4

Effects of Recombinant Leukemia Inhibitory Factor (LIF) on Functional Status of Mouse Embryonic Stem Cells

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1. Introduction

LIF (Leukemia Inhibitory Factor) protein isolated and characterized at early 1990 as a cytokine of the IL-6 family (Williams et al., 1988; Smith et al., 1988; Gearing et al., 1989) is now one of the most popular factors used for investigations with steam cells and embryos (Nagy et al., 1993; Thomson, Marshal, 1998; Metcalf, 2003; Gonzalez et al., 2004; Paling et al., 2004; Ratajaczak et al., 2009), an a potential therapy for oncology and reproductive medicine (Kurek, 2000; Cheng et al., 2001; Gunawardana et al., 2003; Wobus, Boheler, 2005; Guney et al., 2008; Novatny et al., 2009; Aghajanova, 2010). Its molecular structure (amphiphilic protein composed mainly from hydrophobic positively charged amino acids) and molecular mechanism of its action are known in details today (Haines et al., 1999, 2000; Auernhammer, Melmed, 2000; Heinrich et al., 2003; Giese et al., 2005). As to current knowledge, LIF binds with LIF-receptor (LIF-R) of a plasma membrane and with gp130 glycoprotein, a transmembrane transporter causing activation of JAK/STAT3, MAPK and PI3P paths of intercellular transduction of a trigger signal (Hirano et al., 1997; Raz et al., 1999; Auernhammer, Melmed, 2000; Cheng et al., 2001; Park et al., 2003; Paling et al., 2004; Gonzalez et al., 2004; Boniani, Scholer, 2005). Targets genes activated by these signal systems response for proliferation, apoptosis, cell cycles and differentiation.

Acting appropriate receptor complexes (LIF-R-gp130), LIF may cause various cell reactions. In mouse embryonic stem cells (ESC) it blocks processes of spontaneous differentiation and formation of embryoid bodies (EB, analogs of early mammal embryos) maintaining thus the stem cells in pluripotent state *in vitro* (Gearing et al., 1989; Raz et al., 1999; Nagy et al., 1993; Thomson, Marshal, 1998; Wobus, Boheler, 2005). High dependence of mouse ESC lines from LIF is evidenced by the fact that at a LIF-free culture medium the cells spontaneously differentiate into EB loosing thus their pluripotent properties. Main mechanisms of LIF effects, through cell membrane receptors, are comprehensively investigated (Haines et al., 1999, 2000; Heinrich et al., 2003; Gonzalez et al., 2004; Giese et al., 2005) and widely

recognized; nevertheless dynamics of events at early stages of the protein interaction with ESC needs further clarifying. First, LIF is known to have three isoforms one of which (t-LIF) is not secreted but acts within a cell without contacting with outer-cell membrane receptors (Haines et al., 1999); second, mutant mouse ESC lines free of LIF-receptor complex proteins, LIFR and/or GP130, require presence of LIF in a culture medium (Ware et al., 1995; Li et al., 1995; Yoshida et al., 1996; Dani et al., 1998; Boniani, Scholer, 2005). These facts tell some other (not receptor based) mechanisms of LIF mouse ESC regulation.

Possibility of direct effects of recombinant LIF on properties of biological membranes is evidenced by our investigations with bilayer lipid membranes (Borisova et al., 2009), and membranes of intact cells highly dependent on the protein (Lobanok et al., 2008, 2009). We found that LIF acts as a membranotropic agent: it affects viscosity of membrane lipids, surface charge and conductivity of cell membranes. Basing these results, we supposed LIF-mediated changes of cytoplasm membranes structure and functions to be related with maintenance of mouse ESC proliferative activity and pluripotent properties *in vitro.*

2. Molecular mechanisms of LIF's effects on cell membranes

Possibility of non-receptor mechanism of stem cells LIF-regulation has been evidenced by studies with bilayer lipid membranes (BLM). We tried to answer the following questions: 1) what are effects of recombinant LIF proteins on a lipid bilayer and 2) whether mechanism of action varies according to a source of the protein origin (prokaryote or eukaryote). LIF is known to be produced in eukaryotic cells in a secrete form with 32-67 kDa molecular mass (Hilton et al., 1988; Haines et al., 2000; Heinrich et al., 2003); in bacterial cells recombinant LIF molecular masses are not higher than 20 kDa corresponding to theoretical estimations of LIF cDNA nucleotide sequence (Gearing et al., 1989; Hinds et al., 1998). Prokaryotic and eukaryotic LIFs have the same primary structures composed mainly by hydrophobic positively charged amino acids; differences between the recombinant proteins occur during post-translation events (Hinds et al., 1998; Hilton, Gough, 1998; Hirano et al., 2000).

Our studies have demonstrated that eukaryotic LIF(eu) and prokaryotic LIF(pro) effect lipids of cell membranes in similar manner shifting their surface charge towards positive values (fig. 1). Sorption of recombinant LIF on a lipid bilayer depends on its concentration and pH of an external solution. pH 7.0 enhances LIF effects on a surface potential (fig. 1 A). At pH 6.0, effects of the same level need higher concentrations of the protein (fig. 1 Б). From fig.1 one can see that at use of 10 ng/ml and higher LIF, the potential jumps become less pH-dependent evidencing surface-active properties of the protein. From these data it emerges that recombinant LIF in concentrations used routinely for ESC cultivation sorbs at a surface of a lipid bilayer resulting in increase of the membrane positive charge. Calcium ions affect cell membranes in a similar way.

Studies with BLM have shown that all used recombinant LIF proteins, regardless their origin, affect both surface potential (fig. 1) and conductivity of a lipid bilayer (fig. 2-4). A distinctive feature of LIF(pro) at its action on a lipid bilayer is spontaneous conductivity fluctuations without stationary state. At 100 mV, current through a lipid membrane changes chaotically and do not cause stable conductivity levels in a millisecond measurements range (fig.2, *a*); with S-shaped volt-ampere characteristics (fig. 2, *b*).

Chaotic and non-regular character of productivity increase at presence of LIF(pro) is levelled at addition of 10мM $CaCl_2$ into external solution. The observed changes of a lipid bilayer show dependence from presence of Ca^{2+}. At that, bilayer conductivity is decreased and short

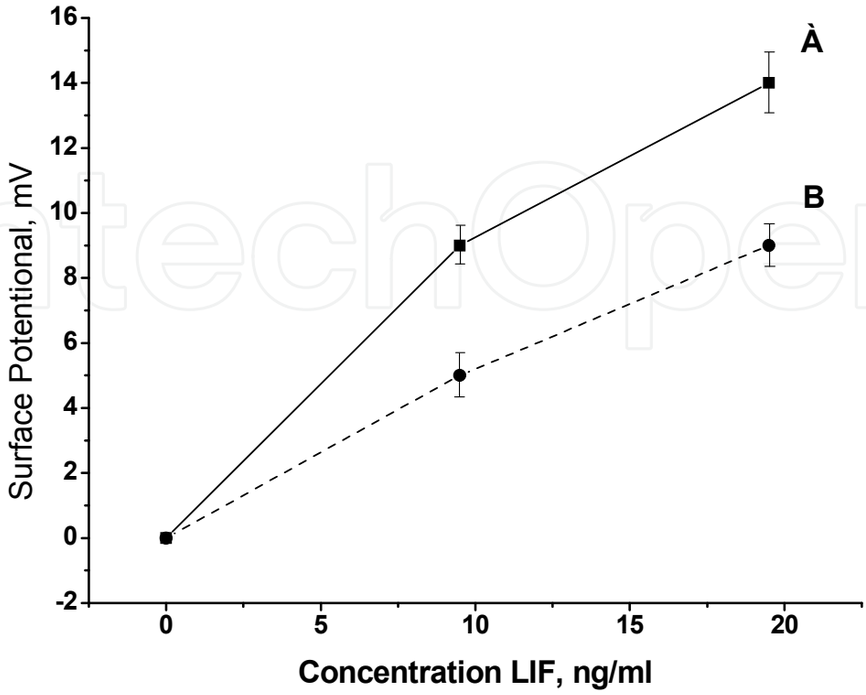

Fig. 1. Effect of mouse recombinant LIF protein from a prokaryotic expression system, LIF(pro), on a surface potential of a bilayer phosphatidylcholine membrane: A – pH 7.0, B – pH 6.0.

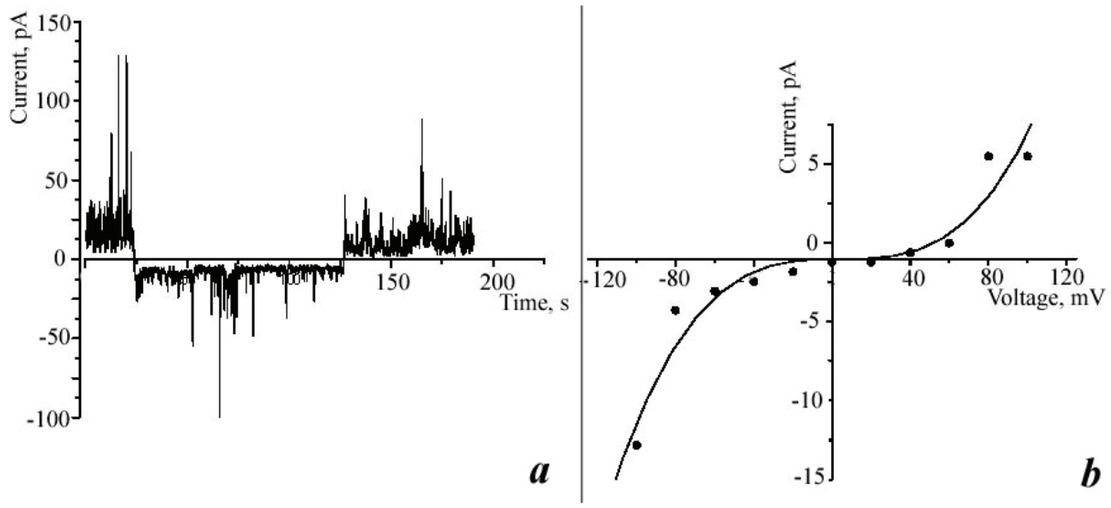

Fig. 2. Current fluctuations (*a*) and current-voltage characteristics (*b*) of a lipid bilayer from phosphatidylcholine (20 mg/ml) in presence 2 ng/ml recombinant LIF(pro). External solution: 1M KCl, 10 мМ $CaCl_2$, 40 мМ Tris-Hepes, pH 7.2; voltage 100 and -100 mV.

current disconnections vanish. No steady-state conductivity and moreover no relatively low rise of it could be approached at use of both LIF(eu) and LIF(pro), hence, effects of the proteins on a bilayer membrane may be evaluated only by quality characteristics of integral current change.

A similar pattern is registered at BLM-testing of a potentially therapeutic protein LIF(eu) isolated from *lif*-transfected Cos-1 cells (Petrova et al., 2006). But in this case current increases by jumps of varying heights; such changes are usually registered when current passes through an ion channel (fig.3 *a*, current jumps 13.6, 2.4, 3.2 and 3.6 pA are pointed with arrows). Heights of current jumps in presence of LIF(eu) depend on ion strength of a external solution: the less is salt concentration the higher is current passing through the current-conducting unit. For example, in 0.1M KCl at 100 mV impressed voltage its mean is 90±20 pA, in 0.2M KCl it is 22±6 pA and in 1M KCl it is 7±1 pA. A mean conductivity of a unit channel depends, besides, on a potential sign: at a negative value a mean amplitude of jumps is higher, than at a positive one (in 0.1M KCl - 175±19 pA and 1M KCl it is 90±20 pA versus 90 and 7 pA, respectively). Presented at fig. 3 *b* data show that in presence of LIF(eu) in 0.1M KCl solutions current depends linearly on voltage; in 0.2M KCl there is a hyperfunction under positive potential and overall current at 100 mV is almost twice greater than at -100 mV. In 1M KCl overall dependence of current has an opposite character but conductivity of the same channel also remains higher under a negative potential (fig. 4).

The presented data tell that recombinant LIF proteins, regardless of a producer (prokaryote or eukaryote), affect a surface potential and conductivity of a lipid bilayer. LIF(eu) has an additional activity: affecting a BLM, it forms stable current-conducting structures, ion channels. Characteristics of current through the channels depend on value and sign of a membrane potential, ionic strength of a surrounding solution, and on a lipid composition of the bilayer. Secreted sequences (20 amino acid residues) which are activated at posttranslational modifications of mature protein may be responsible for formation of the ion channels (Petrova et al., 2006). Bacterial producers have no these systems, so we suppose LIF(pro) to lack channel-forming possibilities. Participation of glycated LIF(eu) sites in forming of current-conducting structures cannot be exempted either, though their function is considered to be stabilisation of molecules and their protection of photolytic degradation (Hinds et al., 1998; Hilton, Gough, 1998; Heinrich et al., 1998).

Thus, electrophysiological studies show a possibility of direct effect of LIF recombinant molecules on lipid matrix of cell membranes involving not only LIF-receptor complex for pass of signals into cells. We suppose modification of cytoplasm membranes by ion-channels forming as one of the mechanisms of LIF regulatory action. This may play an important role in cell-cell interaction and metabolic cooperation of stem cells at their cultivation *in vitro*.

3. Comparison of biological activity of LIF(eu) and LIF(pro) with mouse ESC cultures

Recombinant LIF proteins from pro- and eukaryotic expression systems were tested on cells of R1 line, characterized by their dependence from feeder and LIF in a culture medium presence (Nagy et al., 1993). To prevent effects of feeder cells on ESC, R1 cells were cultivated on 0.1% gelatine cover. This way of cultivation resulted no reliable differences in performances of LIF(pro) and LIF(eu) effects on growth and renovation of R1 line cells were registered (table 1, fig. 5). During 48 h both proteins in 10-13 ng/ml concentrations maintained pluripotent properties of the cells and their relatively high rate of growth in

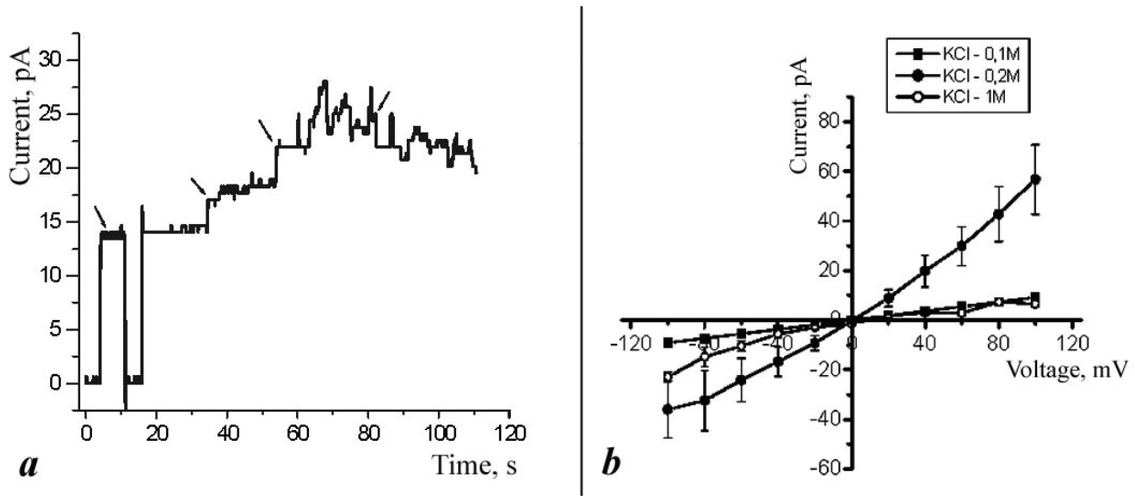

Fig. 3. Current jumps (0.1M KCl; 100 mV) (*a*) and integral current-voltage characteristics at different ionic strengths (*b*); 6.5 ng/ml LIF(eu); phosphatidilcholine membrane.

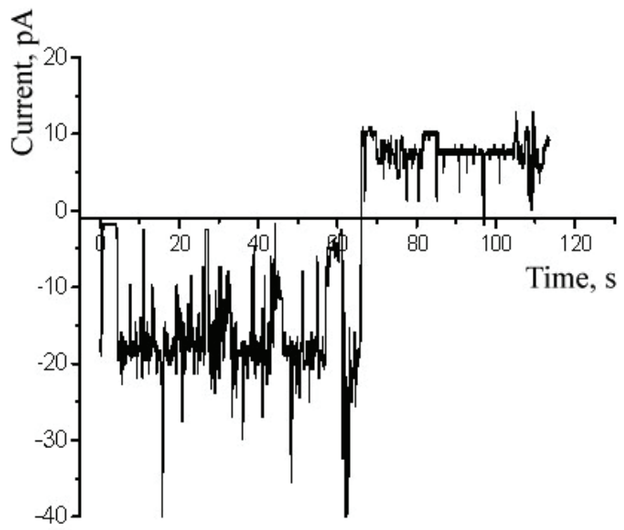

Fig. 4. Current fluctuations at -100 and at 100 mV; phosphatidilcholine membrane (20 mg/ml); 1M KCl; and 19.5 ng/ml LIF(eu).

form of colonies without morphological signs of differentiation and EB formation (table 1, fig. 6 *c*). Correlation between differentiated and non-differentiated (pluripotent) colonies with high endogenous alkaline phosphatase (AP) activity was kept at a high level and did not depend on a source of LIF origin (fig. 5). At the absence of LIF: cell growth was inhibited; time of populations doubling increased (table 1) and EB were found in the medium pointing thus prevalence of embryonic differentiation processes over proliferation ones (fig. 5, fig. 6, *∂*).

Culture medium	cell numbers (x10^5)		growth rate	ESC population doubling time (h)
	48 h	72 h		
LIF(eu)	2.0±0.52	3.4±0.85*	4.0±0.31*	13.6±2.4
LIF(pro)	2.1±0.40	2.1±0.43	3.7±0.25*	13.2±2.7
Control (LIF-free)	1.1±0.20	1.2±0.80	2.4±0.17	22.2±6.3**

Table 1. Comparative proliferative activity of R1 line mouse embryonic stem cells cultivated on 0.1 gelatine in presence of recombinant LIF(eu) and LIF(pro). Notice. Final concentration of LIF proteins in medium: 10-13 ng/ml. * - differences confidence (P≤0.05); **- differences confidence by time of cells populations doubling in LIF-supplemented and LIF-free media (P≤0.01).

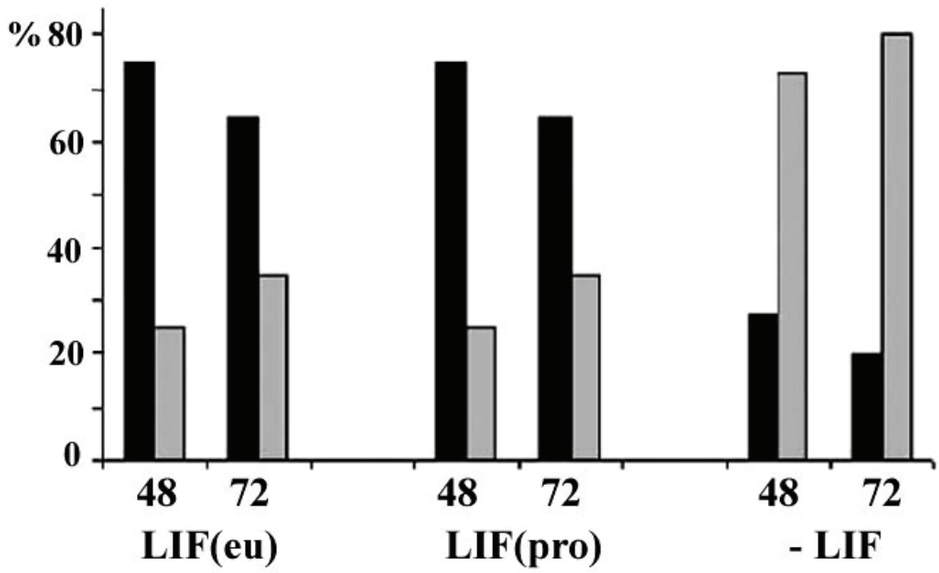

Fig. 5. Percentage of differentiated (□) and non-differentiated (■) colonies of R1 line embryonic cells cultivated on 0.1% gelatine in media with recombinant LIF proteins (10-13 ng/ml).

Regulatory functions of LIF are apparently dose dependent (fig. 7); this was demonstrated by our experiments with techniques of R1 line cells cultivation on a feeder from mouse primary embryonic fibroblasts (PEF) and media supplemented with recombinant LIF protein in various concentrations. These cultivation conditions enhance the general tendency of pluripotent cells: development by colonies without visible signs of spontaneous differentiation and formation of EB. It is well known, that cells of mouse PEF can produce their own LIF (Williams et al., 1988) and can additionally saturate a nutrient medium with this factor. But in spite of such a possibility, use of recombinant LIF in small concentrations (2.5-5.0 ng/ml) and of feeder cultivation does not provide a pluripotent status of R1 line cells in culture completely (fig. 7). To 48 h number of damaged cells in populations with low

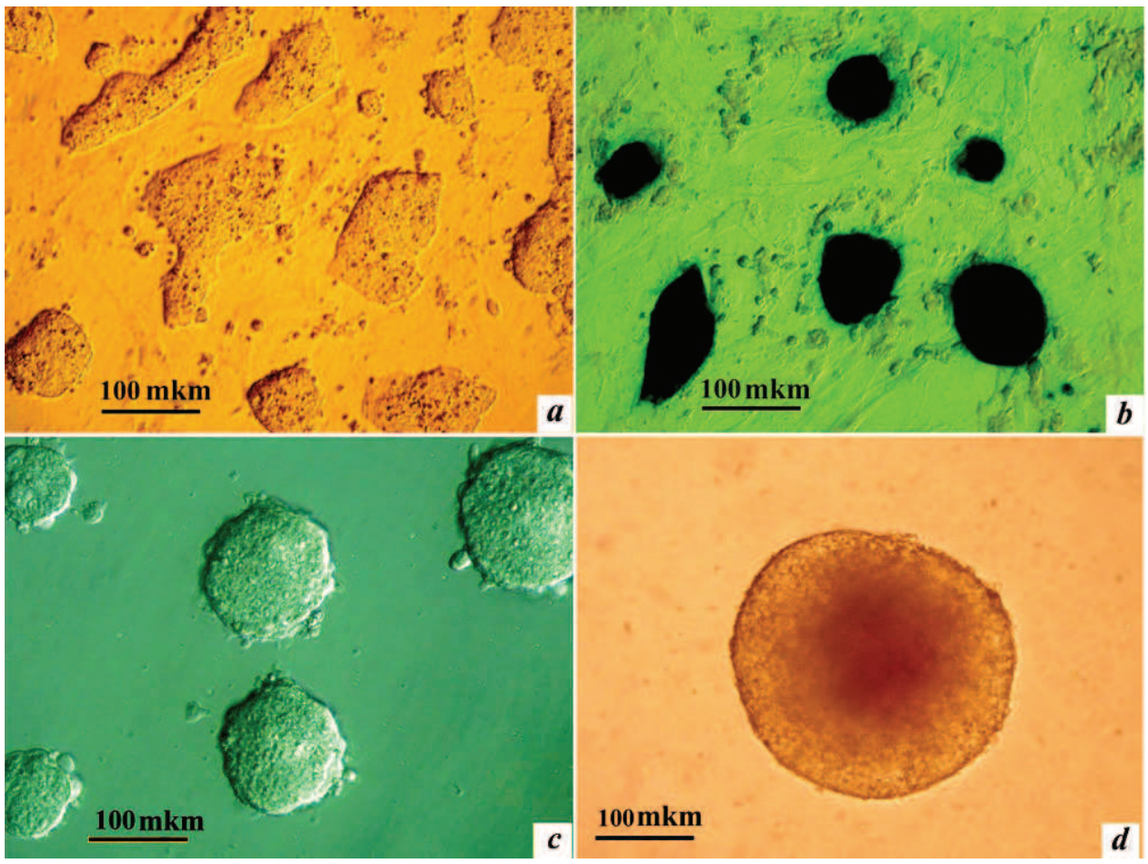

Fig. 6. Morphology of colonies of R1 line embryonic stem cells in a medium with recombinant LIF(pro) after 48 h cultivation on PEF feeder (a) and on 0.1% gelatine (*c*); detecting of alkaline phosphatase activity (b), embryoid body in a LIF-free medium (d).

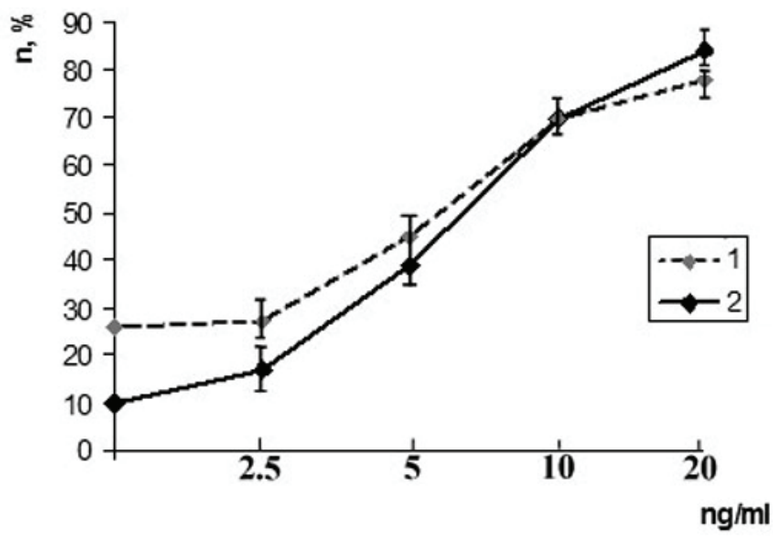

Fig. 7. Dependence of formation of non-differentiated pluripotent colonies of R1 embryonic stem cells from LIF(pro) concentration in 24 (1) and 48 h (2) of PEF feeder cultivation.

content of exogenous LIF protein is increased; rate of cell divisions is inhibited and EB occur (table 2). We registered such a situation at growing of R1 cells on a gelatine support in a LIF-free medium (table 1, fig. 5, 6 *∂*).

LIF concentration (ng/ml)	Cell number (x10^5)		Growth rate	Population doubling time (h)	N/D (%)
	48 h	72 h			
5	19.7±1.2	27.7±1.4	3.9±0.2	12.2±0.9	42.75
10	21.3±0.7*	34.0±1.1*	4.3±0.2	10.0±0,5**	67.45
20	34.3±1.8*	32.3±1.5*	6.9±0.3*	7.0±0.7**	82.90
Control (LIF-free)	15.6±3.1	19.0±8.0	3.1±0.6	15.0±1.8	33.70

Table 2. Proliferative activity and growth of R1 embryonic stem cells colonies in dependence on recombinant LIF(pro) concentration at cultivation on PEF feeder. Notice. N/D - non-differentiated pluripotent colonies of embryonic stem cells with high activity of endogenous alkaline phosphatase; * - reliable increase of proliferative activity and rate of colonies growth under effect of 10-20 ng/ml of LIF (P≤0.05); ** - reliability of differences of cell populations doubling time in media with 10-20 ng/ml LIF and without LIF (P≤0.01).

Stem cells of R1 line require relatively high concentrations of recombinant LIF in a culture medium (10-20 ng/ml) that decreases their doubling times up to 10 hours in average and increases rate of their growth 1.5-2.0 folds relatively cells developing in a LIF-free medium (table 1, 2). At different conditions of R1 cultivation (gelatine or PEF feeder), in the presence of LIF in optimal concentrations, approximately the same share (70-80%) of pluripotent colonies can be registered (fig. 5, table 2). Hence, we may postulate that LIF(pro) and LIF(eu) are equally effective in their support of ESC pluripotency and high proliferative activity *in vitro.* Our observations tell that only R1 stem cells with active proliferation keep their pluripotent potential in the presence recombinant LIF.

4. Effects on cell death (apoptosis) and cell cycles

When cell differentiation is difficult, correlation of two forms of stem cells response, proliferation/cell death, may serve as an essential parameter of their reaction on effects of regulatory LIF protein and reflect self-renewal potential of population. For example, our investigations showed that 5-10 ng/ml concentrations of LIF decrease share of apoptotic cells in R1 population to 2.0% in comparison with 7.2 % following 18 hours of incubation in LIF-free medium (Lobanok et al., 2008, 2009). At 5 ng/ml concentration LIF is more effective as anti-apoptotic agent than at 10 ng/ml. It may be caused by that apoptosis is an active form of cell reaction not only on unfavourable conditions, like deficiency of growth factors in cultivation medium but on their physiological concentrations activating cell growth. In case of R1 ESC this concentration is 10-20 ng/ml LIF (table 1 and 2, fig. 7).

Another essential characteristic of mouse ESC response on effect of LIF regulatory molecules reflecting potential of the cells for renovation and reproduction is distribution of cells by phases of cell cycle. Presented histograms: G1 (content of 2C DNA), S (from 2 to 4C) and

G2+M (4C) tell that if presence 5 and 10 ng/ml of recombinant LIF(pro) presents in a medium, share of R1 cells in S-phase of cell-cycle decreases (fig. 8). A specific feature of mouse ESC lines worth mentioning: their cells spend the most part of their life-time in S-phase, during which DNA synthesis occurs. Contrary to somatic cells of tissue culture, ESC do not need external stimuli to initiate DNA replication processes (Smith, 2001). It can explain why ESC are hardly moved out from cell cycle into G1 or G0 stages.

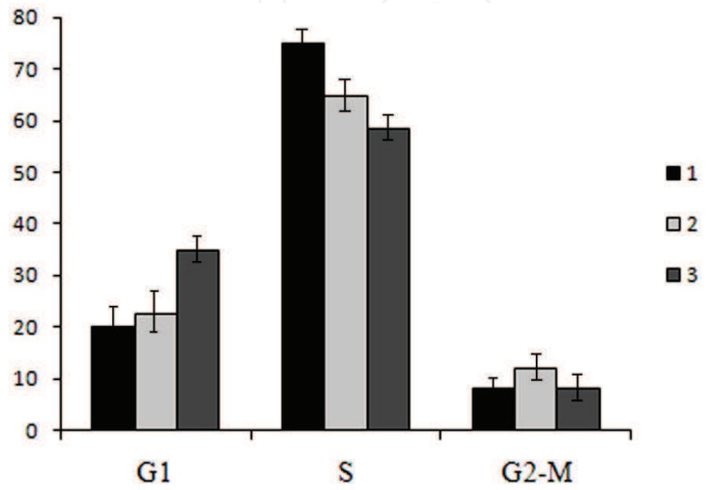

Fig. 8. Effect of recombinant LIF(pro) concentration on distribution of R1 mouse embryonic stem cells by phases of a cell cycle after 18 h cultivation on PEF feeder. 1) Control (LIF-free), 2) 5 and 3) 10 ng/ml LIF in culture medium.

Nevertheless, from fig. 8 one can see that LIF in 10 ng/ml concentration, optimal for ESC, activates these processes. In growing populations of R1 after 18 hours of incubation with 10 ng/ml LIF(pro) share of stem cells in G1 phase increases in comparison with cells that did not get the protein or got it in a smaller concentration (fig. 8). Increase of G1-phase cells number may tell about their spontaneous differentiation into EB and about enhanced production of D-cyclins inhibiting cell division (Savatier et al., 1996). But we must keep in mind that too short G1 period is typical for ESC and therefore mechanisms of their activation may differ somewhat from those of somatic cells (Savatier et al., 1996; Rohwedel et al., 1996; Wianny et al., 1998). For example, some part of mouse ESC keep their ability to differentiate into EB and to grow by colonies in LIF-free medium, but rates of their division become shorter in comparison with growing with LIF (table 1 and 2, fig. 5).

ESC, being cultivated, may stop at one of the cell cycle stages, therefore increase of their number in S synthetic phase does not always reflect proliferative activity of population in whole; this is exemplified by fig. 8. In this connection, we calculated rate of cells in S phase share to G_2+M as S/(G_2+M). The calculations shows that after 18 hours of incubation in a LIF-free medium the most part of R1 cells (more than 70 %) are in S phase and S/(G_2+M) equation reflecting proliferative potential of a culture equals to 10.9. Affected by 5 ng/ml of LIF, the parameter decreases to 4.7, whereas 10 ng/ml decreases it only to 8.3. It may be provided by the following: in presence of 10 ng/ml LIF, mouse ESC pass an S-phase more rapidly and about 10 % of cells stop at a G1 restriction point (fig. 8). Small doses of LIF effects exit of cells from S-phase, increasing thus a proliferative index of a population and share of cells with DNA content ≥2C.

5. Conclusion

Thus comparative analysis of recombinant mouse LIF from pro- and eukaryotic expression systems made in the investigation with bilayer lipid membranes and cultures of ESC (R1 line) showed that both protein types, LIF(pro) and LIF(eu), have membranotropic effects. Independently on an expression system, molecules of recombinant LIF are incorporated into lipid bilayer, causing increase of surface potential and membrane electric conductivity. LIF-induced changes of lipid matrix of cell membranes may play a key role in maintenance of pluripotent properties of mouse ESC *in vitro*. We suppose binary mechanism of LIF actions on both receptors (LIF-R and gp130) and lipid matrix of cell membranes to underlie its functional redundancy. For example, our investigations show that recombinant mouse LIF affects ESC proliferation, apoptosis and cells distribution by cell cycle phases (decreases $S/(G_2+M)$ ratio of a cell cycle and time of ESC population doubling).

6. References

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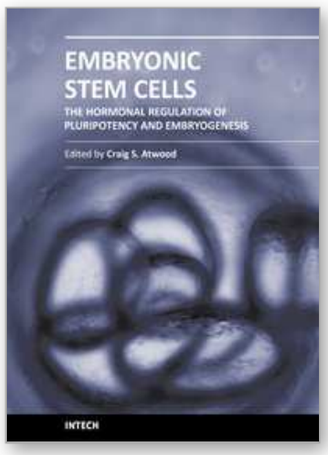

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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

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