Different Effects of Enhanced and Reduced Expression of *pub* Gene on the Formation of Embryoid Bodies by Cultured Embryonic Mouse Stem Cell

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Translated from *Kletochnye Tekhnologii v Biologii i Meditsine,* Vol. **, No. 3, pp. 174-179, September, 2005
Original article submitted March 17, 2005

The effects of *pub* gene on proliferation and initial stages of differentiation of embryonic mouse stem cells were studied *in vitro*. To this end we used enhanced expression of human *pub* gene (*hpub*) and suppression of expression of mouse endogenous *pub* gene with RNA-interference in embryonic stem cells. Proliferative activity of genetically modified polyclonal lines of the embryonic stem cells transfected with plasmids carrying expressing *hpub* gene or plasmids generating small interference RNA to this gene did not differ from that of the control cells. Inhibition of expression of endogenous *pub* gene in embryonic stem cells using small interference RNA 2-fold decreased the formation of embryoid bodies, at the same time additional expression of exogenous *hpub* gene almost 2-fold increased their number in comparison with the control. It was hypothesized that *pub* gene participates in early stages of differentiation of embryonic stem cells leading to the formation of embryoid bodies.

Key Words: *embryonic stem cells; embryoid bodies; RNA-interference; overexpression; differentiation*

Embryonic stem cells (ESC) are capable of self-renewal and differentiation into cells in all three primary germ layers both *in vivo* and *in vitro*. These cells are characterized by enhanced activity of alkaline phosphatase [1,17] and telomerase essential for proliferation [11,16]. Other signs of undifferentiated state of ESC are expression of *POU5f1* gene encoding transcription factor Oct4 [10,11], and expression of specific surface embryonic antigens [12].

Under certain culturing conditions, ESC can spontaneously differentiate into muscle, hematogenic, neuronal, and other cell types [4,8]. Specifically, the ability of ESC to differentiate *in vitro* into cardiomyocytes provides unique possibility to study the processes of cardiogenesis [13]. Animal models showed that ESC-derived cardiomyocytes could form intracardiac transplants with normal contacts to myocardial cells and improve cardiac function [6,7].

Directed differentiation of ESC can be attained by adding various exogenous growth and differentiation factors [6,7] or by genetic modifications [2,3]. ESC obtained as a result of these modifications are characterized by a certain type of directed differentiation and can serve as donors in transplantology and cell therapy.

Enhanced expression of an unknown gene was found in AIDS-infected human and simian lymphomas by subtractive hybridization; this gene was named *KIAAO129* [14,15]. Recent studies showed that human *KIAAO129* gene (*hpub*) is highly homologous (more than 80%) to mouse *pub* gene [5]. It was also shown that the product of *pub* gene is a specific inhibitor of PU.1 transcription factor [5], which plays a key role in hemopoiesis. These data suggest that expression of *pub* gene affects differentiation and proliferation of

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0007-4888/05/1401-0153

the hemopoietic cells [5]. Probably, the *pub* gene product could affect earlier stages of cell differentiation, specifically ESC.

To evaluate whether *pub* gene is involved into the early stages of ESC differentiation, we modified these cells genetically to enhance expression of human *hpub* gene and to suppress expression of endogenous murine *pub* gene. The resulting transfected cultures were used to study proliferation and development of embryoid bodies (EB).

MATERIALS AND METHODS

ESC culturing. The study used ESC from R1 mice kindly provided by Dr. A. Nagy (Mount Sinai Hospital, Toronto). The cells were isolated from blastocytes of agouti-colored mouse line (129/Sv×129/SvJ)F_1. Culturing of ESC was carried out at 37°C and 5% CO_2 in α-MEM medium (Sigma) containing 15% fetal calf serum (FCS, Gibco), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, nonessential amino acids (Gibco), nucleosides, vitamins, and antibiotic gentamycin (20 μg/ml). The feeder layer for ESC consisted of primary fibroblasts obtained from 11-12-day mouse embryos, whose proliferation was blocked with mitomycin C (5 μg/ml). The growth medium for primary fibroblast culture was DMEM (Sigma) supplemented with 10% FCS, 2 mM L-glutamine, and antibiotic gentamicin (20 μg/ml). When ESC were cultured without the feeder layer, the medium was supplemented with leukemia inhibiting factor (LIF, Sigma) to a final concentration of 10 ng/ml, which blocked spontaneous differentiation of these cells. The cells were subcultured and the medium was changed every 3 days.

Induction of ESC differentiation with EB formation. To induce differentiation with EB formation, ESC were isolated from feeder fibroblasts. To this end, the cultures were treated with trypsin and centrifuged. The cell suspension was incubated in a 60-mm Petri dish (Nunc) for 15-30 min. After fibroblasts were fixed to plastic, ESC-containing suspension was transferred to 35-mm Petri dish (Nunc, 200,000 cells per dish) or to 96-well plate (1000 cells per well), and placed into CO_2-incubator (5% CO_2). After 2-4 days formed EB were transferred to gelatin-covered dishes (0.01%, Serva). EB were counted on days 3-4 and 6-8 after appearance of the first EB.

Plasmids used for ESC transfection. To transfect ESC cells, we used *pcDNA3* (Promega) and *plneo* (Invitrogen) plasmids, and vectors based on these plasmids. Vector *pPB* prepared on the basis of *pcDNA3* plasmid contained a 4450 n.p. insertion incorporating full-length cDNA of human *hpub* gene (*KIAAO129*, Blast 2 database number gi 15208662) in *HindIII*/*NotI* sites. Vector *pRNAi* was constructed on the basis of *pclneo* plasmid, where cytomegalovirus (CMV) promoter was replaced with promoter of histon H1 gene to express miRNA (*plneo*). Two synthesized oligonucleotides 5'-GATCTCCCACTGCCAGSTTTCAAACTCTTCAAGAGAGAGTTTGAAAGCTGGCAGTTTTTTGGAAA-3 and 3'-AGGGTGACGGTCGAAAGTTTGAGAAGTTCTCTCTCAAACTTTCGACCGTCAAAAAACCTTTTCGA-5' (inverted sequences are underlined) were annealed and cloned into *plneo* plasmid at *BglII* and *HindIII* sites. In cells this plasmid ensured transcription of small interference RNA homologous to a certain site of *pub* gene. All recombinant plasmids carried geneticin (antibiotic G418)-resistance gene, which ensured selection of transfected cells. The structure of synthesized vectors is shown in Fig. 1 (*a*, *b*).

The plasmid DNA was isolated from transformed cells of *E. coli XL1* on columns using Quiagen kits.

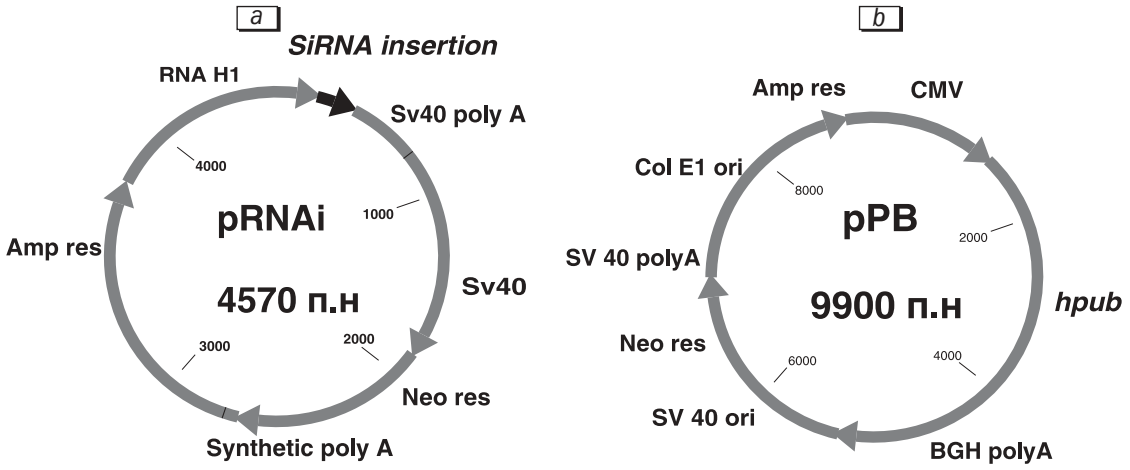

Fig. 1. Structure of the transfection vectors. *a*) vector (pPB) based on pcDNA3 plasmid carrying insertion of cDNA of *hpub* (*KIAAO129*) gene at the restriction sites *HindIII*/*NotI; b)* vector (pRNAi) based on *plneo* plasmid, in which CMV promoter was replaced with histon H1 gene promoter carrying insertion of DNA at the sites *BglII* and *HindIII* to generate miRNA and mRNA of the mouse *pub* gene target.

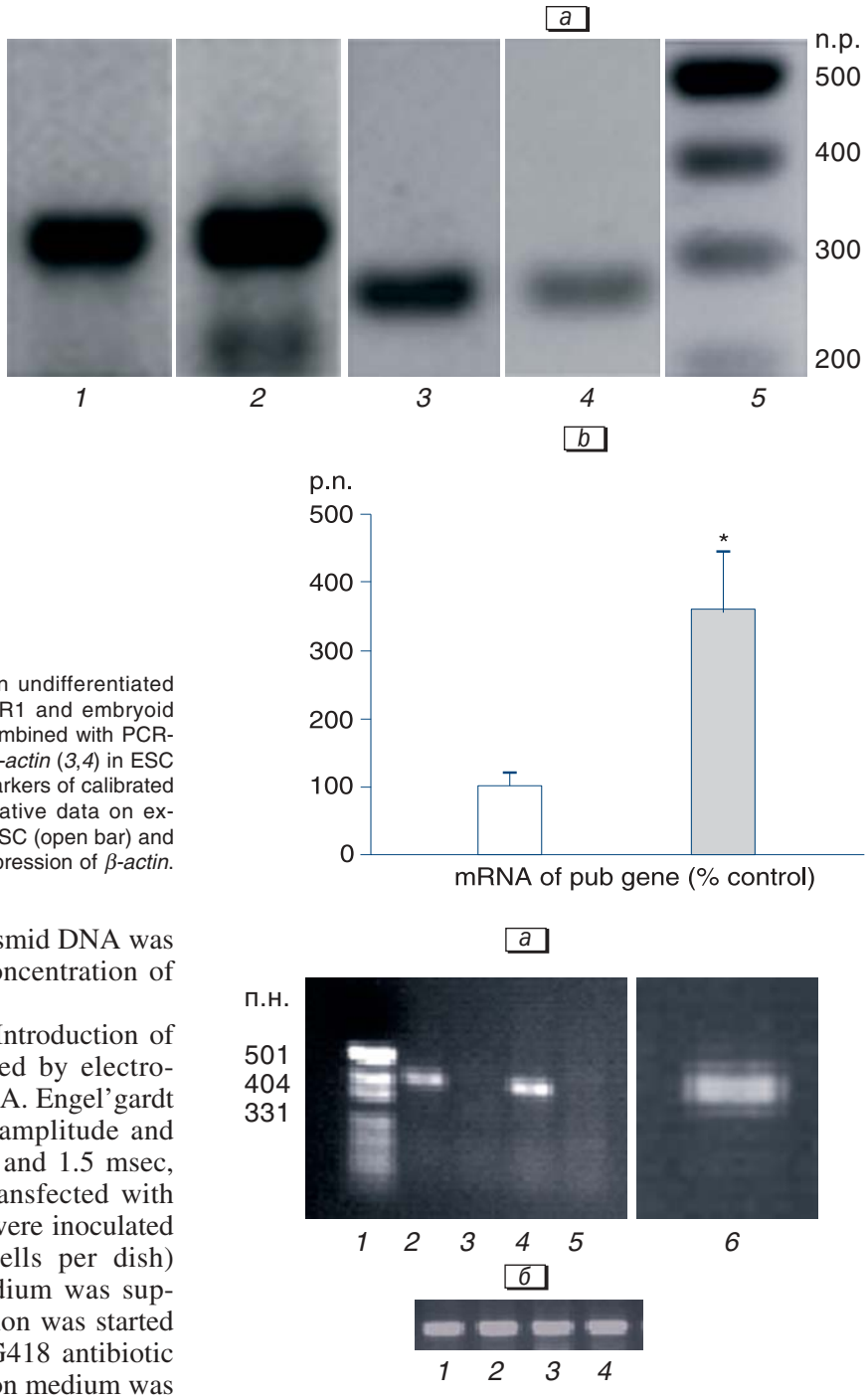

Fig. 2. Expression of endogenous *pub* gene in undifferentiated embryonic stem cells (ESC) from mouse line R1 and embryoid bodies (EB) revealed by reverse transcription combined with PCR-analysis. *a*) expression of genes *pub* (*1*, *2*) and *β-actin* (*3*,*4*) in ESC and EB, respectively. The panel (*5*) shows the markers of calibrated length known as "100 bp DNA ladder"; *b*) Relative data on expression of *pub* gene in murine undifferentiated ESC (open bar) and EB (solid bar). The data were standardized to expression of *β-actin*. $p^*<0.05$.

For transfection of ESC sedimented plasmid DNA was dissolved in sterile water to a final concentration of 2 μg/μl.

ESC transfection and selection. Introduction of plasmid DNA into ESC was performed by electroporation in a SUM4 original device (V. A. Engel'gardt Institute of Molecular Biology). The amplitude and duration of electric pulse were 400 V and 1.5 msec, respectively. One million cells was transfected with 8 μg plasmid DNA. Transfected cells were inoculated into 35-mm Petri dishes (200,000 cells per dish) coated with gelatin (0.01%). The medium was supplemented with LIF (10 ng/ml). Selection was started on post-inoculation day 2 by adding G418 antibiotic (200 μg/ml) to the medium. The selection medium was replaced every 3-4 days. The polyclonal cultures of each transfection variant were taken from dishes on selection day 10 as a pool of G418-resistant clones.

Assessment of *pub* gene expression by reverse transcription PCR. The total RNA of EB and undifferentiated ESC, which were cultured in the presence of LIF without feeder layer, was isolated by the method of phenol-chloroform extraction with the help of a YellowSolve reagent kit (Clonogen) according to the application notes of the producer. The reverse transcription was carried out using Silex kit according to manufacturer's instructions. Total RNA (0.5 μg) was used in the reaction carried out 1 h at 37°C in 20 μl reaction mixture containing 0.05 μ residual HEXA primers and

Fig. 3. Expression of exogenous *hpub* (*KIAA0129*) gene in stable transfected undifferentiated ESC and EB. *a*: expression of exogenous *hpub* (*KIAA0129*) gene; *1*) marker pUC19/Mps1, *2*) ES-hPub, *3*) ES-DNA3, *4*) EB-hPub (EB formed from ES-hPub), *5*) EB-DNA3 (EB formed from ES-DNA3), *6*) positive control: plasmid pPB with inserted human *hpub* (*KIAA0129*) gene. *b*: standardization by glyceraldehyde phosphate dehydrogenase (GAPDH) gene expression. *1*) ES-hPub, 2) ES-DNA3, *3*) EB-hPub, *4*) EB-DNA3.

100 U MMLV reverse transcriptase (molony murine leukemia virus). After the reaction was stopped (10 min at 70°C) cDNA samples were stored at -20°C.

PCR was performed in 25 μl reaction mixture composed of Taq-buffer and 1.5 mM mixture of dNTP, 1.25 U colored Taq-polymerase, H_2O, 0.5 μg cDNA, and 10 pM of each primer. The following primers (Sintol) were used for PCR: 5'-TCATGAAGTGTGA CGTTGACATCCGTAAAG-3' (direct) and 5'-CCTA GAAGCATTTGCGGTGCACGATGGAGG-3' (reverse) for *β-actin* gene, 5'-CCCATTTGGAAGACGC CG-3' (direct) and 5'-AGGGTGGCTCAGCTCCG-3' (reverse) for *pub* gene, 5'-GC AG CA GC AC AT TG AC AA CA-3' (direct) and 5'-TC CA CG AG GC CC TT AA AG AA-3' (reverse) for *hpub* gene (*KIAA0129*).

The conditions for PCR for genes *pub* and *β-actin* were as follows: start, 5 min at 95°C, 25 cycles (*β-actin*) and 43 cycles (*pub*) including 45 sec at 95°C, 45 sec at 62°C (*β-actin*) or at 70°C (*pub*), and 45 sec at 72°C. PCR for genes *hpub* was performed under the following conditions: start, 2 min at 95°C, 30 cycles including 60 sec at 95°C, 45 sec at 60°C and 45 sec at 72°C. The selected primers were chosen for the C-terminal site of *hpub* gene, which was non-homologous to similar site in murine *pub* gene, because *hpub* gene is larger than *pub*. This excluded the possibility of amplification of endogenous murine *pub* gene.

PCR products were separated in 1.5% agarose gel with ethidium bromide visualization. BioDocAnalyze system (Biometra) was used to determine the intensity of luminescence of the products in UV-light (*β-actin*, 284 n.p. and *pub*, 382 n.p.). The software calculated the values of relative quantities of the corresponding cDNA. The data on *pub* gene were normalized relative to β-actin.

Analysis of proliferation of transfected ESC. To assess the growth parameters, the cells were cultured on 35-mm Petri dishes (150,000 per dish) coated with gelatin (0.01%) in the presense of LIF (10 ng/ml). The cells were counted in a Gorjaev chamber on postinoculation day 3. At least 6 dished were analyzed for each line.

The data were analyzed with Sigma Plot software (Jandel Scientific), processed statistically using one-way ANOVA, and presented as *M±SEM*. The differences were significant at $p<0.05$.

RESULTS

At the first stage, reverse transcription-PCR analysis was used to assess expression of endogenous *pub* gene in ESC and EB produced by these cells. This gene was expressed in ESC and EB; expression was much higher in EB (Fig. 2, *a*, *b*).

Transfection of ESC with the corresponding vectors followed by selection resulted in the following cell lines: ES-hPub cells expressing *hpub* gene; ES-DNA3 (control line); ES-PNAi (the line with decreased expression of endogenous *pub* gene; and ES-lneo (control line). In polyclonal line ES-hPub, expression of mRNA of *hpub* gene was observed in the progenitor cells and in EB formed during their differentiation. In control cells and their EB this gene was not expressed (Fig. 3). Reverse transcription combined with PCR revealed no significant decrease in *pub* gene expression in ESC lines transfected with the genetic structure generating interference RNA for this gene.

Comparison of proliferative activity in experimental and control cells revealed no significant difference between the cell lines (Fig. 4, *a*, *b*). These data suggest that the product of *pub* gene plays little role in the regulation of cell cycle in ESC.

Then, we compared the time of formation and the number of formed EB in the cell lines with enhanced

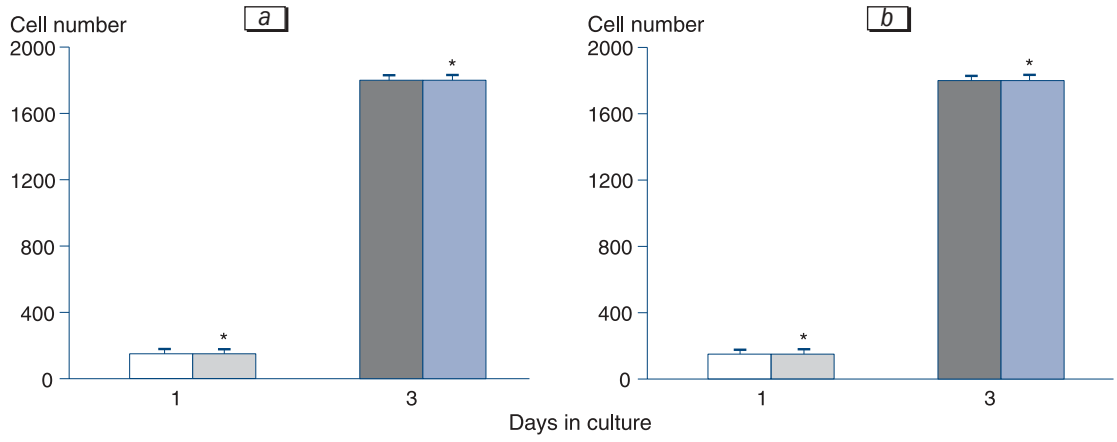

Fig. 4. Effect of enhanced expression of *hpub* gene and potentially reduced expression of *pub* gene on proliferation activity of ESC. *a*) open and dark-grey bars represent ES-DNA3 (control), while light-grey and light-blue bars correspond to ES-hPub. *b*) open and dark-grey bars represent ES-lneo (control), while light-grey and light-blue bars correspond to ES-PNA. The cells were inoculated by 180,000 per a 35-mm dish. The cells were counted on postinoculation day 3. $p^*<0.01$.

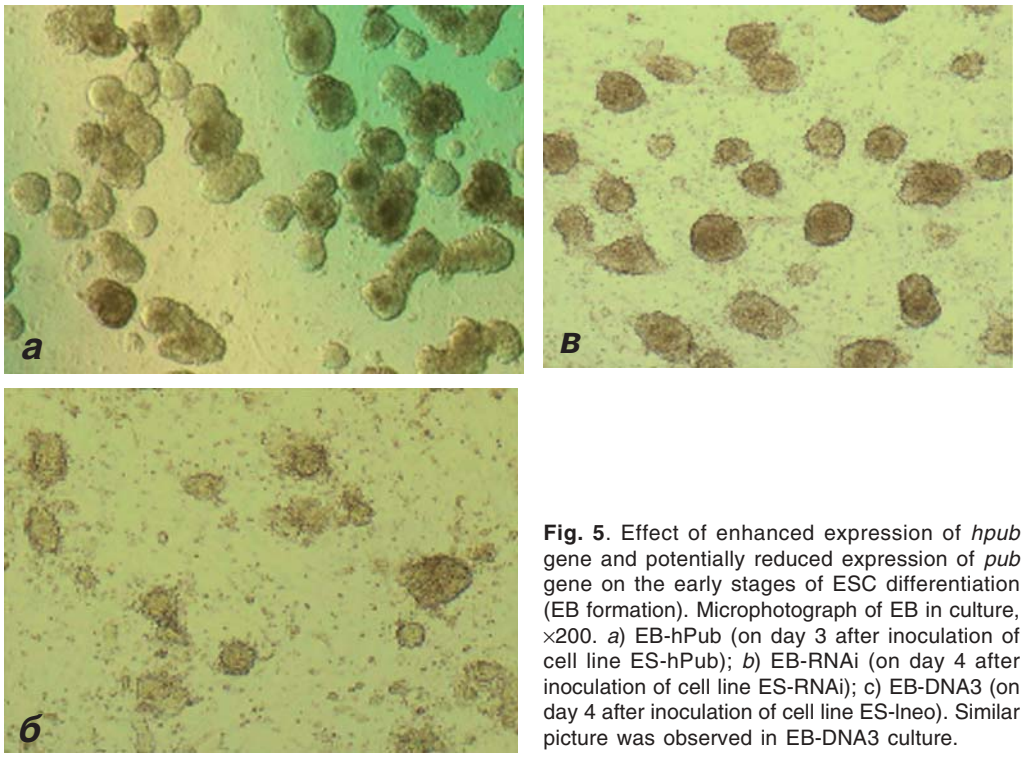

Fig. 5. Effect of enhanced expression of *hpub* gene and potentially reduced expression of *pub* gene on the early stages of ESC differentiation (EB formation). Microphotograph of EB in culture, ×200. *a*) EB-hPub (on day 3 after inoculation of cell line ES-hPub); *b*) EB-RNAi (on day 4 after inoculation of cell line ES-RNAi); c) EB-DNA3 (on day 4 after inoculation of cell line ES-lneo). Similar picture was observed in EB-DNA3 culture.

and potentially reduced expression of *pub* gene. In ES-hPub cells expressing *hpub* gene, EB were formed about a day earlier than in the control cells transfected with pcDNA3 plasmid. In cells with down-regulated expression of *pub* gene (ES-PNA), EB appeared one day later than in the control cells transfected with *plneo* plasmid.

Enhanced expression of gene *hpub* gene in ESC significantly affected the initial stages of cell differentiation and modulated the formation of EB (Fig. 5 and 6). Three days after inoculation of transfected cells under conditions inducing the formation of EB, the number of EB in ES-hPub cell culture about 2-fold surpassed that in control cultures (Fig. 5, *b* and 6, *a*). The difference in the number of EB produced in experimental and control cells persisted on day 6, but decreased to 40% (Fig, 6, *a*). At the same time, 4 and 8 days after *in vitro* culturing of cell line ES-PNAi with potentially decreased expression of *pub* gene, the number of EB produced by these cells was about 2 times lower than in the control. Reverse transcription combined with PCR revealed no significant moderation of *pub* gene expression in ESC cultures transfected with *pRNAi* vector. However, our data on signi-

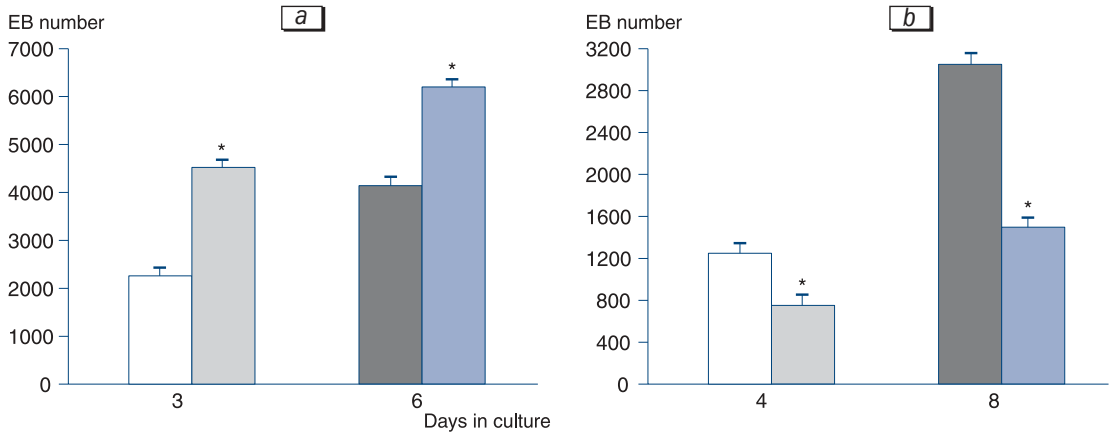

Fig. 6. Histograms of ET numbers in transfected cultures of ESC. *a*) open and dark-grey bars represent EB-DNA3 (control), light-grey and light-blue bars correspond to EB-hPub. EB were counted on postinoculation days 3 and 6. *b*) open and dark-grey bars represent EB-lneo (control), while the light-grey bars correspond to EB-RNAi. EB were counted on postinoculation days 4 and 8. $p^*<0.05$.

ficant decrease in the number of EB produced in these cultures indirectly indicate suppression of this gene. To obtain the direct data on possible suppression of *pub* gene in these cultures, we plan to use Northern blot-hybridization.

Thus, we demonstrated for the first time that *pub* gene can significantly affect the earliest stages of ESC *in vitro* differentiation manifested by production of EB. In the following, we plan to study the effect of the product of this gene on various types of the terminal cell differentiation (muscle, hematopoietic, and nervous) in the transfected ESC.

The authors are grateful to Dr. N. A. Tishchenko for technical help.

The study was supported by the Russian Foundation for Basic Research (grant Nos. 00-04-55055, 04-04-48904) and Presidium of RAS (grant program "Molecular and Cell Biology").

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