Pdgfrb-Cre Targets Lymphatic Endothelial Cells of Both Venous and Non-venous Origins

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Abstract: The Pdgfrb-Cre line has been used as a tool to specifically target pericytes and vascular smooth muscle cells. Recent studies showed additional targeting of cardiac and mesenteric lymphatic endothelial cells (LECs) by the Pdgfrb-Cre transgene. In the heart, this was suggested to provide evidence for a previously unknown nonvenous source of LECs originating from yolk sac (YS) hemogenic endothelium (HemEC). Here we show that Pdgfrb-Cre does not, however, target YS HemEC or YS-derived erythro-myeloid progenitors (EMPs). Instead, a high proportion of ECs in embryonic blood vessels of multiple organs, as well as venous-derived LECs were targeted. Assessment of temporal Cre activity using the R26-mTmG double reporter suggested recent occurrence of Pdgfrb-Cre recombination in both blood and lymphatic ECs. It thus cannot be excluded that Pdgfrb-Cre mediated targeting of LECs is due to de novo expression of the Pdgfrb-Cre transgene or their previously established venous endothelial origin. Importantly, Pdgfrb-Cre targeting of LECs does not provide evidence for YS HemEC origin of the lymphatic vasculature. Our results highlight the need for careful interpretation of lineage tracing using constitutive Cre lines that cannot discriminate active from historical expression. The early vascular targeting by the Pdgfrb-Cre also warrants consideration for its use in studies of mural cells.

Key words: lymphangiogenesis; lymphvasculogenesis; vascular development; mural cell

INTRODUCTION

The Pdgfrb-Cre line (Foo et al., 2006), where Cre recombinase expression is driven by a transgenic fragment of the gene for platelet-derived growth factor receptor β (Pdgfrb), has been used extensively to specifically target mural cells, namely vascular smooth muscle cells, pericytes and hepatic stellate cells; examples are given in ref (Abraham et al., 2008; Foo et al., 2006; Greif et al., 2012; Henderson et al., 2013; Jeansson et al., 2011; Kogata et al., 2009; Siegenthaler et al., 2013; Stenzel et al., 2009; Ye et al., 2009; You et al., 2014). We (Stanczuk et al., 2015) and others (Klotz et al., 2015) recently showed that Pdgfrb-Cre unexpectedly also targets a large proportion of embryonic lymphatic endothelial cells (LECs) in the developing mesentery (Stanczuk et al., 2015) and the heart (Klotz et al., 2015). These observations were made in the context of the startling discovery that LECs in the heart (Klotz et al., 2015), mesentery (Stanczuk et al., 2015), and skin (Martinez-Corral et al., 2015), not only develop through lymphangiogenic sprouting from a venous blood vascular source, which has previously been the only known mechanism for lymphatic vessel formation in mammals (Srinivasan et al., 2007), but also through the assembly of nonvenous derived LEC progenitors.

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FIG. 1. PDGFRB-CRE TARGETS ENDOTHELIUM

A

Y.S hemogenic activity

stage #

E8 E10 E12 E14 E16 E18

FACS analysis of Y.S

B E9 Y.S Pdgfr-Cre;R26-mTmG

PECAM1

DUMG 21.5% 0.2% 17.3%
P.ECAM1

GFP

C E10 Y.S Pdgfr-Cre;R26-mTmG

PECAM1

DUMG 13.8% 1.1% 24.0%
P.ECAM1

GFP

D E12 Y.S Pdgfr-Cre;R26-mTmG

PECAM1

DUMG 74.3% 2.1% 0.1%
P.ECAM1

GFP

E Total Y.S cells

% GFP+

PECAM1 neg

PECAM1 high

E9 E10 E12

F HemEC and EMP

% GFP+

E9 E10

G GFP VEGFR2 CD41

Yolk sac

E9 E10 E12

GFP VEGFR2

FIG. 1.
In the mesentery, our lab traced the non-venous LEC origin to a hemogenic endothelial cell (HemEC) source. This conclusion was based on positive lineage tracing using the endothelial specific \textit{Pdgfrb-CreER}^{22} line induced at embryonic day (E)8-E9, together with positive tracing of E10-E11 induced cKit-CreER^{22} (Stanczuk et al., 2015). cKit is a marker associated with HemECs and HemEC-derived hematopoietic progenitors from all known hemogenic sites of the embryo and the yolk sac (YS; Antas et al., 2013; Medvinsky et al., 2011). In the heart, \textit{Pdgfrb-Cre} induced labeling, together with positive labeling with \textit{Vat-Cre}, a pan-hematopoietic lineage marker, and E7 induced \textit{Csf1r1-MeriCreMer}, which traces YS-derived myeloid cells (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015), was proposed to indicate YS HemEC as the source of cardiac LECs (Klotz et al., 2015).

Based on the highly efficient ability of \textit{Pdgfrb-Cre} to target non-venous derived mesenteric LECs and lymphatic vessels (Stanczuk et al., 2015), along with the suggested YS HemEC origin of the \textit{Pdgfrb-Cre} labeled LECs in the heart (Klotz et al., 2015), we set out to carefully evaluate the suggested ability of \textit{Pdgfrb-Cre} to trace YS-derived HemEC-progenitors (Klotz et al., 2015) and its potential as a specific lineage marker for non-venous derived LEC progenitors. Here we demonstrate that \textit{Pdgfrb-Cre} is not a valid tool for tracing YS HemEC-activity or YS derived progenitors, showing minimal expression in YS ECs before E12 and no tracing of the early YS-derived myeloid lineages. Furthermore, we show that \textit{Pdgfrb-Cre} positive tracing cannot differentiate between non-venous and venous derived LEC progenitors, since it targets the cardinal vein, which is a known source of venous derived LECs. Unexpectedly, our data demonstrate that \textit{Pdgfrb-Cre} is induced in embryonic blood vessels already at E9, and the proportion of Cre-recombined endothelial cells increases during development in both blood and lymphatic vessels. Interpretation of phenotypes caused by \textit{Pdgfrb-Cre}-induced gene deletion must thus take into account also the partial targeting of both BECs and LECs from early embryonic development.

**RESULTS AND DISCUSSION**

To examine if \textit{Pdgfrb-Cre} allows tracing of YS HemEC and YS-derived progenitors we crossed the \textit{Pdgfrb-Cre} mice with the \textit{R26-mTmG} fluorescent reporter line and analyzed YS of embryos during and after the period of hemogenic activity (Fig. 1A) (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Medvinsky et al., 2011). The \textit{R26-mTmG} reporter allows detection of Cre activity by the recombination-induced expression of membrane-bound green fluorescent protein (GFP) and inactivation of the red fluorescent protein Tomato expression (Muzumdar et al., 2007). Because of perdurance of the Tomato protein within the recombined cells, co-expression of Tomato and GFP can additionally help discern recent recombination (i.e. active expression) from lineage tracing (i.e. historical expression).

E9, E10, and E12 embryos were harvested and the YSs were dissected away from the embryo proper and the vitelline artery. After gentle digestion, the YSs were analysed by flow cytometry. Ter119 positive cells (primitive erythrocytes) and dead cells were gated away in one single dump channel (Fig. 1b-d). Analysis of E9 and E10 YSs showed a minimal proportion of GFP\textsuperscript{+} \textit{Pdgfb-Cre}-recombined cells [0.27\% \pm 0.06\% (E9; Fig. 1b,c) and 1.38\% \pm 0.46\% (E10; Fig. 1c,e)], increasing slightly at E12 (2.59\% \pm 0.51\%; Fig. 1d,e). At E9 and E10, the GFP\textsuperscript{+} population contained only PECAM\textsubscript{1}\textsuperscript{high} cells (100\% \pm 0\%; Fig. 1c,e). At E12 both endothelial (PECAM\textsubscript{1}\textsuperscript{high}) (54.3\% \pm 1.8\%) and nonendothelial (PECAM\textsubscript{1}\textsuperscript{low}) (46.6\% \pm 1.8\%) cells showed recombination, likely reflecting expression of \textit{Pdgfrb-Cre} in both YS ECs and mural cells (Fig. 1d,e). Immunofluorescence analysis of the YS confirmed the presence of rare, scattered GFP\textsuperscript{+} ECs in E9 and E10 vasculature (Fig. 1g), while E12 embryos displayed both GFP\textsuperscript{+} ECs and mural cells around larger arteries (Fig. 1g). However, although \textit{Pdgfrb-Cre} did target rare ECs in the YS, analysis of the YS cKit\textsuperscript{−} cell population, which includes both HemECs and YS-derived erythroid/myeloid progenitors (EMPs), did not support specific targeting of HemECs. A very low proportion of GFP\textsuperscript{+} cells was observed both at E9

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**FIG. 1.** \textit{Pdgfrb-Cre} does not target hemogenic endothelium in the YS. (A) Schematic of the \textit{Pdgfrb-Cre} transgene, \textit{R26-mTmG} reporter construct and analyzed embryonic stages. The time frame for YS hemogenic activity is indicated. (B) Gating scheme and representative data for E9 \textit{Pdgfrb-Cre};\textit{R26-mTmG} YSs (n = 5). Dump channel includes dead cells and erythrocytes (Ter119\textsuperscript{+}) (all stages), and macrophages (CD11b\textsuperscript{+} F4/80\textsuperscript{−}) (E10 only). Dot plots from the left; (1) Live, non-erythrocyte gate; (2) Total proportion of GFP\textsuperscript{+} cells in \textit{Pdgfrb-Cre};\textit{R26-mTmG} YSs; (3) Gating of cKit positive cells (HemECs and EMPs); and (4) Tomato and GFP expression within HemEC/EMP population. (C) Gating scheme and representative data for E10 \textit{Pdgfrb-Cre};\textit{R26-mTmG} YSs (n = 3). As described above (B). (D) Gating scheme and representative data for E12 \textit{Pdgfrb-Cre}, \textit{R26-mTmG} YSs (n = 3). Dot plots as above (B and C), cKit\textsuperscript{+} population is adjusted for differences in PECAM1 expression between E9, E10 and E12 YS, to account for downregulation of vascular markers in late EMPs (Gomez Perdiguero et al., 2015). Expression of Tomato and GFP in cKit\textsuperscript{−} HemECs and EMPs is not displayed due to too low cell numbers of these cells at this stage. (E, F) Summary of data from E9, E10, and E12 embryos, showing the proportion of GFP\textsuperscript{+} cells (E) or HemEC/EMPs (F) in the YS. The average percent of GFP\textsuperscript{+} cells within PECAM\textsubscript{1}\textsuperscript{high} and PECAM\textsubscript{1}\textsuperscript{low} populations is shown. The horizontal lines represent mean of all cells (n = 5) or n = 3 (E10 and E12). (G) Whole-mount immunofluorescence of E9, E10 and E12 yolk sacs showing scattered GFP\textsuperscript{+} (green) endothelial cells (arrows, VEGFR\textsuperscript{2}\textsuperscript{−}; red), hematopoietic cells (arrowheads, CD41\textsuperscript{−}; blue), GFP\textsuperscript{+} mural cells (open arrowheads) are present at E12 around larger arteries that show high proportion of GFP\textsuperscript{+} cells at this stage. Scale bars: 100 \textmu m.
Almost no cKit\(^{+}\) cells were present in E12 YSs (Fig. 1d), consistent with the loss of hemogenic endothelial activity by this stage.

To follow up the analysis of \(Pdgfrb\)-Cre expression in the YS we also assessed if \(Pdgfrb\)-Cre can trace early macrophages and monocytes derived from the YS. For this purpose, we performed FACS analysis of \(Pdgfrb\)-Cre;\(R26\)-\(mTmG\) embryos at E10 (Fig. 2a), when the major pool of myeloid cells is YS-derived (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015). In three out of three embryos we could not find any tracing of YS-derived monocytes and macrophages, defined by co-expression of the pan-myeloid marker CD11b and the pan-leukocyte marker CD45 (Fig. 2b). As a control, positive GFP tracing (96.1\%) induced by \(Tie2\)-Cre is shown (Fig. 2c). The historical \(Tie2\)-Cre expression in the YS (E7-E9) is additionally demonstrated by loss of Tomato fluorescence in over 99\% of the GFP\(^{+}\) monocytes/macrophages (Fig. 2c). Taken together, these results exclude the possibility that \(Pdgfrb\)-Cre tracing of the lymphatic vasculature can be explained by a YS origin since the \(Pdgfrb\)-Cre transgene cannot efficiently label YS HemECs or EMPs in E9 and E10 YSs, nor can it trace early E10 monocytes and macrophages that are known to derive from the YS (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015).

Although \(Pdgfrb\)-Cre does not trace YS-derived HemECs, EMPs or YS-derived macrophage lineages, it would still be possible that it specifically targets non-venous-derived LEC progenitors either through tracing of a non-venous derived LEC progenitor cell lineage within the embryo proper or through selective expression in non-venous derived LECs. To assess the specificity of \(Pdgfrb\)-Cre mediated targeting of LECs of different origins, we analysed Cre mediated recombination in the cardinal vein that provides a source of the first venous derived LECs, the peripheral longitudinal lymphatic vessel and the primordial thoracic duct, that are also referred to as the jugular lymph sacs (JLS) (Hagerling et al., 2013; Srinivasan et al., 2007; Yang et al., 2012). Immunofluorescence analysis of E13.5 \(Pdgfrb\)-Cre;\(R26\)-\(mTmG\) embryos showed the expected GFP\(^{+}\) perivascular cells (Foo et al., 2006) (Fig. 3a). In addition, we observed scattered GFP\(^{+}\) ECs within the cardinal vein and widespread labelling of LECs within the JLS (Fig. 3a). This is consistent with recently published data showing \(Pdgfrb\)-Cre mediated targeting of ECs within the cardinal vein, JLS and lymphovenous valves (Turner et al., 2014), but is in contrast to findings of Klotz et al. (2015) reporting absence of \(Pdgfrb\)-Cre mediated recombination within the E10 cardinal vein and E12.5 JLS (Klotz et al., 2015). FACS analysis of endothelial cells (Fig. 3b) further demonstrated limited but detectable \(Pdgfrb\)-Cre induced recombination in the blood vasculature as early as at E9 (3.5\% ± 3.7\% GFP\(^{+}\) ECs, \(n=5\); Fig. 3c). The proportion of Cre-recombined ECs increased at E10 (9.3\% ± 2.0\%, \(n=3\)), reaching a significant labelling of ECs in E12 embryos.
FIG. 3. *Pdgfrb-Cre* cannot differentiate between venous and non-venous derived LEC progenitors. (A) Immunofluorescence of a transverse vibratome section of E13.5 *Pdgfrb-Cre;R26-mTmG* embryo using antibodies against GFP (green), and Endomucin (red; marker of venous EC). Note scattered GFP-labeling of the cardinal vein (CV) and jugular lymph sac (JLS). DA = dorsal aorta. Boxed area is magnified in the small inserts to the right and single channel images are shown. Arrow points to a GFP<sup>+</sup> venous EC, open arrowhead to a GFP<sup>+</sup> LEC and arrowhead indicates a GFP<sup>+</sup> Endomucin<sup>+</sup> mural cell. Scale bars: 50 μm. (B) Gating scheme for analysis of ECs in the E10 embryo proper. After exclusion of dead cells and erythrocytes in the dump channel PECAM<sup>1<sub>high</sub></sup> cells (ECs) are selected while CD45<sup>+</sup> PECAM<sup>1<sub>intermed</sub></sup> immune cells are excluded. (C) Tomato and GFP expression in *Pdgfrb-Cre;R26-mTmG* embryos showing a gradual increase of GFP-labelling of the ECs between E9 and E12 (n = 5 (E9); n = 3 (E10 and E12)). (D) Analysis of Lyve1<sup>+</sup> (venous derived LECs and LEC progenitors) and Lyve1<sup>+</sup> ECs from E10.5 *Pdgfrb-Cre;R26-mTmG* embryos shows Cre recombination in both cell fractions. The Tomato/GFP Dot plots display 1000 event from Lyve1<sup>+</sup> and Lyve1<sup>+</sup> gates.
Separate analysis of Lyve1 ECs (i.e. venous derived LECs and LEC progenitors) and Lyve1- BECs in E10.5 embryos showed similar PDGFRB-Cre induced recombination in both cell fractions (Fig. 3d). In summary, these data demonstrate that PDGFRB-Cre targets embryonic blood vasculature prior to initiation of venous sprouting of lymphatic vessels and shows significant labelling of ECs in the cardinal vein and the JLS. PDGFRB-Cre cannot therefore be used to specifically trace and target non-venous derived LEC progenitors.

The high proportion of ECs (20–25%) targeted by the PDGFRB-Cre transgene in E12 embryos and targeting of both Lyve1+ and Lyve1- ECs in E10.5 embryos indicated a more widespread vascular induction than could be explained by specific PDGFRB-Cre expression in the cardinal vein and developing lymph sacs. This prompted us to further analyze the blood and lymphatic vasculature of the skin, heart and the mesentery/intestine to evaluate if expression of PDGFRB-Cre is specific for LECs and their progenitors. FACS analysis of E15 embryos revealed a high proportion of GFP+ LECs in the skin and heart, as well as significant labelling of LECs in the mesentery/intestine. This suggests that PDGFRB-Cre is a useful tool for tracing and targeting ECs in early embryonic development.
reflect the extent and timing of receptor Vegfr3
et al, 2015) genitors (Stanczuk Cre light of these data, our previous conclusion that tion of lymphatic and blood ECs in multiple organs. In Although we have observed a specific defect in mesen-
teric lymphatic development in embryos with Pdgfrb-Cre-derived LEC progenitors, which we based on posi-
tive lineage tracing with the hemogenic lineage marker cKit-CreER T2 and early induction of the vascular marker Pdgfrb-CreER T2, which labels all major hemogenic ves-
sels but not venous derived LEC progenitors (Stanczuk et al., 2015). In the case of the heart, our data, which excludes YS origin as an explanation for Pdgfrb-Cre labeling of LECs, combined with the lack of endothelial cell tracing (Klotz et al., 2015) that would be expected for HemEC-derived progenitors, calls for further investiga-
tion into the origin of cardiac lymphatic vessels. Our data further highlight that future studies analyzing the effect of Pdgfrb-Cre mediated gene deletion in the mural cells must also take into account deletion in a sig-
nificant portion of both blood and lymphatic ECs from early development. More generally, our results illustrate the inherent limitations of using constitutive Cre lines in lineage tracing experiments, and the careful evalua-
tion needed to differentiate de novo expression from lineage tracing.

METHODS

Mice

Pdgfrb-Cre mice (Foo et al., 2006) were kindly pro-
vided by Ralf Adams (Max Planck Institute for Biomi-
cine, Münster, Germany). R26-mTmG mice (Muzumdar et al., 2007); obtained from the Jackson Laboratory) and Tie2-Cre mice (Koni et al., 2001) were described previously. Staging of E9 and E10 embryos were done by somite counting (sc) and Theiler stage (TS) was determined according to EMAP eMouse Atlas Project (http://www.emouseatlas.org) (Richardson et al., 2014). Data from E9 embryos refer to sc 15-18, TS14; E10 sc 24-26, TS15; and E10.5 sc 34-37, TS17. For embryos older than E11, the morning of vaginal plug detection was considered as E0. All strains were main-
tained and analyzed on C57BL/6j background.

Immunofluorescence

Yolk sac and skin were fixed in 4% paraformaldehyde (PFA) for 2 h at RT, permeabilized in 0.3% Triton-X100 in PBS (PBSTx) and blocked in PBSTx plus 3% milk. Pri-
mary antibodies were incubated at 4°C overnight in blocking buffer. After washing in PBSTx, the samples were incubated with fluorochrome-conjugated second-
ary antibodies in blocking PBSTx plus 3% milk, before further washing and mounting in Mowiol. For visualization of cardinal veins and lymph sacs, 150 μm vibra-
tome cross sections of E13.5 PFA fixed Pdgfrb-Cre;R26-
mTmG embryos were prepared and stained as described above. The following antibodies were used: chicken anti-GFP (Abcam cat 13970), rat anti-mouse Endomucin (Santa Cruz Biotechnology cat sc-65495), goat anti-mouse Neuropilin-2 (R&D Systems cat AF567), rat anti-mouse CD41 (BectonDickinson cat 553847) and goat anti-mouse VEGFR2 (R&D Systems cat AF644).
Collagenase IV 1 mg ml−1

E10 embryos were digested using a lower amount of PECAM1high, dump channelnegative cells. 2. PDPNpositive E15 LECs and BECs were gated in two steps; 1. Sytox blue (Life Technologies) for dead cell exclusion. conjugated with eF450 (eBioscience); together with (M1/70) and red blood cells, anti-TER-119 (TER-119); all phages, anti-F4/80 (BM8), myeoloid cells, anti-CD11b exclude immune cells anti-CD45 (30-F11); macrophages. Immunity 42:665–678.

Cy7, anti-podoplanin (PDPN) eF660 (eBio8.1.1) (both ples were stained with anti-CD31/PECAM-1 (390) PE-Cy7, anti-c-Kit/CD117 (2B8) APC, anti-CD11b (M1/70) PerceC-Cy5.5, anti-c-CD45 (30-F11) APC-eF780. Anti-TER-119 (TER-119) e450 and Sytox blue (Invitrogen; to detect dead cells) were included in the dump channel. For E12 embryos anti-CD11b (M1/70) and anti-F4/80 (BM8) were included in the dump channel as described for E15 samples. The anti-rat/hamster compensation bead kit (Life Technologies) was used for compensation controls, with the addition of Tomato positive tissue and GFP positive tissue for Tomato and GFP compensation. The cells were analyzed on a FACSariaII cell sorter with the FACSDiva software (all from BD biosciences). Data were processed using FlowJo software (TreeStar). Single cells were gated using FSC-A/SSC-A followed by FSC-H/FSC-W and SSC-H/SSC-W in all experiments.

Flow Cytometry

E15 embryonic back skin, heart and mesentery together with intestine were harvested and digested in 4 mg ml−1 Collagenase IV (Life Technologies), DNase I (Roche) 0.2 mg ml−1 in PBS with 10% Fetal calf serum (FCS; Gibco) at 37 °C in a water bath for 20 min. E9 and E10 embryos were digested using a lower amount of Collagenase IV 1 mg ml−1 and 10% FCS, 10–15 min. E12 embryo and YS were digested in 2 mg ml−1 Collagenase IV, 5% FCS (Life Technologies). Digested samples were quenched by adding 2 mM EDTA and filtered through a 70 μm nylon filter (BD Biosciences). Cells were washed with FACS buffer (PBS, 0.5% FCS, 2 mM EDTA) and immediately processed for staining in 96-well plates. Fc receptor binding was blocked by rat anti-mouse CD16/CD32 (93) (eBioscience). Dump channel included markers to exclude immune cells anti-CD45 (30-F11); macrophages, anti-F4/80 (BM8), myeloid cells, anti-CD11b (M1/70) and red blood cells, anti-TER-119 (TER-119); all conjugated with eF450 (eBioscience); together with Sytox blue (Life Technologies) for dead cell exclusion. E15 LECs and BECs were gated in two steps; 1. PECAM1high, dump channelnegative cells. 2. PDPNpositive (LECs) PDPNnegative (BECs). E9 and E10 YS and embryos were stained with anti-CD31/PECAM-1 (390) PE-Cy7, anti podoplanin (PDPN) eF660 (eBio8.1.1) (both from eBioscience). Dump channel included markers to exclude immune cells anti-CD45 (30-F11); macrophages, anti-F4/80 (BM8), myeloid cells, anti-CD11b (M1/70) and red blood cells, anti-TER-119 (TER-119); all conjugated with eF450 (eBioscience); together with Sytox blue (Life Technologies) for dead cell exclusion. E15 LECs and BECs were gated in two steps; 1. PECAM1high, dump channelnegative cells. 2. PDPNpositive (LECs) PDPNnegative (BECs). E9 and E10 YS and embryos were stained with anti-CD31/PECAM-1 (390) PE-Cy7, anti-c-Kit/CD117 (2B8) APC, anti-CD11b (M1/70) Perce-Cy5.5, anti-c-CD45 (30-F11) APC-eF780. Anti-TER-119 (TER-119) e450 and Sytox blue (Invitrogen; to detect dead cells) were included in the dump channel. For E12 embryos anti-CD11b (M1/70) and anti-F4/80 (BM8) were included in the dump channel as described for E15 samples. The anti-rat/hamster compensation bead kit (Life Technologies) was used for compensation controls, with the addition of Tomato positive tissue and GFP positive tissue for Tomato and GFP compensation. The cells were analyzed on a FACSariaII cell sorter with the FACSDiva software (all from BD biosciences). Data were processed using FlowJo software (TreeStar). Single cells were gated using FSC-A/SSC-A followed by FSC-H/FSC-W and SSC-H/SSC-W in all experiments.
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