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Islet1 derivatives in the heart are of both neural crest and second heart field origin

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Abstract

Rationale—*Islet1* (*Isl1*) has been proposed as a marker of cardiac progenitor cells derived from the second heart field and is utilized to identify and purify cardiac progenitors from murine and human specimens for ex vivo expansion. The use of *Isl1* as a specific second heart field marker is dependent on its exclusion from other cardiac lineages such as neural crest.

Objective—Determine if *Isl1* is expressed by cardiac neural crest.

Methods and Results—We used an intersectional fate-mapping system employing the *RC::FrePe* allele which reports dual Flpe and Cre recombination. Combining $Isl1^{Cre/+}$, a SHF driver, and *Wnt1::Flpe*, a neural crest driver, with *Rc::FrePe* reveals that some *Isl1* derivatives in the cardiac outflow tract derive from *Wnt1*-expressing neural crest progenitors. In contrast, no overlap was observed between *Wnt1*-derived neural crest and an alternative second heart field driver, *Mef2c-AHF-Cre*.

Conclusions—*Isl1* is not restricted to second heart field progenitors in the developing heart but also labels cardiac neural crest. The intersection of *Isl1* and *Wnt1* lineages within the heart provides a caveat to using *Isl1* as an exclusive second heart field cardiac progenitor marker and suggests that some *Isl1*-expressing progenitor cells derived from embryos, ES or iPS cultures may be of neural crest lineage.

Keywords

myocardial lineages; second heart field; neural crest; heart development

Introduction

The recent discovery of the second heart field (SHF) has redefined the understanding of mammalian cardiac development.[1] While cells of the first heart field (FHF) form the early

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cardiac tube, the SHF contributes additional cells to the maturing heart through mid-gestation, ultimately forming large portions of the right ventricle, outflow tract and atria.[2–4] SHF precursors have been characterized by expression of the homeobox gene *Islet1* (*Isl1*).[2] *Isl1* has been used to isolate putative cardiac progenitors from embryonic stem (ES) cell and induced pluripotent stem (iPS) cell cultures for the purpose of expansion and possible therapeutic application.[5–10] In vitro studies and in vivo fate-mapping experiments suggest that *Isl1* precursors are tri-potential and give rise to endothelium, smooth and cardiac muscle.[7, 9] Although SHF-specific enhancer elements from several genes have been identified by the analysis of transgenic mice,[3, 11] no other specific markers for SHF precursors have been reported, emphasizing the importance that *Isl1* has played as a specific marker of the SHF in cardiac and stem cell biology.

Surprisingly, inactivation of various factors in SHF using $Isl1^{Cre/+}$ has resulted in mice with congenital heart defects involving the outflow tract (OFT) and aortic arch arteries that are strikingly similar to abnormalities produced by gene manipulation using *Wnt1::Cre* or $Pax3^{Cre/+}$, which drive expression in cardiac neural crest.[12–14] This has led to the suggestion that SHF signals to neural crest via cell-cell interactions or secreted factors. However, an alternative hypothesis is that $Isl1^{Cre/+}$ is not restricted to SHF within the developing heart. To address if $Isl1^{Cre/+}$ may also be expressed in the *Wnt1* lineage of cardiac neural crest in addition to lineages of the SHF, we utilized a dual-fluorescent reporter allele that extends previously established recombinase-based intersectional strategies.[15–17] This dual fate-mapping approach employs a recently developed reporter mouse, *RC::FrePe* (Brust, R.D. and Dymecki, S.M., unpublished data and see ref. 18) that activates expression of enhanced green fluorescent protein (eGFP) within cells that have expressed both Flpe and Cre at any time and in any order within their lineage. In cells that have expressed Flpe alone, mCherry is expressed, while Cre activity alone is not reported. Thus, *RC::FrePe* indicates an intersectional population between expression domains as defined by expression of separate Flpe and Cre drivers.

Methods

A schematic of the *RC::FrePe* allele can be found within a summary of intersectional fate-mapping strategies and methods.[19]

Histology and Immunohistochemistry

Samples were harvested, fixed overnight in 2% formaldehyde and subsequently dehydrated through an ethanol series. Samples were then paraffin embedded and sectioned. For whole-mount staining of *LacZ* expression, samples were lightly fixed with 2% formaldehyde for 30 minutes and then incubated overnight with X-Gal. Antibodies used for immunohistochemistry were anti-dsRed rabbit polyclonal (Clontech), anti-GFP goat polyclonal (Abcam) and anti-αSMA mouse monoclonal 1A4 (Sigma-Aldrich). Quantitation of fluorescence was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Genotyping information can be found in the online data supplement at http://circres.ahajournals.org.

Results

To validate the reporter activity of *RC::FrePe* in neural crest derivatives, two neural crest drivers, *Wnt1::Flpe*[20, 21] and $Pax3^{Cre/+}$[22] were used to assess recombination and reporter activity (Online Figure I A–O). Direct fluorescence of postnatal (P) day 0 *Wnt1::Flpe* $Pax3^{Cre/+}$; *RC::FrePe* hearts demonstrates eGFP expression within the outflow tract where

neural crest derivatives reside (Online Figure I M).[22] Subsequent immunofluorescence confirms eGFP protein expression within the tunica media of the mature OFT, as expected for neural crest derivatives (Online Figure I O). Relatively few mCherry-labeled cells remain after dual Flpe/Cre recombination suggesting that most cardiac neural crest cells that expressed *Wnt1::Flpe* also expressed *Pax3*$^{Cre/+}$ (Online Figure I N). An average of fluorescence from nine stained *Wnt1::Flpe; Pax3*$^{Cre/+}$; *RC::FrePe* outflow tract sections revealed 8.4%±3.6% (mean ± SD) of total staining was mCherry positive, while the majority, 91.6%±3.5%, was eGFP positive. Thus, the indicator allele *RC::FrePe* is sensitive to dual recombination, consistent with previous findings (Brust, R.D. and Dymecki, S.M., unpublished data and see ref. 18). We did not detect any leakiness of eGFP or mCherry expression in these studies (Online Figure I B–E)

We crossed *Isl1*$^{Cre/+}$[23, 24] mice with *Wnt1::Flpe* and *RC::FrePe* mice to detect overlap in *Isl1*- and *Wnt1*-expressing populations (Figure 1A–O). In the absence of Flpe and Cre, only background fluorescence was observed in embryonic (E) day 12.5 embryos (Figure 1A–E). When *Wnt1::Flpe* was expressed in the presence of *RC::FrePe*, mCherry expression was observed by direct fluorescence in the craniofacial region populated by neural crest (Figure 1G, arrowhead) and in dorsal root ganglia (DRG, Figure 1G, arrows). eGFP was not detected (Figure 1H). mCherry+ cells were detected by immunofluorescence in sections through the OFT endocardial cushions that are populated by cardiac neural crest by E12.5 (Figure 1I), while eGFP was not detected (Figure 1J). In the presence of both *Wnt1::Flpe* and *Isl1*$^{Cre/+}$, mCherry expression persisted in craniofacial mesenchyme populated by cranial neural crest (Figure 1L, arrowhead) and eGFP fluorescence was observed in DRGs (Figure 1M, arrows), consistent with the known expression of *Isl1* in neural crest-derived DRGs.[25] Immunofluorescence confirmed expression of both mCherry (Figure 1N) and eGFP (Figure 1O) in sections through the endocardial cushions of the OFT, indicating that at least some cardiac neural crest derivatives in the heart have expressed *Isl1*$^{Cre/+}$ at some time in their development.

A transgenic mouse with an enhancer element derived from the *Mef2c* gene directing expression of Cre recombinase, *Mef2c-AHF-Cre*, is widely used to label SHF derivatives.[11] We crossed this mouse to *Wnt1::Flpe* and *RC::FrePe* but did not detect any evidence for overlap of expression domains at E12.5 (Figure 1P–T). This result indicates that *Mef2c-AHF-Cre* may be more restricted to SHF precursors than is *Isl1*$^{Cre/+}$ and also serves as an important negative control to minimize the chance that leaky expression explains the presence of eGFP in *Wnt1::Flpe* ; *Isl1*$^{Cre/+}$; *RC::FrePe* embryos.

Analysis of P0 hearts of *Wnt1::Flpe*; *Isl1*$^{Cre/+}$; *RC::FrePe* and *Wnt1::Flpe; Mef2c-AHF-Cre*; *RC::FrePe* pups confirms the stark difference between *Isl1*$^{Cre/+}$ and *Mef2c-AHF-Cre* (Figure 2A–J and Online Figure II). Both mCherry and eGFP are evident in the OFT of P0 *Wnt1::Flpe; Isl1*$^{Cre/+}$; *RC::FrePe* hearts (Figure 2B, C) visualized by and eGFP immunofluorescence is evident in the tunica media of the great vessels (Figure 2D) and in the leaflets of the aortic valve (Figure 2E). eGFP expression is not detected in *Wnt1::Flpe*; *Mef2c-AHF-Cre*; *RC::FrePe* hearts (Figure 2H–J). The areas of fluorescence marked by either *Isl1*$^{Cre/+}$ or *Mef2c-AHF-Cre* with *Rc::FrePe* are within the fate-mapped domains marked by these drivers using *R26*$^{LacZ/+}$ (Online Figure II). eGFP expressing derivatives of *Isl1*$^{Cre/+}$ and *Wnt1*-expressing precursors are able to differentiate into smooth muscle, as evidenced by co-expression of eGFP and smooth muscle actin (SMA) in the proximal aorta of *Wnt1::Flpe*; *Isl1*$^{Cre/+}$; *RC::FrePe* pups (Figure 2K–M, arrowheads). eGFP+ cells are observed near the branchial arches of *Wnt1::Flpe; Isl1*$^{Cre/+}$*; RC::FrePe* embryos as early as day E10.5 and within the aortic sac (Online Figure III).

Discussion

These results indicate that *Isl1*$^{Cre/+}$ is not restricted to SHF precursors of the mature heart. Rather, *Isl1*$^{Cre/+}$ labels both SHF precursors and also at least some cardiac neural crest cells. *Isl1* is known to be expressed by other neural crest derivatives, including DRG and cardiac ganglia[23, 25] but the demonstration of *Isl1*$^{Cre/+}$ expression by cardiac neural crest precursors demands re-evaluation of its use as a SHF driver and re-interpretation of some prior studies.

For example, both neural crest and SHF can give rise to smooth muscle. Given our results, it is now unclear if some or all *Isl1*-derivatives in the OFT that express smooth muscle markers are neural crest or SHF derived.[2, 23, 24, 26] Our data suggest that at least some are of neural crest origin. Isolation of *Isl1*-expressing cells from ES or iPS cultures, or from embryos, for the purpose of expanding SHF precursors (see references listed in supporting information in online data supplement)[2, 6, 7, 9, 23] may actually result in the expansion of neural crest cells. Studies in which *Isl1*$^{Cre/+}$ has been used to manipulate gene expression in the SHF, and which have resulted in aortic arch and OFT defects, may need to be re-examined for the possibility of cell autonomous neural crest effects. Experimental approaches using multiple or alternative SHF drivers, such as *Mef2c-AHF-Cre*, and use of dual and intersectional fate-mapping approaches such as the one described here, may define cardiac cell origins and fates more clearly than using the *Isl1* marker alone. Our studies emphasize the need for the identification of more specific molecular markers of SHF precursors.

Novelty and Significance

What is Known?

- Within the developing heart field *Islet1* is postulated as a selective marker of cardiac progenitor cells derived from the second heart field.
- The specificity of *Islet1* as a marker for second heart field is critical to lineage tracing, gene inactivation, and differentiation analyses.
- *Islet1*-derivatives include cells populating the outflow tract, an area patterned and formed by derivatives of the cardiac neural crest and second heart field.

What New Information Does This Article Contribute?

- A reporter mouse *RC::FrePe* allows identification of cells undergoing dual Flpe- and Cre-mediated recombination and sensitively indicates intersection between lineages marked by separate Flpe and Cre drivers.
- *Islet1* is not restricted to second heart derivatives in the heart but is also expressed by a subset of cardiac neural crest cells.
- The intersectional population revealed by *Wnt1::Flpe* ; *Islet1*^{Cre} ; *Rc::FrePe* resides in the cardiac outflow tract and includes smooth muscle cells of the tunica media of the aorta and pulmonary artery.
- Dual fate mapping using an alternative second heart field driver, a *Mef2c* enhancer regulating Cre, does not overlap with neural crest.

Islet-1 is postulated as a marker of cardiac progenitor cells from the second heart field. Numerous studies suggest *Islet1+* cells represent tri-potential precursors of differentiated cardiac tissues including smooth muscle, cardiac muscle and endothelial cells. Using Flpe- and Cre-mediated dual fate mapping, we show that *Islet1* is not restricted to second heart derivatives in the heart but that it is also expressed by a subset of cardiac neural crest cells. Thus, some *Islet1* cardiac derivatives may be neural crest-derived rather than

a multi-potent second heart field precursor. These findings suggest that results based on *Islet1* fate mapping should be interpreted with caution and emphasize the need for additional cardiac lineage tools and markers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

Cre	cyclization recombinase
E	embryonic day
eGFP	enhanced green fluorescent protein
ES	embryonic stem
FHF	first heart field
Flpe	flippase recombinase enhanced
IF	immunofluorescence
iPS	induced pluripotent stem
Isl1	Islet1
Mef2c	myocyte-specific enhancer factor 2C
OFT	outflow tract
P	post-natal day
SHF	second heart field
SMA	smooth muscle actin
Wnt	Wg (wingless) and Int

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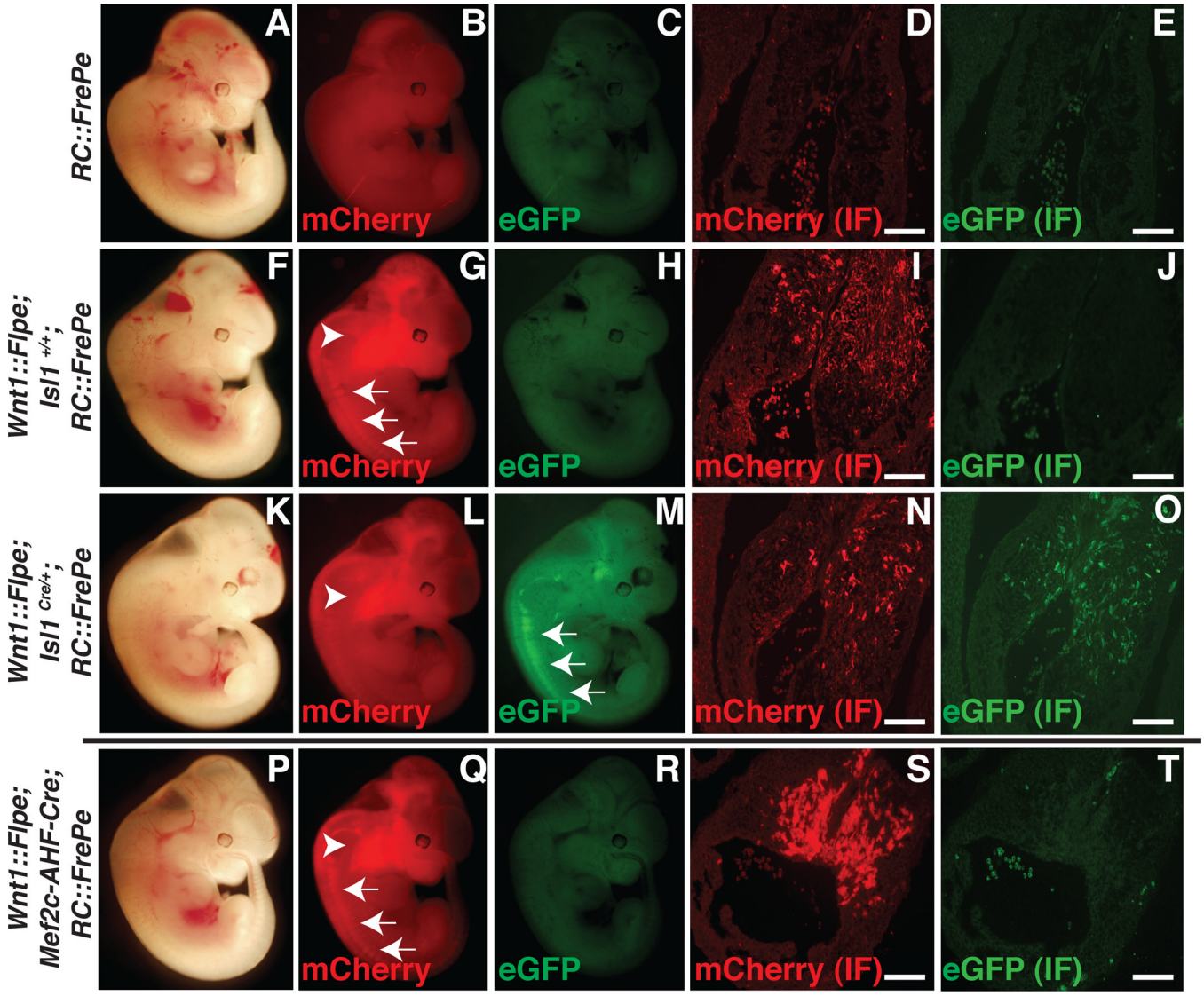

Figure 1. Dual fate mapping identifies *Isl1*$^{Cre/+}$*/Wnt1::Flpe*-derived cells in the heart at E12.5

A–E, E12.5 control *RC::FrePe* embryos **(A–C)** and immunofluorescence (IF) for mCherry **(D)** and eGFP **(E)** of cross sections through cardiac outflow tract **(D,E)**. **F–J,** *Wnt1::Flpe*; *Isl1*$^{+/+}$; *RC::FrePe* embryos. *Wnt1::Flpe*-derived craniofacial neural crest **(G, arrowhead)** and dorsal root ganglia **(G, arrows)** express mCherry, which is also detected by IF in the endocardial cushions of the outflow tract **(I)**. **K–O,** *Wnt1::Flpe; Isl1*$^{Cre/+}$; *RC::FrePe* embryos. mCherry is detected in craniofacial mesenchyme **(L, arrowhead)** and eGFP is now expressed by dorsal root ganglia **(M, arrows)**. mCherry **(N)** and eGFP **(O)** are both detected by IF in the endocardial cushions of the outflow tract. **P–T,** *Wnt1::Flpe; Mef2c-AHF-Cre*; *RC::FrePe* embryos. mCherry is seen in craniofacial mesenchyme **(Q, arrowhead)** and dorsal root ganglia **(Q, arrows)** and by IF in the outflow tract **(S)**. eGFP is not detected **(R, T).** Scale bars: 100μm.

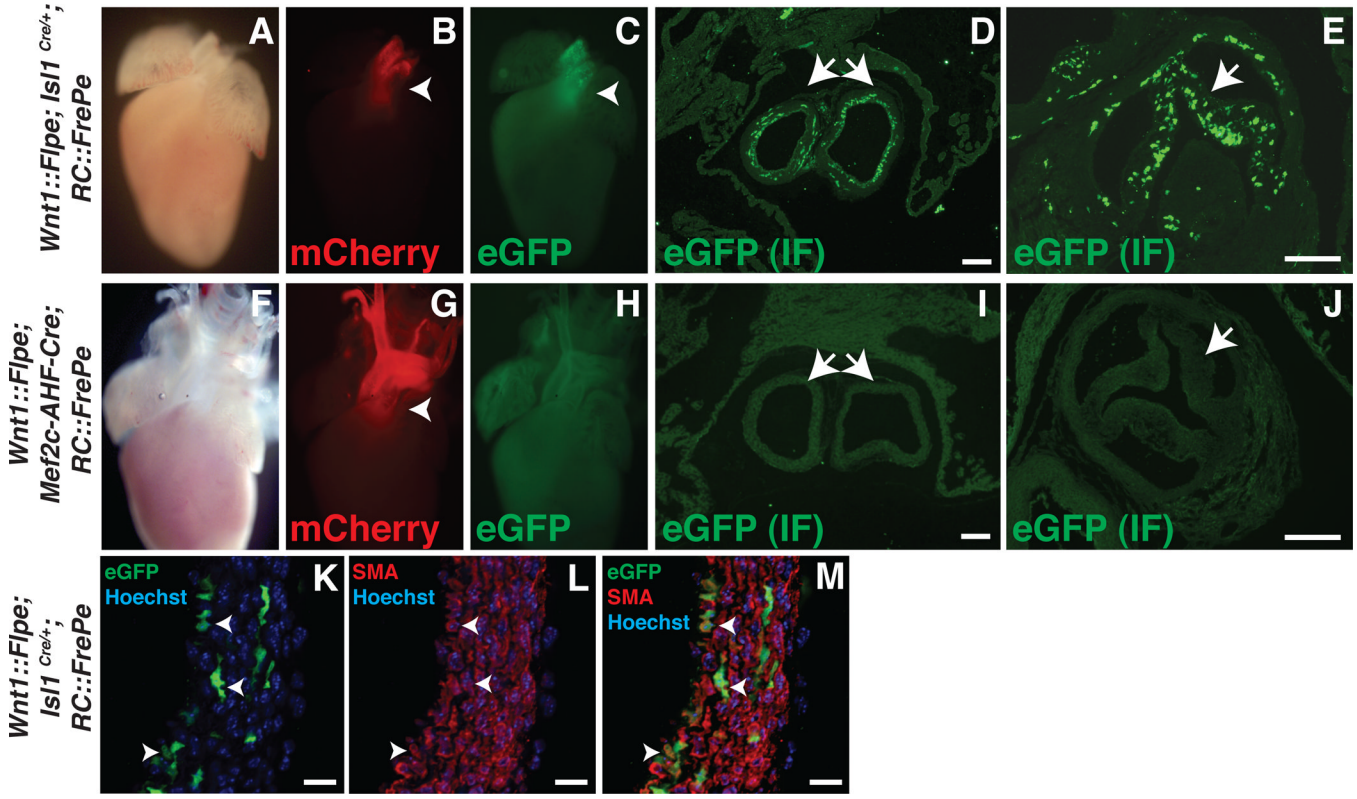

Figure 2. ***Isl*$^{Cre/+}$*/Wnt1::Flpe*-derived cells are present in the newborn heart**

A–E, Post-natal day 0 (P0) *Wnt1::Flpe; Isl1*$^{Cre/+}$; *RC::FrePe* hearts showing mCherry **(B, arrowhead)** and eGFP **(C, arrowhead)** fluorescence in the cardiac outflow tract. **D and E,** Immunofluorescence (IF) for eGFP in transverse sections through the proximal aorta and pulmonary artery **(D)** and aortic valve **(E)** reveals expression in the tunica media of the great vessels **(D)** and in the valve leaflets **(E)**. **F–J,** P0 *Wnt1::Flpe; Mef2c-AHF-Cre*; *RC::FrePe* hearts showing mCherry fluorescence in the outflow tract **(G, arrowhead)**. eGFP is not detected **(H–J)**. **K–M,** Cross section of the aortic wall of *Wnt1::Flpe; Isl1*$^{Cre/+}$; *RC::FrePe* P0 embryo stained by IF for eGFP and smooth muscle actin (SMA). Nuclei appear blue after staining with Hoechst dye. Arrowheads denote cells co-expressing eGFP and SMA. Scale bars in panels **D, E, I, J**: 100μm. Scale bars in panels **K–M**: 14μm.