SUPPLEMENTARY APPENDIX

A population of hematopoietic stem cells derives from GATA4-expressing progenitors located in the placenta and lateral mesoderm of mice

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Supplemental table 1. List of antibodies and other reagents used in this study.

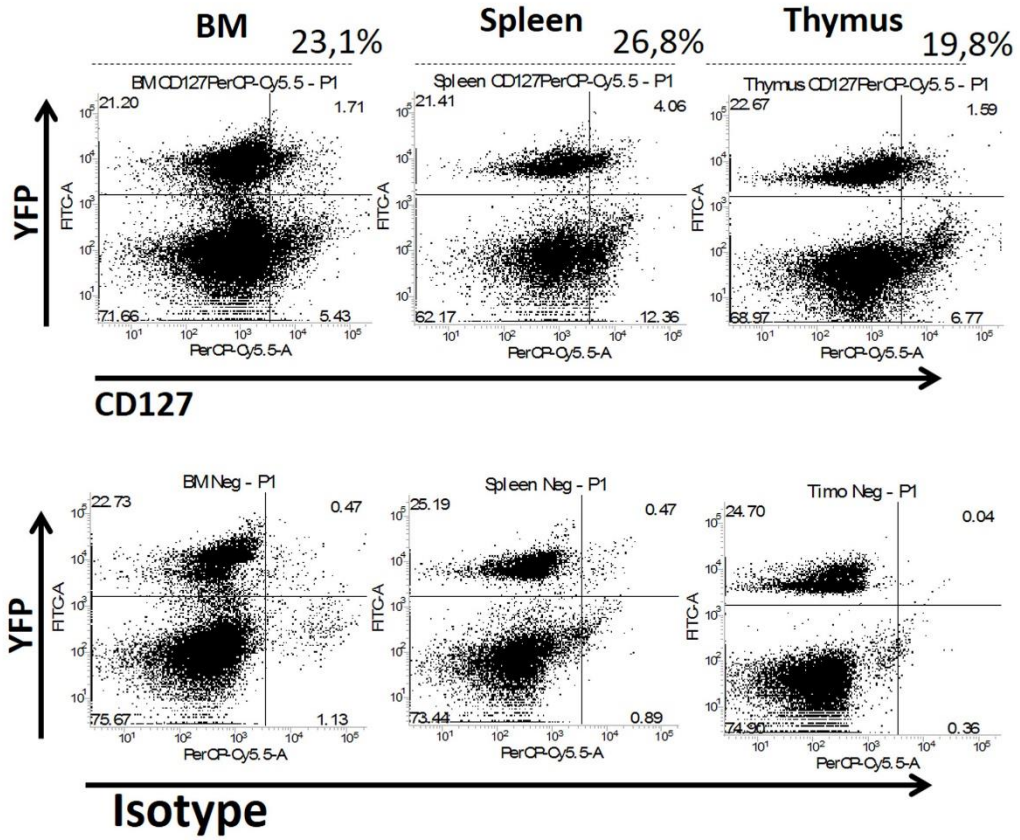
Antibody/reagent	Fluorochrome	Clone	Company	Reference
CD3 Rat- Anti-Mouse	Phycoerythrin	17 A2	BioLegend	100205
CD4 Rat- Anti-Mouse	Cy5	GK 1.5	EBioscience	19-0041
CD8a Rat- Anti-Mouse	APC	53-6.7	EBioscience	17-0081
CD11b Rat- Anti-Mouse/human	Phycoerythrin	M1/70	BioLegend	101207
CD16/CD32 Rat- Anti-Mouse	-	94	EBioscience	16-0161
CD31 Rat- Anti-Mouse	-	Mec13.3	BD Pharmingen	550274
CD31 Rat- Anti-Mouse	APC	390	EBioscience	17-0311
CD31 Rat- Anti-Mouse	Phycoerythrin	390	EBioscience	12-0311
CD34 Rat- Anti-Mouse	eFluor 450	RAM34	EBioscience	48-0341
CD41 Rat- Anti-Mouse	Phycoerythrin	eBioMWReg30	EBioscience	12-0411
CD45 Rat- Anti-Mouse	Phycoerythrin	30-F11	EBioscience	12-0451
CD45 Rat- Anti-Mouse	APC	30-F11	EBioscience	17-0451
CD45R/B220 Rat- Anti-Mouse/human	Phycoerythrin	RA3-6B2	BioLegend	103207
CD73 Rat- Anti-Mouse	Phycoerythrin	eBioTY/11.8	EBioscience	12-0731
CD90.2 (Thy-1.2) Rat- Anti-Mouse	APC	53-2.1	EBioscience	17-0902-82
CD105 Rat- Anti-Mouse	Phycoerythrin	MJ7/18	EBioscience	12-1051-81
CD117 (c-kit) Rat- Anti-Mouse	APC	ACK2	EBioscience	17-1172
CD117 (c-kit) Polyclonal Rabbit Anti-Human	-	-	Dako	A4502
CD127 (Interleukine-7 Receptor alpha) Rat- Anti- Mouse	PerCP-Cyanine5.5	A7R34	EBioscience	45-1271
CD135 (Flt3,FLK2) Rat- Anti- Mouse	PE- Cyanine5	A2F10	EBioscience	15-1351
CD140a (PDGR alpha) Rat- Anti-Mouse	APC	APA5	EBioscience	17-1401
CD309 (VEGFR2, Flk-1) Rat- Anti-Mouse	PerCP/Cy5.5	89B3A5	BioLegend	121917
GATA2 Polyclonal Rabbit Anti-Human	-	-	Thermo Fisher	PA1-100X
GATA4 Rabbit polyclonal	-	-	Santa Cruz Biotechnology	Sc-9053
GFP Chicken polyclonal	-	-	Abcam	Ab13970
IgG1 Rat- Anti-Mouse (Isotype Control)	Phycoerythrin	M1-14D12	EBioscience	dic-15

IgG2a κ Rat- Anti-Mouse (Isotype Control)	Phycoerythrin	eBR2a	EBioscience	dic-21
IgG2a κ Rat-Anti-Mouse (Isotype Control)	eFluor 660	eBM2a	EBioscience	50-4724
IgG/Rat IgG2b/Rat IgG2a Armenian Hamster (Isotype control)	Pacific Blue™	-	BioLegend	133306
IgG Donkey -Anti-Mouse	Cy5	-	Jackson ImmnunoResearch	712-175-150
IgG Donkey Anti-Rat	Cy5	-	Jackson ImmnunoResearch	712-175-150
IgG Donkey Anti-Chicken	FITC	-	Sigma	F8888
Lineage Cocktail (CD3/Ly-6G (Ly-6C)/CD11b/CD45R/Ter-119) Rat Anti- Mouse	Pacific Blue™ Ms	-	BioLegend	133310
Runx1 Anti- Mouse	-	A-2	Santa Cruz Biotechnology	Sc-365644
Sca1 (Ly-6A/E) Rat- Anti-mouse	Phycoerythrin	D7	EBioscience	dic-81
TER119 Rat- Anti-Mouse	Phycoerythrin	TER-119	EBioscience	dic-21
*7-Aminoactinomycin D (7AAD)			Sigma	A9400
*BisBenzimide H (Hoechst)			Sigma	33342

Table S2. Conditional deletion of Gata4 does not impair embryonic hematopoiesis

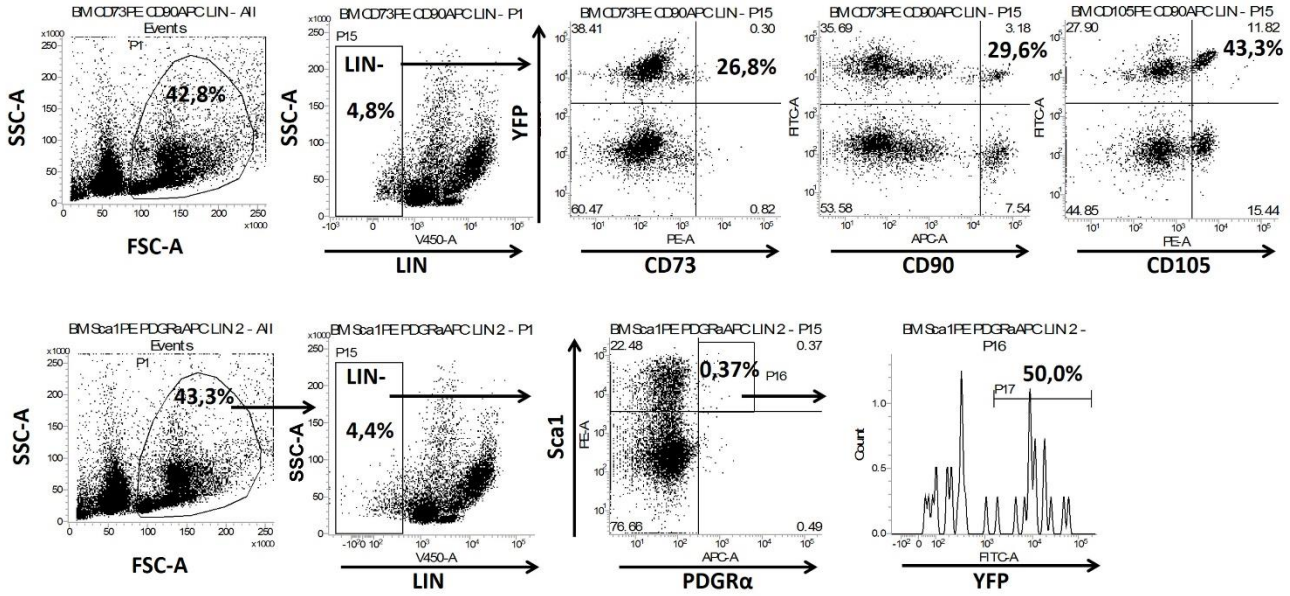
Marker	CD45				Ter119			
Stage	E12,5		E14,5		E12,5		E14,5	
Genotype	Control	Scl^{Cre};$Gata4^{fl/fl}$	Control	Scl^{Cre};$Gata4^{fl/fl}$	Control	Scl^{Cre};$Gata4^{fl/fl}$	Control	Scl^{Cre};$Gata4^{fl/fl}$
% of total cells	6,7±0,5	5,9±0,4	4,6±0,7	4,7±1,7	62,2±3,2	57,2±3,0	67,6±1,9	67,4±0,1

The table shows the frequency of Ter119+ and CD45+ cells in the fetal liver of Scl^{CreERT};$Gata4^{fl/fl}$ embryos, compared with control littermates. E12.5: Two doses of tamoxifen at E9.5 and E10.5 (N=11 controls and 6 mutants). E14.5: Two doses of tamoxifen, at E10.5 and E11.5 (N=7 controls and 2 mutants).



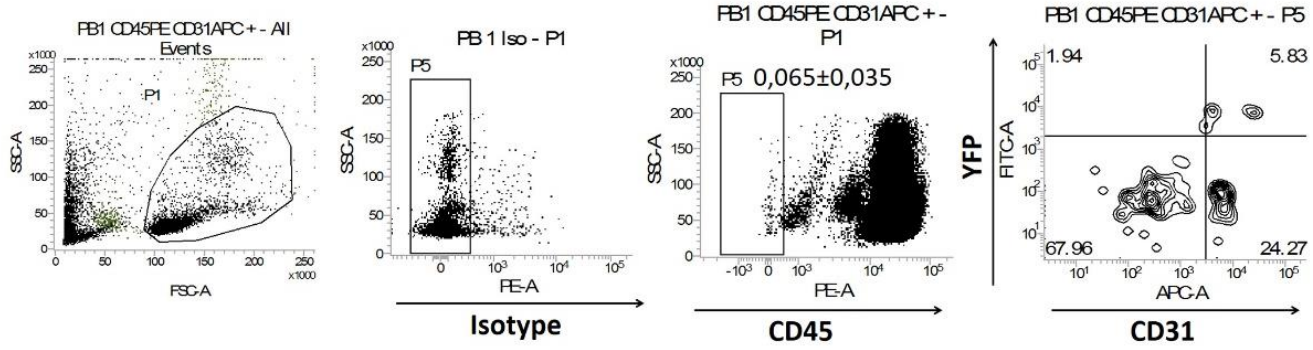
Supplemental figure S1. Localization of lymphoid progenitors (CD127+) in bone marrow, spleen and thymus.

A significant fraction of the $CD127^+$ cells in these tissues were YFP^+. Detailed data of this experiment are shown in table 1.



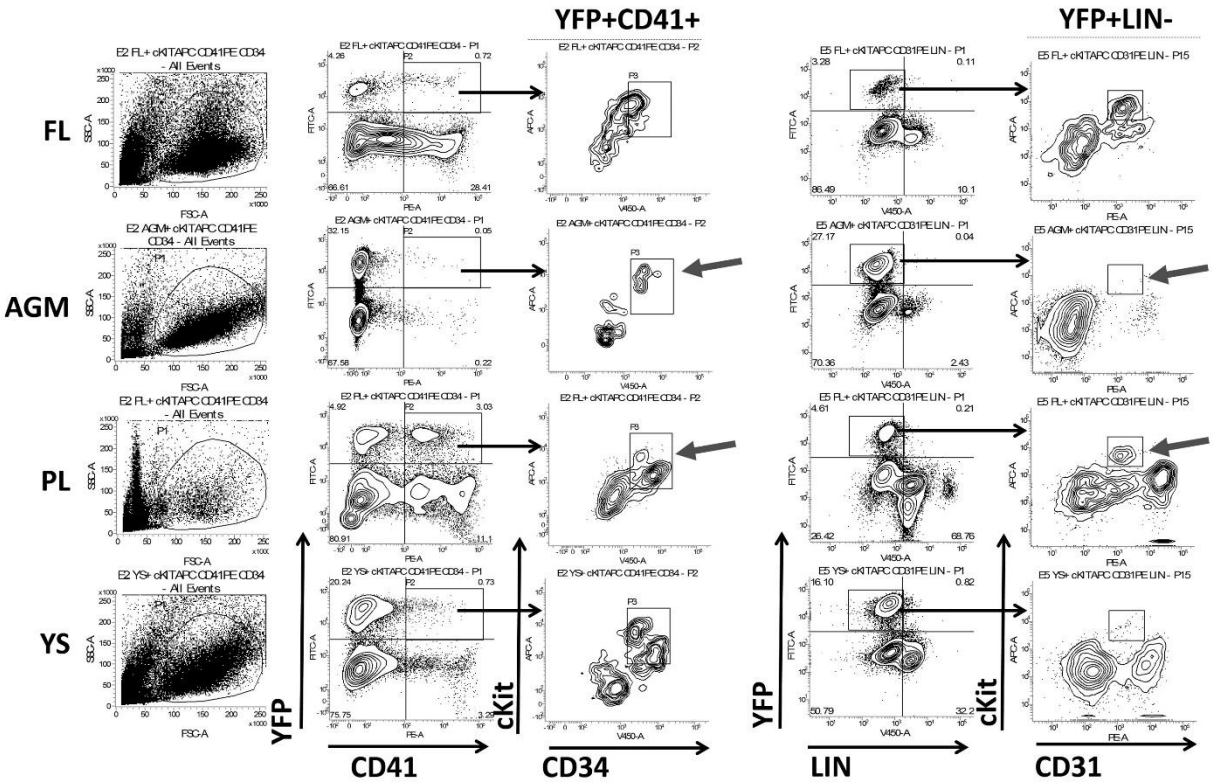
Supplemental figure S2. Mesenchymal stem cell markers in bone marrow from G2^{Cre};R26R^{EYFP} mice.

The percentage of YFP$^+$ cells within the lineage negative, CD73, CD90 and CD105 populations is shown. This percentage was higher than that found in hematopoietic cells. In the bottom row, we identified the Lin-/Sca1$^+$/PDGFRα^+ population, enriched in mesenchymal stem cells (Houlihan et al., 2012). About 50% of this population was YFP$^+$.



Supplemental figure S3. Putative circulating endothelial progenitors in $G2^{Cre}$;$R26R^{EYFP}$ mice.

Putative CD45-/$CD31^+$ circulating endothelial cells or progenitors were identified in lysed blood samples. 28,4% of this population was YFP^+ (mean of two experiments).



Supplemental figure S4. Identification of hematopoietic progenitors in different tissues from G2^{Cre};R26R^{EYFP} mouse embryos.

Two criteria were used to identify the fetal hematopoietic progenitors in tissues from E11.5 embryos, CD41^{+}/cKit^{+}/CD34^{+} and Lin^{-}/cKit^{+}/CD31^{+}. The thick arrows show the YFP+ hematopoietic progenitors identified in the aorta-gonad-mesonephros region and in the placenta with both criteria. These progenitors were far more abundant in the placenta. Detailed data of these experiments are shown in table 3.

SUPPLEMENTAL METHODS

Transgenic mouse lines

The animals used in our research program were handled in compliance with the institutional and European Union guidelines for animal care and welfare and housed in the animal facility of the University of Málaga under controlled standard conditions. The procedure was approved by the Committee on the Ethics of Animal Experiments of the University of Malaga (procedure code 2015-0028). Additional animals were maintained in the CABD animal care facility with the approval of ethical committee of CSIC and Universidad Pablo de Olavide. All embryos were staged from the time point of vaginal plug observation, which was designated as E0.5.

$G2^{Cre}$ mice were generated by cloning an 817 bp fragment containing the conserved region CR2 of the previously identified mouse G2-*Gata4* enhancer into the Cre expression vector to generate $G2^{Cre}$ transgene (Rojas et al., 2005). Gata4 floxed mice have previously been described (Pu et al., 2004).

$G2^{Cre}$ mice were crossed with the reporter line $Rosa26R^{EYFP}$ (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J). The resulting $G2^{Cre/+}$;$R26R^{EYFP}$ mice ($G2^{YFP}$, from now on) constitutively express YFP in all the lineage of the cells where the enhancer G2 has been activated. The tamoxifen-inducible Scl-CreERT mouse line was generated by Dr. Joachim Göthert (Góthert et al., 2005). Tamoxifen (Sigma, T5648) was dissolved in corn oil (10 mg/mL) and injected intraperitoneally at a dose of 0,1 mg/g.

Blood and tissue samples

1) Adult mice

- *Peripheral blood samples*

Samples of peripheral blood were obtained by puncture in the tail vein after induction of vasodilatation by 10 min exposure to a thermal lamp. A few drops of blood were collected in a tube containing 500 µL PBS and 10 mM EDTA on ice. Then, the samples were centrifuged at 1100 rpm for 1 min. Cells were resuspended in 1 mL of erythrocyte lysis buffer (NH_4Cl, $NaHCO_3$, EDTA) and incubated for 30 min at room temperature. The cells were washed with 1mL of PBS containing 5% fetal calf serum (FCS, GE Healthcare, HyClone) and 1% of penicillin (100 U/mL) and streptomycin (100 mg/mL) stock solution (P/S).

- Bone marrow, thymus and spleen samples

For isolation of the bone marrow cells, mice were euthanized by cervical dislocation, and the femur and tibia were dissected. The bone marrow cavity was flushed with 1 mL of PBS/5% FCS/1% P/S. The bone marrow obtained was homogeneized and centrifuged to eliminate the adipocytes.

Spleen and thymus cells were obtained by mechanical homogeneization of these tissues in PBS/5%/FCS/1%P/E (García-Ortega et al., 2010).

- Tissue samples from non hematopoietic organs

Adult mice were euthanized and perfused with PBS through a cannula inserted into the left ventricle. The organs were dissected and fixed in 2% paraformaldehyde for five hours. After washing in PBS, the organs were incubated in sucrose solution in PBS, 15% for two hours and 30% overnight at 4ºC. Then, the tissue was transferred to sucrose:OCT and pure OCT, and snap frozen in liquid nitrogen-cooled isopentane. 10 µm sections were obtained in a cryostate and stored at -20ºC.

2) Embryos

- Whole embryo processing

For fluorescent reporter expression analysis and immunofluorescence the embryos were fixed in 2% fresh paraformaldehyde solution in PBS for 2-4 h, and processed as above described for the adult organs.

- Tissue samples from fetal hematopoietic organs (AGM, liver, placenta)

Fetal hematopoietic organs (AGM, liver and placenta) were dissected in D-PBS with $Ca^{2+}Mg^{2+}$ (Sigma-Aldrich) supplemented with 5% FCS and 1% P/S. This medium was also used for the next steps. Ectoplacental cones together with the allantois were obtained by fine dissection of E8.0 embryos. AGM, placenta and ectoplacental cones were treated with 0.1% collagenase solution (Sigma-Aldrich) in D-PBS for 15-20 min at 37ºC. Fetal liver did not required collagenase treatment. The samples were homogenized by repeated pipetting, washed and filtered. The cells obtained in this way were counted and used for flow cytometry, Methocult assays and transplants.

Immunohistochemistry and immunocytochemistry

Frozen sections were thawed and incubated with blocking solution (SBT, 16% sheep serum, 1% bovine serum albumin, 0,1% Triton X-100 in PBS) for 30 min. Then, the sections were incubated with the primary antibodies diluted in SBT, overnight at 4ºC. After 3x10 min washes in Tris-PBS, the sections were incubated for 1 h with the Cy5-

conjugated secondary antibodies and the nuclear counterstaining DAPI (4,6 diamidino-2-phenylindole) in SBT. After washing the sections were mounted in glycerol:PBS and observed in a LEICA-SP5 II confocal microscope.

RT-PCR

Bone marrow cells obtained from the femur were washed in PBS and homogenized in 1mL of Tri Reagent (Sigma) for 10 min. AGM and fetal liver were used as positive controls. Total RNA was extracted with chloroform using Tri Reagent Protocol. Isolated RNA was used in a RT-PCR for b actin and Gata4, using the following primers:

b- ACTIN R: 5'-GTCGCCTTCACCGTTCCAGT
b- ACTIN F: 5'-CCTGGCCTCACTGTCCACCT
GATA4 R: 5'-TTGGAGCTGGCCTGCGATGTC
GATA4 F: 5'-CACTATGGGCACAGCAGCTCC

Flow cytometry

A maximum amount of $10x10^6$ cells were resuspended in 100 µl of PBS containing 5% FCS and 1% P/S (working solution). Fluorochrome-conjugated antibodies were added and the cells were incubated for 30 min on ice. After washing, the labelled cells were analyzed in a FACSverse flow cytometer.

For fluorescent activated cell sorting, the labelled cells were resuspended in 1 mL of working solution and sorted in a MoFlo cell sorter. The separated cell populations were collected in D-PBS+$Ca^{2+}Mg^{2+}$, containing 10% FCS and 1% P/S. After centrifugation at 1500 rpm for 5 min the cells were counted and resuspended in D-PBS 1%FCS 1%P/S for transplant or *in vitro* analysis.

Colony assay in Methocult

To analyze the presence of myeloid and erythroid hematopoietic progenitors the cells obtained from tissue homogenization (see above) were cultured in the semisolid medium Methocult (GF-M3434, Stem Cell Technologies Inc. Vancouver, Canada). This assay was performed as described (Cañete et al., 2016). Ectoplacental cones were previously incubated for 5 days in 24-well multidishes (Nunc) containing 1 mL of RPMI 1640, 15% FCS, 1% P/S, 1% HEPES, and 5×10^{-5} M 2-beta-mercaptoethanol (Corbel et al., 2007).

Cell concentration was always adjusted to a maximum of $2x10^4$ cells/100µl. This volume was well mixed with 2 mL of thawed Methocult. After 10 min (to eliminate bubbles) the cell suspension was placed in a 6-well plate and incubated at 37ºC with 5% CO_2. After 7-14 days the colonies were photographed. When the fluorescence of the

cells had to be detected, the colonies were picked and adhered to a slide via cytospin.

Adult bone marrow cells transplant into irradiated adult mice

Female C57Bl/6 x CBA mice, 3-4 months old were exposed to 500 + 500 RADS of X-Rays (CP-60, FAXITROM, Canada) with two hours of difference between doses (Sánchez et al., 2001). Five millions of bone marrow cells were suspended in 300µL of D-PBS+1%FCS+1%P/S and injected in the tail vein.

Adult bone marrow and embryonic cells transplant in newborn mice treated with Busulfan.

Busulfan (1, 4-butanediol dimethysulphonate) is a myeloablative agent used to improve the efficiency of a hematopoietic graft in newborn mice. We follow the protocol of García-Ortega et al. (2010). Busulfan (B-2635, Sigma-Aldrich) stock solution is prepared suspending 12.5 mg of busulfan in 250 µL of DMSO, and adding 12,5 mL of PBS pre-heated at 50ºC. Pregnant females are injected i.p. by days 17 and 18 of gestation with a 15 mg busulfan/Kg dose. Newborns at stage P1 are injected through the facial vein with the cells suspended in 50 µL of D-PBS+1%FCS+1%P/S (Sands and Barker, 1999).

Statistical analysis

Quantitative data were always expressed as mean±SEM. Statistical comparison of values was performed using the Student's t-test.

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