Original Article

Leukaemia Inhibitory Factor Inhibits Cardiomyogenesis of Mouse Embryonic Stem Cells via STAT3 Activation

(leukaemia inhibitory factor / embryonic stem cell / cardiomyogenesis / STAT3 / serum-free)

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Abstract. The leukaemia inhibitory factor is a cytokine that exhibits pleiotropic activities in a wide range of cell types. There are evidences that leukaemia inhibitory factor-regulated signalling pathways are involved in cardiomyogesis and maintenance of cardiomyocytes. In the present work we studied the effect of leukaemia inhibitory factor on cardiomyogenesis of embryonic stem cells together with the role of serum-born factors. We showed that leukaemia inhibitory factor had an inhibitory effect during both the induction and progression phases of cardiomyogenesis of embryonic stem cells. The leukaemia inhibitory factor-mediated inhibition of cardiomyogenesis was abolished by inhibitors of STAT3 activity. These results suggest that leukaemia inhibitory factor-activated STAT3 is responsible for the inhibition of cardiomyogenesis in embryonic stem cells.

Introduction

Mouse embryonic stem (ES) cells represent unique cell types derived from the inner cell mass of pre-implantation blastocyst. The defining feature of ES cells is their self-renewal and ability to follow multiple differentiation pathways both *in vivo* and *in vitro* (Evans and Kaufman, 1981; Martin, 1981). Maintenance of mouse ES (mES) cells depends on the activation of glycoprotein 130 (gp130) receptor-regulated signalling pathways, which is generally mediated by binding of the leukaemia inhibitory factor (LIF) (Rathjen et al., 1990; Niwa et al., 1998). However, this pathway also plays a very important role during embryogenesis itself. LIF is a pleiotropic cytokine that belongs to the interleukin 6 (IL-6) cytokine family, which is secreted by various cell types and mediates a variety of cellular responses depending on the target tissue and cell lineage (Shellard et al., 1996; Seiler et al., 2001). Following ligand-induced dimerization of the receptor β and the gp130 receptor, gp130 is phosphorylated by Janus kinases (JAKs), which are constitutively associated with it. The activated JAKs then phosphorylate signal transducers and activators of transcription (STATs), which subsequently dimerize and translocate to the nucleus, where they transactivate LIF-responsive genes. In parallel, a different part of the gp130 receptor serves as a docking site for SRC homology phosphatase 2 (SHP2), which recruits growth factor receptor-bound 2 (GRB2) and leads to the activation of rat sarcoma (RAS) >> mitogen-activated protein kinase (MAPK) (Taga, 1997), (Seiler et al., 2001). During development, LIF (or other IL-6 family cytokines)-mediated gp130 activation plays a significant role from early embryogenesis to adulthood. Depletion of *JAK2* or *STAT3* genes leads to early embryo mortality (about 5–7 dpc) and depletion of the gp130 receptor leads to embryo death during day 12.5 dpc due to the failure of development of the heart and other organs (summary in Boiani and Schöler, 2005). Some IL-6 cytokines (LIF, CT-1, IL-11) also induce cardiac hypertrophy and protect cardiomyocytes from death (summarized in Seiler et al., 2001; Fischer and Hilfiker-Kleiner, 2007).

Similarly to *in vivo* development, a significant role of JAK-STAT signalling has also been shown during cardiomyogenesis *in vitro*. It was reported that induction of cardiomyogenesis of pluripotent mES cells is dependent on the JAK2 and STAT3-mediated signalling pathway (Foshay et al., 2005; Rajasingh et al., 2007). If LIF is the principal inductor of gp130>JAK2>STAT3 signalling, we would expect it to stimulate JAK2>STAT3-dependent cell differentiation. Thus, in the present study we tested the effect of LIF on induction of ES cell cardio-

Received November 16, 2009. Accepted May 14, 2010.

This work was supported by grants from the Czech Science Foundation 301/08/0717 and 204/09/H058.

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Abbreviations: EB – embryoid bodie, ES – embryonic stem, FCS – foetal calf serum, GADPH – glyceraldehyde-3-phosphate dehydrogenase, dpc – days post coitum, GRB2 – growth factor receptor-bound 2, IL-6 – interleukin 6, ITS – insulin, transferrin selenium, JAK – Janus kinase, LIF – leukaemia inhibitory factor, MAPK – mitogen-activated protein kinase, MEK – MAP/ERK kinase, mES – mouse ES, MHC – myosin heavy chain, RAS – rat sarcoma, SDS – sodium dodecyl sulphate, SHP2 – SRC homology phosphatase 2, STAT – signal transducer and activator of transcription, SV – Stattic V.

myogenesis. Surprisingly, LIF inhibits cardiomyogenesis both during its induction phase and during advanced cardiomyocyte differentiation. Moreover, our results demonstrated that the LIF-mediated inhibition of cardiomyognesis was mediated via the STAT3 signalling pathway.

Material and Methods

Culture and differentiation of ES cells

Undifferentiated mouse ES cells (mES D3 cells; (Doetschman, et al., 1985) were adapted to feeder-free culture and routinely grown in Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Carlsbad, CA) supplemented with 15% foetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 100 mM non-essential amino acids (Gibco-Invitrogen), 0.05 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco-Invitrogen), and 1000 U/ml recombinant leukaemia inhibitory factor (LIF; Chemicon International, Temecula, CA). The differentiation of ES cells to embryoid bodies (EB) was performed by hanging drop technique and/or direct culture of ES cells on bacteriological dishes in complete ES media with or without LIF – here mentioned as induction phase of culture (Krejci, et al., 2008). DMEM-F12 (1 : 1) supplemented with insulin, transferrin, selenium (ITS, Gibco-Invitrogen), and antibiotics as above was used as serum-free medium. Seven days growing EB were transferred into tissue culture dishes with appropriate media and supplements – here mentioned as differentiating phase of culture. Fresh medium was replaced every two days. Beating cell colonies were scored and their morphology was documented by photography. LIF concentration used in all the presented experiment was 2.5 ng per one ml of media. The specific STAT3 inhibitor Stattic V (2 μM; Merck-Calbiochem, Darmstadt, Germany) and specific MEK inhibitor (ERK signalling) UO126 (5 μM; Sigma-Aldrich) were employed for inhibition of the mentioned signalling pathways.

qRT-PCR analysis

Total RNA was extracted by the RNeasy Mini Kit (QIAGEN, Valencia, CA). Complementary DNA was synthesized according to the manufacturer's instructions for M-MLV reverse transcriptase kit (Sigma-Aldrich). qRT-PCR was performed in Light-cycler Roche using the following programme: initial activation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s, annealing temperature (see below) for 10 s, and 72 °C for 10 s. Gene expression for each sample was expressed in terms of the threshold cycle normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as we described previously (Bryja, et al., 2008). Primers and PCR conditions were as follows (primer sequence/annealing temperature):
Pou5f1 (Oct-4) (5'-CGTTCTCTTTGGAAAGGTGTTC-3', 5'-GAACCATACTCGAACCACATCC-3'/59 °C),
Nanog (5'-AAGCAGAAGATGCGGACTGT-3',5'-GTGCTGAGCCCTTCTGAATC-3'/59 °C),
Sox2 (5'-GCGCCCTGCAGTACAACTCC-3', 5'-TTCGCAGTCCAGCCCTCACA-3'/66 °C),
GATA1 (5'-GAAGCGAATGATTGTCAGCA-3', 5'-TTCCTCGTCTGGATTCCATC-3'/60 °C),
Fgf5 (5'-CTGTACTGCAGAGTGGGCATCGG-3', 5'-GACTTCTGCGAGGCTGCGACAGG-3'/57 °C),
Pax6 (5'-TGCCCTTCCATCTTTGCTTG-3', 5'-TCTGCCCGTTCAACATCCTTAG-3'/57 °C);
Gapdh (5'-AAGGGCTCATGACCACAGTC-3',5'-CATACTTGGCAGGTTTCTCCA-3'/61 °C).

Immunoblot analysis

For immunoblot analysis, cell samples were prepared as follows: ES cells and/or EB were washed twice with PBS (pH 7.2) and lysed in sodium dodecyl sulphate (SDS) lysis buffer (50 mM Tris-HCl, pH 7.5; 1 % SDS; 10 % glycerol). Protein concentrations were determined using DC Protein assay kit (Bio-Rad, Hercules, CA). Lysates were supplemented with bromphenol blue (0.01%) and β-mercaptoethanol (1 %), and incubated for 5 min at 95 °C. Equal amounts of total protein (10 μg) were subjected to 8 or 10 % SDS-PAGE. After being electrotransferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA), proteins were immunodetected using appropriate primary and secondary antibodies, and visualized by ECL-Plus reagent (Amersham Pharmacia Biotech, Piscataway, NJ) according to manufacturer's instructions. The antibodies used were as follows: rabbit polyclonal antibodies against ERK (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-STAT3 (Cell Signaling Technology, Danvers, MA), phospho-ERK (Cell Signaling Technology), and mouse monoclonal antibody against β-tubulin (Santa Cruz Biotechnology), STAT3 (Santa Cruz Biotechnology), and myosin heavy chain (the hybridoma MF20, developed by Drs. Donald and Fischman, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, http://www.uiowa.edu/~dshbwww)). After immunodetection, each membrane was stained by amidoblack to confirm equal protein loading.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was assessed by one-way ANOVA. The values of $P < 0.05$ were considered to be statistically significant.

Results

Differentiation of ES cells to beating cardiomyocytes – effects of serum and LIF

ES cells were differentiated as described above in Material and Methods. In short, during the first set of

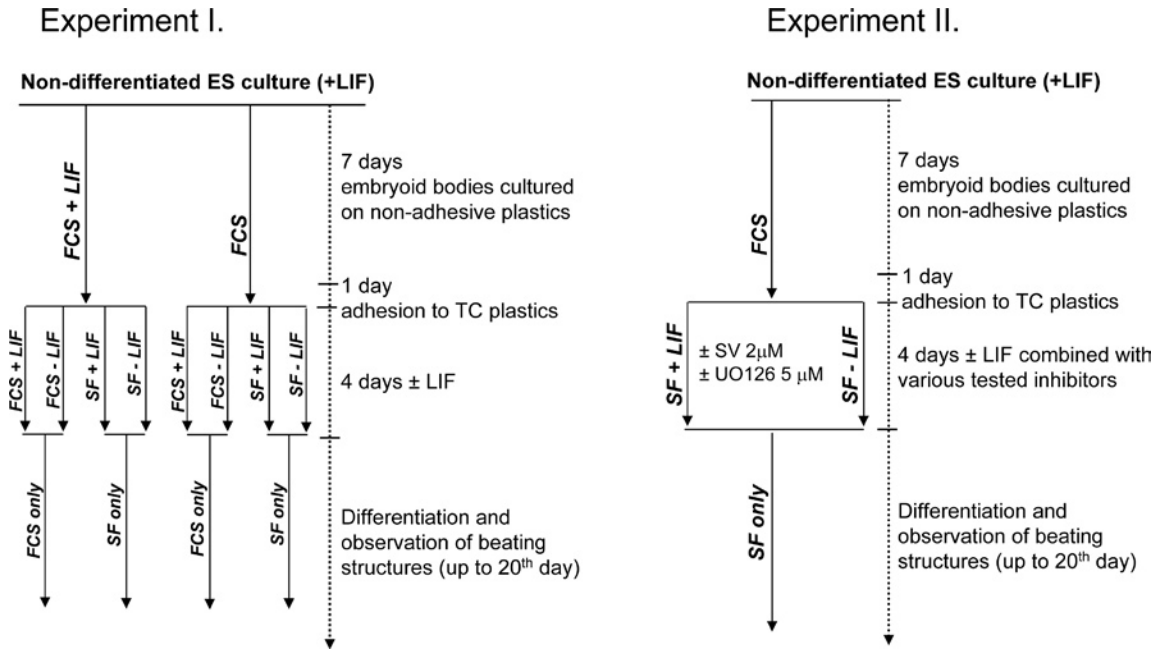

Fig. 1. Experimental designs. The differentiation process requires several changes of cultivation conditions. FCS – complete ES media containing 15 % of foetal calf serum; SF – serum-free media.

experiments EB were formed in complete media in the presence or absence of LIF. The EB were seeded on a gelatinized tissue culture plastic dish on day 7. After 24 h, when the EB were firmly attached to culture dishes, the medium was replaced with appropriate media for the following differentiation. The differentiation media contained serum or were serum-free with ITS supplement, and the attached EB were cultured with or without LIF during the first four days of differentiation. The complete experimental design of this part is depicted in Fig. 1. The only significant difference of EB formed in the presence of LIF was their more compact morphology (Fig. 2a). The dense roundish morphology of EB formed in the presence of LIF was sustained even after attachment of EB to the dish surface. This phenomenon was observed both in serum-supplemented and serum-free conditions (Fig. 2b). Beating colonies supplementary data were evaluated on a daily basis. The first beating colonies appeared approximately on day 12–13 from the beginning of EB formation; the maximum number of beating colonies was observed from day 18 to day 22. Although EB in complete media quickly expanded, their

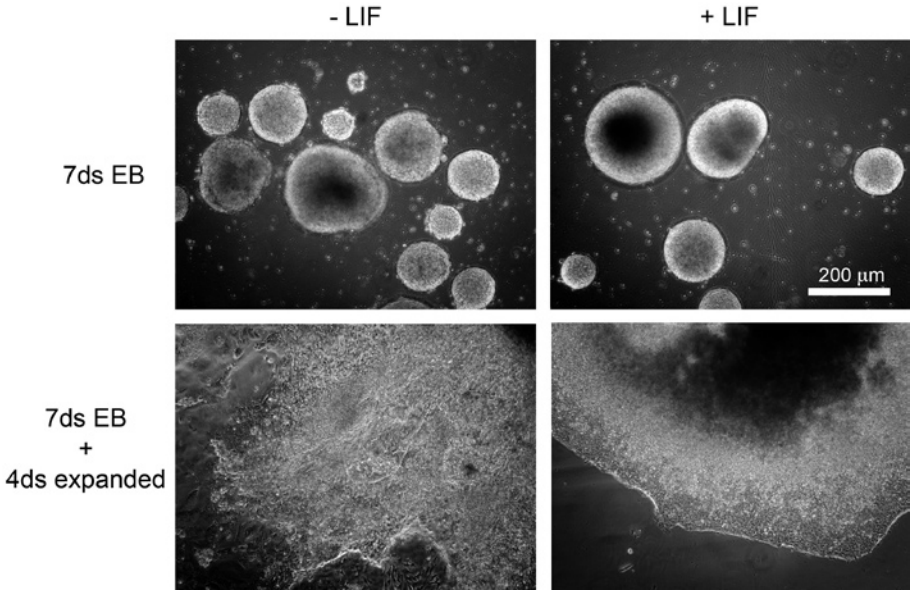

Fig. 2a. Morphology of floating embryoid bodies (EB) on day 7 in the presence or absence of LIF compared with morphology of the same EB on day 11 of differentiation. According to the experimental designs (Fig. 1) EB were transferred to adherence-prone plastics and expanded in serum-free media.

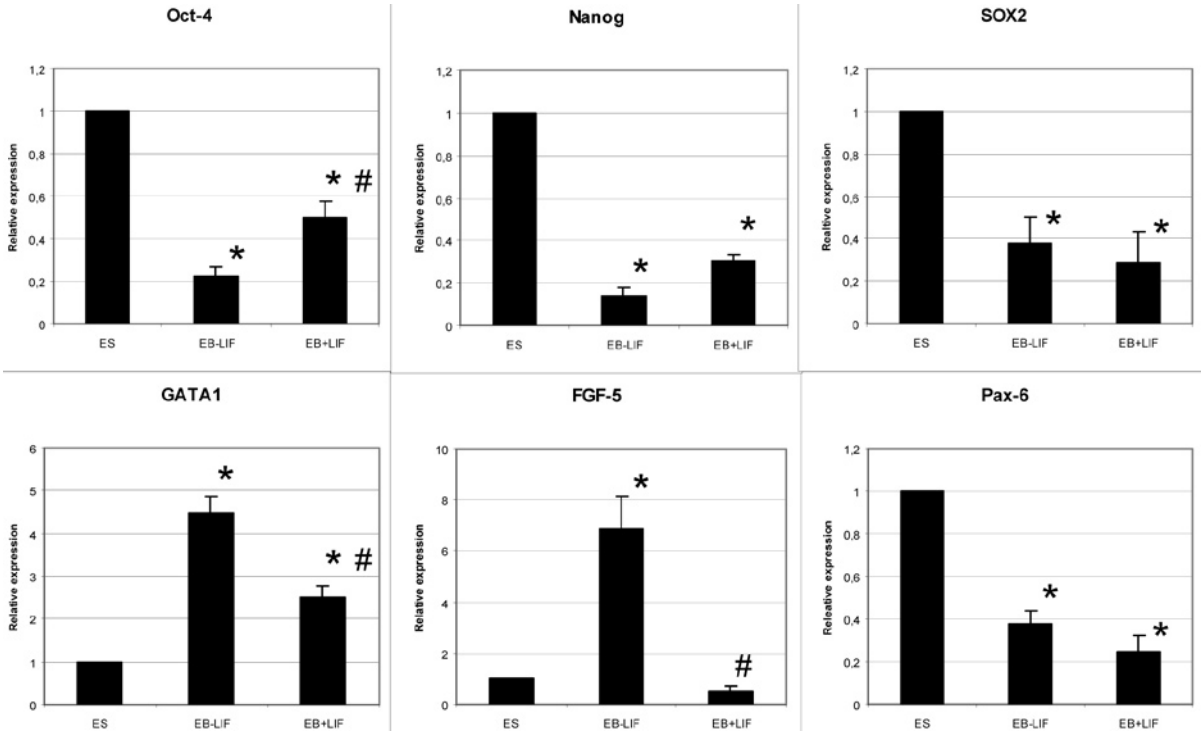

Fig. 2b. qRT-PCR analysis of the level of differentiation status-specific mRNA in non-differentiated (ES) cells and 7 days old EB cultured in the absence (EB-LIF) or presence (EB+LIF) of LIF. The level of mRNA for genes associated with pluripotency (*Pou5f1 (Oct4)*, *Nanog*, and *Sox2*) and early lineages commitment (*Gata1*, *Fgf5*, and *Pax6*) are shown. * marks statistically significant difference between ES and EB, # marks statistically significant difference between EB-LIF and EB+LIF in three independent experiments, $P < 0.05$

differentiation to beating cardiomyocytes was less efficient compared to their counterparts differentiating in serum-free ITS media. When colonies expanded in the serum-free media, beating structures were easy to identify due to their specific 'bunch-like' morphology (Fig. 3). In the presence of the serum, the beating structures were not clearly demarcated from other parts of the expanded colony (not shown). The presence of LIF in induction phases of the culture fully inhibited the formation of beating cardiomyocytes under all conditions. The presence of LIF during the expansion phase did not inhibit cardiomyogenesis of EB in the presence of serum. However, LIF significantly inhibited cardiomyogenesis when the EB were expanded in serum-free conditions (Fig. 4).

Effect of LIF on ES cell differentiation within EB

To evaluate the ES cell differentiation within EB we determined the expression of mRNA in pluripotent cells

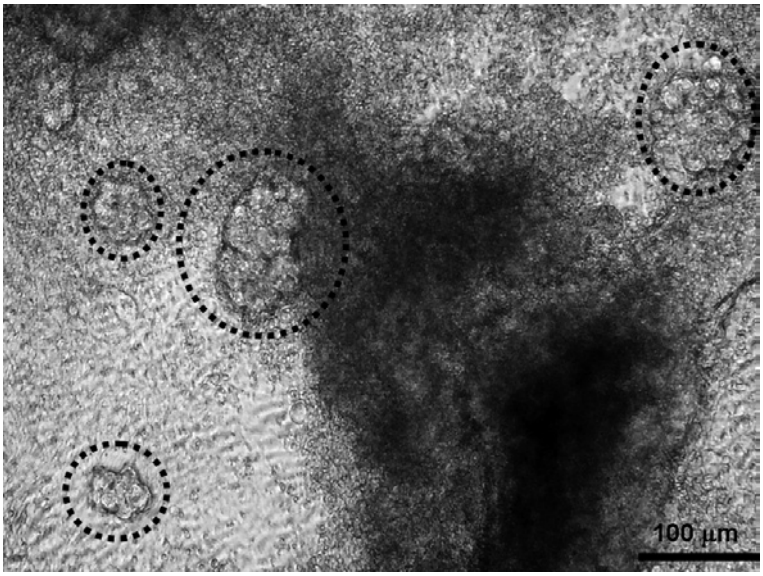

Fig. 3. Morphology of beating 'bunch-like' colonies. EB formed in complete media without LIF were expanded in serum-free media up to 20th day. The beating colonies are highlighted by dashed circle.

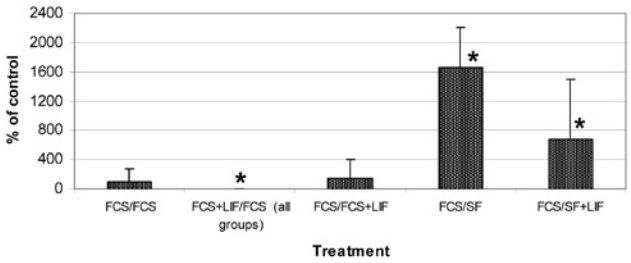

Fig. 4. Various culture conditions affect the number of beating colonies in the culture. Culture conditions are assigned as medium for EB formation/medium for adhered EB expansion. The number of beating colonies in samples cultivated in serum-containing complete media in all the steps of the differentiation process was taken as 100 % and control. For details see Fig. 1a. * $P < 0.01$.

(*Pou5f1 (Oct4)*, *Nanog*, and *Sox2*) and mRNA characterized for early embryonic cell lineages (*Gata1*, *Fgf5* and *Pax6*). The results are presented in Fig. 2b. Formation of EB led to down-regulation of all three genes expressed in pluripotent ES cells, in case of *Nanog* and *Sox2* independent of the presence of LIF. *Pou5f1* was significantly more down-regulated in the absence of LIF. Expression of *Fgf5* was strongly up-regulated in EB without LIF and unaffected in the presence of LIF. The *Gata1* mRNA level was also significantly increased, but it was lower in the presence of LIF. Expression of *Pax6* mRNA was similarly decreased in both cases. Thus, we can conclude that LIF-induced partial inhibition of differentiation within EB did not occur in all the committed lineages generated here.

Response of EB to LIF treatment

We also analysed the responsiveness of 8-day-old EB to LIF treatment using the immunoblot technique. The ES cells and attached EB were starved in serum-free, ITS-free media for 6 h. After starvation, LIF was added to these cultures for 20 min, cells were lysed and subjected to Western blot analysis. Results in Fig. 5 clearly show that LIF-induced STAT3 and ERK phosphorylation in ES cells and EB formed both in the absence and presence of LIF. The total level of STAT3 and the level of phosphorylated STAT3 proteins were higher in ES cells than in EB. Thus, ES cells and EB were both responsive to LIF/gp130 activation.

The mechanism of LIF-mediated suppression of cardiomyogenesis in differentiating ES cells

Attached EB (differentiated for eight days as given above) were transferred to serum-free media and treated with the specific STAT3 inhibitor Stattic V or MEK>ERK inhibitor UO126. After addition of the mentioned inhibitors, the particular experimental groups were supplemented with LIF for the following four days. The concentrations of drugs employed in our study were selected on the basis of their effectiveness and low toxicity (not shown and Pachernik et al., 2007). The detailed experimental design is depicted in Fig. 1b. We discovered that the STAT3 inhibitor Stattic V did not affect cardiomyogenesis itself, but that it significantly reversed the LIF-mediated inhibition of cardiomyogenesis. Inhibition of ERK signalling using the MEK specific inhibitor UO126 blocked cardiomyogenesis irrespective of the presence of LIF (Fig. 6). LIF inhibition of cardiomyogenesis, its reversion by the STAT3 inhibitor Stattic V, and the negative effect of ERK signalling inhibitor UO126 were also documented by detection of myosin heavy chain (MHC) expression using Western blot analyses. MHC is extensively expressed in the heart and in our experiments its expression co-located with areas of beating cardiomyocyte structures. In experimental groups with intensive cardiomyogenesis (group without drugs or LIF, and in groups with Stattic V or with Stattic V plus LIF) we detected high expression of MHC. In experimental groups with inhibited cardiomyogenesis (group with LIF alone and UO126) we detected only a low level of MHC (Fig. 6).

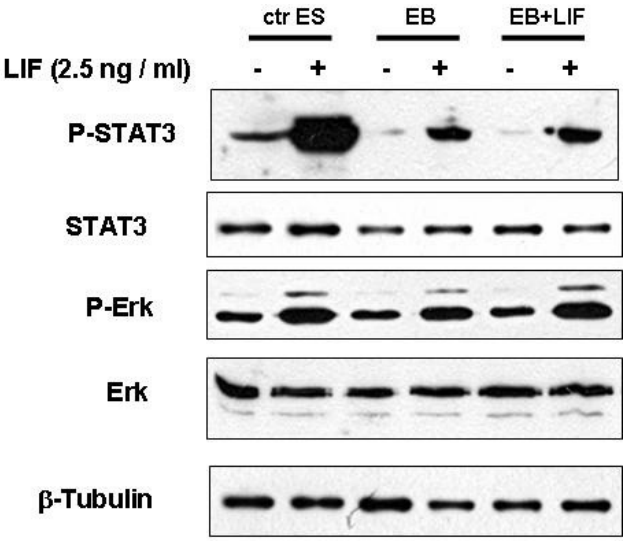

Fig. 5. Induction of STAT3 and ERK phosphorylation by LIF in ES cells and 7 days old EB formed in the presence or absence of LIF. The cells were 6 h starved in serum-free media without any supplement before treatment. After 20 min of LIF treatment, the cells were subjected to Western blot analysis using phospho-specific antibodies. The detection of β-tubulin protein was used as an equal loading control.

Discussion

Strategies of tissue replacement and cell therapy in medicine require preparation of specific cell precursors or progenitors. Embryonic stem cells represent a potentially promising source of such progenitors (Keller, 2005). To obtain a high-quality and homogenously differentiated cell population, we need to know mechanisms of their differentiation to the desired cell type. This study is focused on the mechanism of ES cell differentiation to cardiomyocytes. Cardiomyogenesis in

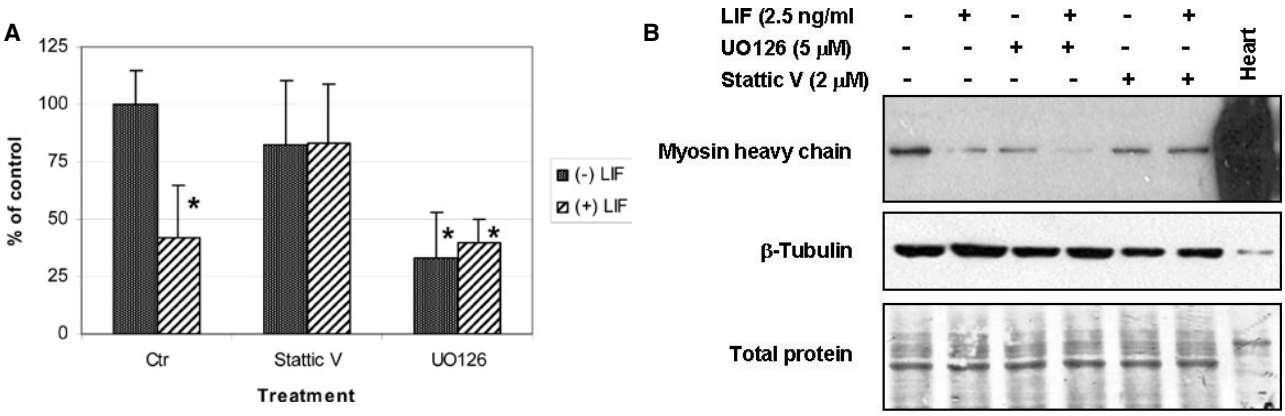

Fig. 6. Effect of LIF and inhibitors of ERK and STAT3 signalling on the number of beating colonies and myosin heavy chain expression. (A) The EB formed in the presence of serum and expanded in serum-free media were cultivated both in the presence and absence of LIF or specific inhibitors of ERK and STAT3 signalling. The number of beating colonies in the sample of EB formed in complete serum-containing media and expanded in serum-free media without LIF or inhibitors was taken as control (Ctr). The experiment was performed according to the experimental design II – for details see Fig. 1. * $P < 0.05$. (B) Level of myosin heavy chain expression in cells treated as described in A. The lysate of mouse adult heart cells serves as a positive control. The detection of β-tubulin protein and amidoblack staining of total protein were used as equal loading controls. Ten μg of protein lysate per lane was used in samples obtained from cell cultures, whereas only 1 μg of protein was used for positive control (Heart).

ES cells may be easily induced in 6- to 8-day-old non-adherent culture (containing formed EB) followed by further expansion of EB in adherent culture (Boheler et al., 2002; Wei et al., 2005). Most research groups differentiated ES cells to cardiomyocytes in complete, serum-containing media. However, it has also been reported that if cardiomyogenesis is to be effective, high-quality foetal bovine serum is required (Boheler et al., 2002). In our experiments we also explored the effects of the serum. Although we used various high-quality ES-tested FBS from two suppliers, cardiomyogenesis was relatively low compared to the serum-free conditions (Fig. 3). Cardiomyogenesis of ES cells under serum-free condition was also described by another research group (Murashov et al., 2005). Differentiation of cardiomyocytes in serum-free conditions is thus an easier, cheaper method, which is also independent of the serum batch. Moreover, the serum-free conditions allow more defined treatment of cells without unpredictable interaction with undefined compounds of the serum. Unfortunately, we were not able to form EB in serum-free media during the initial step (formation of EB) of differentiation due to extensive cell death under these conditions (not shown). Thus, the presence of serum or serum-replacement substances in culture media is essential for the initial step of differentiation if EB formation is required.

Recent studies with genetically manipulated mice and pluripotent cell lines have shown that the gp130/STAT3 signalling plays a significant role in cardiomyocyte differentiation both *in vivo* and *in vitro* (see Introduction). We hypothesize that gp130 signalling activated by its ligands (e.g. LIF) has a pro-cardiomyogenic effect. Therefore, we analysed the effect of LIF both during the initial phase of differentiation (EB formation) and the following expansion phase when EB were attached to the surface, expanded and further differentiated. Surprisingly, we observed a negative effect of LIF/gp130 activation on cardiomyogenesis in ES cells. LIF abolished cardiomyogenesis both in the induction and expansion phases of differentiation. However, when LIF was present only in the expansion phase together with the serum, the inhibition effect of LIF on cardiomyogenesis was not observed due to the more pronounced suppressing effect of the serum. Furthermore, we do not suppose that the effect of LIF described above is caused by the general ability of LIF to maintain the undifferentiated status of ES cells. Our observations (for example Fig. 2b) and previously published data showed that LIF down-regulated differentiation of specific populations within EB, but did not block differentiation generally (Murray and Edgar, 2001). The negative effect of LIF on ES cardiomyogenesis was also described previously (Bader et al., 2000). Bader and co-workers used a differentiation protocol similar to the present study. The major difference is their use of complete serum-containing media in each step of ES cell differentiation. However, the final cell response to treatment by various growth factors or drugs may be greatly dependent on the presence or absence of serum or other factors in culture media as illustrated by our results presented here and also by other authors (Pachernik et al., 2002; Rajasingh et al., 2007). On the basis of our and Bader's results we concluded that LIF-activated gp130 signalling inhibited cardiomyogenesis of ES cells independent of any serum-borne factors. Thus, LIF-activated gp130 signalling may be generally inhibitory for early steps of cardiomyogenesis.

Next, we were interested in the mechanisms causing the observed LIF effect. Generally, STAT3 and ERK signalling pathways are two major downstream effectors of activated gp130 (Heinrich et al., 2003). Inhibition of STAT3 signalling using Stattic V alone not only had no effect on cardiomyogenesis, but this drug reversed its LIF-mediated inhibition. On the other hand, inhibition of ERK signalling using UO126 inhibited cardiomyogenesis under all tested conditions. Therefore, we concluded that LIF/gp130-mediated inhibition of ES cardiomyogenesis occurred via STAT3 activation. Previously it was observed that depletion of JAK2 (direct upstream activator of STAT3) or inhibition of JAK2 activation by JAK2 inhibitor AG490 leads to suppression of cardiomyogenesis of ES cells (Foshay et al., 2005). This discrepancy has not been explained until now. However, we may speculate that various roles of JAK2 and STAT3 are dependent on their posttranslational modification (Yang and Stark, 2008). Foshay and co-workers did not employ any gp130>JAK2>STAT3 inducers such as LIF that we used in our present study (Foshay et al., 2005). It may be possible that JAK2 acts through alternative downstream effectors (Chung et al., 2006). The explanation of these results of *in vitro* study versus *in vivo* observations is more difficult. There are no doubts that gp130 signalling plays a role in cardioprotection against various stresses by promoting cardiomyocyte survival, inducing compensatory hypertrophy and preserving the cardiac function. However, gp130 signalling also affects angiogenesis, which may influence cardiomyocytes and the heart in general (summary in Fischer and Hilfiker-Kleiner, 2007). There are also very important differences between differentiating cardiomyocyte progenitors and pre-mature/mature cardiomyocytes. All these questions require further detailed studies to be answered. Further, the development of mesoderm and its derivatives requires a functional ERK signalling cascade under the control of the tyrosine kinase receptor (e.g. FGF receptors, etc.) (Deng et al., 1994; Esner et al., 2002). The indispensability of ERK signalling for mesoderm development represents a plausible explanation for the negative effect of UO126, specific inhibitor of ERK signalling, on cardiomyogenesis presented in this manuscript.

In conclusion, we described the inhibitory effect of LIF on cardiomyogenesis in ES cells. We also observed that the LIF negative effect was mediated through STAT3 signalling, but not through ERK signalling. Even though the positive role of gp130 signalling in the maintenance of cardiomyocytes has been well documented, activation of gp130 signalling is not a generally applicable approach to the improvement of differentiation or expansion of cardiomyogenesis from ES cells.

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