Interleukin-3 promotes hemangioblast development in mouse aorta-gonad-mesonephros region

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Supplementary Design and Methods

Embryo dissections

C57BL/6 mice were mated overnight. Noon of the day when a vaginal plug was detected was designated as E0.5. At the time point indicated, mice were killed, and embryos were dissected free of decidual tissues and Reichter's membrane. Somite-stage embryos were staged by counting somite numbers. The embryonic tissues were treated with 0.1% type I collagenase (Sigma, St. Louis, MO, USA) at 37°C for 1 h (for the AGM region, placenta and yolk sac) or 1-1.5 h (for E7.5 embryos), and then dispersed into single-cell suspension. Cell counts and viability were estimated by trypan blue exclusion.

Hematopoietic colony-forming cell assay

The cytokine cocktail for the hematopoietic colony-forming cell (CFC) assay included rmSCF (50 ng/mL), rmIL-3 (10 ng/mL), rhIL-6 (5 ng/mL), rhIL-11 (10 ng/mL), rmGM-CSF (10 ng/mL), rhTpo (20 ng/mL), and rhEpo (3 U/mL). Primitive hematopoietic colonies were scored on day 4 to 7 of culture, whereas definitive erythroid colonies were counted after 3 to 5 days of incubation. CFU-GM, BFU-E and CFU-Mix were counted after 7 to 10 days of culture.

Online Supplementary Table S1. Oligonucleotide primers used for nested RT-PCR.

Genes	Primer sequence	Tm (°C)	Size (bp)
Brachyury	first set$_1$ 5'-TGCTGCCTGTGAGTCATAAC-3'		
	first set$_2$ 5'-CAGATTGTCTTTGGCTACTT-3'	55	883
	nest set$_1$ 5'-CTCTAGCCAGTATCCCAGTC-3'		
	nest set$_2$ 5'-TATCCATACAGTTGACTTCC-3'	60	812
Runx1	first set$_1$ 5'-ACTTCCTCTGCTCCGTGCTA-3'		
	first set$_2$ 5'-GTCCACTGTGATTTTGATGGC-3'	57	307
	nest set$_1$ 5'-ACTCACTGGCGCTGCAACAA-3'		
	nest set$_2$ 5'-AAGCTCTTGCCTCTACCGCT -3'	57	208
Flk-1	first set$_1$ 5'-CTTGATTTCACCTGGCACTCT-3'		
	first set$_2$ 5'-TACTTCACAGGGATTCGGACT-3'	56	275
	nest set$_1$ 5'-CACCTGGCACTCTCCACCTTC- 3'		
	nest set$_2$ 5'-GATTTCATCCCACTACCGAAAG-3'	61	239
HPRT	first set$_1$ 5'-GGGGGCTATAAGTTCTTTGC-3'		
	first set$_2$ 5'-TCCAACACTTCGAGAGGTCC-3'	56	313
	nest set$_1$ 5'-GTTCTTTGCTGACCTGCTGG-3'		
	nest set$_2$ 5'-TGGGGCTGTACTGCTTAACC-3'	57	247

In vitro *tube formation assay*

Individual blast colonies were plucked and transferred to single wells of 96-well plates containing Iscove's modified Dulbecco's medium with 10% fetal bovine serum, 10% horse serum, rhbFGF (10 ng/mL), rhVEGF-165 (5 ng/mL), rhIGF-I (10 ng/mL), rmSCF (50 ng/mL), rmIL-3 (10 ng/mL), rhIL-11 (10 ng/mL), rhEpo (3 U/mL), ECGS(100 μg/mL), L-glutamine (2 mM) and 0.45 mM MTG. After 4 days of incubation, non-adherent cells were removed and the adherent cells were inoculated with endothelial growth medium-2 (EGM2, Cambrex, Walkersville, MD, USA) for a further 6 days. They were then trypsinized and replated into single wells of 48-well plates precoated with Matrigel (Biosciences BD, Bedford, MA, USA). Tube-like and network structures were documented after 12-24 h of culture. Measurements were taken in four independent fields. For the LDL uptake assay, the adherent cells were cultured in the presence of 10 μg/mL DiI-Ac-LDL (Biomedical Technologies, Inc., Stoughton, MA, USA) for 4 h. Tube-like structures were fixed in 4% paraformaldehyde for 30 min, and were incubated overnight at 4°C with primary antibodies against α-SMA (Sigma) or calponin (Sigma). They were then washed three times and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc.) for an additional 60 min at room temperature.

Flow cytometry

For flow cytometry analysis, the non-adherent cells in the co-culture were harvested and stained for 30 min at 4°C with FITC-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies. Antibodies recognizing CD19, B220, IgM, CD3, CD4 and CD8 were purchased from eBioscience (San Diego, CA, USa). Antibodies against TCRβ chain and NK1.1 were purchased from BD Biosciences

Online Supplementary Table S2. Oligonucleotide primers used for RT-PCR.

Genes	Primer sequence	Tm (°C)	Size (bp)
IL-3RβC	Sense 5'-TGAGCTATAACCACATCCAGA-3'	56	727
	Anti-sense 5'-GGAGTTGGTCCCTCTACTTGA-3'		
IL-3Rβ	Sense 5'-CCTCCAATCCTCAACCAGACC-3'	58	676
	Anti-sense 5'-CATTGGGAAGTTGCTCTGTGG-3'		
IL-3Rα	Sense 5'-CAACTTCACCGTCTTCCTG-3'	59	649
	Anti-sense 5'-CTCTGACCTCGACTTGACC-3'		
HPRT	Sense 5'-GTTCTTTGCTGACCTGCTGG-3'	57	247
	Anti-sense 5'-TGGGGCTGTACTGCTTAACC-3'		

(PharMingen, Franklin Lakes, NJ, USA). Flow cytometry was performed using a FACScalibur instrument (BD Biosciences, San Diego, CA, USA).

Magnetic sorting

Single-cell suspension from the AGM region was labeled with purified anti-mouse CD31 (BD Pharmingen), purified anti-mouse endomucin, biotin-conjugated CD105 (endoglin) or PDGFRβ monoclonal antibodies (eBioscience), then incubated with anti-rat or anti-biotin microbeads. Magnetic sorting was performed according to the manufacturer's protocol with processing through the MS-separation column attached to a Mini-MACS separation unit (Miltenyi Biotech).

Immunofluorescence staining

Blast colony cells were stained with monoclonal antibodies against Sca-1 (PE-conjugated, eBioscience), Ter-119 (PE-conjugated, eBioscience), or F4/80 (FITC-conjugated, eBioscience) in phosphate-buffered saline containing 0.1% bovine serum albumin at 4°C for 30 min. For adherent cells, they were fixed in 4% paraformaldehyde for 10 min, and were incubated overnight at 4°C with primary antibodies against CD45 (eBioscience). They were then washed three times and incubated with F4/80 (FITC-conjugated, eBioscience), FITC-conjugated goat anti-rat IgG (Southern Biotechnology Associates, Inc. Birmingham, AL, USA) for an additional 60 min at room temperature.

Western blotting

Total proteins of AGM cells were harvested: 60 μg of proteins were loaded on 12% sodium dodecyl sulfate polyacrylamide gel for electrophoresis. Immunoblotting was performed using anti-Stat5, anti-Phospho-Stat5, anti-Erk1/2, anti-Phospho-Erk1/2 and anti-actin antibodies (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions.

Other reagents

Oncostatin M was obtained from R&D Systems (Minneapolis, MN, USA). IL-3-blocking antibody and IgG1 were purchased from BD PharMingen (San Diego, CA, USA). The MEK1/2 inhibitor PD98059 was purchased from Promega (Madison, WI, USA) and the JAK2 inhibitor AG490 from Sigma.

Phase contrast and fluorescence imaging

Phase contrast images were collected using a Nikon TE2000-U (Tokyo, Japan) inverted microscope with Nikon 4×/0.13 NA, 10×/0.30 NA, 20×/0.45 NA Plan Fluor objective lenses. Images were acquired using a digital camera (Cool SNAP 5.0; Roper Scientific, Tucson, AZ, USA). Fluorescence and confocal images were collected using a Zeiss microscope (LSM 510 META system, Germany). Composite images were assembled in Adobe Photoshop version 7.0 (Adobe Systems, Munich, Germany).

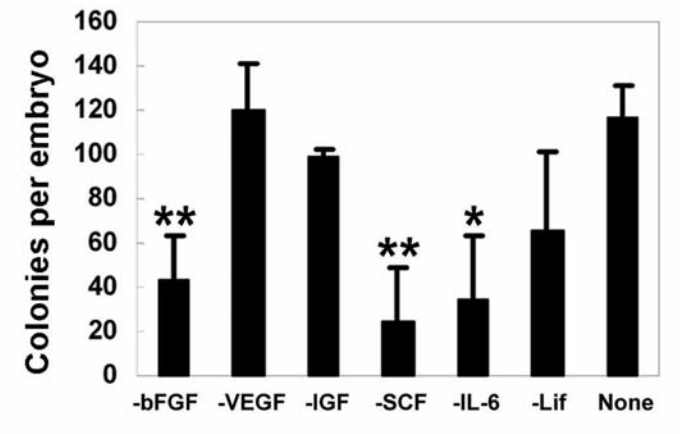
Online Supplementary Table S3. **Oligonucleotide primers used for real-time PCR.**

Genes	Primer sequence	Tm (°C)	Size (bp)
HPRT	Sense 5'-GCTGGTGAAAAGGAC CTCT-3' Anti-sense 5'-CACAGGACTAGAACACCTGC-3'	60	249
Flk-1	Sense 5'-CAGAACACCAAAAGAGAGGAACG-3' Anti-sense 5'-TGACAGGAGTGGAGATAGAGGAA-3'	60	193
Scl	Sense 5'-GGCAAGAAACAGGAGTGAGAA G-3' Anti-sense 5'-GCAAGGAAGACACAGAGGAAGA-3'	60	162
Runx1	Sense 5'-ACTCACTGGCGCTGCAACAA-3' Anti-sense 5'-AAGCTCTTGCCTCTACCGCT -3'	60	208
Gata2	Sense 5'-GCG AAAACCAAACTGCATAAGC-3' Anti-sense 5'-CTGTCTCCCAGAAACCAAGAGC-3'	60	235
Gata1	Sense 5'-AGCCTATTCTTC CCCCAAGTT T-3' Anti-sense 5'-GGTTCCTCGTCTGGATTCCATC-3'	60	371
vWF	Sense 5'-GTGTACCACGAGGTCATCAACG-3' Anti-sense 5'-GAGGGCACAAAGGTCAGGAG-3'	60	164

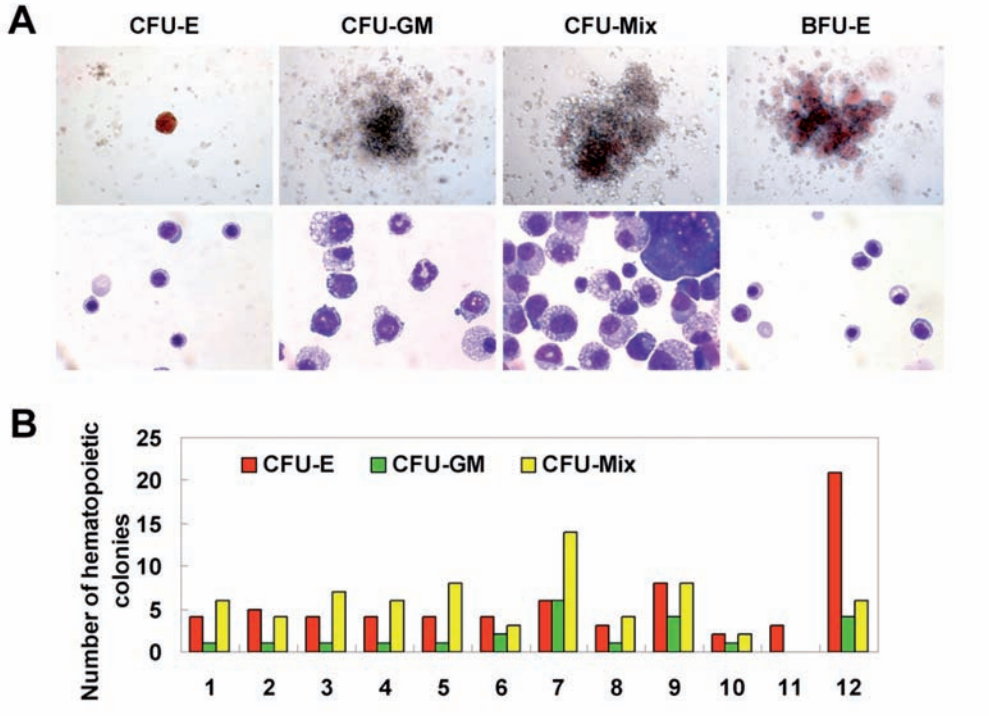
Online Supplementary Table S4. **Lymphoid potential of blast colonies from the E10.5 mouse AGM region.**

	CD19⁺ (%)	CD3⁺/TCRβ⁺ (%)
1	+	8.31
2	14.09	5.74
3	5.04	5.87
4	+	3.92
5	+	4.92
6	13.1	1.2

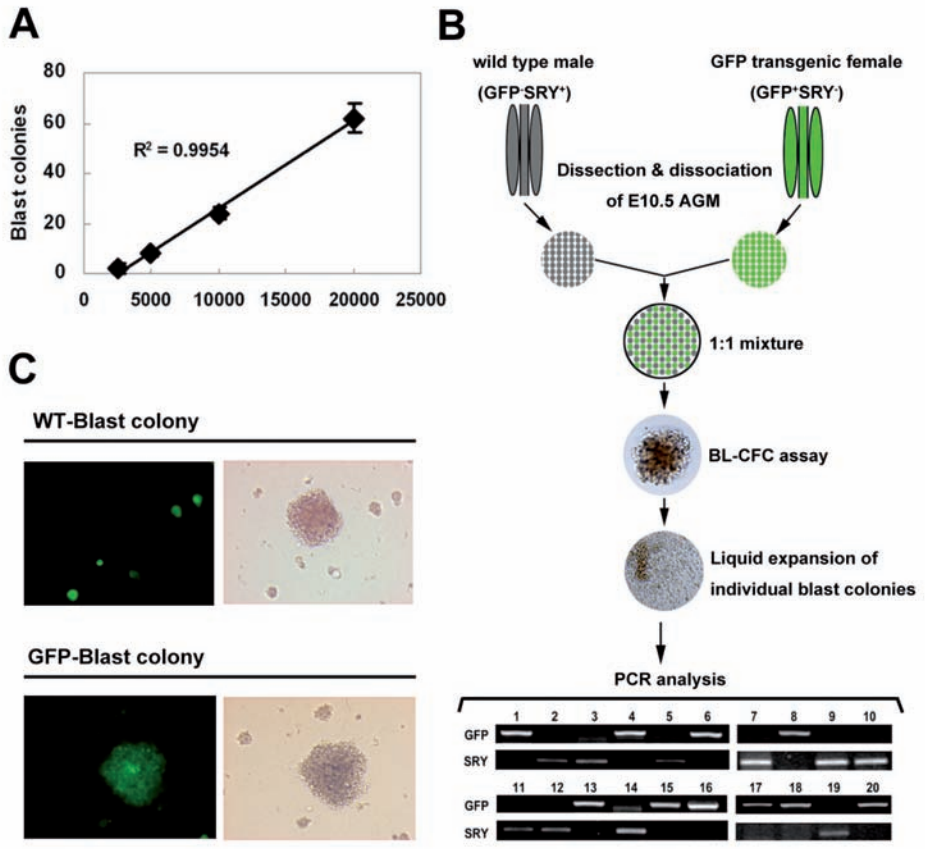
+ indicates that the CD19⁺ cells were determined by fluorescent microscopy rather by than flow cytometry.



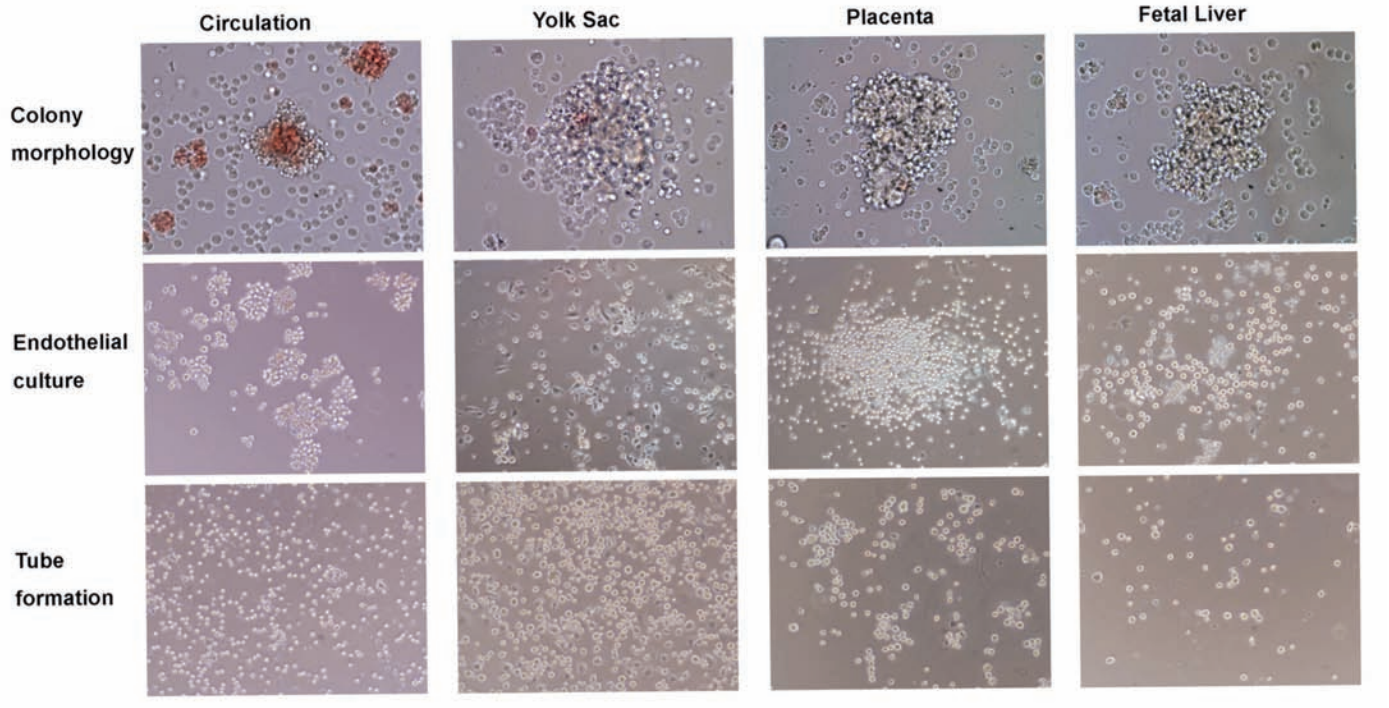
Online Supplementary Figure S1. Roles of cytokines in the growth of blast colonies. Cytokines were eliminated one by one from the original formula in a sequential fashion to determine the roles of cytokines in colony formation. SCF and bFGF had the most dramatic effect on colony growth ($P<0.01$). The removal of IL-6 had a modest effect on the number of colonies ($P<0.05$). The results represent means ± s.e.m. Significance was determined using the Student's t-test: *, $P<0.05$; **, $P<0.01$, compared with the control data.



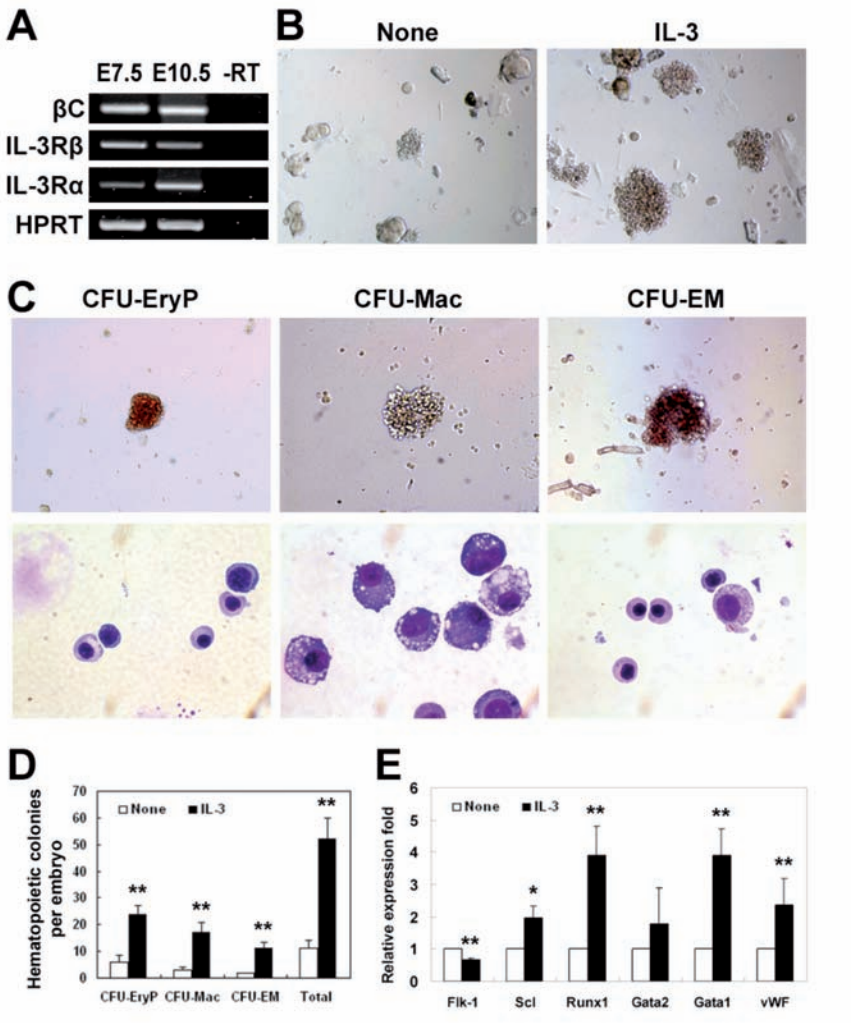
Online Supplementary Figure S2. Hematopoietic potential of BL-CFC derived from the E10.5 mouse AGM region. (A) CFU-E, CFU-GM, CFU-mix and BFU-E derived from blast colonies. (B) Quantification of hematopoietic colonies generated from 12 representative blast colonies. Original magnification: ×100 (A: upper panels), and ×1000 (A: lower panels).



Online Supplementary Figure S3. Clonality of the AGM-derived blast colonies. (A) The number of colonies was directly proportional to that of AGM cell input, indicating that a single cell corresponds to the growth of a colony. (B) Cell mixing analysis. An equal number of cells from the AGM region of male wild type (Sry^+GFP^-) and female GFP transgenic (Sry^-GFP^+) mice were mixed, and after 4 days of culture, colonies were picked out and expanded individually to determine their genomic DNA content. Among 20 representative colonies analyzed, ten displayed exclusively the Sry^+GFP^- genotype and the remaining ten Sry^-GFP^+. (C) Representative blast colonies from wild type (top) and GFP transgenic AGM region (bottom). Fluorescence (left), bright field (right).



Online Supplementary Figure S4. Tissue distribution of BL-CFC. Identification of BL-CFC in various blood-forming tissues of mid-gestation embryos, including the circulation, yolk sac, placenta and fetal liver. Single-cell suspensions from these tissues yielded only canonical hematopoietic colonies, which failed to expand as adherent layers and were incapable of forming tube-like structures in Matrigel. Original magnifications: ×100 (middle and bottom panel); ×200 (top panel).



Online Supplementary Figure S5. IL-3 promotes primitive hematopoiesis in E7.5 embryos. (A) Transcripts of two types of IL-3 receptors (IL-3Rα/βC, IL-3Rα/βIL-3) were detected by RT-PCR in E7.5 embryos and the E10.5 AGM region. (B) Morphology of hematopoietic colonies derived from single-cell suspension of E7.5 embryos with or without IL-3 treatment for 12 h. (C) General (upper) and cellular (lower) morphology of CFU-EryP, CFU-M and CFU-EM derived from E7.5 embryos. CFU-EryP indicates colonies of primitive erythrocytes; CFU-Mac, colonies of macrophages; CFU-EM, mixed primitive erythroid/macrophage colonies. (D) Number of hematopoietic colonies per E7.5 embryo with or without IL-3 treatment for 12 h. (E) Real-time PCR analysis of CFC cultures derived from E7.5 embryos to examine IL-3-mediated molecular changes. Original magnification: ×100 (B; C, upper panel), ×1000 (C, lower panel). The results represent means ± s.e.m. Significance was determined using the Student's t- test: *, $P<0.05$; **, $P<0.01$, compared with the control data.