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A Synthetic Fragment of Leptin Increase Hematopoietic Stem Cell Population and Improve Its Engraftment Ability

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ABSTRACT

Several studies have shown the important actions of cytokine leptin that regulates food intake and energy expenditure. Additionally, the ability to modulate hematopoiesis has also been demonstrated. Previous reports have shown that some synthetic sequences of leptin molecules can activate leptin receptor. Herein, decapeptides encompassing amino acids from positions 98 to 122 of the leptin molecule were constructed to evaluate their effects on hematopoiesis. Among them, the synthetic peptide $Lep_{110-119}$-NH_2 (LEP F) was the only peptide that possessed the ability to increase the percentage of hematopoietic stem cells (HSC). Moreover, LEP F also produced an increase of granulocyte/macrophage colony-forming units and activated leptin receptor. Furthermore, LEP F also improves the grafting of HSC in bone marrow, but did not accelerate the recovery of bone marrow after ablation with 5-fluorouracil. These results show that LEP F is a positive modulator of the in vivo expansion of HSC and could be useful in bone marrow transplantation. J. Cell. Biochem. 116: 1334–1340, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: LEPTIN; SYNTHETIC PEPTIDES; HEMATOPOIETIC STEM CELLS; HEMATOPOIESIS

Leptin is a cytokine secreted by adipose tissue, which is known to regulate changes in food intake [Schwartz et al., 1996; Sahu, 2003] and energy expenditure [van Dijk, 2001]. However, leptin has a variety of other functions including the regulation of hematopoiesis [Umemoto et al., 1997; Fantuzzi and Faggioni, 2000; Claycombe et al., 2008]. Leptin acts through activation of leptin receptor, a type I cytokine-receptor family member that does not possess intrinsic kinase activity, but relies on activating Janus kinases (Jaks), which are constitutively bound to the receptor intracellular domains. In particular, the binding of leptin to its receptor activates the Jak2/Stat-3 pathway [Li and Friedman, 1999; Mancour et al., 2012]. The expression of leptin receptors was identified in murine and human bone marrow cells [Bennett et al., 1996; Cioffi et al., 1996; Umemoto et al., 1997], and their activation can produce proliferation of hematopoietic stem cells (HSC) and myeloid progenitors [Umemoto et al., 1997].

Based on the leptin molecule three-dimensional structure, synthetic fragments have been prepared. Among them, Ac-Lep_{92-115}-NH_2 and Ac-[Ser^{117}]-$Lep_{116-140}$-NH_2 were found to be the most potent compounds. Both molecules were able to promote Fos immunoreactivity in the hypothalamus of rats in a similar manner to that observed with the whole leptin [Oliveira et al., 2005]. Moreover, their sequences have been recognized by the leptin

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receptor present in human pituitary HP-75 cell lineage [de Oliveira et al., 2008]. In other studies involving the use of leptin-based fragments, [D-Leu[4]]-OB3 and OB3 regulated the energy balance and glucose homeostasis, and also activated Stat-3 [Grasso et al., 1997, 2001; Novakovic et al., 2013; Lin et al., 2014].

Recently we have observed that Ac-[Ser[117]]-$Lep_{116-140}$-NH_2 could also stimulate expansion of HSC [Dias et al., 2013]. HSC is capable of giving rise to all of the cells in the immune system. Thus, the ability to modulate this population of cells may be an important clinical tool because, in some situations, bone marrow ablation may occur, such as after chemotherapy for transplantation.

Therefore, owing to the importance of HSC population in bone marrow reconstitution, we evaluated the effects of decapeptide fragments of leptin from the region that encompass the amino acids from positions 98 to 122 of murine leptin receptor on HSC. We observed that the synthetic fragment, Ac-$Lep_{110-119}$-NH_2, exhibited activity in hematopoiesis, increasing the number of HSC and improving HSC grafting.

MATERIALS AND METHODS

PEPTIDE SYNTHESIS, PURIFICATION, AND CHEMICAL CHARACTERIZATION

All leptin-derived peptide fragments (Table I) were synthesized manually using the Merrifield methodology with the t-Boc strategy on an MBHA resin, as previously described [Dias et al., 2013; Oliveira et al., 2005]. The dry protected peptidyl-resins were exposed to anhydrous hydrogen fluoride in the presence of 10% methoxybenzene or ethanedithiol at 0°C for 1.5 h. The crude peptides were precipitated with anhydrous diethyl ether, separated by filtration, extracted from the resin with 50% acetic acid in H_2O and lyophilized. The crude lyophilized peptides were purified in two steps by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a Waters Associates system (Model Prep 600) using linear gradients (slope 0.33% B/min). Briefly, they were loaded on a Jupiter C_{18} preparative RP-HPLC column (25 mm × 21.2 mm, 15 µm particle size, 300 Å pore size) at a flow rate of 10.0 ml/min and eluted with solvent A (TEAP, pH 2.25) and solvent B (25%/TEAP, pH 2.25/CH_3CN) with detection at 220 nm. Selected fractions were collected and converted to their trifluoroacetic acid salt by loading on a preparative column as mentioned above and eluting using solvents A (0.1% trifluoroacetic acid/H_2O) and B [0.1% trifluoroacetic acid in CH_3CN/H_2O (75:25)] at a flow rate of 7.0 ml/min. Selected fractions containing the purified peptide were pooled and lyophilized. The purified peptides were characterized by RP-HPLC and liquid chromatography–electrospray ionization mass spectrometry (LC/ESI-MS). RP-HPLC was performed on a Waters system using a linear gradient of 5–95% B for 30 min of CH_3CN in two aqueous buffers: TEAP, pH 2.25 and 0.1% trifluoroacetic acid, at 1.5 ml/min, on a Jupiter C_{18} column (150 mm × 4.60 mm, 5 µm particle size, 300 Å pore size) at 214 nm. The LC/ESI-MS data were obtained using a Waters instrument, model 3100, coupled with a Waters Alliance model 2695 system using a Waters Nova-Pak C_{18} column (2.2 mm × 150 mm, 3.5 µm particle size, 60 Å pore size), solvents A (0.1% trifluoroacetic acid/H_2O) and B [0.1% trifluoroacetic acid in CH_3CN/H_2O (75:25)], gradient: 5–95% B for 30 min, at 214 nm and mass range: 300–2000 *m*/*z*.

MURINE HEMATOPOIETIC CELL EXTRACTION

The 3-month-old female C57BL/6 mice used in this study were supplied by the INFAR/ Universidade Federal de São Paulo (UNIFESP) Animal Facility. Green Fluorescent Protein (GFP) mice (C57BL/6-Tg(act-EGFP)C14-Y01-FM131Osb) were purchased from the Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME) from UNIFESP, São Paulo. All of the experiments were approved by the Animal Care Ethics Committee of UNIFESP (number 0307-12) and were conducted in accordance with the Brazilian Guide for the Care and Use of Laboratory Animals (federal law number 11794/2008), which is equivalent to the National Institutes of Health guidelines. Mice were maintained at 25°C on a reversed 12-h light:12-h dark cycle with ad libitum access to water and food. Animals were treated with synthetic peptides diluted in phosphate-buffered saline (PBS). Three different doses (0.5, 1.0, or 3.0 mg/kg) were evaluated when administered intraperitoneally (i.p.) in a volume of 100 µl using a 29-gauge needle for three consecutive days. The control animals received only the vehicle. The experiments were performed 24 h after the last dose was given. Bone marrow cells from the femurs were subsequently collected in PBS. The resulting solution were centrifuged and counted in a Neubauer chamber.

COLONY-FORMING UNIT GRANULOCYTE-MACROPHAGE (CFU-GM) ASSAY

Bone marrow cells were collected from the mouse femurs and suspended in Iscove's Modified Dulbecco's Medium (Invitrogen,

TABLE I. Peptide Sequence of the Synthetic Leptin Fragments Studied

Fragment	Name	Sequence
LEP A	Ac-$mLep_{98-122}$-NH_2	Ac-A-H-D-L-E-N-L-R-D-L-L-H-L-L-A-F-S-K-S-C-S-L-P-Q-T-NH_2
LEP B	Ac-$mLep_{98-107}$-NH_2	Ac-A-H-D-L-E-N-L-R-D-L-NH_2
LEP C	Ac-$mLep_{101-110}$-NH_2	Ac-L-E-N-L-R-D-L-L-H-L-NH_2
LEP D	Ac-$mLep_{104-113}$-NH_2	Ac-L-R-D-L-L-H-L-L-A-F-NH_2
LEP E	Ac-$mLep_{107-116}$-NH_2	Ac-L-L-H-L-L-A-F-S-K-S-NH_2
LEP F	Ac-$mLep_{110-119}$-NH_2	Ac-L-L-A-F-S-K-S-C-S-L-NH_2
LEP G	Ac-$mLep_{113-122}$-NH_2	Ac-F-S-K-S-C-S-L-P-Q-T-NH_2

USA). Twenty thousand cells were mixed in 1 mL of MethoCult GF M3534 (Stem Cell Technologies, USA) and were supplemented according to the manufacturer's instructions. The leptin fragment (10^{-7} M) was added to MethoCult. The CFU assay was performed by placing cells in a 35-mm Petri dish. Cells were cultured in humidity chamber at 37°C with 5% CO_2 for 7 days. At the end of the incubation period, colonies of more than 50 cells were counted using an inverted microscope at 20× magnification.

IMMUNOLABELING

Hematopoietic populations were labeled with antibodies as described previously [Leon et al., 2011; Nogueira-Pedro et al., 2011; Nogueira-Pedro et al., 2014]. The following monoclonal antibodies were used to define the different populations of hematopoietic cells: Ter-119 (Ly-76), Mac-1 (M1/70), CD34 (RAM34), FLK-2 (A2F10), FcγRlow (2.4G2), IL-7R (SB199), Gr-1 (RB6-8C5), B220 (RA3-6B2), c-Kit (2B8), Sca-1 (Ly-6A/E-D7), CD3-APC (145-2C11), CD90.1 (Thy1.1-HIS51), F4/80 (BM8) and CD3-PE (145-2C11). Cells were incubated with the antibodies for 20 min and then washed. To identify HSC, whole bone marrow cells (3×10^6/sample) were labeled with a mature lineage (Lin) antibody cocktail (Gr-1-PE, Mac-1-PE, CD3e-PE, Ter-119-PE and B220-PE), CD90.1 (Thy1.1.)-FITC, FLK-2-PE, Sca-1-PE/Cy7 and c-Kit-APC for 20 min. Cells were then washed. Mac-1^+Gr-1^+ cells were defined as myeloid cells. Mac-1^+F4/80^+ was defined as monocytic progenitors. $B220^+$ as defined as B cells. Ter-119^+ cells were defined as erythroid cells. HSCs were defined as Lin^-FLK-2^-Sca-1^+c-Kit^+Thy1.1^{low} [Barbosa et al., 2011]; common myeloid progenitors (CMP) as Lin^-IL-$7R^-$c-Kit^+Sca-$1^-$$CD34^+Fc\gamma R^{low}$, granulocyte-macrophage progenitors (GMP) as Lin^-IL-$7R^-$c-Kit^+Sca-$1^-$$CD34^+Fc\gamma R^{high}$ and megakaryocytic-erythroid progenitors (MEP) as Lin^-IL-$7R^-$c-Kit^+Sca-$1^-$$CD34^-Fc\gamma R^{low}$. All

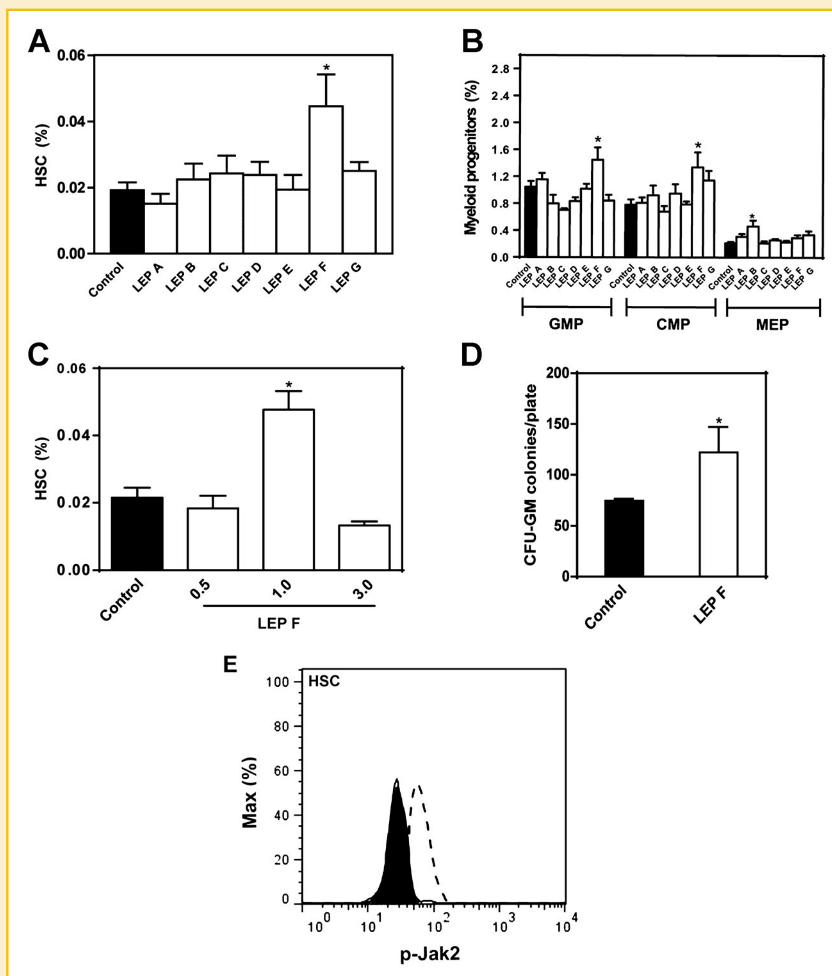

Fig. 1. LEP F increased primitive hematopoietic populations. Mice were treated i.p. with 1 mg/kg of the peptides for three consecutive days. The hematopoietic population from the bone marrow was labeled with monoclonal antibodies and analyzed by flow cytometry. A: Percentage of HSC. B: Percentage of myeloid progenitor cells: common myeloid progenitor (CMP), granulocyte–macrophage progenitors (GMP) and megakaryocytic–erythroid progenitor (MEP). C: Percentage of HSC using different concentration of LEP F. D: The capacity to form primitive CFU-GM was evaluated by cultivating 2×10^4 whole bone marrow cells in methylcellulose for 7 days in the presence of 10^{-7} M LEP F. A–D: The data are expressed as the mean ± SEM of six independent experiments. $^*P < 0.05$, ANOVA (A–C) or *t*-test (D). E: Activation of the leptin receptor in HSCs. The activation of the leptin receptor by LEP F was evaluated by phosphorylation of Jak2. Bone marrow cells were stimulated by 10^{-7} M LEP F for 30 min. HSCs were identified as described previously. Solid line: Unstimulated. Dotted line: Stimulated by LEP F. The data are representative of the results of three experiments.

antibodies were purchased from Becton Dickinson (USA). All cytometry analysis in this study were performed on a FACSCalibur (Becton Dickinson, USA) flow cytometer using Cell Quest (Becton Dickinson, USA) and FlowJo software (Tree Star Inc., USA).

JAK2 PHOSPHORYLATION

Phosphorylation of Jak2 associated to leptin receptor activation was quantified by flow cytometry as previously described [Dias et al., 2013]. Whole bone marrow cells (3×10^6 cells/sample) were obtained from mouse femurs and stimulated with 10^{-7} M LEP F or murine leptin (Sigma-Aldrich, Germany) for 30 min. Then, the cells were fixed with 2% paraformaldehyde for 30 min. Cells were washed with 0.1 M glycine in PBS, permeabilized with 0.001% Triton X-100 in PBS for 15 min and washed with PBS. Subsequently, the cells were incubated for 4 h with 1:100 anti-phospho-Jak2 (Cell Signaling Technology, USA). After incubation with the primary antibody, the samples were incubated with 4 μg/ml goat anti-rabbit IgG Alexa Fluor 488-conjugated secondary antibody (Invitrogen, USA) for 40 min. To identify of HSC (c-Kit$^+$Sca-1$^+$Lin$^-$FLK-2$^-$, Thy1.1 antibody was not used) population the antibodies described above were used after protein labeling.

ANALYSIS OF BONE MARROW FOR CHIMERISM AND 5-FLUOROURACIL TREATMENT

Whole bone marrow cells of GFP transgenic mice were stimulated with leptin fragment for 3 days, as described above. After the stimulation period the hematopoietic cells GFP$^+$ (5×10^6 cells) were injected into wild-type sub-lethally irradiated mice (6 Gy). The frequency of GFP$^+$ cells in each hematopoietic population (HSC, Gr-1$^+$Mac-1$^+$, B220$^+$) was quantified after 8 weeks post-transfer using a FACSCalibur flow cytometer [Barbosa et al., 2011; Nogueira-Pedro et al., 2014].

For 5-fluorouracil (5-FU) treatment, mice received a single i.p. injection of 5-FU (150 mg/kg of body weight) [Presley et al., 2005]. Then, after 24 h animals were treated with LEP F or vehicle for 3 days. Animals were killed after 6 or 14 days of the treatment with LEP F and bone marrow content was evaluated.

STATISTICAL ANALYSIS

All data are expressed as the mean ± standard error of the mean (SEM). Student's *t*-tests were used to compare the data from two groups. Statistical analyses were performed using an analysis of variance (ANOVA) and Bonferroni's post hoc test for multiple comparisons among groups. Differences were considered significant when $P < 0.05$.

RESULTS

MODULATION OF HEMATOPOIESIS BY A SYNTHETIC FRAGMENT OF LEPTIN

To evaluate the action of the synthetic leptin fragments (see Table I), the animals were i.p. treated with the peptide solution (1 mg/kg per day for 3 days). Initially the percentage of HSC and myeloid progenitor cells from the bone marrow were quantified. Quantification of primitive hematopoietic cells showed that LEP F induced a 2.3-fold increase in the percentage of HSC (Fig. 1A) and the total number of HSC (Control: 1.2×10^4; LEP F: 2.4×10^4), but it did not change the percentage of the other hematopoietic cells, such as myeloid cells (Gr-1$^+$Mac-1$^+$) and erythrocytes (Ter-119$^+$) (Supporting information Fig. S1A and C). However, LEP C reduced the Mac-1$^+$F4/80$^+$ population (Supporting information Fig. S1B). The other peptides promoted a slight reduction in the Ter-119$^+$ population (Supporting information Fig. S1C).

Additionally, LEP F also induced an increase in CMP and GMP populations (Fig. 1B). A significant increase in MEP population caused by LEP B was also observed (Fig. 1B). When 0.5 or 3.0 mg/kg doses of LEP F were employed, no significant increase in the number of HSCs was observed (Fig. 1C). To verify the action of LEP F in HSC, bone marrow cells were cultivated in the presence of 10^{-7} M LEP F in supplement methylcellulose medium. The number of CFU-GM increased in the cultures treated with LEP F (Fig. 1D). We also evaluated the effect of 10^{-7} M of LEP F on the phosphorylation of

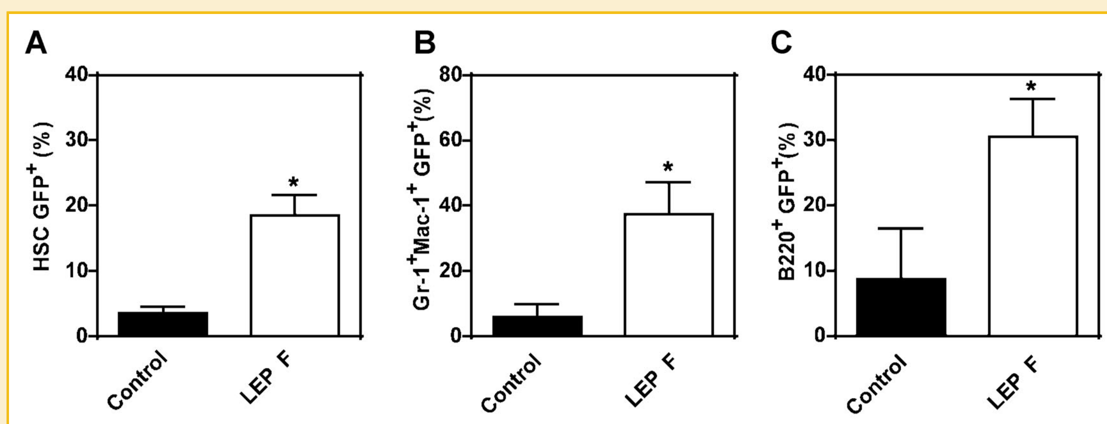

Fig. 2. LEP F fragment increase the engraftment of HSC. Bone marrow cells were extracted from GFP mice previously stimulated by 3 days with LEP F or vehicle, and then transferred to non-lethally irradiated mice. Eight weeks later percentage of GFP$^+$ cells was quantified in each hematopoietic population: (A) HSC, (B) Gr-1$^+$Mac-1$^+$ and (C) B220$^+$. Data are expressed as the mean ± SEM. $^*P < 0.05$. *t*-Test. n = 6.

Jak2, which is classically stimulated by leptin receptor activation [Li and Friedman, 1999; Mancour et al., 2012]. We observed that LEP F was able to induce the phosphorylation of Jak2 in the HSC population (Fig. 1E). Furthermore, LEP F did not change the cell viability of c-Kit$^+$ cells (Supporting information Fig. S2). These data clearly show that LEP F modulates HSC and myeloid progenitors.

LEP F IMPROVES THE ENGRAFTMENT ABILITY OF HSC

As described above, LEP F was able to increase HSC and myeloid progenitors. In order to verify whether these increase of HSC and of myeloid progenitors, induced by LEP F, can improve the engraftment ability of HSC, we tested its action on a bone marrow transplantation model.

Animals were sub-lethally irradiated and received bone marrow from GFP$^+$ mice donor previously treated, or not, with LEP F for 3 days. The treatment with LEP F improved the engraftment ability of HSC, myeloid cells (Gr-1$^+$Mac-1$^+$) and lymphoid B cells (B220$^+$) (Fig. 2).

SYNTHETIC FRAGMENTS OF LEPTIN ONLY INCREASE HSC AFTER ABLATION OF BONE MARROW

Additionally, a chemotherapy 5-FU was used to ascertain whether the use of LEP F could accelerate bone marrow recovery after 6 or 14 days. 5-FU is a thymidylate synthase inhibitor that blocking DNA synthesis producing thrombocytopenia and bone marrow ablation [Myers et al., 1975]. A reduction of the number of bone marrow cells were observed in animals treated with 5-FU (Fig. 3A). The treatment with LEP F did not increase the total number of bone marrow cells neither after 6 days (Fig. 3A) or 14 days (Fig. 3E) after the treatment with 5-FU. However, an increase in HSC population was observed after 6 days in the 5-FU + LEP F group (Fig. 3B). After 14 days the population of HSC returned to normal values in the 5-FU group and a slight elevation of HSC population was observed in the 5-FU + LEP F group (Fig. 3F). Furthermore, all other hematopoietic cell populations returned to their normal levels after 14 days (Fig. 3C, D, G, H).

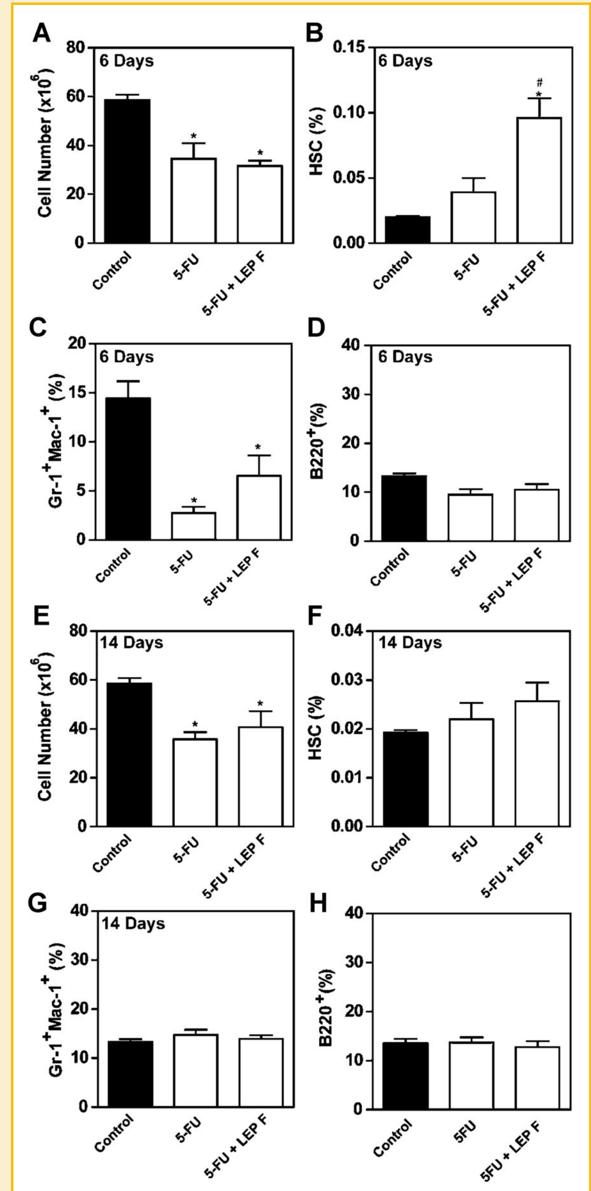

Fig. 3. Effect of LEP F fragment after the treatment with 5-FU. Mice received a single i.p. injection of 150 mg/kg 5-FU. After 24 h the animals were treated i.p. with 1 mg/kg of the LEP F or vehicle for 3 consecutive days. After 6 (A–D) or 14 (E–H) days bone marrow content was evaluated. (A and D) Total number of cells by femur. Percentage of HSC (B and F), Gr-1$^+$ Mac-1$^+$ (C and G) and B220$^+$ (D and H). Data are expressed as the mean ± SEM. $^{*,\#}P < 0.05$. ANOVA test. *, statistical analysis was performed against a control group. $^\#$, statistical analysis was performed against a group treated with 5-FU. n = 6.

DISCUSSION

Leptin regulates several and important physiology functions; thus, knowledge about how leptin receptor is activated is important to understand, to assist the design of new strategies for activation. Previously, several reports have shown that a region encompassing the amino acids from 98 to 122 of the leptin molecule is important for its activity. Some short fragment sequences on this region apparently possess bioactivity similar to intact leptin [Grasso et al., 1997, 2001; Oliveira et al., 2005, 2012; de Oliveira et al., 2008; Martins et al., 2009; Leinung and Grasso, 2012; Dias et al., 2013; Novakovic et al., 2013; Lin et al., 2014].

Herein we evaluated different synthetic decapeptides confined in to the Ac-Lep$_{98-122}$-NH$_2$ region. Among them only LEP F showed the ability to modulate positively the hematopoiesis by increasing the percentage of HSC (Fig. 1A) and the number of myeloid colonies (Fig. 1D), and improve HSC engraftment (Fig. 2). These results are in agreement with previous reports that have shown the ability of leptin to increase the proliferation of HSCs and CFU-GMs in vitro [Umemoto et al., 1997]. Intriguingly, LEP A (Ac-Lep$_{98-122}$-NH$_2$) the longest fragment that contains the entire sequence tested in this study was not able to modulate the HSC population. This finding could be due to the conformational features assumed by this long fragment. In fact, some peptide sequences composed of 20 amino acids are not always able to activate the leptin receptor [de Oliveira et al., 2008; Dias et al., 2013]. Additionally, when the full-length leptin molecule was tested, neither HSC percentage or CFU-GM

colonies were affected, but the activation of leptin receptor was observed by phosphorylation of Jak2 (Supporting information S3).

Moreover, the engraftment ability of HSC into bone marrow was tested after treatment with LEP F. Untreated mice exhibited a low rate of chimerism, which is common in sub-lethally irradiated mice [Leon et al., 2011; Nogueira-Pedro et al., 2014], but the HSC population was raised by the treatment with LEP F (Fig. 2). The fragment caused an elevated chimerism in HSC and in the mature populations (Gr-1^{+}Mac-1^{+} and $B220^{+}$) (Fig. 2). The increase of HSC percentage and the total number of this population, increase the CFU-GM formation and its ability to improve the grafting of HSC in bone marrow suggesting that LEP F produces an increase of HSC self-renewal. HSC is the most important cell in the hematopoietic system since it can differentiate in all mature cell populations. In some clinical situations heterologous and autologous transplantation of HSC is necessary such as plastic anemia, myelodysplastic syndromes and in some kinds of leukemias [Boyd and Bhatia, 2014]. The success of bone marrow transplantation is directly related with the number of HSC capable of colonizing the recipient. Thus, the effect of LEP F to improve this capability of HSC would be helpful in bone marrow transplantation. Another situation in which the increase of HSC can be important is in peripheral mobilization of HSC used in autologous transplant, since HSC are only collected when a specific percentage is present in peripheral blood [Sousa et al., 2014]. In the bone marrow ablation model using 5-FU, LEP F promoted the increase of HSC, but was not able to accelerate bone marrow recovery (Fig. 3).

In conclusion, among the synthetic fragment tested in this study, only LEP F fragment was able to promote HSC expansion and improve HSC grafting showing that this new lead peptide could be useful in bone marrow transplantation.

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