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Lipocalin-2 controls the expression of SDF-1 and the number of responsive cells in bone

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ABSTRACT

Lipocalin-2 (LCN2) is a member of the lipocalin family, small secreted proteins functioning as modulators of many different physiological processes including cell differentiation, proliferation and apoptosis. LCN2 expression is also up-regulated in several pathological conditions, including inflammation and cancer. LCN2 synthesis has been described in epithelia, bone and cells of the immune system. Despite its wide expression the role of LCN2 remains to be fully elucidated. To better understand the role of this lipocalin in the bone/bone marrow system we generated transgenic mice over-expressing LCN2 specifically in bone under the control of a type I collagen promoter. In the bone marrow of these transgenic mice we observed an increased expression of SDF-1 that correlated with an increased number of CD34+/CXCR4+ (SDF-1 receptor) cells. To some extent, this appeared due to an enhanced cell proliferation rate. The higher level of the factor synthesis and the increased number of cells expressing its receptor was maintained during animal aging. Our results show that LCN2 could play a role in determining the number of CD34+/CXCR4+ precursor cells in the bone marrow thus contributing to the control of the bone marrow microenvironment.

1. Introduction

Lipocalins are a family of small secreted proteins that bind with high affinity to lipophilic molecules such as fatty acids, cholesterol, retinoids and prostaglandins [1].

Lipocalin-2 (LCN2), also named SIP24/24p3, NRL, and NGAL in mouse, rat, and human respectively [2], is a member of this family that was originally described as an acute-phase protein in the liver [3]. It is up-regulated after LPS stimulation in macrophages [4], in the lungs of treated mice [5] and in chondrocytes via p38/NF-kappa B activation [6]. The human orthologue, NGAL, is secreted from specific granules of activated human neutrophils [7] and it is induced in a variety of epithelial cells during inflammation [8].

LCN2 is also a component of the innate immune system and part of the acute-phase response to infection limiting bacterial growth by sequestering iron [9–11]. More recently it has been shown that LCN2 binds iron and deliver it to the cells through a small molecular weight siderophore [12–14]. In particular LCN2 recognizes a transmembrane receptor, recently cloned, which is internalized in the cell by endocytosis [11,15]. LCN2 is variously expressed in different types of cells where it can induce apoptosis/survival [16]. During inflammation, LCN2 is produced in different organs, such as liver [3] and lung [5]. LCN2 is highly expressed during tissue involutions of postpartum uterus [17] and of mammary gland during weaning [18]. Finally several bone marrow cells secrete large amounts of the protein during their differentiation [19,20].

Close interactions link bone tissue and blood cells. In adult life, osteoblasts, responsible for osteogenesis, and hematopoietic cells, responsible for hematopoiesis, are closely associated at the endosteal surface in the bone marrow (BM), forming the so-called osteoblastic niche. There is an increased evidence that osteoblasts are active components of this niche and that they synthesize and secrete several molecules controlling proliferation, homing, quiescence and survival of hematopoietic stem cells [21,22].

One of the most important bone niche factor, is the Stromal Derived Factor 1 (SDF-1), a chemokine of the C-X-C family involved in the recruitment of hemopoietic precursors, and playing a major role both during tissue repair and in the maintenance of the bone marrow hematopoietic niche [21]. SDF-1 was also found increased during inflammatory processes and cancer [23–26].

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We identified LCN2 as one of the osteoblast factors secreted in bone (manuscript in preparation). To investigate whether the increased synthesis of LCN2 could affect the hematopoietic compartment within the bone marrow in an in vivo system, we generated a transgenic mouse over-expressing LCN2 specifically in osteoblasts. In these transgenic mice we observed an increased number of CD34 positive cells expressing the SDF-1 receptor CXCR4 and we concluded that LCN2 could modulate the bone marrow microenvironment by controlling the expression of both the SDF-1 factor and the number of responsive cells.

2. Materials and methods

2.1. Generation and screening of transgenic mice

The full-length cDNA of the mouse LCN2 was cloned between the HindIII and EcoRI site of the mouse pro alpha 1 collagen 1 promoter plasmid containing a bovine polyadenylation addition site (Bpa). The transgene was injected into the pronuclei of FVB fertilized eggs and implanted into the CD1 pseudopregnant foster mothers. Three transgenic (tg) lines (tgA, tgB and tgC) were obtained and tg mice were identified by both PCR analysis, using the primers LCN2 forward 5′-ACCACGGACTACAACCAGTTCG 3′ and reverse, in the Bpa region to amplify only the transgene, 5′-GGAGGGGCAAACAACA GATGG 3′ and by Southern blot analysis of EcoRI digested genomic DNA, hybridized with a transgene specific probe (not shown).

Mice were bred and maintained at the Institution's animal facility. The care and use of the animals were in compliance with laws of the Italian Ministry of Health and the guidelines of the European Community.

2.2. ELISA and Western blot

BM was obtained from femurs of a pool of three wild type (wt) and three transgenic (tg) mice respectively. Bones were flushed with 500 μl of PBS. After centrifugation, an equal amount of total proteins of the supernatant, measured by Bradford assay, was assessed to determine SDF-1 level by ELISA and LCN2 by Western blot. SDF-1 was measured both in BM supernatants and in serum using the mouse SDF-1α ELISA immunoassay (R&D System, Minneapolis, MN) following manufacturer's instructions.

For LCN2 detection in calvaria and BM supernatant equal amount of proteins, measured by Bradford assay were loaded on a 12% reducing SDS–PAGE. After electrophoresis, the gel was blotted to a BA85 nitrocellulose membrane (Schleicher and Schuell GmbH, Dassel, Germany) according to the procedure described by Towbin et al. (1979). The blot was saturated for 16 h with 5% non-fat cow milk in TTBS buffer and incubated over-night at 4 °C with the anti-rabbit LCN2 (kindly provided by Dr. M. Nilsen-Hamilton). After washing, the detection was performed by a conjugated HRP-anti-rabbit IgG (Amersham Pharmacia Biotech) using the ECL Western blotting detection reagents (Amersham Pharmacia).

For LCN2 detection in the serum 1 μl of serum was loaded on a 12% reducing SDS–PAGE and processed as described above.

2.3. Metabolic iron radiolabeling and FPLC

To test the ability of LCN2 to bind iron, cultured undifferentiated osteoblasts from tg mice were washed with PBS and transferred into serum free medium containing $Fe^{55}Cl_3$ (20 μC) in the presence or absence of apo-enterobactin in equimolar concentration respect to the Iron. After three days of culture, medium was harvested and dialyzed against PBS, concentrated in a Centriprep-10 (Amicon Inc., Beverly, MA, USA) and loaded onto a Superdex-75 FPLC column (1 × 30 cm; Amersham Pharmacia Biotech, Milano, Italy) equilibrated with PBS. Flow rate was 0.5 ml/min. Fractions of 0.5 ml were collected and aliquots of each fraction were counted for radioactivity and analyzed by Western blot for LCN2 content.

2.4. Cell stimulation

Osteoblasts were obtained from wt mice. Briefly, calvaria from one month aged mice were isolated and cleaned. Bone chips were digested with sequential solutions of collagenase I (0.6 mg/ml), and Trypsin (0.5 mg/ml) in Ringer solution for 15, 20 and 30 min. Supernatants were discarded and the pellet was processed with two additional successive digestions of 50 and 60 min with collagenase I. The obtained cells were washed and plated in DMEM medium with 10% FBS.

Undifferentiated cells were stimulated with different concentration of recombinant LCN2 (generous gift of Dr. M. Nilsen-Hamilton) and recovered after 22 h to determine SDF-1 mRNA level by quantitative PCR.

2.5. Real-time RT-PCR

Total RNA was extracted from both tissue and osteoblast primary cultures of calvaria from wt and tg mice. Briefly both tissue and cells were homogenized in Tryzol (Sigma Aldrich) and RNA was obtained according to manufacture's protocol. Total RNA was digested with DNAase in order to eliminate contamination of genomic DNA. Specific mRNA levels were measured by semiquantitative or real-time quantitative RT-PCR using the PE ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured in parallel as endogenous control. The sequences of forward and reverse primers and of the TaqManTM fluorogenic probes were designed using the Primer Express 1.5 software as follows: GAPDH: forward 5′-TGTGTCCGTCGTGGATCTG-3′, reverse 5′-GATG CCTGCTTCACCACCTT-3′, probe 5′-TETTGATGTCATCATACTTGGCAG GTTTCTCCA-TAMRA-3′ SDF-1: forward 5′-AGCACAACAGCCCAAAGG AC-3′, reverse 5′-CTTGCATCTCCCACGGATG-3′, probe 5′-FAM-TTCCA GTAGACCCCCGAGGAAGGC-TAMRA-3′ LCN2 Tg: forward 5′-ACCA CGGACTACAACCAGTTCG-3′, reverse 5′-GGAGGGGCAAACAACAG ATGG-3′.

All probes were located at the junction between two exons. Relative transcript levels were determined from the relative standard curve constructed from stock cDNA dilutions, and divided by the target quantity of the calibrator according to the manufacturer's instructions.

2.6. Flow cytometry

BM was flushed from femur and tibiae of tg and wt mice. After washing with PBS, nucleated cells were separated by centrifugation with Ficoll (Sigma Aldrich). After an additional wash with PBS the expression of CD34 and CXCR4 was detected with a FITC-anti-CD34 (BD Biosciences) and with the PE-anti-CXCR4 (clone 2B11/CXCR4 BD Biosciences). An isotypic control antibody was used to exclude false positive cells. After staining, cells were analyzed by FACS.

2.7. BrdU incorporation and immunohistochemistry

BrdU incorporation was performed by intraperitoneally injecting tg and wt mice with 50 μg BrdU/gram of body weight 90 min before sacrifice. BM was collected and either embedded in Tissu Col (Baxter AG) gel or used for CD34+ cell isolation with immunomagnetic beads (Miltenyi) and a FITC-anti-CD34 (BD Pharmingen) followed by inclusion in Tissu Col (Baxter AG). BrdU was detected

by immunohistochemistry with a monoclonal anti BrdU antibody, according to the manufacturer's protocol (Roche). Several random images were taken from slides from three mice per condition and the positive cells were counted in percentage.

2.8. Statistical analysis

Statistical significance of differences observed during ELISA, quantitative RT-PCR, flow-cytometry, and cell counts were assessed by two-tailed *t* test. A chi-square test was used to determine statistical significance of differences observed during BrdU incorporation assay.

3. Results

3.1. Generation of transgenic mice over-expressing LCN2 in bone

To investigate the role of the LCN2 in the bone microenvironment, we generated three tg mouse lines (tgA, tgB and tgC) expressing the coding region of the murine LCN2 under an osteoblast specific modified type I collagen promoter, that is turned on at the beginning of osteoblast differentiation [27] (Fig. 1A). In order to screen transgenic mice and to discriminate genomic contamination in PCR amplification, construct-specific primers were designed to anneal to different exons. The tg mice of the three lines were all viable and fertile, and they expressed higher level of LCN2 mRNA with respect to the wt mice (data not shown). Fig. 1B shows the results obtained with the tgA line. As of the three lines the tgA line displayed the highest level of LCN2 mRNA expression (data not shown) we decided to focus on it our subsequent analyses. Therefore, the data thereafter shown refer to the tgA line unless otherwise specified.

Western blot analysis of proteins from calvaria pool of three animals, confirmed that this tissue expressed LCN2 and this protein was in a higher amount in tg with respect to wt mice (Fig. 1C).

3.2. SDF-1 expression in bone of wt and tg mice over-expressing LCN2

The level of SDF-1 mRNA, determined by real-time PCR, was higher in calvaria of tgA and tgB mice when compared to wt animals ($p < 0.05$) (Fig. 2A). The supernatant of BM flushed from tg mice displayed an amount of SDF-1 protein, measured by ELISA assay, about 2.9-folds higher than the BM supernatant from wt mice ($p < 0.005$) (Fig. 2B).

While local increase of SDF-1 was observed in calvaria of tg animals, this chemokine was not detectable in the serum by ELISA assay, suggesting a normal systemic SDF-1 concentration in these mice (data not shown). On the contrary LCN2 was up-regulated, besides in the bone tissue, both in the BM supernatant and in the serum of tg when compared with control mice as detected by Western blot analysis and ELISA assay respectively (Fig. 2C and D). To evaluate a possible specific effect of LCN2 in SDF-1 induction, we stimulated undifferentiated osteoblast primary cell cultures with different concentrations of either recombinant LCN2 (Fig. 2E) or LCN2 isolated from conditioned medium of cultured osteoblasts from tg mouse calvaria (not shown). In both experiments SDF-1 expression increased in a dose dependent manner related to the concentration of LCN2 added to the medium.

Given the capacity of LCN2 to bind iron through bacterial siderophores [11–13,15], we tested this capacity in the LCN2 purified from the osteoblast conditioned medium. Cells were cultured in presence of $Fe^{55}Cl_3$ with or without the bacterial siderophore enterobactin. Fig. 3 shows FPLC analysis. In particular with the black line is depicted the amount of protein and with the gray line the radioactive iron presence in the different fractions. Western blot performed with the antibody against LCN2 indicates the presence of the protein in the fractions from 20 to 24. Taken together the assays showed that LCN2 released from osteoblasts of tg mice at T0 was able to bind iron (arrow), but only when enterobactin was added to the culture medium (Fig. 3).

3.3. Isolation and characterization of bone marrow CD34 positive cells in mice over-expressing LCN2

We observed an age-dependent increase of CD34+ cell number within the BM of both wt and tg mice. In particular a sharp increase between 8 and 11 months was noticeable, followed by a decrease at 17 months (Fig. 4A). At all tested ages, CD34+ cells percentage was constantly significantly higher in tg mice ($p < 0.05$ comparing tg mice aged 4, 8, and 11 months with same-age wt animals) (Fig. 4A). Also tgB line displayed an increased number of CD34 positive cells although it did not reach the statistical significance (data not shown) that could be explained with a possible not sufficient increase of SDF in the BM supernatant observed in this line. The increased number of CD34+ hematopoietic progenitor cells in the bone marrow appeared to be due to an increased cell proliferation rate in tg mice, as resulted from the in vivo BrdU incorporation (Table 1). A higher BrdU incorporation was observed both in the unfractionated BM from tg mice and in the BM subpopulation of CD34+ cells isolated with immunomagnetic beads (Table 1).

CD34+ cells isolated from the BM of 11 months-old mice were further analyzed by FACS for the expression of the SDF-1 receptor CXCR4. An increase in the double positive CD34/CXCR4 was noticed in the BM from tg mice (Fig. 4B). Moreover, despite the increase in the double positive CD34/CXCR4 in the BM, the percentage of this cellular subpopulation in the peripheral blood remained almost unchanged (data not shown).

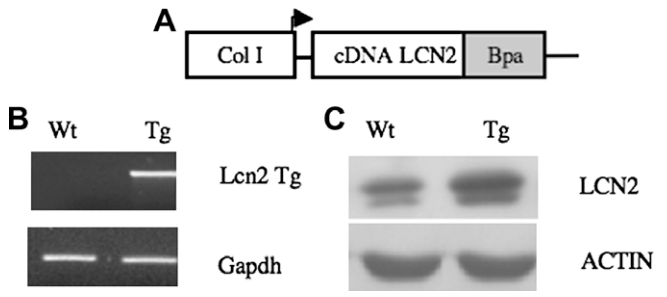

Fig. 1. Over-expression of LCN2 in tg mouse line A (tgA). (A) Transgene construct showing the LCN2 cDNA under the modified type I collagen promoter and with a Bovine Poliadelination Site (Bpa); (B) semiquantitative RT-PCR of transgene expression in calvaria of wt and tg mice. Specific primers for the transgene were used. GAPDH primers were used to normalize the PCR; (C) Western blot analysis of LCN2 on homogenized calvaria of wt and tg mice. The same amount of proteins was loaded on each lane.

4. Discussion

An enhancement of LCN2 expression has been shown in several physiological and pathological processes, but a clear role for this protein has not been described. Several in vitro cell culture systems had been proposed to study LCN2 function [9,19,21,28]. Nevertheless, although a mouse model of LCN2 deficiency has been generated [29,30], no animal models with LCN2 over-expression have been reported. Given our finding that LCN2 is expressed in bone, to elucidate LCN2 function at least in the skeletal system, we generated a transgenic mouse over-expressing LCN2 in bone.

In this last decade several papers showed the importance of the bone marrow microenvironment as a niche that controls at the same time hematopoiesis and skeletal turnover. Endosteal surface

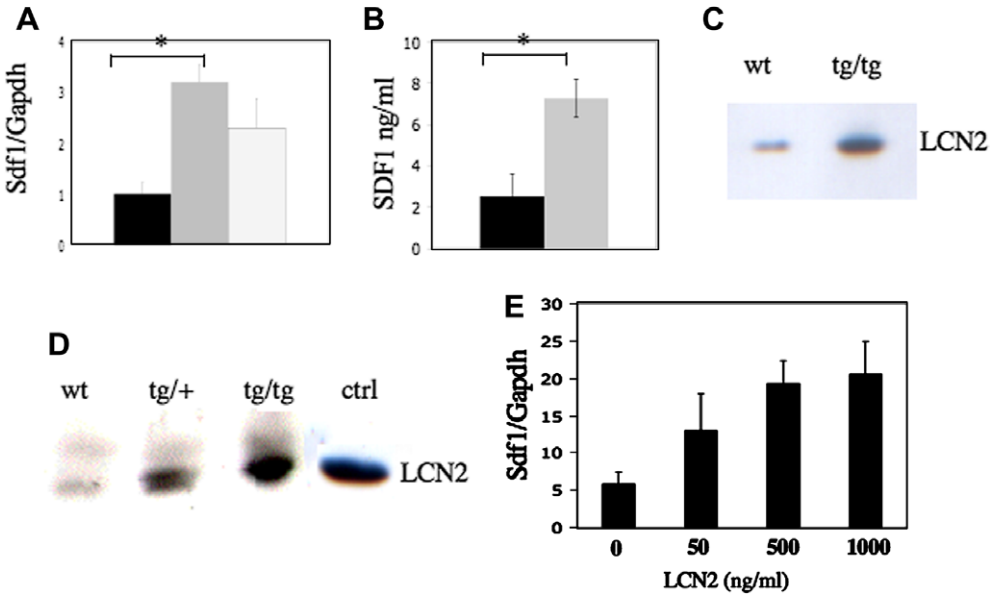

Fig. 2. Induction of SDF-1 expression in bone of tg mice and LCN2 detection in the serum. (A) Quantitative RT-PCR expression of SDF-1 mRNA in wt and tg mice calvaria. Black columns represent wt mice, gray column tgA and white column tgB mouse line. Data are mean ± SD, $n = 3$. *Indicates $p < 0.05$. (B) ELISA assay for detection of SDF-1 protein in equal amount of total proteins from the supernatant of flushed BM of normal and tgA mice. Data are mean ± SD, $n = 3$. *Indicates $p < 0.05$. (C) Western blot analysis for the detection of LCN2 in the BM supernatant of wt, tgA/tgA mice. Equal amount of protein of a pool of three mice was loaded on each gel lane. (D) Western blot analysis for the detection of LCN2 in the serum of wt, tgA/+, tgA/tgA mice. Equal amount of serum was loaded on each gel lane; conditioned media of MC615 cellular line, stimulated with LPS, were loaded as a control (ctrl). (E) Quantitative RT-PCR for SDF-1 mRNA in undifferentiated osteoblasts primary cell culture obtained from wt mice calvaria stimulated with recombinant LCN2, at different concentration for 22 h. Data are mean ± SD, $n = 3$.

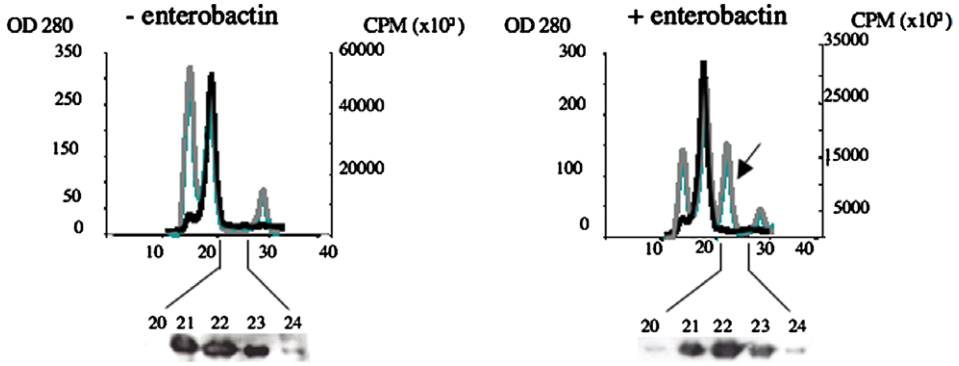

Fig. 3. Binding ability of LCN2 to iron. Serum free conditioned media of tg undifferentiated osteoblasts cultured with $Fe^{55}Cl_3$ in the presence (right panel) or in the absence of enterobactin (left panel) were loaded on the FPLC column. Black lines indicate the protein amount as measured in OD_{280} and the gray lines the amount of radioactive iron present in the different fractions of the column expressed in CPM. In particular fractions 20–24 eluted from the FPLC were assayed by immunoblot with anti LCN2 polyclonal antiserum shown below each graph. The addition of enterobactin to the culture enabled LCN2 to bind iron, as shown by the arrow, indicating the appearance of a gray peek of cpm in fractions 21, 22, 23 where LCN2 is also present.

and bone marrow vasculature are important components of this niche. In particular osteoblasts and bone marrow cells share soluble factors, adhesion molecules and membrane receptors that influence stem cell proliferation, differentiation and functions [21,22,31,32]. Several physiological and pathological conditions can modify the bone marrow microenvironment. The interaction between SDF-1, a chemokine of the C-X-C family, expressed by endosteal osteoblasts and its receptor CXCR4, expressed by hematopoietic stem cells, could regulate survival, anchorage and quiescence of stem cells [33–36].

In this manuscript we reported that LCN2 over-expression increased SDF-1 expression in bone tissue and in bone marrow of tg mice and resulted in a higher percentage of CD34+ cells in the bone marrow, expressing the SDF-1 receptor CXCR4. SDF-1 increase was specifically induced by LCN2. In fact, two different sources of LCN2, recombinant and purified from conditioned culture medium, induced in vitro the same effects on normal stimulated osteoblasts. Moreover, also the other tg line, obtained by independent microinjection, displayed mRNA increase for SDF-1 as shown by real-time PCR. In particular the fact that LCN2 from conditioned medium bound radioactive iron only with the addition of the siderophore suggested that the iron-lacking form of LCN2 (apo-LCN2) could induce the SDF-1 increase.

The tg mice presented a higher concentration of the LCN2 in the serum. Therefore they can be considered a good in vivo model for future study on the systemic effect of LCN2 over-expression. On the contrary, despite the local SDF-1 increase, no SDF-1 was found in the serum of tg mice. This could correlate with the absence of a statistically significant increase of the CD34+/CXCR4+ peripheral cell population. However, we found an increment with age of CD34+ cells in the BM. The expression of CD34 marker in these cells reflects a dynamic condition that changes during the develop-

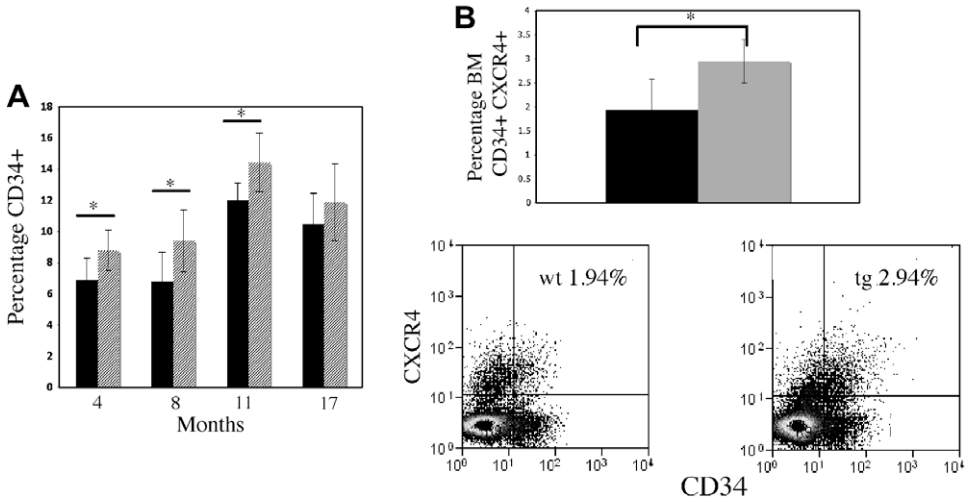

Fig. 4. Hematopoietic progenitor analysis in LCN2 over-expressing mice. Black columns represent wt mice and gray column tg line. (A) FACS analysis on BM of wt and tgA mice at different ages showing the percentage of CD34+ cells. Data are mean ± SD, $n \geqslant 5$. *Indicates $p < 0.05$; (B) BM percentage of double positive CD34+/CXCR4+ from wt and tg mice at 11 month age. Data are mean ± SD, $n = 5$. *Indicates $p < 0.05$. Lower panels in (B) are representative dot plots of CD34+ and CXCR4+ in tg and wt mice.

Table 1
Percentage of BrdU positive cells in total BM cells of wt and tg mice. Cell counting was performed on several sections of three different mice for each condition.

	BM cell			
	n cell tot	*n* cells BrdU+	% cells BrdU+	*p*
wt	1324	114	8.6	
tg	1206	177	14.7	<0.0001
	BM CD34+ cells			
	n CD34+ tot	*n* CD34+ BrdU+	% CD34+ BrdU+	*p*
wt	4520	706	18.5	
tg	3055	872	36.5	<0.0001

mental stages and the kinetic state of cells. In particular, although CD34 was considered the best-known marker of hematopoietic stem cells (HSC), the acquisition of this membrane antigen reflects the activation/kinetic state of HSC and it is reversible [28]. In the BM dormant and proliferating/activated HSC coexist. Self-renewing HSC are CD34+ and blood cell progenitors arise from these cells during normal homeostasis [30,37,38]. The majority of stem cells in the BM of normal adult mice are CD34− [39], but they can acquire the CD34 marker under different conditions. Treatment with 5-fluorouracil (5-FU) or interleukin 11 (IL11), mimicking stress conditions, leads to the activation of cells with the acquisition of the CD34 marker. LCN2 could represent a factor mediating the induction of CD34 expression and stress molecules. Furthermore, we showed that the increment of CD34+ cells was due, to some extent, to an enhanced proliferation rate of this cell population in the BM of tg mice. In our tg mice CD34+ cells remained in the BM, and their number was not altered in the peripheral blood showing a local effect of LCN2.

In conclusion, here we described LCN2 as a molecule able to modify bone marrow microenvironment and hematopoiesis. Further studies are needed to clarify the molecular mechanisms involved in the function of LCN2 in modulating the bone hematopoietic niche.

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