-Myc} alleles. The iMOS alleles produce an initial 3:1 EYPF:ECFP mosaic\textsuperscript{28}. The iMOS\textsuperscript{WT} allele only expresses the fluorescent reporters and is used as control. The iMOS\textsuperscript{T1-Myc} allele is similar but overexpresses Myc in the EYPF cells. We analyzed the contribution of the EYPF population to the myocardium in E14.5 hearts.

In histological sections, we found that EYPF-Myc cells in iMOS\textsuperscript{T1-Myc} hearts colonized a larger area than that colonized by their EYPF-wild type equivalent population in the iMOS\textsuperscript{WT} mice (Fig. 1A–B, E–G). These results were confirmed by confocal microscopy (Fig. 1H). The greater contribution of the EYPF-Myc population was exacerbated at P0 (Fig. 1C–D), indicating that the EYPF-Myc cells continued their differential expansion during all cardiac prenatal development. The overcolonization by Myc-overexpressing cells did not induce any change in heart morphology or embryonic development in general.

We noticed that the expansion of the EYPF-Myc population involved the appearance of large patches of EYPF cardiomyocytes that were not observed in the control mosaics (Fig. 1A–D). To further characterize this phenomenon we determined the differential enrichment of the EYPF population within the Wt1Cre-recombined ventricular cardiomyocyte compartment and in the non-cardiomyocyte compartment. We found that the EYPF population was moderately expanded in the non-cardiomyocyte compartment of E14.5 iMOS\textsuperscript{WT} hearts in comparison with iMOS\textsuperscript{WT} hearts (Fig. 2A–B, C–E). In the cardiomyocyte compartment, we observed a non-significant expansion of the EYPF-Myc population over that observed in the control mosaics in the inter-ventricular septum (Fig. 2D). The IVS was the only region in which we could detect Wt1Cre-recombined cardiomyocytes in control mosaics (Not shown). In iMOS\textsuperscript{T1-Myc} mosaics, however, we found extensive presence of iMOS\textsuperscript{WT} cardiomyocytes in the RV and LV free walls, which were exclusively EYPF (Fig. 2C, E). These results suggested that most of the observed over-colonization of the ventricular myocardium is due to the appearance of EYPF cardiomyocytes in the free walls of the ventricles. This population is strictly dependent on the presence of Myc overexpression, as it was not observed in the control mosaic and did not include the ECFP population in the iMOS\textsuperscript{T1-Myc} hearts. To determine the relevance of differential proliferation in the colonization of the ventricles by EYPF-Myc cardiomyocytes, we analyzed the frequency of Ph3\textsuperscript{+} cardiomyocytes in the EYPF\textsuperscript{+} and EYPF populations of the Wt1Cre-recombined iMOS\textsuperscript{T1-Myc} mosaics. We detected higher proliferative activity of the EYPF-MYC cardiomyocytes, in agreement with the observed over-colonization of the myocardium by this population (Fig. 2I).

**Myc-overexpression in the WT1-Cre lineage increases epicardial cell migration and invasiveness, and promotes the progressive colonization of EPDC compartments.** We then studied whether the expansion of EYPF-Myc cells resulted in an overall increase in the number of EPDCs. For this, we established primary cultures from the LV at E14.5 and determined EYPF frequency in the cardiomyocyte and non-cardiomyocyte compartments (Fig. 2E, G). We found a moderate and non-significant increase of the overall abundance of EYPF cells within the non-cardiomyocyte compartment (Fig. 2H). In contrast, the cardiomyocyte compartment showed a ~10-fold increase in EYPF cell frequency (Fig. 2H). These results show that while EYPF-Myc cells expand at the expense of ECFP cells within the EPDC compartment, they do not increase the overall contribution of EPDCs to the myocardium. In contrast, Myc overexpression modifies the contribution of the Wt1Cre lineage in the ventricles to extensively colonize the cardiomyocyte compartment.

To determine the sequence of enrichment in Myc cells during the development of the epicardial lineage, we analyzed the contribution of the EYPF population to the total iMOS\textsuperscript{WT} population in the different regions colonized by this lineage. We found that the epicardium, the subepicardial space and the myocardium were all enriched in EYPF-Myc cells (Fig. 3A–C). To confirm these results we studied primary epicardial cultures from E10.5 ventricle explants and found that the EYPF compartment colonized close to 100% of these cultures (Fig. 3D–F). These results indicate the ability of Myc to expand epicardial cells at the expense of wild type cells both in vitro and in vivo from the earliest stages of epicardial development. We also observed that in vivo the enrichment in the sub-epicardial space was increased with respect to that observed in the epicardium, suggesting a higher invasive capacity of Myc-overexpressing cells. To investigate this behavior, we established a co-culture assay, exposing an explanted epicardium-less E9 WT ventricle to an E10.5 iMOS\textsuperscript{WT} ventricle covered by epicardium (Fig. 3G–J). In these assays, the epicardial cells migrated out from the “donor” ventricle and eventually reached the “receptor” ventricle (Fig. 3G–J). We performed these experiments using either Wt1Cre-recombined iMOS\textsuperscript{T1-Myc} or iMOS\textsuperscript{WT} donors and observed higher migratory activity of the EYPF-Myc cells and a capacity to invade the receptor myocardium that was not observed for the EYPF-wild type cells (Fig. 3G–K).

We finally studied the contribution of the EYPF cells to the coronary vessels in the postnatal heart (P0) (Fig. 3L–O). At this stage we found variable contribution of iMOS\textsuperscript{WT}-EYPF\textsuperscript{+} cells to the vessel layers, with limited
contribution to the intima (endothelium), strong contribution to the media (smooth muscle) and intermediate to the adventitia (fibroblasts) (Fig. 3L,M,P). The contribution was within a similar range for arteries and veins (Fig. 3P). We then examined the contribution of EYFP+ in iMOST1-Myc mosaics (Fig. 3P) and found no increase in the contribution to the intima, non-significant increase for the adventitia and venous media; however, we found a very significant increase in the contribution to the arterial media. Similarly, we found a very important enrichment in EYFP-MYC endothelial cells in the microvasculature (Fig. 3P). We then studied whether the EYFP-Myc population had displaced the EYFP-CFP population in the WT1Cre-derived large vessel cell populations and endothelium of the microvasculature. We determined the enrichment in EYFP+ cells within the iMOST1-Myc-derived cells (Fig. 3N,O) and found that the enrichment in all large vessel layers and endothelium of the microvasculature was close to 100% (Fig. 3Q) and clearly above that observed for the epicardium and

Figure 1. Enhanced myocardial contribution by Myc-overexpressing cells. (A–B’) Confocal images from histological sections of iMOSWT (WT) (A) and iMOSWT-Myc (MYC) (B) hearts at E14.5 induced with Wt1-Cre. (A’-B’) show magnification of the boxed areas shown in (A,B). (C-D’) Confocal images from histological sections of P0 iMOSWT (WT) (C) and iMOSWT-Myc (MYC) (D) recombined with Wt1-Cre. (C’,D’) show magnification of the boxed areas shown in (C,D). (E–G) Quantification of the percentage of recombined area detected in iMOSWT (WT) and iMOSWT-Myc (MYC) at E14.5 and P0 in the RV (E), IVS (F) and LV (G). (H) Cytometer histogram plot showing Counts (X axis) versus FITC laser (EYFP detection) in iMOSWT (WT) and iMOSWT-Myc (MYC) E14.5 whole digested hearts (upper panel). Lower panel shows quantification on EYFP population related to the total amount of live cells detected on the cytometer for iMOSWT (WT) and iMOSWT-Myc (MYC) E14.5 hearts. Graphs in E, F, G, H show means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Bar, 200 μm in A, B, C, D and 50 in A’, B’, C’, D’ n ≥ 5 hearts for (A-G) and n ≥ 7 for (H).
These results indicate that Myc promotes the progressive expansion of the epicardial lineages starting at the colonization of heart surface and continuously during the colonization of the myocardium and large vessels. This overcolonization takes place mostly at the expense of the WT epicardium-derived compartment, as only mild expansion of the overall WT1Cre lineage was observed for the media and the intima, with the notable exception of the arterial smooth muscle layer and the endothelium of the microvasculature, in which Myc cells expanded further and replaced the non-EPDC compartment.

Wt1Cre-derived cardiomyocytes likely derive from early extra myocardial Wt1Cre-expressing cells and not from established epicardium. To investigate the origin of EYFP cardiomyocytes in the Wt1Cre-recombined iMOSWT (WT) hearts, we induced recombination using a tamoxifen (TM)-inducible Cre driver (WT1CreERT2). This line has been reported before to induce limited recombination of the epicardium and to contribute to the cardiomyocyte lineage in one study but not in another. Here we optimized the tamoxifen administration protocol to obtain around 50% recombination of the E14.5 epicardium. We administered TM at E9.5, which produced epicardial iMOS recombination at E10–10.5 (not shown), however contribution of iMOS cells to cardiomyocytes at E14.5 was neither observed in the control, nor in the Myc-expressing mosaics, even though EPDCs were detected (Fig. 4A,B'). These results suggested that Wt1Cre-derived cardiomyocytes do not derive from the epicardium and are in agreement with the absence of cardiomyocyte differentiation from Wt1Cre-recombined iMOSWT newborn hearts. Frequencies were normalized to that observed in the EYFP cardiomyocyte population. Graphs in (C–E,H,I) show means ±SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Bar, 200μm in A, B, and 50 in (A',B',A'',B'') n ≥ 5 hearts.
(Fig. 4C,D'), indicating that Myc overexpression does not enhance differentiation to cardiomyocytes in this assay (Fig. 4E).

We then investigated other possible origins of the Wt1Cre-labeled cardiomyocytes. Since cardiomyocytes are observed in the IVS of the Wt1Cre-recombined iMOSWT hearts, we considered the possibility that cell competition could stimulate the colonization of the ventricular free walls by expanding the original septal cardiomyocyte population. To explore this possibility, we recombined iMOSWT and iMOST1-Myc hearts with the AHFCre line, which produces recombination in the RV and IVS. Although we could observe local expansion of the EYFP-Myc population in E14.5 hearts, we did not detect colonization of the LV free wall as previously detected when using the Wt1Cre line (Fig. 4F,G), which discards septal cardiomyocytes as the origin of the LV free wall contribution of the Wt1Cre lineage.

We observed that the Wt1Cre line also recombined some hematopoietic cells and endocardial cells from early stages of cardiac development (Fig. 4H,H'). To explore whether these lineages could generate the Wt1Cre-recombined iMOSWT-Myc cardiomyocytes, we used the Tie2Cre line, which recombines all endothelial and...
blood lineages in the mouse embryo. We did not observe any contribution of the Tie2Cre lineage to the cardiomyocytes in E14.5 iMOST1-Myc hearts (Fig. 4H, H'), which discarded blood or endothelial precursors as the origin of Wt1Cre-derived cardiomyocytes.

We then studied the timing and site of appearance of Wt1Cre-recombined cardiomyocytes in iMOSWT and iMOSiOST-Myc hearts. We did not detect any iMOS+ cardiomyocytes before E9 (data not shown, N = 5), which discards recombination in early cardiac mesodermal precursors. The first EYFP cardiomyocytes were detected in both iMOSWT and iMOSiOST-Myc hearts between E9 and E9.5 before colonization by epicardial cells (Fig. 5A, B'). At this stage, the frequency of hearts containing EYFP cardiomyocytes was similar in iMOSWT and iMOSiOST-Myc hearts and the localization of EYFP cardiomyocytes in different regions of the developing heart is very variable but similar in control and Myc mosaics (Fig. 5C). These results show that recombination of the cardiomyocyte lineage is induced by Wt1Cre in a scattered and non-stereotyped manner in both iMOSWT and iMOSiOST-Myc hearts and that Myc overexpression does not affect the initial pattern of recombined cardiomyocytes. In addition, these results show that the recombined cardiomyocytes should derive from Wt1Cre-expressing cells before the establishment of the epicardium. To confirm this possibility, we induced Cre activity in the Wt1CreERT2 line by TM injection at E8.0 and observed scattered recombined cardiomyocytes at E9.5 (Fig. 5D), contrary to what was observed following induction at later stages, in which no recombined cardiomyocytes were detected (Fig. 4A, B').

We then tried to identify the original cells in which the recombination was produced and specifically whether cardiomyocytes undergo Cre recombination in situ. The fact that two different Cre lines driven by Wt1 regulatory sequences led to early cardiomyocyte recombination suggested that this activity could correspond to endogenous Wt1 expression. To explore this possibility, we studied in detail Wt1 protein expression in early cardiac formation, with a special emphasis on the stages in which Wt1Cre recombination was first observed (Fig. 6). The first Wt1+ cells were observed around E9 in the pericardial and proepicardial regions (Fig. 6B, B' and data not shown), however, we did not detect any Wt1-positive cardiomyocyte between E8 and E14.5 (Fig. 6A, B' and data not shown). To determine whether, independently of Wt1 expression, the Wt1Cre and Wt1CreERT2 lines used here produced Cre protein expression directly in cardiomyocytes, we performed antibody staining of Cre and CreERT2 proteins (Fig. 6C–F'). While Cre and CreERT2 could be clearly detected in the epicardium and pro-epicardium, both...
proteins were completely absent from cardiomyocytes between E8 and E10 (Fig. 6C–F’). Altogether our results suggest that the observed recombined cardiomyocytes derive from extra-myocardial WT1Cre-expressing cells before the establishment of the epicardium.

Discussion

We reported here the ability of Myc moderate overexpression to provoke homeostatic cell population shifts during the development of the epicardial lineage. Myc-induced preferential expansion took place from the first stages of epicardial development, being observable in primary cultures of E10.5-explanted epicardial cells, and further increased at various stages of epicardium and epicardial-derived cells development, including the myocardium invasion and the colonization of the vasculature. The tissues colonized by EPDCs have multiple origins, with EPDCs contributing to only a fraction of these tissues. Interestingly, in most cases we observed that the expansion of EYFP-MYC cells occurred within the EPDC compartment of the colonized tissues, without displacing cells from other sources than EPDCs. Exceptions to these observations were the microvasculature endothelium and the arterial smooth muscle, where we observed clear overexpansion of the EPDC compartment with displacement of cells from other origins. Interestingly arterial coronary smooth muscle, but not venous coronary smooth muscle receives a contribution from the neural crest31, suggesting MYC-stimulated EPDCs may be able to displace neural crest smooth muscle precursors but not those of different origins. Similarly, large part of the arterial coronary endothelium is produced by budding of well-established endothelial sacs from the endocardium32, while the microvasculature is produced by other vasculogenic/angiogenic mechanisms. These differences in the mechanisms for the generation of endothelial populations may then underlie the different colonizing activity of Myc-stimulated EPDCs.

In part, the expansion of EYFP-MYC cells could take place by cell competition, however Myc-induced cell competition in mouse development involves close interactions between cells that are expected to take place in established tissues, like fully-colonized epicardium or vascular layers. In the case of epicardium-EPDC development, a very important additional step could be involved in the enrichment; cell migratory and invasive ability. In fact, we observed that Myc cells showed enhanced migratory and myocardium-invasive ability in explant co-culture assays and the complete colonization of the vasculature by Myc-overexpressing cells may involve an enhanced migratory ability. These observations suggest that stimulating epicardial cell
activity through homeostatic Myc overexpression may enhance the potential reparative ability of epicardium and epicardial-derived cells. In addition, we observed widespread colonization of the ventricular free walls by \textit{Wt1Cre}\textsuperscript{-}\textit{derived cardiomyocytes in Myc mosaics but not in control mosaics. However, a detailed study in cultured explants failed to show any ability of Myc to stimulate cardiomyocyte differentiation. Furthermore, \textit{Wt1CreERT2}\textsuperscript{-recombined epicardial cells failed to contribute to the cardiomyocyte lineage, even when Myc was overexpressed. Given the ability of Myc to expand cardiomyocyte populations in the developing myocardium, even very minor initial contributions from the epicardial lineage to cardiomyocytes would have been expanded and easily detected. Our results thus strongly support the inability of \textit{Wt1}\textsuperscript{+} epicardial cells to contribute to the cardiomyocyte lineage during development\cite{12}, consistent with observations in other vertebrate models\cite{33}. Based on our Cre and CreERT2 expression analysis, we suggest that Cre activity in \textit{Wt1Cre}-expressing precursors before epicardium adhesion to the ventricles could be the origin of cardiomyocyte lineage colonization, however we cannot completely exclude
in situ cardiomyocyte recombination driven by undetectable levels of Cre protein expressed in cardiomyocytes. Our study of the Wt1Cre and Wt1CreERT2 lines thus suggests the exclusive use of the latter when aiming for targeting Wt1 epicardial-derived lineages, with the caveat that also endocardial/endothelial cells express Wt1 and undergo Cre-recombination in the WT1CreERT2 line\(^1\). Surprisingly, the initial pattern of Wt1Cre recombination in cardiomyocytes is similar in control and Myc mosaics, however, at E14.5, cardiomyocytes in the free ventricular walls are only detectable in the Myc mosaics, while labeled cardiomyocytes in the IVS are observed in both. This pattern of colonization, and the in vitro studies reported here, indicate that Myc does not regulate the incorporation of Wt1Cre lineages to the cardiomyocyte pool, but differentially expands this initial population. Interestingly, the epicardium is essential for proper IVS formation and has an important cellular contribution to the IVS in different species\(^3\), however contribution to the IVS cardiomyocytes has not been observed in Quail-Chick transplantation experiments\(^4\).

The overcolonization of the ventricles by EYFP-MYC cardiomyocytes in Myc mosaics continues progressing until birth with up to 50% colonization (in some specimens) of the left ventricle free wall cardiomyocytes, while it remains barely detectable in control mosaics. Interestingly, the initial recombination pattern seems to affect more the derivatives of the first heart field (FHF) (See Fig. 1A), however at later stages, colonization of the RV progresses to reach the levels observed in the LV. Very likely this effect is related to the observation that Wt1Cre-promoted cardiomyocyte recombination takes place mostly during early cardiac stages, when only FHF cardiomyocytes are in place. In fact, using the AhHRCre line, we observed expansion of second heart field (SHF) derivatives into the FHF-derived myocardium (Fig. 4G), indicating that Myc enhancement confers cardiomyocytes the ability to violate the boundary between FHF and SHF.

Myc-driven phenotypically silent cell competition in cardiomyocytes has been previously reported, however the results reported here highlight the extraordinary ability of cell competition to stimulate small cardiomyocyte populations to colonize extensive areas of the myocardium. Our results suggest that cell competition stimulation could be useful for enhancing epicardial activation and cellular contributions to the myocardium. In addition, they highlight an extraordinary capacity of cell competition to expand scarce cardiomyocyte populations without altering cardiac anatomy.

Methods

Mouse Strains. All experiments were performed using mice (Mus musculus) of a mixed background that were maintained and handled according to the recommendations of the CNIC Ethics Committee, the Spanish laws and the EU Directive 2010/63/EU for the use of animals in research. The experimental protocols involving animals were approved by the CNIC and Universidad Autónoma de Madrid Committees for “Ética y Bienestar Animal” and the area of “Protección Animal” of the Comunidad de Madrid with reference PROEX 220/15.

iMOS Mouse lines have been previously described\(^2\). Homozygous iMOS females were mated with males carrying different Cre lines; Wt1Cre\(^9\), Mef2C-AHFCre\(^3\), Tie2Cre\(^8\) and Wt1Cre ERT2\(^10\) to generate embryos or pups. Mice were genotyped by PCR. To induce recombination in iMOS;Wt1CreERT2 mice, tamoxifen was administered by oral gavage (4 mg/mL). The Wt1GFP knockin line\(^2\), in which the exon 1 of one Wt1 allele has been replaced by the GFP sequence, was also used as an independent, reporter for active Wt1 transcription.

Embryonic epicardial explants. We followed previously described methods\(^3\). Briefly, to isolate epicardial cells from E10.5 and E11.5 embryos, hearts were dissected and the ventricles were each cut into two pieces. Each piece was placed with the epicardial outermost part facing down onto a gelatin covered MatTek Glass bottom Dish (0.1%Gelatin in PBS). These myocardial pieces were cultured in DMEM containing 10% FBS and 1% Penicillium streptomycin. After 24 to 48 hours, epicardial cells had migrated from the explant and form a monolayer and the myocardial explant was removed using forceps. Epicardial cells were left to grow for 5 days, at which point they were visualized by confocal microscopy and/or fixed for immunostaining in 2%PFA overnight at 4 °C. Coexpression assays were performed in a similar way but adding a E9.0 heart in the dish close to the epicardial explant without contacting it.

Embryonic proepicardial explants. This method is extensively described in\(^3\). Embryos for proepicardium explants were harvested at E9.0. The proepicardium was identified just under the heart as a “grape-like” clustering of cells. Heart tube was removed and discarded and proepicardial cells were removed. Proepicardial explants were cultured in DMEM 10% FBS 1% Penicillin-Streptomycin for 48 h on gelatin coated MatTek Glass bottom dishes.

Confocal microscopy. Histological sections and epicardial explants were imaged with a Nikon A1R confocal microscope using 405, 458, 488, 568 and 633 nm wavelengths and 20x/0.75 dry and 40/1.30 oil objectives. Areas occupied by EYFP and ECFP cells and EYFP and ECFP cell number were quantified using the threshold detection and particle analysis tools of the Image J, (NIH, http://rsb.info.nih.gov/ij). To calculate the relative frequency of ECFP cells the percentage of ECFP cells in each embryo was divided by the average percentage in iMOS\(^1,2\) mosaic. ECFP was scored by subtracting the information of EYFP from the anti-GFP staining which detects both fluorescent proteins.

Immunofluorescence. Embryos were obtained at different gestational stages, fixed overnight at 4 °C in 2% paraformaldehyde in PBS, gelatin embedded and cryosectioned. Embryonic epicardial and proepicardial explants were fixed in 2% PFA and whole stained. Primary antibodies used were Wt1 (ab89901 abcam), Living colors Rabbit polyclonal anti GFP antibody (Clontech), c-tnT (MS-295 Thermo Scientific), PECAM-1 (553370 BD Pharmingen™ clone MEC 13.3), SM22a (ab14106 abcam), SMA (C6198 Sigma), MF20 (DSHB), PH3 (06-570 Millipore), Cre (69050 Millipore) and ER (ab27595 abcam). For coronary vessel characterization, intima was detected with PECAM-1, media with SM22a and adventitia cells were identified by their mesenchymal...
appearance and their disposition surrounding the media. Veins and arteries were identified by their position within the ventricle (veins more superficial and arteries more internal) and by the thickness and shape of the cells of the media, which are thin and fusiform in veins and thick and cuboidal in arteries. Identification with specific antibodies was not possible due to the need to detect 4 different channels in our experiments. In experiments in which both ECFP+ and EYFP+ positive cells were characterized, we found that ECFP was difficult to detect after section processing. We therefore detected all FP's with anti-GFP immunofluorescence and EYFP through its native fluorescence. Cells positive for anti-GFP and negative for EYFP were considered ECFP+.

**Flow cytometer.** Isolated cardiac cells from embryonic hearts were analyzed by flow cytometry to quantify EYFP population (ECFP was undetectable by flow cytometry). Propidium Iodide was added to the cells to assess viability (1:5000). An LSR Fortessa 4L Flow Cytometer was used for the analysis (Laser wavelengths 488, 640, 405, 561). For the analysis, FACS DIVA and FlowJo softwares were used.

**Statistical analysis.** To compare average percentages of ECFP cells between more than two groups, the Kruskal-Wallis test was used (assuming non-normal distributions). For comparisons of two groups a Man-Whitney U-test was used. All comparisons were made using Prism statistical software.

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Author Contributions

Designed the studies: C.V.d.C., G.L. and M.T. Undertook the experimental work: C.V.d.C., R.C., G.L., R.S. and C.C. Analysed the data C.V.d.C., G.L. and M.T. Contributed to figure and manuscript preparation: C.V.d.C., G.L., R.C., R.M.C. and M.T.

Additional Information

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Erratum: Myc overexpression enhances epicardial contribution to the developing heart and promotes extensive expansion of the cardiomyocyte population

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The original version of this Article contained a typographical error in the title, where:

“Myc overexpression enhances of epicardial contribution to the developing heart and promotes extensive expansion of the cardiomyocyte population”.

Now reads:

“Myc overexpression enhances epicardial contribution to the developing heart and promotes extensive expansion of the cardiomyocyte population”.

This has now been corrected in the HTML and PDF versions of this Article.