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The migration of bone marrow-derived non-hematopoietic tissue-committed stem cells is regulated in an SDF-1-, HGF-, and LIF-dependent manner

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Abstract

Introduction: Recently we identified in bone marrow (BM) by employing chemotactic isolation to SDF-1 gradient combined with real time RT-PCR analysis a mobile population of CXCR4$^+$ BM mononuclear cells that express mRNA for various markers of early tissue-committed stem cells (TCSCs). In this study we evaluated whether TCSCs respond to other motomorphogens, such as hepatocyte growth factor (HGF) and leukemia inhibitory factor (LIF).
Materials and Methods: We again employed chemotactic isolation combined with real-time RT-PCR analysis to assess whether murine and human BM contain TCSCs that respond to HGF and LIF gradients. We also evaluated expressions of HGF and LIF in damaged organs.
Results: We noted that the number of TCSCs is highest in BM from young (1- to 2-month-old) mice and decreases in 1-year-old animals. Murine and human TCSCs 1) respond to HGF and LIF gradients in addition to an SDF-1 gradient, 2) reside in populations of BM-derived non-hematopoietic CD45$^-$ cells, and 3) are released (mobilized) from BM into the peripheral blood (PB) during tissue injury (e.g. after partial body irradiation).
Conclusions: These findings further support our theory of the BM as a "hideout" for TCSCs and we suggest that their presence in BM tissue should be considered before experimental evidence is interpreted simply as transdifferentiation/plasticity of hematopoietic stem cells. Since we demonstrated that not only SDF-1, but also HGF and LIF are upregulated in damaged tissues, we postulate that CXCR4$^+$ c-Met$^+$ LIF-R$^+$ TCSC could be mobilized from the BM into the PB, from which they are subsequently chemoattracted to damaged organs, where they play a role in tissue repair/regeneration.

Key words: CXCR4-SDF-1, c-MET-HGF, LIF-R-LIF, stem cell plasticity, stem cell mobilization, regeneration.

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INTRODUCTION

It has been suggested that bone marrow (BM)-derived hematopoietic stem cells transdifferentiate into tissue-specific stem cells (the so-called phenomenon of stem cell plasticity), but the possibility of committed tissue-specific stem cells pre-existing in the BM has not been given sufficient consideration [21, 24, 27, 36, 46]. We have recently demonstrated using SDF-1 chemotaxis combined with real-time RT-PCR analysis of cells that these early tissue-specific CXCR4$^+$ cells reside in normal murine BM and can be enriched by chemotaxis to an SDF-1 gradient [31, 49, 52, 53]. We also found that the expression of mRNA for SDF-1 is upregulated in damaged heart, kidney, and liver [31, 52]. Hence our findings provide a new perspective on BM not only as a home for hematopoietic stem cells, but also as a "hideout" for already differentiated CXCR4-positive tissue-committed stem/progenitor cells (TCSCs) that follow an SDF-1 gradient, could be mobilized into peripheral blood (PB), and might later take part in organ/tissue regeneration. This evidence prompted us to formulate

a novel hypothesis of circulation of CXCR4-positive TCSCs in the PB and their competition for SDF-1-positive niches in various organs/tissues [29, 52, 53]. Furthermore, our results supported the concept of the presence of heterogeneous populations of TCSCs in various organs [39, 46, 60, 65].

In the current study we provide new evidence in support of our hypothesis on the circulation of TCSCs and their competition for common niches. In order to explain the organ-specific homing of these cells, we evaluated whether other axes besides the SDF-1-CXCR4 axis could also be involved in regulating their trafficking. Based on the roles that both hepatocyte growth factor (HGF) [1, 4, 20, 41, 54, 58, 61, 62, 69] and leukemia inhibitory factor (LIF) [5, 23, 33, 44, 59, 72] play in the development of muscle, the liver, and the central nervous system, we hypothesized that, in addition to the SDF-1-CXCR4 axis, the HGF-c-MET and LIF-LIF-R axes could also potentially be involved in directing the mobilization, circulation, and homing to various organs of TCSCs. We investigated whether these factors 1) induce a migratory phenotype in murine pluripotent embryonic stem (ES) cells and 2) chemoattract TCSCs from a suspension of fetal liver- and adult BM-derived mononuclear cells.

Our work reported here provides further evidence that BM contains a population of early muscle, liver, and neural TCSCs which are enriched in young animals in a fraction of non-hematopoietic CD45-negative BM cells and can be mobilized into the PB during organ injury. Moreover, in addition to the SDF-1-CXCR4 axis, the HGF-c-MET and LIF-LIF-R axes play an important role in the trafficking/tissue distribution of these cells.

MATERIALS AND METHODS

Cell culture of ES cells

Cells of the murine ES cell line D3 (American Type Culture Collection, Rockville, MD, USA) were kept in permanent culture as described with the addition of LIF (5 ng/ml) to maintain their pluripotent characteristics. The medium was changed twice per week.

Collection of embryos and embryonic liver

To obtain age-defined embryos, female BALBc mice (The Jackson Laboratory, Bar Harbor, ME, USA) 12–24 weeks of age were placed in cages with individually caged male mice. Four hours later, a time defined as day 0 of gestation, the females were removed and examined for the presence of a vaginal plug. At the indicated time intervals post-coitus, the pregnant female mice were euthanized in a CO_2 chamber and the fetuses surgically excised. The fetal livers were microdissected from the embryos. The livers were triturated with a syringe, then filtered through a 40-μm-mesh strainer. Whole embryos were dispersed by triturating 20 times in a 1-ml syringe, then rinsed through a 40-μm-mesh strainer. All preparations were assessed for cell counts and viability using trypan blue exclusion on a hemocytometer.

Murine and human BM cells

Murine mononuclear cells (MNCs) were isolated from BM flushed from the femurs of pathogen-free 2 week-old and 1-, 2-, 6-, and 12-month-old female BALBc mice (The Jackson Laboratory, Bar Harbor, ME, USA) and enriched for light density MNCs by Ficoll-Paque centrifugation. Sca-1$^+$ cells were isolated by employing paramagnetic mini-beads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's protocol [55].

Light-density BM MNCs were obtained from four cadaveric BM donors (aged 52–65 years) and, if necessary, depleted of adherent cells and T lymphocytes (A^-T^- MNCs) as described [55]. The cells were enriched for CD34$^+$ cells by immunoaffinity selection with MiniMacs paramagnetic beads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's protocol and as described by us in detail [68]. The purity of the isolated CD34$^+$ cells was determined as >98% by FACS analysis. For some experiments we isolated CXCR4$^+$/CD45$^+$, CXCR4$^+$/CD45$^-$, CXCR4$^-$/CD45$^+$, and CXCR4$^-$/CD45$^-$ BM MNCs by employing FITC-α--CD45 and PE-α-CXCR4 MoAb (Pharmingen, Palo Alto, CA, USA) and a MoFlo cell sorter (Cytomation, Dako CA, USA) as described [66]. Briefly, cells were stained for 30 min at 4°C, washed twice, sorted, and spun down immediately after sorting to isolate RNA using the Qiagen RNA isolation kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. All studies were approved by the human ethics committees of the Universities of Louisville and Cracow.

Hematopoietic assays

For cell proliferation assays, murine BALBc or human sorted BM MNCs were plated in serum-free methylcellulose cultures in the presence of granulocyte--macrophage colony-stimulating factor plus interleukin-3 for colony-forming unit (CFU) granulocyte-macrophage colonies, erythropoietin plus stem cell factor for burst-forming unit erythroid colonies, and thrombopoietin for CFU-megakaryocytic colonies as described [50]. Using an inverted microscope, the murine hematopoietic colonies were scored on day 7 and the human hematopoietic colonies on day 12.

For the CFU-S assays, female BALBc mice (4–6 weeks old) mice were irradiated with a lethal dose of γ-irradiation (900 cGy). After 24 h the mice were transplanted by tail-vein injection with 1×10^4 CXCR4$^+$CD45$^+$ or CXCR4$^+$CD45$^-$ cells obtained from syngeneic mice. On day 12 their spleens were removed and fixed in Tellysyniczky's fixative and the CFU-S colonies were counted on the surface of the spleen using a magnifying glass as previously described [68].

Chemotactic isolation

We used a new method we devised for chemotactic isolation [29, 52]. Briefly, after the isolation of murine BM-, PB- and spleen-derived MNCs, the cells were re-suspended in RPMI with 0.5% bovine serum albumin (BSA) and equilibrated for 10 min at 37°C. The lower chambers of Costar Transwell 24-well plates, 6.5-mm diameter, 5-μM-pore filter (Costar Corning, Cambridge, MA, USA) were filled with 650 μl of RPMI with 0.5% BSA containing SDF-1 (200 ng/ml) or HGF (10 ng/ml) or LIF (50 ng/ml) or with media alone (control). Cell suspension aliquots of 100 μl were added to the upper chambers. The plates were incubated at 37°C, 95% humidity, 5% CO_2, for 5 h and evaluated under an inverted microscope. Cells from the lower chambers were collected and their numbers scored by FACS analysis (FACScan Becton Dickinson) as described [68].

Adhesion assay

Ninety-six-well microplates were coated with 0.5% gelatin in phosphate-buffered saline (PBS) and left at 4°C overnight as described [19, 37]. Following two washes with PBS, the remaining sites were blocked with 1% BSA for 1 h at 37°C and excess BSA was removed by washing. ES D3 cells cultured in DMEM with 0.5% BSA were treated with SDF-1 (300 ng/ml), HGF (10 ng/ml), and LIF (50 ng/ml) for 10 min. The cells (5×10^4/ml) were then added to gelatin-coated 96-well plates and incubated at 37°C in humidified 5% CO_2 conditions for 1 h. The plate was washed twice with PBS to remove unbound cells. The attached cells were then fixed for 5 min with methanol at room temperature and stained with Hema solution (Fisher Diagnostics, Middletown, VA, USA).

Transmembrane chemotaxis

ES D3 cells were seeded into 6-well plates in DMEM medium containing 15% fetal bovine serum. When the cells had adhered to the dish bottom, the medium was changed and the cells were made quiescent for 48 h with 0.5% BSA in DMEM. Cells were detached with 0.5 mM EDTA, washed in DMEM medium, resuspended in DMEM medium with 0.5% BSA, and seeded into the upper chambers (Costar Transwell) at a density of 10^5 in 200 μl. The lower chamber was filled with SDF-1 at 200 ng/ml, HGF at 10 ng/ml, or LIF at 50 ng/ml concentrations and the 0.5% BSA/DMEM medium was used as a negative control. The directional movement of the cells across an 8-μm-pore polycarbonate membrane towards the gradient of SDF-1, HGF, or LIF concentration was evaluated as described [19, 37]. Before assay, the membranes were covered with 50 μl of fibronectin (50 μg/ml) for 2 h at 37°C and overnight at 4°C. To determine whether the migration was stimulated by the gradient of the chemoattractant, in some samples HGF, SDF-1, or LIF was also added into the upper chamber to equalize the difference in concentration between the chambers. After 48 h the insert was removed from the transwell. Cells remaining in the upper chamber were scraped off with cotton wool and the cells that had transmigrated were counted either on the lower side of the membrane or on the bottom of the transwell.

Phosphorylation of intracellular pathway proteins

Western blots were done on extracts prepared from the ES D3 cell line (1×10^7 cells) which were kept in DMEM medium containing low levels of BSA (0.5%) to render the cells quiescent. The cells were then divided and stimulated with optimal doses of HGF (10 ng/ml), SDF-1 (300 ng/ml), and LIF (50 ng/ml) for 10 min at 37°C, and then lysed (for 10 min) on ice in M-Per lysing buffer (Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA). The extracted proteins were subsequently separated on a 10% SDS-PAGE gel and the fractionated proteins transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) as previously described [50, 68]. Phosphorylation of the intracellular kinases 44/42 MAPK (Thr 202/Tyr 204), AKT (protein kinase B) and STAT (Tyr 691) was detected using commercial mouse phospho-specific MoAb (p44/42) or rabbit phospho-specific polyclonal antibodies (all from New England Biolabs, Beverly, MA, USA) with horse radish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG as a secondary antibodies (Santa Cruz Biotech., Santa Cruz, CA, USA). Equal loading in the lanes was evaluated by stripping the blots and re-probing with appropriate MoAbs: p42/44 anti-MAPK antibody clone #9102 and anti-AKT antibody clone #9272 (New England Biolabs). The membranes were developed with an ECL reagent (Amersham Life Sciences, Little Chalfont, UK), dried, and subsequently exposed to film (HyperFilm, Amersham).

Fluorescent staining of the actin cytoskeleton

For the visualization of the actin cytoskeleton, cells were cultured for 12 h on glass coverslips in RPMI-1640 medium and supplemented with 0.5% BSA in the presence (10 ng/ml HGF, 200 ng/ml SDF-1, or 50 ng/ml LIF) or the absence of these chemokines as described [19, 37]. The cells were subsequently fixed in 3.7% paraformaldehyde/Ca- and Mg-free PBS for 15 min. They were permeabilized by 0.1% triton X-100 in PBS for 1 min at RT and stained with Alexa-phalloidin at a concentration of 500 ng/ml for 1 h. The stained cells were examined using a BX51 fluorescence microscope (Olympus America, Melville, NY, USA) equipped with a charge-coupled device camera (Olympus America, Melville, NY, USA). Each staining was repeated three times.

RT-PCR

Total RNA was isolated using RNeasy Mini Kit (Quiagen Inc., Valencia, CA, USA). mRNA (0.5 μg) was

reverse-transcribed with 500 U of Moloney murine leukemia virus reverse transcriptase. The resulting cDNA fragments were amplified using 5 U of *Thermus aquaticus* (Taq) polymerase. Primer sequences for CXCR4 were forward primer: 5'-CGG CGG CGG AAC TGC TAC GAA-3' and reverse primer: 5'-GGG GCG GGG GCG GAA ACT T-3', for c-met were forward primer: 5'-GGG TCG CTT CAT GCA GGT TGT GGT-3' and reverse primer: 5'-ATG GTC AGC CTT GTC CCT CCT TCA--3', for LIF-R were forward primer: 5'-GGG TCG CTT CAT GCA GGT TGT GGT-3' and reverse primer: 5'-ATG GTC AGC CTT GTC CCT CCT TCA-3'.

Real-time RT-PCR

For the analysis of Myf5, MyoD, myogenin, GFAP, nestin, α-fetoprotein, and CK19 mRNA levels, total mRNA was isolated from cells with the RNeasy Mini Kit (Quiagen Inc.). mRNA was reverse-transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Detection of Myf5, MyoD, myogenin GFAP, nestin, α-fetoprotein, CK19, and β-actin mRNA levels was performed by real-time RT-PCR using an ABI PRISM® 7000 Sequence Detection System (ABI, Foster City, CA, USA). A 25-μl reaction mixture contains 12.5 μl SYBR Green PCR Master Mix, 10 ng of cDNA template, 5'-ACC ATG GAT CGG CGG AAG G-3' forward and 5'-AAT CGG TGC TGG CAA CTG GAG-3' reverse primers for human Myf5; 5'-CTA GGA GGG CGT CCT TCA TG--3 forward and 5'-CAC GTA TTC TGC CCA GCT TTT--3' reverse primers for murine Myf5; 5'-CGG CGG CGG AAC TGC TAC GAA-3' forward and 5'-GGG GCG GGG GCG GAA ACT T-3' reverse primers for human MyoD; 5'-GGA CAG CCG GTG TGC ATT-3' forward and 5'-CAC TCC GGA ACC CCA ACA G-3' reverse primers for murine MyoD; 5'-AGC GCC CCC TCG TGT ATG-3' forward and 5'-TGT CCC CGG CAA CTT CAG C-3' reverse primers for human myogenin; 5'-GGA GAA GCG CAG GCT CAA G-3' forward and 5'-TTG AGC AGG GTG CTC CTC TT-3' reverse primers for murine myogenin; 5'-GTG GGC AGG TGG GAG CTT GAT TCT-3' forward and 5'-CTG GGG CGG CCT GGT ATG ACA-3' reverse primers for GFAP; 5'-GCA GGC CAC TGA AAA GTT CC-3' forward and 5'-TTC TCC TGC TCC AGG GCT T-3' reverse primers for nestine; 5'-CTG GAT GTG GCC CAT GTA CA-3' forward and 5'-ATC CAG CAC ATC TCC TCT GCA-3' reverse primers for α-fetoprotein; 5'--CAT GCG AAG CCA ATA TGA GGT-3' forward and 5'-TCA GCA TCC TTC CGG TTC TG-3' reverse primers for CK19; 5'-GGA TGC AGA AGG AGA TCA CTG-3' forward and 5'-CGA TCC ACA CGG AGT ACT TG-3' reverse primers for β-actin; 5'-CGT GAG GCC AGG GAA GAG T-3' forward and 5'-GA TGA GCA TGG TGG GTT GA-3' reverse primers for murine SDF-1. Primers were designed with Primer Express software. The threshold cycle (Ct), i.e. the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was subsequently determined. Relative quantitation of Myf5, MyoD, myogenin, GFAP, nestin, α-fetoprotein, and CK19 mRNA expression was calculated with the comparative Ct method. The relative quantitation value of the target, normalized to an endogenous control β-actin gene and relative to a calibrator, is expressed as $2^{-\Delta\Delta Ct}$ (fold difference), where ΔCt equals the Ct of the target genes (Myf5, MyoD, myogenin, GFAP, nestin, α-fetoprotein, CK19) minus the Ct of the endogenous control gene (β-actin), and ΔΔCt equals the ΔCt of the samples for the target gene minus the ΔCt of the calibrator for the target gene.

To avoid the possibility of amplifying the contamination of DNA, 1) all the primers for the real-time RT-PCR were designed with an intron sequence inside cDNA to be amplified, 2) reactions were performed with appropriate negative controls (template-free controls), 3) a uniform amplification of the products was rechecked by analyzing the melting curves of the amplified products (dissociation graphs), 4) the melting temperature (T_m) was 57–60°C, the product T_m was at least 10°C higher than the primer T_m, and finally 5) gel electrophoresis was performed to confirm the correct size of the amplification and the absence of unspecific bands.

Models of organ injury

Chemotherapy model. Female BALBc mice (4–6 weeks old) were exposed to cycloposphamide (200 mg/kg i.p.). Four animals in each group were sacrificed 6 h after exposure and mRNA was subsequently isolated from their BM MNCs.

CCl_4 exposure model. Acute CCl_4 liver and kidney injury was induced in BALBc mice by the i.p. injection of a single dose of carbon tetrachloride (CCl_4 0.8 ml/kg) diluted to 100 μl with corn oil (Sigma, St. Louis, MO, USA). Four animals in each group were sacrificed at various times after exposure (6, 24, and 72 h) and mRNA was subsequently isolated from the kidney and liver.

Irradiation model. Female mice BALBc mice were irradiated by 800 cGy. In some animals the femora and tibias were protected from irradiation by lead shields.

Statistical analysis

Arithmetic means and standard deviations of our FACS data were calculated on a Macintosh computer PowerBase 180 using Instat 1.14 (GraphPad, San Diego, CA, USA) software. Data were analyzed using the Student's *t*-test for unpaired samples. Statistical significance was defined as $p<0.05$.

RESULTS

Pluripotent ES cells express functional CXCR4, c-MET, and LIF receptors

The LIF-LIF-R axis plays an important role in regulating the biology of ES cells. Hence we asked whether

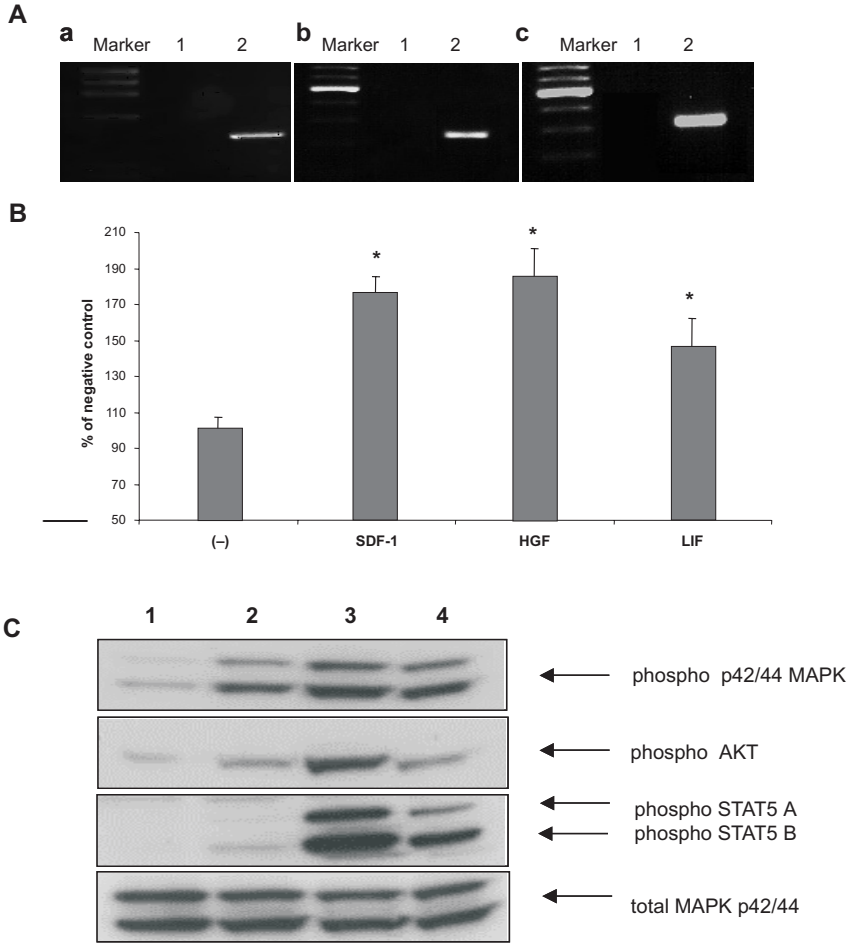

Fig. 1. Murine ES cells express functional CXCR4, c-Met, and LIF-R. **Panel A:** lanes 2 – expression of mRNA for CXCR4 (**a**), c-Met (**b**), and LIF-R (**c**) was evaluated by RT-PCR in the murine ES-D3 cell line. Lanes 1 – negative RT-PCR reactions (DNA instead of mRNA). Experiments were repeated three times with similar results. **Panel B:** chemotaxis of ES-D3 cells to medium alone (–), SDF-1 (300 ng/ml), HGF/SF (10 ng/ml), or LIF (50 ng/ml). The data were pooled together from three independent experiments (n=12). Data are expressed as means ±SD. *p<0.00001 compared with control. **Panel C:** phosphorylation of AKT. MAPKp42/44, STAT-1, 3- and -5 in ES-D3 cells. Cell were made quiescent and then stimulated with medium alone (lane 1), or for 10 min by SDF-1 (lane 2), LIF (lane 3), or HGF/SF (lane 4). Experiments were repeated three times with similar results.

murine ES cells, in addition to functional LIF-R [5, 44], also express functional CXCR4 and c-MET receptors. Figure 1A shows that ES D3 cells express all these receptors at the mRNA level. Since ES cells responded to stimulation by SDF-1, HGF, and LIF by phosphorylation of MAPK p42/44, AKT, and STAT-5A and STAT-5B proteins (Fig. 1C), these receptors are functional on these cells. Furthermore, studies of chemotaxis (Fig. 1B) and intracellular staining of actin cytoskeleton (not shown) revealed that all these factors chemoattracted ES D3 cells and reorganized intracellular actin filaments in these cells. Thus it appears that all three axes, CXCR4-SDF-1, c-MET-HGF, and LIF-R-LIF, are already involved in regulating the motility of pluripotent ES cells in the early stages of embryogenesis.

Chemotactic isolation reveals the presence of early muscle, liver, and neural TCSCs in fetal murine liver that respond to SDF-1, HGF, and LIF

In our previous study we showed that the BM contains TCSCs that could be isolated by means of an SDF-1 chemotactic gradient [28, 52]. Therefore we asked whether early TCSCs are already present in the fetal liver, a major hematopoietic organ in mammals in the second trimester of gestation. We also asked whether, in addition to SDF-1, these cells would be chemoattracted to LIF and HGF gradients.

To address this question, day-11 fetal murine liver-derived MNCs were exposed in the transwells to the SDF-1, HGF, and LIF gradient. We found that day-11 fetal murine liver already contains a population of small migrating cells that express markers for early muscle cells and that these cells are chemoattracted by LIF and SDF-1. Accordingly, mRNA for MyoD in fetal liver-derived MNCs separated by SDF-1 and LIF gradients was expressed 17±3-fold and 109±11-fold more, respectively, than control input fetal liver-derived MNCs. We also found that fetal liver MNCs contained a population of cells expressing early neural markers which are chemoattracted to LIF. Accordingly, mRNA for GFAP or nestin in fetal liver-derived MNCs separated by a LIF gradient was expressed ~4-fold more than control input fetal liver-derived MNCs. At the same time we observed an up to ~160-fold enrichment in mRNA for early liver cells (CK19) in cells isolated by SDF-1, HGF, or LIF chemotactic gradients compared with control input fetal liver-derived MNCs (not shown). Thus, fetal liver during the second trimester of gestation, in addition to being the "home" of HSPCs, also contains cells committed to muscle and neural tissues. Moreover, early liver precursor cells responded to SDF-1, HGF, or LIF gradients.

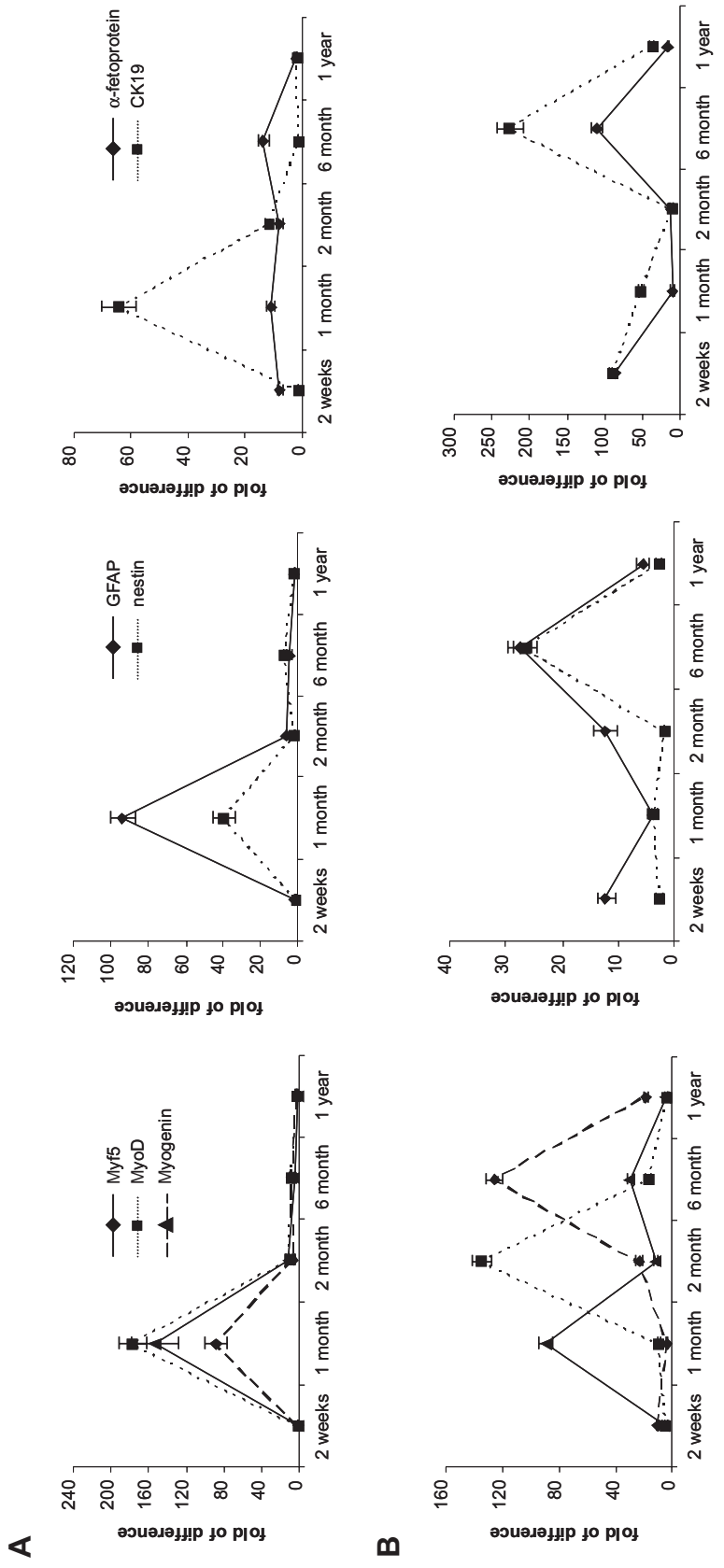

Fig. 2. Age-dependent real-time RT-PCR analysis of the expression of mRNA for early muscle, neural, and liver markers in murine BM MNCs isolated by chemotaxis to SDF-1, HGF, or LIF. BM cells were isolated from the lower transwell-chambers after chemotaxis to SDF-1 (**panel A**), HGF (**panel B**), and LIF (**panel C**) and the expressions of mRNA for early muscle (Myf5, MyoD, myogenin; left panels), neural (GFAP, nestin; middle panels), and liver (α-fetoprotein, CK19; right panels) TCSCs were compared between the same number of cells from the input and lower chamber by employing real-time RT-PCR. The data were pooled together from three independent experiments (12 mice/point). Data are expressed as means ± SD.

Chemotactic isolation to SDF-1, HGF, and LIF gradients reveals the presence of TCSCs in the BM

Next we evaluated the presence of cells expressing early skeletal muscle, neural, and liver markers in murine BM MNCs. To address this issue we isolated BM MNCs derived from 2 weeks-old and 1-, 2-, 6-, and 12-month-old mice that responded to SDF-1, HGF, or LIF gradients and evaluated these cells for the expression of markers for early TCSCs using real-time RT-PCR.

Figure 2 shows that mRNA for these cells was easily detectable in BM isolated from animals older than 2 weeks. This expression was highest in 1- to 2-month-old mice, which corresponds with the time of their rapid growth in body mass. It subsequently decreased with age, and finally these cells were poorly detectable in 1-year-old animals. Figure 2 also shows that the chemotactic response of these cells to SDF-1 (Fig. 2, panel A), HGF (Fig. 2, panel B), or LIF (Fig. 2, panel C) varies with the age of the mice. While the strongest response to SDF-1 was observed for BM MNCs derived from 1-month-old animals (Fig. 2, panel A), the highest responsiveness to HGF and LIF was observed for BM MNCs isolated from 2- to 6-month-old mice (Fig. 2, panels B and C, respectively).

Murine and human early TCSCs do not possess hematopoietic potential and are CD45$^-$

Since CXCR4 is widely expressed by both primitive and more differentiated hematopoietic cells, it is not surprising that the total number of BM MNCs that responded by chemotaxis to SDF-1 was ~100-fold higher compared with cells collected after exposure to HGF or LIF gradients. However, in contrast to cells isolated by an SDF-1 gradient, cells collected after chemotaxis to HGF or LIF neither grew any hematopoietic colonies *in vitro* nor formed day-11 CFU-S-derived spleen colonies *in vivo* after transplantation into lethally irradiated syngeneic littermates (not shown). This indicates that cells isolated by HGF and LIF gradients do not contain a potential admixture of HSPCs. Thus these data suggest that the expression of early tissue markers in cells isolated by HGF or LIF gradients is not related to the presence of some HSPCs which could express mRNA for these markers.

Next, to determine the relationship between TCSCs expressing markers for early muscle, endothelial, liver, and neural cells and HSPCs in cells isolated by an SDF-1 chemotactic gradient, we used a two-step purification strategy. In the first step the adherent cell-depleted BM MNCs were isolated by chemotaxis to SDF-1 and in the second step the cells present in the lymph-gate of the cells isolated by the SDF-1 gradient were sorted by FACS into a population of CD45$^+$ (hematopoietic) and CD45$^-$ (non-hematopoietic) cells (Fig. 3, panel A). We found, using real-time RT-PCR for markers of early TCSCs, that the expression of these markers strongly correlates with a population of non-hematopoietic CXCR4$^+$ CD45$^-$ cells (Fig. 3, panel B). To further confirm the non-hematopoietic character of the CXCR4$^+$ CD45$^-$ cells, we tested their ability to grow hematopoietic colonies *in vitro* and to form spleen colonies *in vivo* after transplantation into lethally irradiated mice (10^4 cells/animal). We found that these cells, in contrast to CXCR4$^+$ CD45$^+$ cells, do not grow hematopoietic colonies *in vitro* and do not form CFU-S colonies in lethally irradiated mice (not shown).

In parallel studies on human non-adherent BM MNCs, we also found that human BM also contains a population of small cells that show chemotaxis to SDF-1, HGF, and LIF that express early markers for skeletal, liver, and neural markers (Fig. 4). Like murine cells, these human cells (except for those isolated by chemoattraction to an SDF-1 gradient) did not grow hematopoietic colonies *in vitro* (not shown). Furthermore, by using a FACS sorter we isolated populations of CXCR4$^+$CD45$^+$, CXCR4$^+$CD45$^-$, CXCR4$^-$CD45$^+$, and CXCR4$^-$ CD45$^-$ cells from the lymph gate of the human adherent cell-depleted (A$^-$) BM MNCs. These cell populations were subsequently compared by real-time RT-PCR for expression of mRNA for early TCSCs. Table 1 shows that the expression of early tissue-committed markers correlated with the fraction of human non-hematopoietic A$^-$ BM MN CD45$^-$ cells. This expression was mainly detectable in a fraction of CXCR4$^+$ CD45$^-$ cells.

In control experiments, to exclude the possibility that stimulation of cells with SDF-1, HGF, or LIF may affect the expression of early tissue-committed cell genes, we examined whether stimulation by SDF-1, HGF, or LIF for 5 h (corresponding to the time of chemotactic isolation of murine or human BM MNCs) does not influence the expression of genes examined in this study, and no effects was found (not shown).

Thus our data suggest that both murine and human BM MNCs contain a population of small mobile TCSCs (different from the CD45$^+$ HSPCs) that respond to HGF and LIF gradients in addition to an SDF-1 gradient [28, 29, 30, 32, 52].

mRNA for SDF-1, HGF, and LIF is upregulated in damaged organs

To test the hypothesis that CXCR4$^+$, c-MET$^+$, and/or LIF-R$^+$ TCSPCs could be released/mobilized from BM into the PB during organ injury and subsequently chemoattracted to the damaged peripheral tissues by SDF-1, HGF, and/or LIF gradients, we evaluated the expression of mRNA for these factors in 1) BM damaged by cyclophosphamide and 2) liver and kidney after toxic injury by exposure to CCl_4. Figure 5 shows that mRNA for SDF-1, HGF, and LIF is strongly upregulated in damaged BM. We observed high expressions of mRNA for these factors 6 h after exposure to cyclophosphamide (Fig. 5, panel A), and all these factors were upregulated in kidney. Both LIF and HGF were also highly upregulated in liver after exposure to

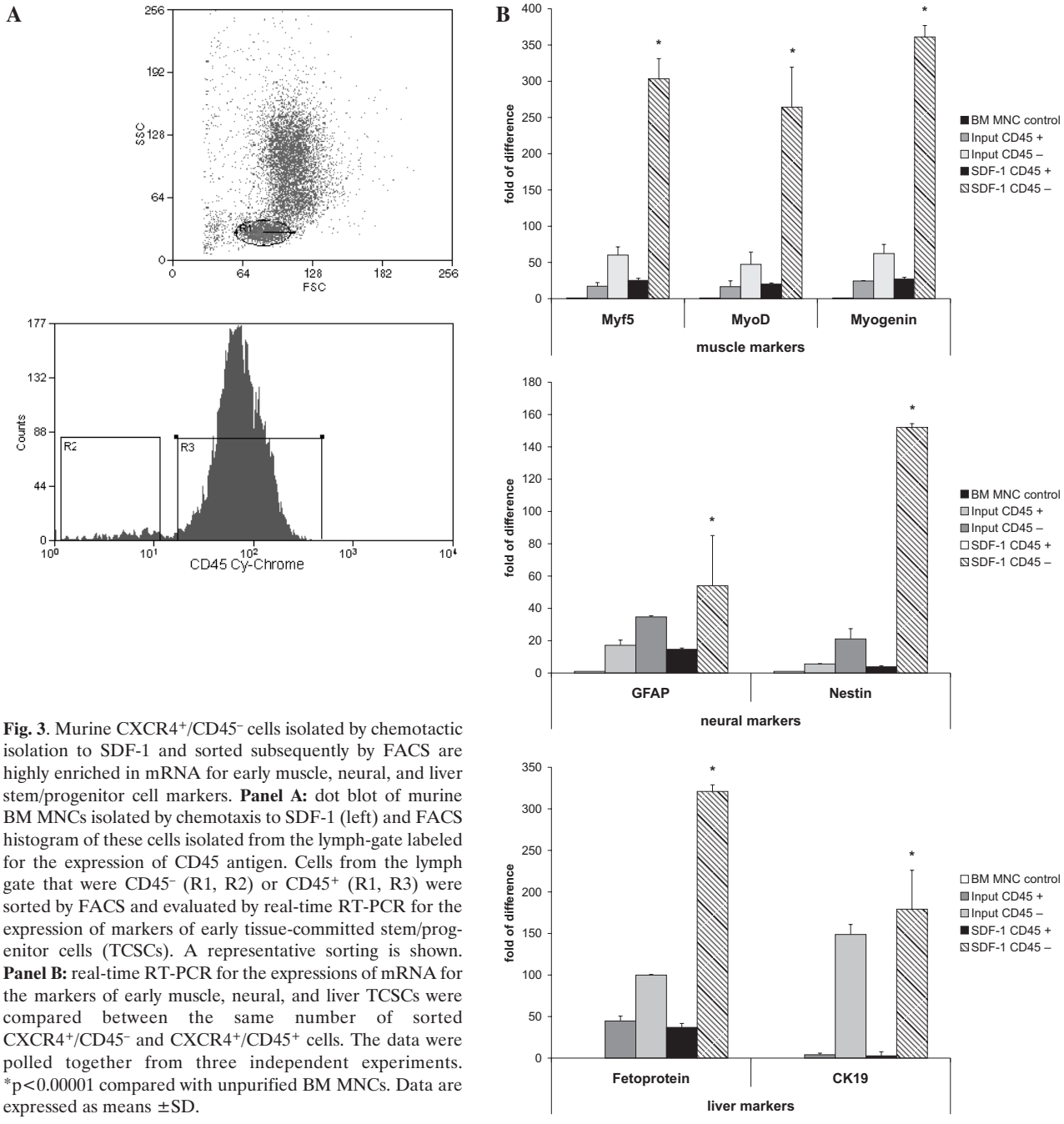

Fig. 3. Murine CXCR4$^+$/CD45$^-$ cells isolated by chemotactic isolation to SDF-1 and sorted subsequently by FACS are highly enriched in mRNA for early muscle, neural, and liver stem/progenitor cell markers. **Panel A:** dot blot of murine BM MNCs isolated by chemotaxis to SDF-1 (left) and FACS histogram of these cells isolated from the lymph-gate labeled for the expression of CD45 antigen. Cells from the lymph gate that were CD45$^-$ (R1, R2) or CD45$^+$ (R1, R3) were sorted by FACS and evaluated by real-time RT-PCR for the expression of markers of early tissue-committed stem/progenitor cells (TCSCs). A representative sorting is shown. **Panel B:** real-time RT-PCR for the expressions of mRNA for the markers of early muscle, neural, and liver TCSCs were compared between the same number of sorted CXCR4$^+$/CD45$^-$ and CXCR4$^+$/CD45$^+$ cells. The data were polled together from three independent experiments. *$p<0.00001$ compared with unpurified BM MNCs. Data are expressed as means ±SD.

CCl_4 (Fig. 5, panel B). This suggests that the damaged tissues may overexpress HGF and LIF in addition to SDF-1, which may mobilize into the PB and subsequently chemoattract circulating TCSCs into damaged areas.

Early muscle, liver, and neural tissue-committed stem cells are mobilized into the PB after partial body irradiation

To address whether early TCSCs could be mobilized into the PB during tissue injury we employed a model of irradiation-induced tissue damage. Mice were irradiated by 800 cGy. In some animals the femora and tibias were protected from irradiation by lead shields. Interestingly, these irradiation experiments, combined with real-time RT-PCR analysis, revealed that the number of circulating cells expressing early muscle, liver, and neural markers increased in the PB only in those animals whose long bones were protected from lethal irradiation. This suggested that during tissue injury caused by irradiation, these cells were released into the PB from the protected BM cavities. This was confirmed by our finding that an increase in the number of these circulating cells in the PB parallels their decrease in the BM cavities protected from irradiation (Fig. 6). This suggests that circulating

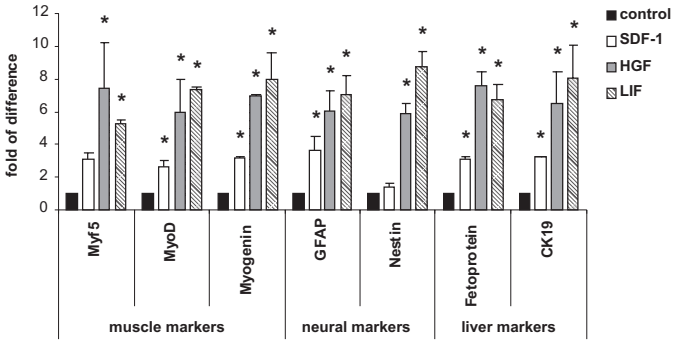

Fig. 4. CXCR4$^+$, c-Met$^+$, and LIF-R$^+$ cells that express markers of muscle and neural progenitor cells are present in human BM. Human BM MNCs isolated after chemotaxis to SDF-1, HGF or LIF were compared for the expressions of mRNA for early muscle (Myf5, MyoD, myogenin), neural (GFAP, nestin), and liver (fetoprotein, CK19) TCSC markers. The data were polled together from four independent experiments. *$p<0.00001$ compared with control. Data are expressed as means ±SD.

Table 1. Real-time RT-PCR analysis of the expression of early muscle, neural, and liver markers among different populations from equal numbers of sorted human A$^-$ BM MNCs (n=3 donors)

	Muscle markers Σ ±SD (Myf5, MyoD)	Neural markers Σ ±SD (GFAP, nestin)	Liver markers Σ ±SD (fetoprotein, CK19)
CXCR4$^+$CD45$^+$	17.14±1.12	13.15±0.85	12.37±0.98
	16.56±0.98	5.60±0.67	4.03±0.05
CXCR4$^+$CD45$^-$	123.35±5.58	34.71±2.65	99.89±4.65
	369.56±7.25	21.09±3.01	148.49±6.28
CXCR4$^-$CD45$^+$	0.01±0.001	1.03±0.02	1.02±0.01
	1.23±0.01	1.46±0.01	3.36±0.02

TCSCs were mobilized from the BM protected from irradiation. This observation further supports our hypothesis that BM contains a mobile pool of TCSCs that play a role in the regeneration of damaged tissues. However, final proof of this will require further study in selected tissue regeneration models.

DISCUSSION

Using a strategy of chemotactic separation combined with real-time RT-PCR, we recently reported that the BM harbors a population of small non-adherent CXCR4-positive cells that express mRNA for early muscle, neural, cardiac, and liver TCSPCs [28, 31, 52]. These non-adherent BM human and murine CXCR4$^+$ cells were enriched in CD34$^+$ and AC133$^+$ populations and in Sca-1$^+$ cells, respectively [52]. We postulated that these TCSCs "hide out" in the BM and could be mobilized into the bloodstream during stress or tissue injury [28, 31, 52]. We envisioned the following sequence of events: 1) the expression of SDF-1 is upregulated in damaged organs/tissues, 2) CXCR4$^+$ TCSCs are mobilized from tissues (e.g. BM) and circulate in the PB, and 3) TCSCs circulating in the PB are chemoattracted to the damaged tissues/organs for regeneration via the SDF-1-CXCR4 axis.

These observations, first made on BM tissue, caused us to question the phenomenon of so-called de-differentiation/plasticity of adult stem cells. We developed an alternative hypothesis based on the assumption that circulating stem cells may compete for common tissue-specific niches, which results in their lodging in various organs [29, 30, 32]. Thus the heterogeneity of stem cells

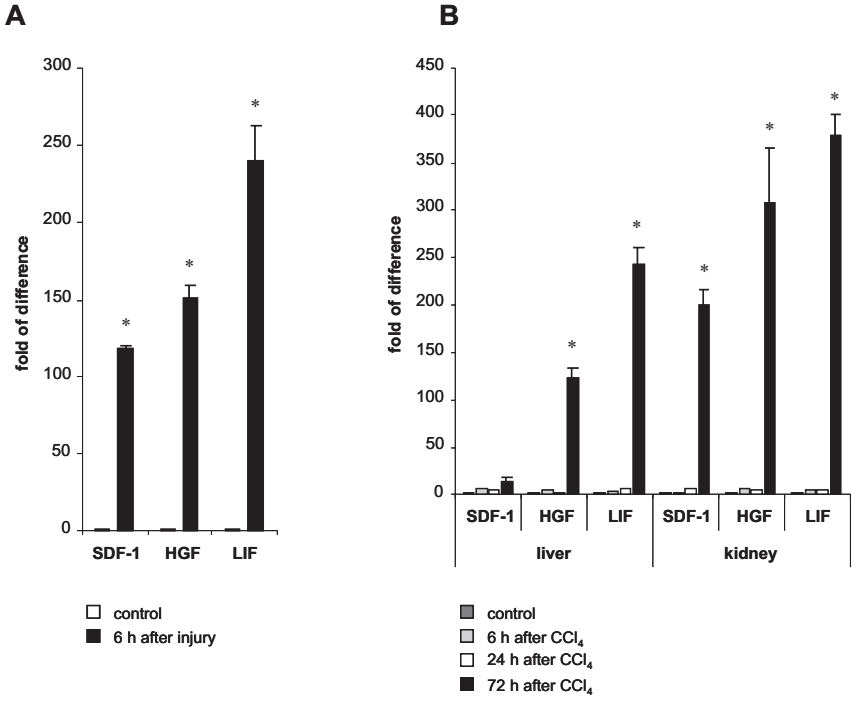

Fig. 5. Expression of SDF-1, HGF, and LIF is upregulated in murine cyclophosphamide-exposed BM and CCl_4--damaged kidney and liver. Changes in the expression of mRNA for SDF-1 between normal and damaged tissues after BM exposed to cyclophosphamide (**panel A**) or CCl_4-damaged kidney and liver (**panel B**) evaluated by real-time RT-PCR. The data were polled together from four independent experiments. *$p<0.00001$ compared with unpurified BM MNCs. Data are expressed as means ±SD.

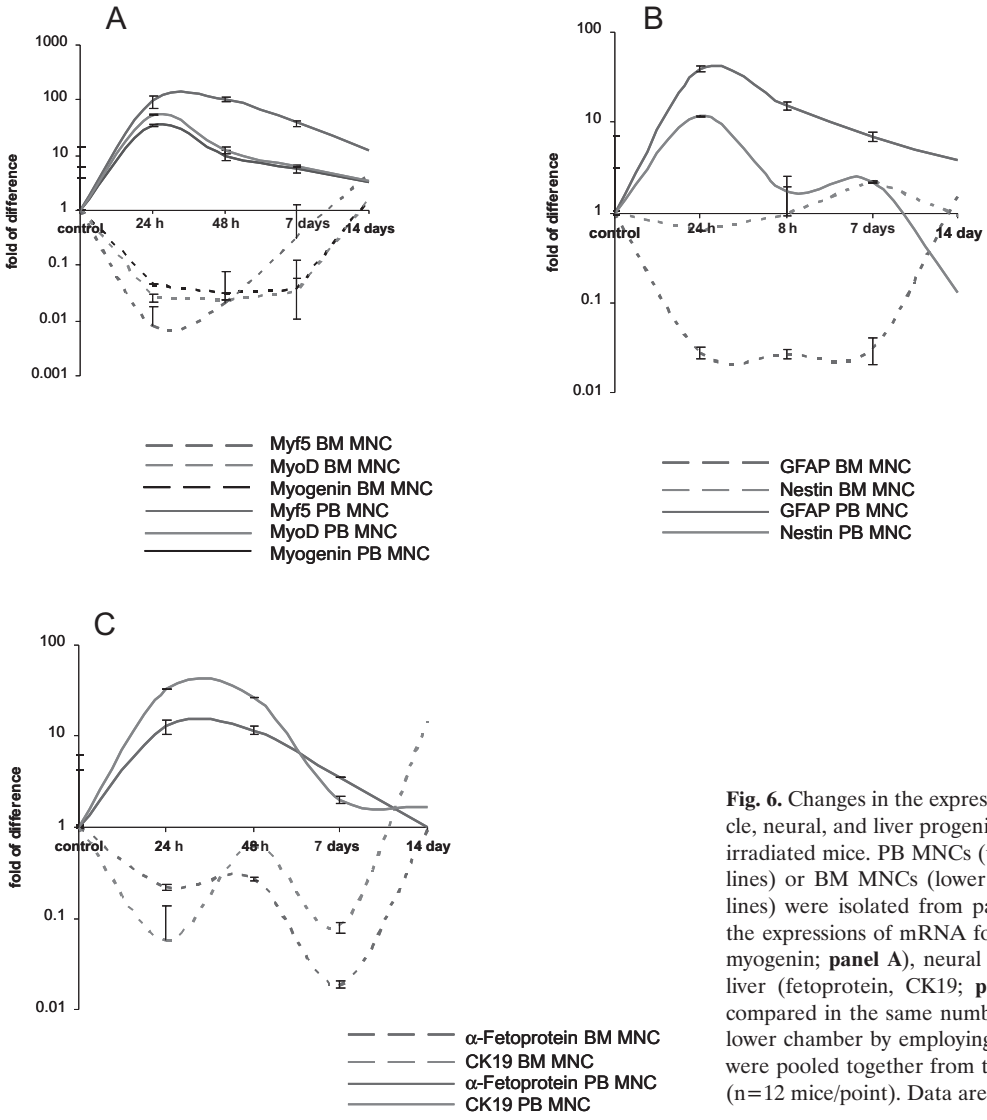

Fig. 6. Changes in the expressions of markers for early muscle, neural, and liver progenitors in PB and BM of partially irradiated mice. PB MNCs (upper parts of the graphs solid lines) or BM MNCs (lower parts of the graphs – dashed lines) were isolated from partially irradiated animals and the expressions of mRNA for early muscle (Myf-5, MyoD, myogenin; **panel A**), neural (GFAP, nestin; **panel B**), and liver (fetoprotein, CK19; **panel C**) TCSC markers were compared in the same number of cells from the input and lower chamber by employing real-time RT-PCR. The data were pooled together from three independent experiments (n=12 mice/point). Data are expressed as means ±SD.

in various organs (e.g. BM, muscles), rather than the plasticity of adult tissue-committed stem cells, could explain why it is possible to grow muscle cells from BM or hematopoietic colonies from muscle tissue [42, 47]. These data may also explain why cells expressing early neural markers were found among murine BM-derived Sca-1$^+$ CD34$^+$ cells [15] and why human CD34$^+$ cells cultured *in vitro* were found to express liver-specific markers [11].

In our hypothesis of the circulation of tissue-specific stem cells in the PB, BM takes center stage (Fig. 7) [51, 52]. The well-developed vessel system of BM as well as its secretion of SDF-1 and several other chemoattractants and cell-survival factors provides a unique microenvironment for chemoattracting various circulating cells. Accordingly, BM is the preferred site to which cells of breast and prostate cancers or pediatric sarcomas (e.g. rhabdomyosarcoma, neuroblastomas, nephroblastoma, retinoblastoma, and hepatoblastoma) metastasize, by means of the SDF-1-CXCR4 axis [6, 7, 14, 37, 45]. Since functional CXCR4 is expressed on skeletal muscle stem/progenitor satellite cells [54], primordial germ [2, 8], neural [3, 35, 40, 56, 71], kidney epithelial [22], retinal pigment epithelial [7], and liver oval stem cells [16, 26], all of these TCSCs can be chemoattracted to BM by SDF-1. In our hypothesis of stem cell circulation and competition for common niches, we also suggest the involvement of other potential chemoattractants that, in addition to SDF-1, could play a role in the preferential homing of circulating TCSC to their corresponding organs/tissues.

Here we focused on the potential contribution of HGF and LIF to this phenomenon, both important motomorphogens during embryonal development [1, 20, 25, 33, 59, 61, 69]. First we demonstrated that all three factors (SDF-1, HGF, and LIF) induce chemotaxis and the migratory-specific rearrangement of β-actin fibers in murine pluripotent ES cells. This supports the concept that CXCR4, c-MET, and LIF-R are already functional on the most primitive populations of stem cells. Next we demonstrated that these cells are present in day-11 murine fetal livers and that BM

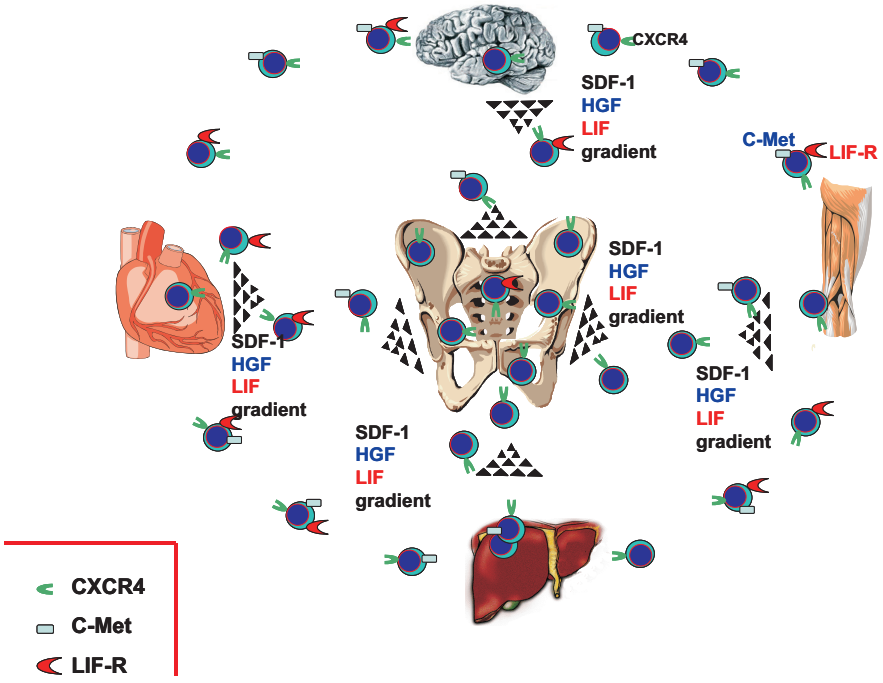

Fig. 7. Our hypothesis postulating the circulation of tissue-committed CXCR4$^+$, c-Met$^+$, and LIF-R$^+$ cells. The concept presented in this paper is based on the assumption that TCSCs circulate in the PB. In fact, PB has been shown to contain, in addition to HSPCs, circulating endothelial progenitor cells, stromal cells, and a poorly defined population of fibrocytic cells. Here we provide new evidence that the PB, in addition to HSPCs and endothelial progenitors, contains other circulating TCSCs. Although the percentage of early cells for muscle, liver, or neural tissue circulating in the PB is much lower than that of early HSPCs, these mobilized circulating tissue-specific cells may play an important role in tissue repair following injury. We postulate that mobilization of these cells occurs during tissue damage/injury (e.g. heart infarct, stroke, toxic liver damage). We hypothesize that CXCR4$^+$, c-Met$^+$, and LIF-R$^+$ cells after mobilization into the PB may be subsequently chemoattracted to the damaged tissues. In this context, BM tissue becomes a "hideout" not only for hematopoietic stem cells, but also for already differentiated circulating CXCR4$^+$ c-Met$^+$ and LIF-R$^+$ tissue-specific stem/progenitor cells. These cells are deposited early in development and, as result of this, reside in BM, and we hypothesize that they could play an important role in tissue/organ repair as a mobile reserve pool of tissue-committed progenitors. Whether these cells also originate in the BM from some more generic pluripotent stem cells requires further studies.

derived from young 1- to 6-month-old animals also contains TCSCs that can be isolated by chemotactic gradients to SDF-1, HGF, and LIF. Furthermore, our tissue injury data demonstrates that mRNA for these factors is highly upregulated in damaged tissues. This suggests that these factors play an important role in chemoattracting TCSCs that are mobilized from their niches into the PB by tissue/organ damage-related stress.

Furthermore, in this study we identified several new important characteristics of BM-derived TCSCs. First we found that these cells accumulate in the BM of intensively growing animals (1–2 months of age), which corresponds to the teenage years in humans [32, 51, 63]. This suggests that the number of circulating tissue-specific stem/progenitor cells is highest in rapidly growing individuals and we assume that at this time these cells are efficiently "deposited" as a reserve pool of cells for organ repair in BM tissue. The number of these cells subsequently decreases with the age of the animals and, as a result, are scarcely detectable in 1-year-old mice. These findings could explain why the regeneration process becomes less efficient in older individuals. We propose that in addition to telomere shortening in dividing stem cells, an age-related decrease in TCSCs that are deposited during development, for example in the BM, may explain the aging process [29, 30]. This concept, however, requires further study in appropriate animal models. Since we also found tissue-committed stem cells in human BM, further studies are planned to examine whether their number in human marrow decreases with age as in mice.

Next we found that the responsiveness of these cells changes somewhat during murine development (Fig. 2). While cells isolated from younger mice (1–2 months old) respond better to SDF-1, cells from older animals (2–6 months old) responded better to HGF and LIF gradients. The molecular basis of this age-related shift to different chemoattractants requires further study. This effect could be dependent on age-related changes, for example in receptor-responsiveness and/or selection of subsets of cells that preferentially respond to HGF or LIF or other age-related changes in signaling processes. To support this, it was reported that the homing properties of HSPCs in response to SDF-1 vary with age [67].

In this study we also provide evidence that the tissue-committed cells "hiding/residing" in the BM expressing early tissue markers are different from HSPCs. In the previous study we postulated that these cells are proba-

bly non-hematopoietic despite the fact that when they are isolated from human BM they express the CD34 and AC133 antigens and when isolated from murine BM they express the Sca-1 antigen [52]. Here we provide more extensive evidence showing that these cells 1) do not express the CD45 antigen, 2) do not grow hematopoietic colonies *in vitro*, and 3) do not form *in vivo* day-11 spleen colonies (CFU-S) in lethally irradiated littermates. Moreover, because these cells are $CD34^+$ and $AC133^+$ and are enriched in a non-adherent population of BM-derived cells, they are also not mesenchymal stem cells, despite being $CD45^-$.

Furthermore, our experiments with partial body irradiation of mice demonstrated that early TCSCs could be mobilized from the BM during tissue injury. Moreover, when present in the PB they may follow chemotactic gradients of SDF-1, HGF, or LIF and play an important role in tissue/organ regeneration. Supporting this are experiments using several models of tissue injury (e.g. irradiation, cyclophosphamide, CCl_4) that demonstrate that increased expression of mRNA for these factors occurs in injured tissues. However, we cannot exclude the involvement of other factors that affect the trafficking of these cells. To support this, vascular endothelial growth factor has been recently demonstrated to chemoattract early neural progenitor cells [70].

We are aware, however, that some issues require further clarification. First, we do not know the origin of the cells in the BM that are expressing markers for early TCSCs. These cells could be chemoattracted to the BM from the peripheral tissues or, alternatively, could originate locally from a population of early BM-residing stem cells that theoretically could be precursors both for hematopoietic and other tissue-committed unipotent stem cells. The presence of such a hypothetical generic pluripotent stem cell population residing in peripheral tissues has been postulated by several investigators [17, 36, 39, 46, 65]. However, the fact that the number of TCSCs decreases with age argues against their continuous supply from a putative population of multipotent stem cells residing in the BM. Our data shows that TCSCs accumulate in the BM tissue of young mice during rapid body growth. This suggests that they could migrate via the circulation into BM from peripheral tissues. Again, even if we assume that all or even a proportion of these early TCSCs home from the circulation into the BM, their final fate is still not known. Because the number of these cells decreases with age, it is likely that their expansion potential is limited. We also cannot rule out the possibility that has been shown for early hematopoietic stem cells which express mRNA for multiple lineages before being locked into a myeloid or a lymphoid fate [10]. A population of BM-residing early tissue-committed/pluripotent stem cells that we have isolated by means of an SDF-1-gradient could potentially express mRNA for multiple lineages before being "developmentally locked" into the various cell lineages. Our data, however, strongly suggest that these cells are distinct from populations of HSPCs. Finally, we are aware that because we worked with a population of FACS-sorted cells, further studies on more highly purified cell populations expressing tissue lineage-specific markers as well as studies at the single-cell level are needed to better characterize these cells. Experiments are under way in our laboratory to address these questions.

In conclusion, these findings further support our theory of the BM as a "hideout" for circulating TCSCs that could thus be isolated from the BM or mobilized PB and subsequently expanded for clinical use in tissue/organ regeneration. We further suggest that the implications of the existence of these cells in the BM should be considered before experimental evidence is interpreted simply as indicating the transdifferentiation or plasticity of adult stem cells. Thus our work provides an explanation as to why it is possible to grow early muscle, neural, liver, endothelial, renal tubular, and even pancreatic cells from BM mononuclear cells [9, 12, 13, 18, 34, 38, 43, 48, 57, 64]. Finally, the fact that the number of these cells decreases in the BM with age provides a new insight on aging as a process entailing the progressive loss of this population of mobile circulating tissue-committed stem cells. We envision that this newly identified regeneration-facilitating mechanism developed and remained preserved during the evolution in mammals.

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