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ORIGINAL ARTICLE

Organ-injury-induced reactivation of hemangioblastic precursor cells

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Early in mammalian development, the stem cell leukemia (SCL/TAL1) gene and its distinct 3′ enhancer (SCL 3′En) specify bipotential progenitor cells that give rise to blood and endothelium, thus termed hemangioblasts. We have previously detected a minor population of SCL (+) cells in the postnatal kidney. Here, we demonstrate that cells expressing the SCL 3′En in the adult kidney are comprised of CD45+ CD31− hematopoietic cells, CD45−CD31+ endothelial cells and CD45−CD31− interstitial cells. Creation of bone marrow chimeras of SCL 3′En transgenic mice into wild-type hosts shows that all three types of SCL 3′En-expressing cells in the adult kidney can originate from the bone marrow. Ischemia/reperfusion injury to the adult kidney of SCL 3′En transgenic mice results in the intrarenal elevation of SCL and FLK1 mRNA levels and of cells expressing hem-endothelial progenitor markers (CD45, CD34, c-Kit and FLK1). Furthermore, analysis of SCL 3′En in the ischemic kidneys reveals an increase in the abundance of SCL 3′En-expressing cells, predominantly within the CD45 (+) hematopoietic fraction and to a lesser extent in the CD45 (−) fraction. Our results suggest organ-injury-induced reactivation of bone marrow-derived hemangioblasts and possible local angioblastic progenitors expressing SCL and SCL 3′En.
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Introduction

The vascular system develops in close association with the hematopoietic lineage in vertebrate embryos. Vasculogenesis refers to *in situ* formation of primitive blood vessels from endothelial precursors or angioblasts.[1] Endothelial precursor cells have been identified in adult bone marrow and peripheral blood.[2,3] These endothelial precursor cells have been shown to share properties with embryonic angioblasts;[4,5] they have the capacity to migrate, proliferate and differentiate *in situ* into mature endothelial cells and to colonize sites of active neovascularization.

Despite recent progress in the identification of signaling molecules and cell surface receptors that regulate blood vessel formation, little is currently known about the precise transcriptional events that govern endothelial fate. The stem cell leukemia (SCL) gene (also known as TAL1) encodes a basic helix–loop–helix protein that is commonly activated in T-cell leukemia.[6] SCL was found to be essential for normal embryonic and definitive hematopoiesis as well as for proper vascular development;[7–10] SCL-deficient mice die *in utero* between E9.5 and 10.5 with no evidence of blood formation and with a severe defect in vascular development.[9,10] SCL is also important for the commitment of the common precursor of both hematopoietic and endothelial lineages, the hemangioblast. During embryogenesis, SCL is one of the first markers detected in isolated mesodermal cells that give rise to angioblasts while its expression is downregulated in the endothelium of mature vessels.[11,12] In zebrafish development, SCL is expressed in a population of putative hemangioblasts. Ectopic SCL expression greatly increased the number of hemangioblasts and also resulted in excessive blood and endothelial development at the expense of other mesodermal cell fates.[13] In this model organism, SCL (+) progenitors ultimately migrate and localize to the kidney, the definitive site of hematopoiesis.[14] More recent studies have shown a combined critical role for FLK-1, a receptor tyrosine kinase (VEGFR2), and SCL, in hemangioblast specification.[15,16] Specifically, it was suggested that specification within FLK-1-expressing mesoderm is regulated by SCL expression.[17,18] Importantly, FLK-1-expressing mesoderm contributes to the circulatory system including vasculogenesis of the embryonic kidney.[19]

A 3′ element (the 5245 bp SCL +18/19 enhancer or SCL 3′En) localized 19 kb downstream of exon 1 of the SCL gene has a remarkable specificity in regulating SCL expression in the hematopoietic and angioblastic compartments.[20,21] Within this fragment, a 644 bp core +19 enhancer was subsequently defined and shown to be active at a single time point in embryonic blood and endothelium.[22] During embryonic development, the SCL 3′En is selectively active in a subset of mesodermal cell lineages, including endothelial and hematopoietic stem cells and progenitors.[20,21] These cells express c-Kit and CD34, both associated with hematopoietic progenitors. In adult mice, the 3′En is active in the vast majority of hematopoietic stem cells and progenitors present in bone marrow[20] and recent evidence suggests also expression in some adult endothelial cells.[23] Unlike the +18/19 enhancer whose activity was assessed in transgenic mice carrying a LacZ/+18/19 reporter construct, the +19 core enhancer was active only in adult mice when linked to the eukaryotic reporter gene human placental alkaline phosphatase (PLAP)[23].

We have recently shown that SCL expression and the activity of the SCL 3′En are developmentally regulated in the mammalian kidney.[24] The early and specific expression of SCL 3′En in the metanephric mesenchyme suggests a role in the differentiation and growth of the mammalian kidney, especially in specifying angioblastic progenitors. Interestingly, although postnatal SCL message was abrogated, we observed SCL 3′En

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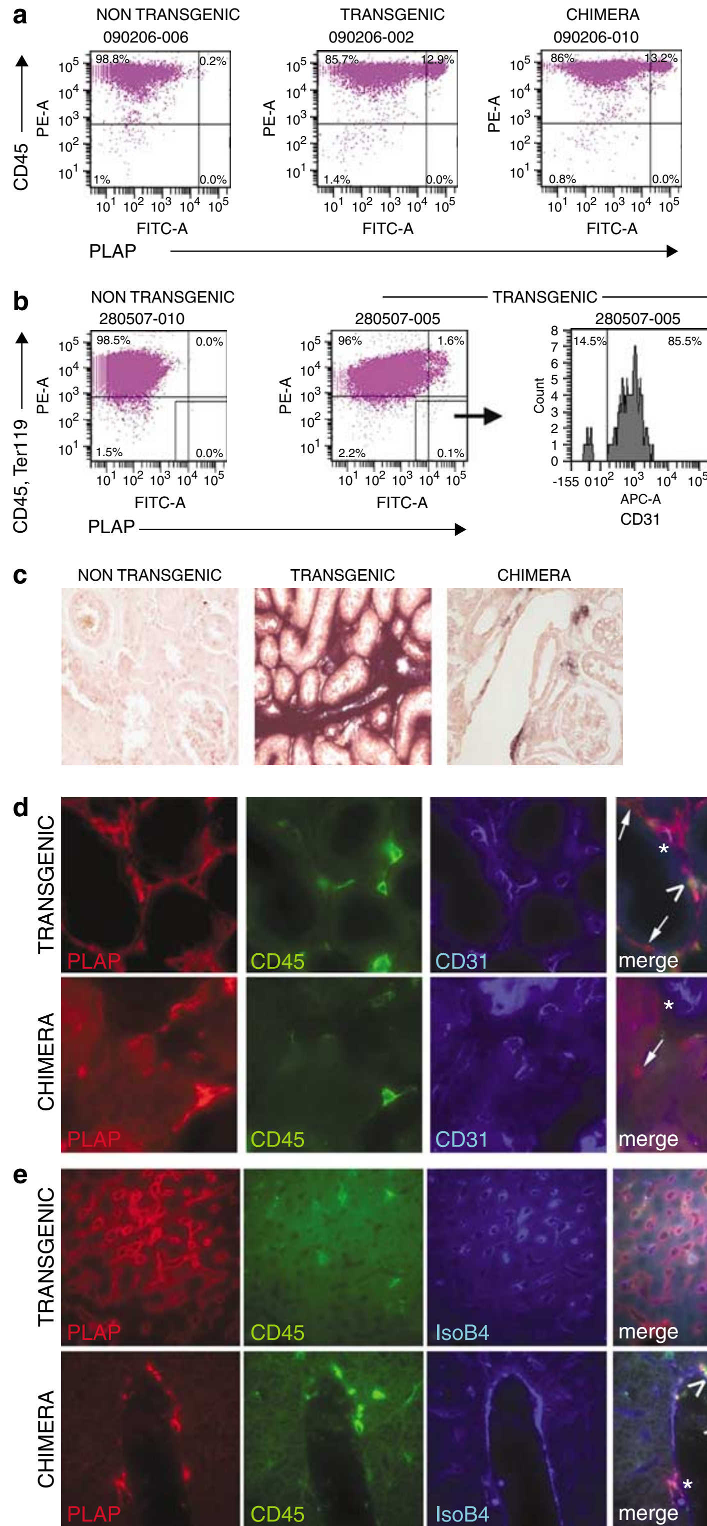
(+) cells resident in the adult kidney.[24] Renal developmental pathways have been shown to be activated during adult tissue regeneration,[25,26] providing possible links for both processes. In addition, blood and marrow-derived cells have been recently suggested to have vasculogenic potential during kidney damage.[27,28] We have therefore hypothesized that the expression of SCL characterizing embryonic kidney will re-appear during regeneration of adult kidney integrating bi-potential information needed for blood and endothelial development.

Methods

Animals

All mice used were of the (CBA × C57BL/6) background. SV/PLAP/19 transgenic mice expressing the human alkaline



phosphatase gene under the regulatory elements of the SCL 3′En (+19 region) and SCL +6E5/lacZ/3′En transgenic mice (+18/19 region) were described previously.[20–23] A breeding pair of +6E5/lacZ/3′En transgenic mice was kindly provided by AR Green, Cambridge Institute for Medical Research. Mice were placed under conventional housing and diet in a pathogen-free (SPF) environment. All animal procedures were approved by the local committee for care and use of laboratory animals and were performed according to governmental and international guidelines on animal experimentation.

Newborn transplantation assays

To generate chimeric mice we followed the protocol published by Yoder *et al.*[29] with modifications. In brief, pregnant females are injected with busulfan (Sigma-Aldrich, St Louis, MO, USA) to sublethally condition the newborn pups for high-level donor cell engraftment. Busulfan is prepared at $1\,mg\,ml^{-1}$ in 2% dimethylsulfoxide in phosphate-buffered saline (PBS). The pregnant dam is given an intraperitoneal injection of $15.5\,mg\,kg^{-1}$ of busulfan on the morning of days 17 and 18 of pregnancy. Pups are born on day 19 and injected in the facial vein on day 20 according to the protocol described by Sands and Barker.[30]

Bone marrow cells were obtained from SCL-+19 PLAP transgenic mice and prepared as previously described.[23] Bone marrow cells (5×10^6 cell) suspension is injected in 50 μl of D-PBS 1% fetal calf serum. When primary recipient animals have reached 1 month of age, blood is retrieved from the tail and subject to PLAP-PCR blood chimerism analysis. Positive chimeras are killed between 4 and 6 months. For fluorescence-activated cell sorting (FACS) analysis, bone marrow cells from transgenic mice were stained according to the protocol described in the following section as previously described.[23] Cells were stained with the antibodies PLAP, Ter119PE, CD45PE and CD31bio followed by incubation with anti-rabbit-FITC and stretavidine-cy5.

Hematopoietic chimerism analysis

To test the contribution of donor hematopoietic stem cells to different hematopoietic lineages, long-term transplanted mice were killed at 4–6 months post transplant. A week before killing, peripheral blood was drawn from the tail of the recipient mice into 0.3% EDTA in PBS and red blood cells lysed with Tris-buffered ammonium chloride (9:1 dilution of 0.16 M NH_4Cl/0.17 M Tris pH 7.6). Leukocytes were pelleted, stained for PLAP (AbD Serotec, Oxford, UK) and CD45-PE (clone 30-F11, BD Biosciences Pharmingen, San Jose, CA, USA) followed by incubation with sheep anti-rabbit biotin-conjugated antibody (AbD Serotec) and streptavidin-FITC conjugate (BD Biosciences Pharmingen). Cells were analyzed by flow cytometry (FACS) using a FACSAria flow cytometer (BD Biosciences) according to the protocol previously described.[23] Peripheral blood chimerism was assessed by quantification of the percentage of PLAP+ cells compared to the levels in SV/PLAP/19 transgenic and nontransgenic mice. To confirm multilineage hematopoietic engraftment, animals with high levels of peripheral blood PLAP+ cells were killed and genomic DNA was isolated from spleen, lymph nodes, thymus and bone marrow or FACS-sorted cells and analyzed by PCR for the presence of the PLAP transgene as published.[23]

Ischemia/reflow experiments

Eight-week-old SCL +6E5/lacZ/3′En transgenic mice were used for ischemia/reperfusion (I/R) injury according to the protocol previously described.[31] Mice were anesthetized with $100\,mg\,kg^{-1}$ ketamine and $10\,mg\,kg^{-1}$ xylazine injected intraperitoneally, and a flank incision was made. For unilateral I/R, the left renal pedicle was clamped for 40 min using a vascular clamp (Fine Science Tools Inc., Foster City, CA, USA). The abdomen was covered with gauze moistened in PBS, and the mice were maintained at 37°C using a warming pad. After 40 min, the clamp was removed and reperfusion was confirmed visually. Mice were killed at 24 h, 48 h, 1 week, 2 weeks and 4 weeks after injury was performed and kidneys were processed for histological (hematoxylin and eosin and sirius red staining) and molecular analysis.

Flow cytometry of renal cells

Intact and ischemic kidneys were washed extensively with sterile PBS to remove contaminating debris and red blood cells. Kidneys were then diced and treated with 0.075% collagenase (type D; Sigma-Aldrich, St Louis, MO, USA) diluted in PBS for 60 min at 37°C with gentle agitation. The collagenase was inactivated with an equal volume of culture medium (DMEM/10% fetal calf serum/1% penicillin–streptomycin), the dissolved tissue further minced and then centrifuged for 10 min at low speed. The cellular pellet was resuspended in culture medium and sequentially filtered through 70 and 40 μm mesh filters to remove debris and cell segments. Cell suspensions were treated with cold ACK buffer (0.15 M potassium-ammonium chloride buffer) to remove remaining red blood cells. Flow cytometric analysis was performed using a modified FACScan (BD

Figure 1 Analysis of bone marrow-derived chimeras. Newborn mice were transplanted with total bone marrow cells derived from SV/PLAP/+19 transgenic mice and high-level reconstituted chimeric mice analyzed at 4–6 months post transplant. (**a**) Flow cytometric profiles of peripheral blood are shown for CD45 and hPLAP expression for transgenic, nontransgenic and chimeric mice. All PLAP+ cells coexpress CD45. (**b**) Flow cytometric profiles of bone marrow are shown for CD45/Ter119, hPLAP and CD31 expression for nontransgenic and transgenic mice. Expression of CD31 in the gated PLAP+CD45−Ter119− population is represented in the histogram. Percentages of total cells (10^6 events) are shown in each quadrant. Percentage of cells out of the gated PLAP+CD45−Ter119− population is shown in the histogram. (**c**) Histochemical detection of hPLAP activity (purple stain) in paraffin sections of kidney derived from nontransgenic, transgenic and chimeric mice. Expression is detected in the vascular network of transgenic mice and cells with endothelial and fibroblastic appearance in the chimera. (**d**) Immunofluorescence images of kidney sections from SV/PLAP/+19 PLAP transgenic (upper panel) and SV/PLAP/+19 bone marrow-derived chimeric mice (lower panel) co-stained with anti-PLAP (red), anti-CD45 (green) and anti-CD31 (blue) antibodies. Three types of cell are identified in the merged image: PLAP+CD45−CD31− (arrows), PLAP+CD45−CD31+ (asterisk), PLAP+CD45+CD31− (arrowheads). (**e**) Immunofluorescence images of liver sections from SV/PLAP/+19 PLAP transgenic (upper panel) and SV/PLAP/+19 bone marrow-derived chimeric mice (lower panel) co-stained with anti-PLAP (red), anti-CD45 (green) antibodies and isolectine B4 (blue). Two types of cells are identified in the merged image of chimeric liver: PLAP+CD45−CD31+ (asterisk) and PLAP+CD45+CD31− (arrowheads). PLAP, placental alkaline phosphatase.

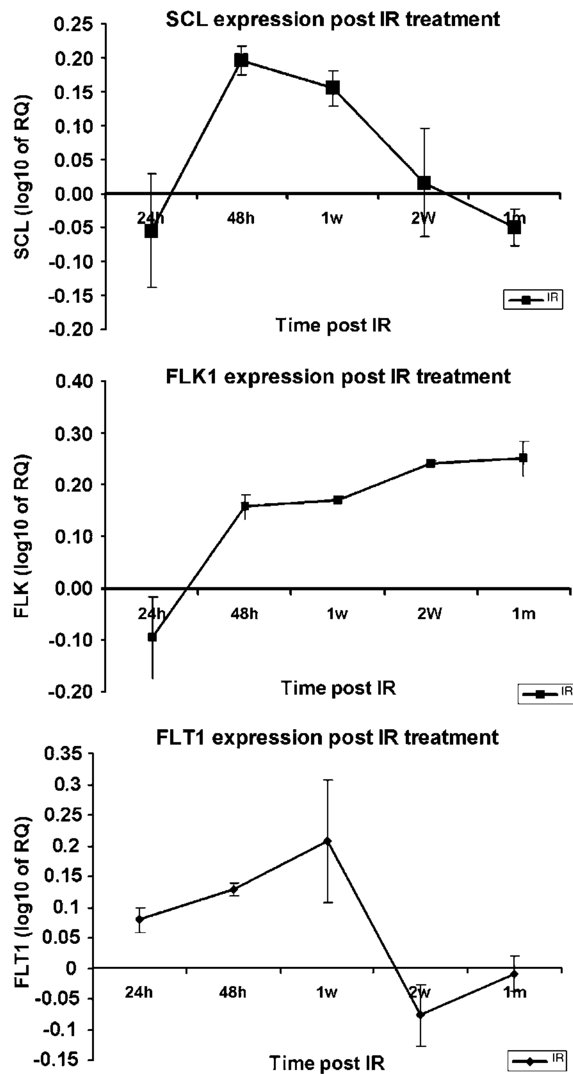


Figure 2 SCL, FLT1 and FLK1 gene expression following I/R injury to murine kidneys. Quantitative RT-PCR was performed for SCL, FLK1 and FLT1 at consecutive time points after ischemia. Three mice were analyzed for each time point. Fold expression (calculated as $\log_{10}$ of RQ) denotes the relative expression compared with sham kidneys. Data were calculated as average$\pm$s.d. Significant induction of SCL and FLK1 mRNA is noted with time: SCL, $P<0.05$, 48 h and 1w compared to time point 24 h; FLK1, $P<0.05$, 48 h, 1 week, 2 weeks and 1 m compared to time point 24 h. SCL, stem cell leukemia.

Biosciences). Fluorescence data were collected using 3-decade logarithmic amplification on $25–50\times10^3$ viable cells, as determined by forward light scatter intensity. Cells were labeled with CD45−, CD34−, c-Kit- and Flk-1-PE (BD Pharmingen). PE-rat IgG2a was used as isotype control.

Real-time PCR

cDNA was synthesized using Omniscript® Reverse Transcriptase (Qiagen, Hilden, Germany) on total RNA. Real-time PCR of mouse samples was performed using an ABI7900HT sequence detection system (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA) in the presence of SYBR-green (SYBR green PCR kit; Qiagen). This fluorochrome incorporates stoichiometrically into the amplification product, providing real-time quantification of double-stranded DNA PCR product. The following primers were designed, to amplify an 80–120 bp fragment with 50–65 °C annealing temperature: FLT1, 5′-AAGCGGTTCACCTGGACT GA-3′ and 5′-CCTTGCTTTTACTCGCTATTCTCA-3′; FLK1, 5′-GTG GTCTTTC GGTGTGTTGCT-3′ and 5′-TCTCCTACAAAATT CTTCATCAATCTTG-3′; SCL, 5′-CATGTTCACCAACAACAA CCG-3′ and 5′-GGTGTGAGGACCATCA GAAATCTC-3′; *β-actin*, 5′-CCTGTATGCCTCTGGTCGTA-3′ and 5′-CCATCTCC TGCTCGAAGTCT-3′. The relative initial amount of mRNA of a particular gene was extrapolated from a standard curve. For standard curve determination, we used a pool of all the samples, serially diluted in four $\log_2$ steps and run in parallel to the samples. The total volume of each reaction was 20 μl, containing 300 nM of each forward and reverse primer and 125 ng of cDNA. Appropriate negative controls were run for each reaction. All of the reactions were performed in triplicate. Optimization of the real-time PCR reaction was performed according to the manufacturer's instructions. For each analysis, transcription of the gene of interest was compared with transcription of the housekeeping gene *β-actin*.

Immunohistochemistry

Animals were perfused with Tris-buffered saline and tissues placed in zinc fixative (BD Pharmingen, San Diego, CA, USA) for 72 h, dehydrated in ethanol series, cleared in xylene, processed into paraffin wax at 60° C for a total of 40 min and embedded. Sections (5–6 mm) were placed on electrostatically charged slides (VWR International) and rehydrated. Histochemical detection of PLAP in tissue sections was performed as described.[23] For inhibition of endogenous phosphatases, the sections were incubated in pre-heated PBS at 75° C for 35 min. After a 10-min wash in AP buffer (0.1 M Tris-HCl pH 9.5, 0.1 M $MgCl_2$, 0.1 M NaCl), the slides were transferred into AP staining solution (0.1 M Tris-HCl pH 9.5, 0.05 M MgCl2, 0.1 M NaCl and BCIP/NBT substrate, Roche, Basel, Switzerland) and incubated in the dark at room temperature for 1 h. Tissue sections were counterstained with brazilin (Anachem, Luton, UK), dehydrated in ascending concentrations of ethanol, cleared in PronaHistoclear II (Pronadisa, National Diagnostics) and mounted in DPX (BDH, VWR Poole, UK). For immunofluorescence staining, after re-hydration 5 μm sections were blocked with 20% goat serum for 2 h followed by incubation with rabbit anti-PLAP (1:10 dilution; AbD Serotec) and rat anti-CD31 antibodies (1:20 dilution; BD Biosciences), followed by anti-rabbit Alexa 568 (1:500) and anti-rat Alexa 647 (1:500) (Invitrogen Molecular Probes, Eugene, OR, USA). After several washes in PBS, slides were fixed in neutral-buffered formalin 4% (Merck, Darmstadt, Germany) for 5 min, washed and incubated with rat anti-CD45 (BD Biosciences), followed by staining with anti-rat Alexa 488. All primary antibody incubations were performed for 2 h and secondary antibody incubations for 1 h. IsolectineB4 was also used for endothelial staining. Tissue sections were stained overnight with biotinylated isolectineB4 B4 (L2140; Sigma) followed by incubation with streptavidin Cy5.[32] Controls were performed by omitting the primary antibody at different steps of the staining procedure. Immunofluorescence image analysis was performed using a Delta Vision system with an Olympus IX-71 microscope (Applied precision; Issaquah, WA, USA). Halogen lamp and appropriate filter combinations were used (excitation filters 470/40, 555/28 and 640/20; emission filters 528/38, 617/73 and 685/40). The images were taken using a UPLAN FL N $\times$40/1.30 oil objective).

For detection of SCL lacZ/3′En, paraffin-embedded sections were labeled with immunoperoxidase applying rabbit anti-

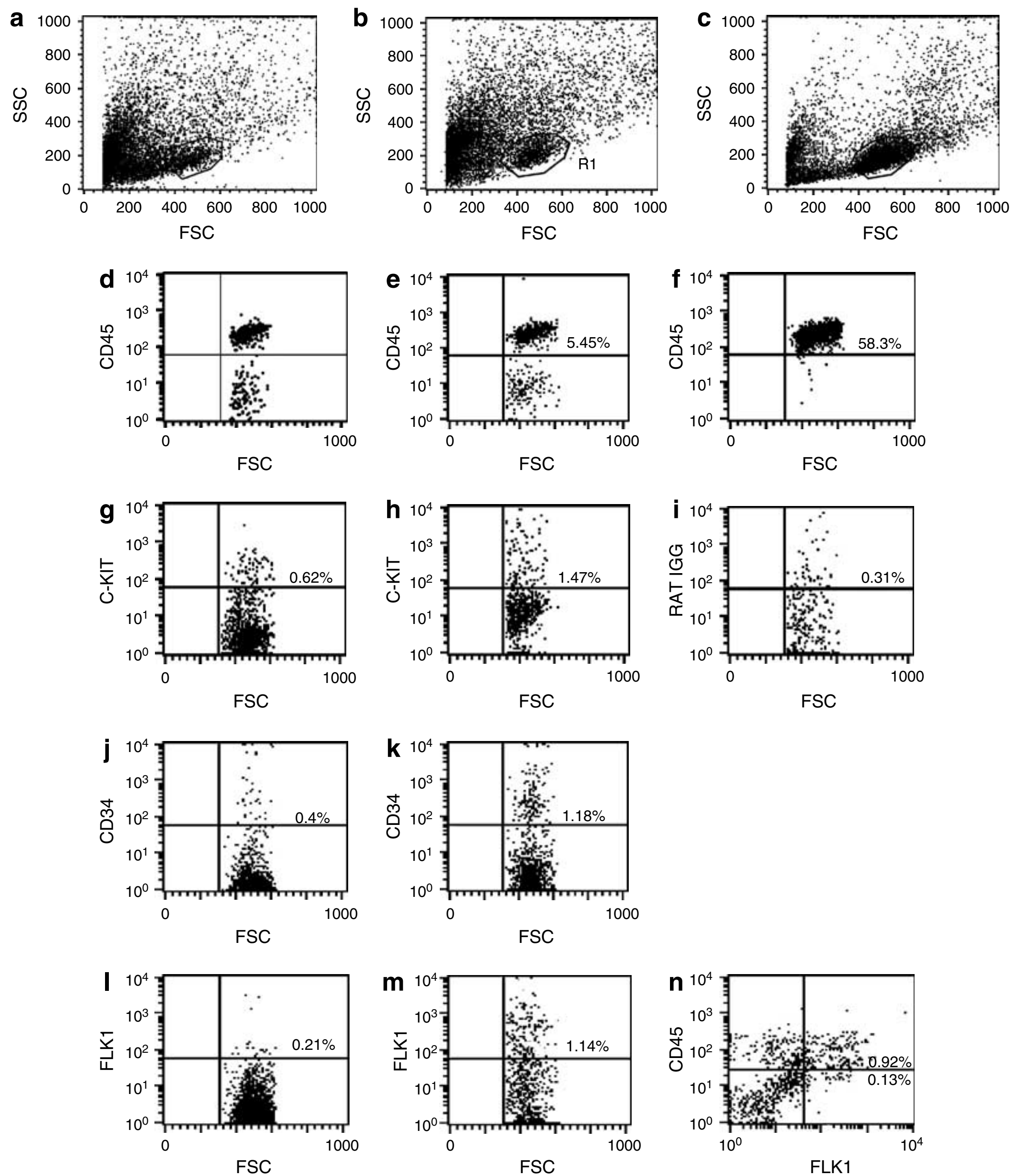
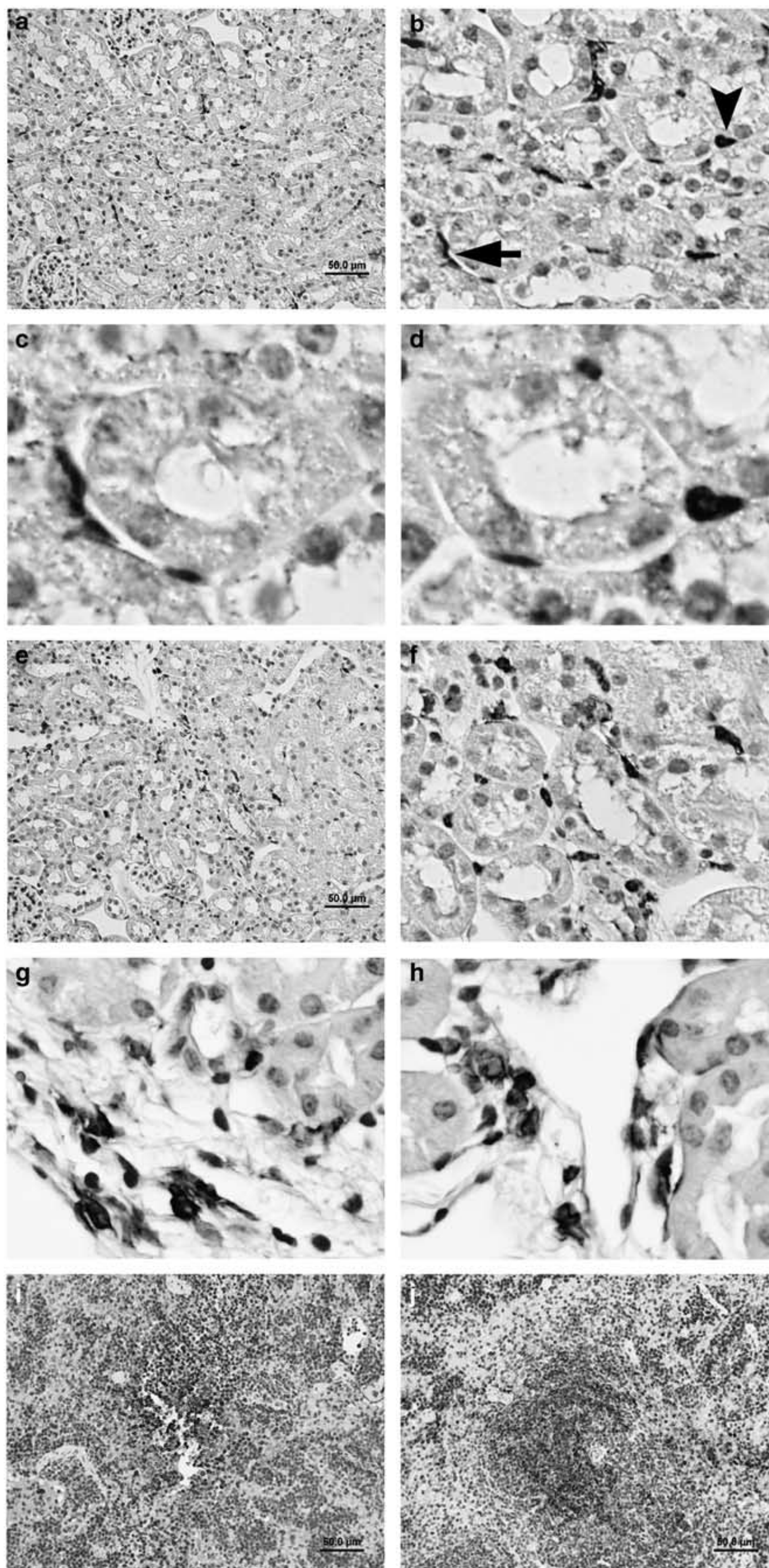


Figure 3 Hematopoietic progenitor cell surface markers in the ischemic kidneys. Flow cytometry analysis of (**a**) pre-ischemic renal, (**b**) post-ischemic renal and (**c**) spleen cell suspensions defines the renal 'lymphogate', with similar forward (FSC)/side scatter (SSC) parameters as spleen cells. Within the 'lymphogate' the majority of cells (**d**, **e**; pre- and post-ischemic kidney, respectively) or all of the cells (**f**, spleen) express the pan-hematopoietic marker CD45. (**g–m**) Analysis of hematopoietic cell surface marker expression within the renal 'lymphogate' in intact (**g**, **j**, **l**) compared to ischemic (**h**, **k**, **m**) kidneys shows elevation in expression. (**n**) A representative image verifying the hematopoietic origin of FLK1 (+) cells (double positive for CD45). (**i**) Isotype control. Percentage of total cells is shown in each panel. Experiments were repeated at least three times.

beta-galactosidase (Abcam, Cambridge, MA, USA; 1:2000, overnight incubation) and secondary reagent system, DAKO EnvisionTM + System, HRP (DAKO, Glostrup, Denmark), using DAB as chromogen.[31] Nuclei were counterstained with hematoxylin. Controls were performed by omitting the primary β-galactosidase antibody or by substituting the primary antibodies with goat IgG isotype.

Magnetic cell sorting

A double-step immunomagnetic staining technique was used to target the hematopoietic fraction for the immunomagnetic separation step following kidney digestion. The primary antibody used was a mouse anti-CD45 PE (BD Pharmingen) and the secondary antibody was anti-PE magnetic cell sorting (MACS) microbeads (Miltenyi Biotec, Auburn, CA, USA). At least three cycles of magnetic selection were used for cell purification as previously described.[31] For MACS, the labeling was performed on $3–5\times10^6$ kidney cells by incubating in PE-conjugated CD45 antibodies and a second incubation in magnetic PE microbeads according to the supplier (Miltenyi Biotech). For magnetic separation, we used mini-MACS columns (Miltenyi Biotech), applying $3–5\times10^6$ labeled cells on one column.[31] Both the positive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and negative fractions were applied to a second column before subjecting the cells to FACS analysis.

Results

Origin of SCL 3′En-expressing cells in the adult kidney

We have previously demonstrated that the high expression of SCL during nephrogenesis is abrogated in the adult kidney. Nevertheless, we could identify SCL 3′En-expressing cells in the adult renal interstitium.[24] The SCL 3′En driving β-galactosidase as a reporter gene is detected in hematopoietic cells and vasculature during development. However, a high degree of variation in reporter gene expression is observed in the vasculature of adult tissues, possibly due to the interaction of the regulatory elements of the 3′En and the lacZ reporter gene.[23] To enhance the detection of all the potential kidney cells expressing the SCL 3′En, we used the SV/PLAP/19 transgenic mice containing the +19 core enhancer element of the SCL 3′En. This construct reproducibly directed strong expression in adult hematopoietic and endothelial cells.[23] Transgene expression can readily be detected in peripheral blood (Figure 1a, middle panel). Detection of PLAP reporter gene by NBT staining on transgenic kidney sections showed high level of expression in the interlobular spaces, coinciding with the vascular network (Figure 1c, middle panel). To determine the nature of SCL3′En-expressing cells, we performed triple staining immunofluorescence for PLAP, CD45 and CD31 molecules. While some of the PLAP+ cells express CD45, the majority of PLAP+ cells are negative for CD45 and of endothelial (CD31+) or more fibroblastic appearance (CD31−) (Figure 1d, upper panel). Accordingly, three types of cell populations can be identified CD45+CD31− hematopoietic cells, CD45−CD31+ endothelial cells and CD45−CD31− interstitial cells. To determine if SCL 3′Enh cell subsets can be derived from the bone marrow, we generated bone marrow chimeras by infusing SCL 3′En bone marrow cells obtained from SV/PLAP/19 transgenic mice into pre-conditioned wild-type newborn hosts. FACS analysis of peripheral blood for expression of the reporter gene PLAP demonstrated stable chimerism at 5 months post transplant (Figure 1a, right panel). Engraftment of bone marrow-derived cells in the kidneys was analyzed in positive chimeras by histochemistry and immunostaining for PLAP. We identified cells of endothelial, fibroblastic and round blood morphology in kidney sections from chimeric mice stained with NBT for PLAP detection (Figure 1c, right panel). To further determine the nature of these cells, we performed co-immunostaining for PLAP, CD45 and CD31. Similarly, staining of chimeric kidneys revealed PLAP-positive cells that coexpress CD45 as well as PLAP+CD45−CD31− and PLAP+CD45−CD31+ cells (Figure 1d, lower panel). Thus, all types of resident SCL 3′En+ cells in the postnatal kidney can be derived from the bone marrow. To determine whether contribution of transplanted bone marrow cells into blood vessels following transplantation represents a more widespread phenomenon, we performed staining of the chimeric liver. To identify endothelial cells we used isolectine B4,[32] as CD31 could not be homogenously identified in the liver sections (Figure 1e, upper panel). The few donor-derived cells observed were predominantly localized around big vessels of the portal area, mostly composed of PLAP+CD45+IsoB4− cells and few PLAP+CD45−IsoB4+ (Figure 1e, lower panel). We could only identify one fibroblastic cell that was PLAP+CD45−IsoB4− in the 20 donor-positive liver fields, whereas this population was easily identified in the chimeric kidney (Figure 1e, lower panel).

Having identified PLAP+CD45−CD31− cells in the chimeric kidney, we determined whether donor bone marrow cells contain a similar fraction (Figure 1b). Transgenic bone marrow cell suspension analysis by FACS showed that the percentage on PLAP+ not expressing blood markers (CD45/Ter119) is low, around 0.1% (control not transgenic 0.0%). Both CD31+ (85%) and CD31− (14%) can be identified within the PALP+CD45/ter119− fraction (Figure 1b). Thus, although in a very small proportion all three populations can be identified in the bone marrow.

SCL, FLK1 and FLT1 mRNA expression in regenerating kidneys

We next determined whether intra-renal SCL mRNA is induced following I/R injury to the kidneys of SCL 3′En transgenic mice. Histopathology of adult kidneys 24–48 h after ischemia showing lumen obliteration with loss of brush border as well as the disappearance of nuclei and necrosis of tubular cells has been previously demonstrated.[28] We analyzed SCL transcript levels along with the VEGF receptors FLK1 and FLT1, which have been shown to participate in the angiogenesis of ischemic limb and heart[33] at consecutive time points after ischemic injury. Real-time PCR demonstrated an early decline followed by rapid upregulation of SCL mRNA, peaking 48 h after ischemia (Figure 2). This response declined thereafter and transcript levels returned to baseline at 2 weeks after ischemia. Similarly, FLK1 showed an initial reduction in transcript levels followed by significant elevation. In contrast to SCL, FLK1 mRNA continued to increase and peaked at 4 weeks post-ischemia. FLT1 mRNA, although mildly elevated at 1 week, did not achieve statistical significance and returned to baseline levels afterwards. Thus, ischemic injury induces rapid and transit induction of intra-renal SCL mRNA followed by a prolonged and significant induction of FLK1.

Elevation of hem-endothelial progenitor cells in the ischemic kidney

To determine whether postnatal induction of SCL after ischemic injury is associated with a systemic response involving recruitment of subsets of cells into the kidney bearing hem-endothelial progenitor markers, both ischemic and intact kidneys were digested and analyzed by flow cytometry for the expression of CD45, CD34, c-Kit and FLK1 at early (48 h) and late time points (4 weeks) (Figure 3). Analysis of the cell suspension after the addition of ACK for removal of RBCs in pre- and postischemic

Figure 4 *In situ* localization of lacZ/SCL 3′En in kidneys subjected to I/R injury. Immunostaining for LacZ was performed on intact kidneys (**a–d**) and kidneys 48 h after injury (**e–h**). (**a**) Basal expression of SCL LacZ/3′En; magnification, ×40. Expression is localized to endothelial-appearing cells ((**b**) arrow, original magnification ×40; (**c**) original magnification ×100) and fibroblast-like cells ((**b**) arrowhead, original magnification ×40; (**d**) original magnification ×100). (**e**) Expression of SCL 3′En is more prominent in ischemic kidneys (magnification, ×40), with similar localization of LacZ to elongated cell types (**f**, original magnification ×40), but also to clusters of more round cells residing in close proximity to the renal tubules in the perivascular space (**g** and **h**, original magnification ×40). Analysis of SCL 3′En expression in the spleen of mice subjected to I/R renal injury reveals prominent staining (**i**) compared to intact spleen (**j**), (**i**, **j**) magnification ×40. SCL, stem cell leukemia.

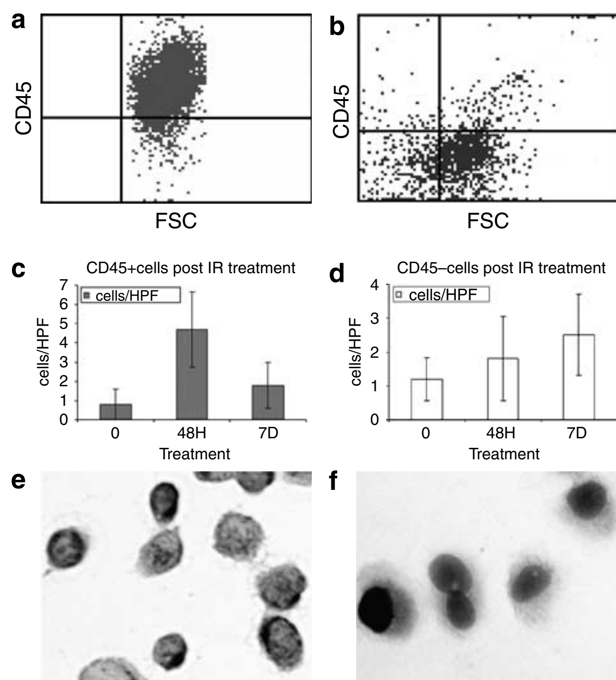

Figure 5 Increase in the number of SCL 3′En cells in ischemic kidneys. MACS sorting was performed on cell suspension from intact and ischemic kidneys separating hematopoietic CD45 (+) and non-hematopoietic CD45 (–) cell fractions. FACS analysis of the sorted fractions showing enriched (**a**) CD45-expressing hematopoietic population and (**b**) cell fraction lacking CD45. Immunostaining of these cell fractions for lacZ/SCL 3′En (5 high-power fields/kidney in three kidneys of each group) demonstrates (**c** and **d**) significant increases in the number of SCL 3′En-positive cells within the CD45 (+) and CD45 (–) cell fractions at 48 h (CD45 +) and 7 days (CD45–) after renal I/R injury. In ischemic kidneys, positively stained cells in the CD45 (+) fraction appear as mononuclear (large nuclei, small cytoplasm) (**e**, original magnification ×100) and in the CD45 (–) fraction as bearing larger cytoplasm (**f**, original magnification ×100). FACS, fluorescence-activated cell sorting; MACS, magnetic cell sorting; SCL, stem cell leukemia.

kidneys, and comparison to spleen showed a cluster of cells compatible with lymphocytes, which were gated ('lymphogate') (Figures 3a–c). CD45 expression in both kidneys and spleen is clearly demonstrated in this gate (Figures 3d–f, respectively). Nonspecific antibody binding estimated by isotype control staining was approximately 0.3% (Figure 3i). While c-Kit, CD34 and FLK1 expression in intact kidneys was just above or below that of isotype control (0.6%±0.09, 0.4%±0.066 and 0.3%±0.07), we found that in ischemic counterparts, 1.32%±0.14, 1.1%±0.11 and 1.02%±0.085 of the total cells were positive for c-Kit, CD34 and FLK1, respectively ($P<0.05$ in all instances) (Figures 3g–m). Moreover, most of the FLK1-expressing cells were double positive for CD45 (Figure 3n). These changes in cell composition within the renal 'lymphogate' were not observed at 4 weeks after I/R injury (data not shown). Thus, acute ischemic injury results in the early increase of cells expressing hematopoietic and endothelial progenitor markers in the adult kidney.

Changes in SCL lacZ/3′En (+) cells in the kidney following I/R injury

Following the observation that I/R injury induces expression of SCL mRNA and elevation of putative hem-endothelial progenitor cells, we determined the *in situ* expression of SCL 3′En in ischemic +6E5/lacZ/3′En transgenic kidneys. We chose to analyze the presence of β-galactosidase (LacZ) as the advantages of LacZ immunostaining for reporter gene detection specifically following I/R kidney injury have been recently demonstrated.[34] Also the reduced level of β-galactosidase-expressing cells in SCL/lacZ/3′En transgenic kidneys[23] should allow detection of an increase in interstitial progenitor SCL 3′En-expressing cells.

As shown for the SV/PLAP/19 transgenic kidneys, we found endogenous expression of SCL lacZ/3′En in the intact adult kidney (Figure 4a). Expression was mostly localized to cells appearing as mature endothelial (Figures 4b (arrow) and c) and fibroblast-like cells (Figures 4b (arrowhead) and d). At 48 h after renal injury, immunostaining of the SCL 3′En in the ischemic kidneys was more prominent compared with control intact kidneys. Similar to the intact kidneys, in the ischemic counterpart, we found expression of LacZ in endothelial-appearing and fibroblast-like cells (Figures 4e and f). However, more abundant were interstitial round cells positively stained for SCL 3′En. These clusters of SCL 3′En-positive cells were identified in the ischemic kidney in close proximity to renal tubules in the perivascular spaces (Figures 4g and h). Interestingly, I/R injury resulted in increased SCL 3′En expression in the spleen, suggestive of a systemic response involving migration of SCL 3′En (+) cells (Figures 4i and j).

To quantify the change in SCL 3′En-expressing cells and to determine whether hematopoietic or non-hematopoietic SCL 3′En-expressing cells are elevated in the adult kidney after I/R injury, we sorted the CD45 (+) hematopoietic fraction and the CD45 (–) non-hematopoietic fraction from the adult kidney by labeling cells with CD45-PE antibody and then applying the MACS system with anti-PE microbeads (Figures 5a and b). Immunostaining of the CD45 (+) sorted cells for LacZ and counting the number of positive cells demonstrated that a very small fraction of LacZ-expressing cells was present in the suspension obtained from the native kidney (0.8±0.8 cells/HPF×100) (Figures 5C). Analysis of the sorted fraction obtained from injured kidneys, 48 h and 7 days post-ischemia, demonstrated a significant elevation in the number of LacZ-expressing cells (large nuclei, small cytoplasm), of approximately six-fold (4.7±1.96 cells/HPF×100, $P=0.001$), followed by decline in cell number (Figures 5c and e). In contrast, immunostaining in the CD45-negative fraction revealed significantly increased LacZ (+) cells, bearing larger cytoplasm, only at 7 days after I/R injury (2.5±1.2 vs 1.2±0.64 cells/HPF×100, $P>0.05$) (Figures 5d and f). This elevation was modest compared to that observed for the hematopoietic cells. Thus, I/R injury induces rapid elevation of SCL 3′En-positive cells of hematopoietic origin.

Discussion

Reporter gene expression driven by the SCL 3′En characterizes the bipotential embryonic mesodermal progenitor, the hemangioblast, which gives rise to blood and blood vessels.[13,35] In adult life, bone marrow contains endothelial precursors with phenotypic and functional characteristics of embryonic hemangioblasts.[36] Given that SCL functions in hem-endothelial differentiation, it was surprising to find resident interstitial SCL 3′En-expressing cells in the adult kidney.[24] We now characterized these cells among which are hematopoietic (CD45+CD31–), mature endothelial (CD45–CD31+), and surprisingly a relatively large fraction of nonendothelial non-hematopoietic

cells (CD45−CD31−). Because only SCL 3′En^{+}CD45^{+} cells were observed in the peripheral blood, the latter do not represent circulating cells. In addition, all types of intra-renal SCL 3′En (+) cells can be traced back to a bone marrow origin, using genetic tagging in bone marrow chimeric mice, supporting the idea that at least some of the SCL 3′En cells, including the CD45-negative population, are not of intrinsic renal origin. Although in a very small proportion, all three populations of SCL 3′En-expressing cells can be identified in the bone marrow, indicating that the different fractions of intra-renal SCL 3′En (+) cells may be derived from three different types of progenitors in the bone marrow (that is hematopoietic stem cells , angioblasts and mesenchymal stem cells). Currently, we cannot discriminate if these populations expand or differentiate from a single progenitor population, such as the HSCs, previously identified in the PLAP+ fraction.[23]

Clearly, the bone marrow contributed to formation of mature endothelial cells in peri-tubular capillaries of the kidney, representing postnatal vasculogenesis. Identification of donor-derived endothelial cells in the liver suggesting that this phenomenon is widespread and not limited to the kidney is in agreement with previous reports.[37] Nevertheless, the near absence of bone marrow-derived SCL3′En + population negative for blood and endothelial markers in the chimeric liver demonstrates this fraction to be specifically associated with the kidney.

The finding of resident SCL 3′En (+) cells in the kidney that lack hem-endothelial differential markers is intriguing. One possibility is that the PLAP+CD31−CD45− cells are myofibroblastic cells as the SCL3′En is also expressed in vascular smooth muscle cells associated to big vessels in the kidney.[38] Indeed, analysis by immunofluorescence showed clear coexpression of the muscle-associated antigen α-actin and PLAP in arteries (data not shown). However, α-actin expression was almost undetectable in the capillary network of the kidney, supporting the idea that the CD45−PLAP+ cells are not of muscular nature. We favor the idea that these cells may signify local angioblastic/hemangioblastic progenitors that have migrated from areas of blood formation during development[14] and in adulthood from the bone marrow. This observation would parallel findings in skeletal muscle connective tissue where resident progenitor cells, shown to promote neovascularization, were tracked back to a bone marrow origin.[39]

Recent studies have addressed the roles of bone marrow-derived progenitor cells in the repair of ischemic injured kidneys.[40,41] While the ability of bone marrow cells to repopulate renal tubular cells is controversial,[34] contribution to repair of endothelial injury seems more promising. We have recently suggested a role for human CD34+ hematopoietic stem cells (enriched for the endothelial progenitor marker CD133) in vasculogenesis and not tubulogenesis, after their engraftment in ischemic murine kidneys.[28] Similar findings of peri-tubular vascularization were reported in postischemic kidneys[34] and more progressive renal injury.[42] Furthermore, in a different model system, Rookmaaker *et al*.[27] induced murine experimental glomerulonephritis and showed that whole murine bone marrow-derived cells (not fractioned into stem cell subtypes) participates in endothelial repair in injured glomeruli. In addition, intrarenal injection of culture modified bone marrow-derived angiogenic cells reduced endothelial injury in experimental glomerulonephritis.[43] Thus, the vasculogenic potential of bone marrow-derived cells, previously shown in heart infarct or retinal neovascularization,[44,45] can be demonstrated in injured kidneys.

All these studies however did not examine the involvement of endogenous hematopoietic-angioblastic progenitors after renal injury. We show that acute I/R results in rapid induction of SCL mRNA followed by the VEGF receptor FLK1. These results are in agreement with previous studies showing SCL as an upstream transcriptional regulator of Flk-1,[17] suggesting that the transient expression of SCL possibly induces the sustained expression of FLK1. SCL induction is associated with the elevation of cells expressing hematopoietic/endothelial progenitor markers and SCL 3′En-expressing cells in the kidney. Immunostaining showing accumulation of round SCL 3′En (+) cells in the interstitial spaces and cell sorting of the CD45 cell fraction suggests a rapid influx of hematopoietic-angioblastic progenitors after renal injury. Thus, while in the intact kidney CD45+ SCL 3′En(+) cells represent a small fraction of the SCL 3′En-expressing cells, they predominate in the early phase after ischemia (48 h). The recently documented SDF-1α/CXCR4 gradient promoting migration of blood and marrow cells into the ischemic kidney supports these findings.[46]

However, it is also possible that the intra-renal bone marrow-derived SCL 3′En (+) cells present in the intact adult kidney are induced to proliferate after ischemic injury, resulting in SCL induction. This is more likely to be relevant to the non-hematopoietic SCL 3′En (+) cells, which showed a modest but significant increase in number a week after ischemia. It will be of interest to determine if there is a distinct functional difference and role for the previously resident SCL 3′En (+) cells in the kidney versus the influx of SCL 3′En (+) cells from the bone marrow. Interestingly, Brunet de la Grange *et al*.[47] have revealed a major role for SCL in specification of adult hematopoietic myeloid progenitors, a type of hematopoietic cell that has been recently shown to stimulate angiogenesis in adult tissues via secretion of angiogenic growth factors.[48] Indeed, Huss *et al*.[49] showed that intravenous infusion of SCL(+) multipotent adult progenitor cell clones in a hind-limb ischemia transplant model supported muscle repair via enhanced local arteriogenesis rather than induction of new blood vessel formation. In this regard, further characterization, purification and functional analyses of specific SCL 3′En cell subsets could be informative.

Finally, considering into that SCL 3′ enhancer is dispensable for hematopoietic development,[50] it remains to be determined whether the observed 3′ enhancer-mediated upregulation of SCL in injured kidneys is mandatory for vascular repair.

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