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Preparation of bioactive soluble human leukemia inhibitory factor from recombinant Escherichia coli using thioredoxin as fusion partner

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A Novel Production and Purification Process for Soluble Human Leukemia Inhibitory Factor as Fusion Protein from Recombinant *Escherichia coli*

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Abstract

Leukemia Inhibitory Factor (LIF) is a polyfunctional cytokine with numerous regulatory effects in vivo and in vitro. In stem cell cultures it is the essential media supplement for the maintenance of pluripotency of embryonic and induced pluripotent stem cells. With regard to large scale cultures of these cells, LIF is needed in high quality and quantity and represents the major costs (90%) of the culture media. In this report, we describe a novel production and purification process for human LIF (hLIF) from recombinant *E. coli* cultures. hLIF was cloned into pET32b-trx and expressed as soluble protein in fusion with thioredoxin. After purification based on membrane adsorber technology, the fusion protein underwent cleavage by specific TEV protease. Released, soluble hLIF was subsequently purified by cation exchange chromatography and successfully tested for its biological activity using suspension cultures of murine embryonic and induced pluripotent stem cells. Our novel protocol for the production of recombinant hLIF is very suitable and effective for the production of low soluble proteins through expression in fusion with the solubilizing partner thioredoxin.

1 Introduction

Leukemia Inhibitory Factor is a polyfunctional cytokine displaying numerous effects on target cells. It was first characterized as an inducer of macrophage differentiation while it inhibits the growth of murine myeloid leukemia M1 cells [1], [2]. In vivo, it regulates the survival of neurons [3], [4] and acts as regulator of hematopoiesis [5]. In $LIF^{-/-}$ knock out mice it has been demonstrated that this cytokine is essential for blastocyst implantation [6]. Currently, LIF is in phase II clinical trial being developed as agent assisting the implantation of the embryo into the uterus in women who fail to become pregnant [7]. Furthermore, LIF is able to maintain the pluripotency of murine embryonic stem cells [8], [9] and is therefore extensively used in pluripotent stem cell culture. Due to their capacity of unlimited proliferation and ability to differentiate into cell types of all three germ layers, pluripotent stem cells are considered as highly interesting for both cell based therapies and the use in the pharmaceutical industry for drug screening and drug target identification. In order to facilitate the investigations of these cells for the mentioned applications, controlled, reproducible and scalable culture systems are needed to generate high amounts of undifferentiated cells [10], [11].

Besides the requirement of suitable culture systems cytokines such as LIF play a major role in large scale cultures of pluripotent stem cells. With commercially available LIF, we estimated this cytokine representing 90 % of the costs of the culture medium. To overcome this limitation it is desirable to have a source of highly purified and biological active LIF in large quantities.

Production and purification of LIF from recombinant *E. coli* have previously been reported. Gearing et al. [12] expressed murine and human LIF in a glutathione-S-transferase based fusion construct while Samal et al. [13] reported the expression of mature human LIF as insoluble inclusion bodies with subsequent refolding procedures. In this work we describe the development of a novel production and purification process for recombinant human LIF (hLIF). Mature 20 kDa hLIF contains six cysteine residues all being part of three disulfide bonds. In our approach, hLIF is expressed as soluble protein

in fusion with thioredoxin. Bacterial thioredoxin has been found to act as a strong enhancer of the solubility of eukaryotic proteins [14], [15] and is considered to actively promoting the disulfide bond formation of appropriate substrate proteins [16]. After purification of the soluble fusion protein, it was further processed by cleavage with highly active TEV protease that resulted in a complete digest of the fusion protein releasing soluble hLIF. Finally, bioactivity of the purified hLIF was tested using suspension cultures of pluripotent stem cells.

2 Materials and Methods

1.1 Construction of expression vector pET32b-trx-his-tev-hLIF

A hLIF cDNA encoding the mature 180 amino acid polypeptide was introduced downstream to the thioredoxin and His-tag coding sequence of pET32b-trx (Novagen). Additionally, oligodeoxynucleotides (gag aat ctt tat ttt cag gga) encoding a TEV protease cleavage site were inserted directly 5´ to the hLIF cDNA replacing the origin thrombin cleavage site of the pET32b-trx vector.

1.2 Expression of recombinant hLIF in fusion with thioredoxin

For the expression of the fusion protein an *E. coli* BL21 (DE3) (Novagen) host strain was used. Expression cultures were inoculated with 2 % of over night grown preculture and performed in shaking flasks in TB medium at an agitation rate of 150 rpm. In order to increase the solubility of the fusion protein the cultivation temperature was set to 23°C. When the culture reached an optical density (O.D.) of 0.6 protein expression was induced by 0.5 mM IPTG. The bacteria were cultivated for further eight hours and then harvested by centrifugation at 4000 g for 10 minutes. Bacterial cell pellets were resuspended to an O.D. of 10 in binding buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl) and passed four times through a microfluidizer (Microfluidics) for cell disruption. Soluble cell protein was obtained after centrifugation at 17000 g for 30 minutes and analysed by SDS PAGE.

1.3 Purification of trx-his-tev-hLIF fusion protein

The overexpressed fusion protein was purified using a Sartobind IDA 75 membrane adsorber module (Sartorius-Stedim Biotech) [17], [18]. In brief, after binding of Zn^{2+} (0.5 M in 500 mM NaAc pH 4.5) to the membrane, soluble cell protein was loaded. The membrane was then washed with binding buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl) and washing buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl) before a pre- elution step was performed with 50 mM Tris-HCl pH 8.0 and 100 mM imidazole. A final elution of tightly bound protein was achieved with elution buffer containing 250 mM imidazole

and 100 mM NaCl in 50 mM Tris-HCl, pH 7.5. Fractions containing the trx-his-tev-hLIF fusion protein were pooled and directly used for proteolytic cleavage. Protein concentration was determined by densitometry [19] using BSA as standard. For analysis, AlphaEaseFC Stand Alone Software was used.

1.4 Proteolytic cleavage of trx-his-tev-hLIF fusion protein

The purified fusion protein was cleaved by treating with recombinant TEV_{SH} protease in a substrate to protease ratio of 10:1 (w/w). Cleavage was performed at 4°C in properly diluted 20 x TEV buffer (50 mM EDTA in 1 M Tris-HCl, pH 7.5) for 16 h.

1.5 Purification of hLIF after proteolytic cleavage

The released hLIF was purified from the protein mixture using a Sartobind S 75 membrane adsorber module (Sartorius-Stedim Biotech). After equilibration of the membrane with binding buffer (100 mM NaCl in 50 mM Tris-HCl, pH 7.0), the protein sample was loaded. The membrane was then washed with washing buffer before elution of the hLIF protein was performed with 1000 mM NaCl in 50 mM Tris-HCl, pH 7.25. Protein concentration of hLIF containing fractions was determined by densitometry [19] using BSA as standard and analysing scanned SDS gels with AlphaEaseFC Stand Alone Software. To detect endotoxin levels, the hLIF protein solution was analysed by LAL Test (Endosafe®-PTS™, Charles River Laboratories) according to the manufacturer´s instruction. Finally, the hLIF protein was supplemented with 0.0002 % BSA (w/v), aliquoted and stored at - 80°C.

1.6 Expression and purification of TEV_{SH} protease

Recombinant TEV protease was expressed using TH24:TEV plasmid kindly provided by Helena Berglund [20]. The plasmid encodes a highly soluble mutant form of TEV protease referred as to TEV_{SH} with a carboxy terminal His-tag (6 x His). Cultures of transformed *E. coli* Rosetta pLysS (Novagen) were carried out essentially as described

previously [20]. After cell disruption and separation, TEV_{SH} protease was purified using a Sartobind IDA 75 membrane adsorber module (Sartorius-Stedim Biotech) with immobilized Zn^{2+}. After equilibration of the membrane with binding buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl) the soluble protein fraction containing overexpressed TEV_{SH} protease was loaded. The membrane was then washed with binding buffer before the target protein was eluted with a linear gradient from buffer A (50 mM Tris- HCl pH 7.5, 100 mM NaCl, 100 mM imidazole) to buffer B (50 mM Tris- HCl pH 7.5, 100 mM NaCl, 500 mM imidazole in 50 % glycerol). TEV_{SH} protease containing fractions were pooled, aliquoted and stored at -20°C. Protein concentration was determined by densitometry [19] using BSA as standard. Scanned SDS gels were analysed with AlphaEaseFC Stand Alone Software.

1.7 Biological testing of purified hLIF with murine ES and iPS cells

Cell culture

The murine embryonic stem (ES) cell line Brachyury and the murine induced pluripotent stem (iPS) cells YM100 (clone M15) were routinely cultured and expanded feeder cell free as floating cell spheres. The culture medium was composed of DMEM (Gibco) supplemented with 15 % foetal calf serum (HyClone), 0.2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 0.1 mM non-essential amino acid stock (all from Gibco) and 1000 units/ ml (10 ng/ ml) leukemia inhibitory factor (Millipore). The suspended cell spheres were passaged every three to four days by dissociating them into single cells with 0.025% trypsin and reseeding with a cell density of $2 \cdot 10^4$ cells/ ml (Brachyury cells) and $4 \cdot 10^4$ cells/ ml (YM100 cells).

Cell numbers were determined daily using hemocytometer counts with trypan blue exclusion. Additionally, glucose and lactate concentrations in the culture media were determined with an YSI 2700 Select analyzer (YSI Incorporated Life Sciences, Yellow Springs, US) to follow the metabolic activity.

Flow cytometry

The expression of the pluripotency marker SSEA-1 was analysed on the single cell level via flow cytometry. For this purpose cell spheres were harvested, washed with PBS and trypsinated for 1 min at 37° C. Single cells were recovered by centrifugation at 200 x g for 5 min, washed twice in cold PBS supplemented with 2 % FCS (PAA Laboratories GmbH) and resuspended to a concentration of 10^5 cells / 100 µl for each test. For specific SSEA-1 staining, titrated quantities of FITC- or PE-conjugated anti mouse SSEA-1 IgM κ antibodies (all from BD Biosciences, Heidelberg, Germany) were added in a volume of 20 µl to 100 µl cell suspension. Negative control staining was performed using matched FITC- and PE-conjugated isotype antibodies (all BD Biosciences). After storage for 20 minutes at room temperature in the dark, 400 µl of PBS supplemented with 2 % FCS as well as 1 µl propidium iodide (50 µg/ml in PBS) were added. The cell suspension was then analyzed in an EPICS XL/MCL flow cytometer (Beckman Coulter GmbH). Living cells were gated in a dot plot of forward versus side scatter signals and according to propidium iodide exclusion. At least, 10,000 gated events were acquired on a LOG fluorescence scale. Positive staining was defined as the emission of a fluorescence signal exceeding levels obtained by > 99% of cells from the control population stained with matched isotype antibodies.

Apoptosis was assayed using the Annexin V detection kit (BD Biosciences) according to manufacturer's instructions. Histograms and density plots were generated using the software WinMDI 2.8 (Joseph Trotter).

3 Results

1.8 Expression and purification of hLIF

Cloning of hLIF fusion gene

Figure 1.A shows the constructed vector for the hLIF expression. PET32b-trx plasmid (Novagen) containing a thioredoxin encoding sequence followed by a His-tag was used. Downstream of this sequence we introduced the TEV protease cleavage site and the hLIF cDNA to generate a 945 base pair open reading frame under the control of a T7 Promoter (Figure 1.A). The resulting fusion protein is depicted in a schematic representation in Figure 1.B. Correct assembly of the fusion gene and introduction into the pET32b-trx vector backbone was verified by nucleotide sequencing. Transformation of competent *E. coli* BL21 host cells (Novagen) was performed according to manufacturer's instructions.

Overall production and purification process of hLIF

Figure 2 gives an overview of the overall production process. For hLIF expression the transformed *E. coli* BL21 cells were cultivated in 100 ml TB medium in shaking flasks at a low temperature (23°C). Figure 3.A illustrates the fact that with our expression system a substantial amount of fusion protein (trx-his-tev-hLIF) was obtained in the soluble fraction. Following cell disruption and separation, the soluble protein fraction was processed via Immobilized Metal Chelate Affinity Chromatography. The filtrated protein solution was loaded on a membrane adsorber module (Sartorius-Stedim Biotech) with immobilized Zn^{2+}. The high purity of the obtained fusion protein was demonstrated via SDS-PAGE using silver staining (Figure 3.B, lane 1).

Subsequent proteolytic cleavage of the purified fusion protein was carried out using TEV_{SH} protease produced in recombinant *E. coli* as previously described [20]. The TEV_{SH} protease was similarly purified via IMAC using membrane adsorber technology. Cleavage of the fusion protein was performed using a substrate to protease ratio of 1:10 (w/w). In Figure 3.B, lane 2 the released His-tagged thioredoxin (14 kDa) and the hLIF protein (20 kDa) following proteolytic digest can be seen. A complete digest was achieved after

16 hours at 4 °C demonstrating the high efficiency of this protease. No further stabilizing agents such as glutathione were necessary to maintain the solubility of the released hLIF. The last purification step consisted in the separation of hLIF from the protease and thioredoxin by cation exchange chromatography. The cleavage products were directly loaded on a membrane adsorber module functionalized with sulfonic acid. The elution fractions containing the purified hLIF were pooled. The quality of the final product can be seen in Figure 3.B, lane 3. We estimated the purity being 95 % by SDS PAGE and silver staining. With this production and purification process 0.4 mg of hLIF per 100 ml bacterial culture broth could be obtained. The hLIF protein solution was essentially free of endotoxins (< 4 EU/µg) as determined by LAL test.

1.9 Biological testing of hLIF

The biological activity of the purified hLIF was proved using suspension cultures of murine embryonic and induced pluripotent stem cells. The activity of our material was compared to the activity of a commercially available LIF protein (ESGRO, Millipore). Each cytokine was added to the culture media to a final concentration of 10 ng/ml respectively. As a negative control, cells were cultivated in the absence of LIF.

Maintenance of murine ES cells Brachyury in suspension cultures

Erlenmeyer flasks containing 25 ml medium (n=4) were inoculated with 2 x 10^4 cells / ml each and shaken orbitally at an agitation rate of 110 rpm. The cultures were monitored daily by cell number determination and measurement of glucose and lactate concentrations.

Figure 4.A shows growth curves and viability of the cells cultivated in the three different culture media. Whereas cells proliferated normally in the presence of both control (ESGRO LIF) and purified LIF (*E. coli* LIF TCI), the negative control cultures showed a significantly reduced proliferation. These results are in line with the metabolic activity illustrated here by glucose consumption and lactate production of the cells (Figure 4.B). The viability assayed by trypan blue exclusion (Figure 4.A) and Annexin V apoptosis

assay (Figure 5.A and B) was comparable for both LIF cultures. In contrast, cultures without LIF displayed a significant lower viability (Figure 4.A) and massive apoptosis was demonstrated via Annexin V assay (Figure 5.C). It was not possible to maintain the Brachyury ES cell line in long term culture without LIF throughout subsequent passaging.

With regard to growth and viability, the purified hLIF is comparable with commercially available ESGRO LIF. The maintenance of the cell's pluripotency however is another important aspect for the characterization of the purified cytokine.

SSEA-1 expression of Brachyury ES cells

Maintenance of pluripotency over long term suspension cultivation in the presence of the purified hLIF was evaluated by expression analysis of the pluripotency marker SSEA-1. The expression level of the surface molecule was determined via flow cytometry using a monoclonal antibody labelled with FITC. In Figure 6 the histograms representing SSEA-1 expression of Brachyury ES cells derived from each cultivation set up are shown. Cells cultivated throughout numerous passages (> 21) in the presence of our hLIF retained high SSEA-1 expression levels (> 94 % positive cells) fully comparable with the positive control (Figure 6.A and B). Cells cultivated without LIF showed a decrease in SSEA-1 expression (only 77 % positive cells) already after two passages (Figure 6.C).

Maintenance of iPS cells in suspension cultures

Additionally, the activity of LIF was tested using static suspension cultures of induced pluripotent stem (iPS) cells. As observed for the Brachyury ES cells, iPS cells cultured with the purified and control LIF throughout several passages did not show proliferation or viability differences (data not shown). Cell expression of both pluripotency markers Oct-3/4 and SSEA-1 was monitored simultaneously via multicolor flow cytometry. Oct-3/4 expression was quantified according to GFP fluorescence, as the cells contain a GFP transgene under the control of the endogenous Oct-3/4 promoter (Reference U. Martin). SSEA-1 surface expression was detected using a monoclonal antibody labelled

with PE. The expression of both markers was simultaneously measured in a three colors experiment using propidium iodide for dead cell exclusion. In Figure 7 Oct-3/4 versus SSEA-1 expression of cells cultivated with control LIF (A), the purified LIF (B) and without LIF (C) can be seen.

No significant differences with regard to pluripotency marker expression could be observed between iPS cells cultivated in the control and purified LIF (Figure 7.A and B) over five continuous passages. All cells remained positive for both markers and maintained a high expression level comparable to the original population at passage 0 (data not shown). In contrast, a dramatic decrease of Oct-3/4 expression was observed in the negative control leading to the apparition of an Oct-3/4 negative cell population Figure 7.C, upper left quadrant) and to a less extend of SSEA-1 expression (Figure 7.C, lower left quadrant). Interestingly, reduced SSEA-1 expression could be only observed in cells which already lost Oct-3/4 expression, suggesting that the latter may be the first down regulated marker during the loss of pluripotency of iPS cells. As observed with the Brachyury ES cells, it was not possible to maintain the iPS cells in long term suspension culture without LIF throughout subsequent passaging.

This experiment demonstrated that the purified LIF can be used for suspension cultures of both murine ES and iPS cells.

4 Discussion

In this report we describe a novel production and purification process for human Leukemia Inhibitory Factor from recombinant *E. coli* cultures. We designed an expression vector where hLIF is expressed in fusion with a His-tagged thioredoxin. In between the two protein encoding sequences a cleavage site specifically recognized by TEV protease was inserted. When cultured at a low temperature (23°C) the *E. coli* cells expressed the fusion protein both in the soluble and insoluble fraction. The soluble protein fraction was then processed by Immobilized Metal Affinity Chromatography where the fusion protein could be obtained with high purity. Subsequent proteolytic cleavage with TEV_{SH} protease resulted in the release of thioredoxin containing 6 of the 7 amino acids of the protease cleavage site and soluble hLIF that contained the last amino acid of this sequence. This additional amino acid (glycine) at the amino terminus of the recombinant protein did not affect its activity.

After further purification by cation exchange chromatography the released hLIF could be obtained as pure, soluble protein. We assume the solubility being the result of correct disulfide bond formation promoted by the fusion partner thioredoxin. In our hands, simple non- optimized *E. coli* shaking flask cultures resulted in 0.4 mg of hLIF per 100 ml of culture broth that is sufficient to prepare 40 litres of stem cell media.

As demonstrated, the purified hLIF was active in maintaining the proliferation potential of murine embryonic and induced pluripotent stem cells throughout numerous passages and retained their pluripotency. We first present suspension cultures of pluripotent stem cells for the biological testing of LIF and demonstrate their suitability since the feeder cell free conditions allow for a strong and immediate response of the ES and iPS cells to the absence or presence of LIF already after one to two passages.

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6 References

1. Gearing, D.P., et al., *Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF).* Embo J, 1987. **6**(13): p. 3995-4002.
2. Gough, N.M., et al., *Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor.* Proc Natl Acad Sci U S A, 1988. **85**(8): p. 2623-7.
3. Cheema, S.S., et al., *Leukemia inhibitory factor prevents the death of axotomised sensory neurons in the dorsal root ganglia of the neonatal rat.* J Neurosci Res, 1994. **37**(2): p. 213-8.
4. Cheema, S.S., et al., *Leukaemia inhibitory factor rescues motoneurones from axotomy-induced cell death.* Neuroreport, 1994. **5**(8): p. 989-92.
5. Metcalf, D., N.A. Nicola, and D.P. Gearing, *Effects of injected leukemia inhibitory factor on hematopoietic and other tissues in mice.* Blood, 1990. **76**(1): p. 50-6.
6. Chen, J.R., et al., *Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis.* Endocrinology, 2000. **141**(12): p. 4365-72.
7. Metcalf, D., *The unsolved enigmas of leukemia inhibitory factor.* Stem Cells, 2003. **21**(1): p. 5-14.
8. Williams, R.L., et al., *Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells.* Nature, 1988. **336**(6200): p. 684-7.
9. Smith, A.G., et al., *Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides.* Nature, 1988. **336**(6200): p. 688-90.
10. Amit, M., Itskovitz- Eldor, J., *Methods of expanding embryonic stem cells in a suspension culture* 2008, Patent, International Publication Number WO/2008/ 015682 A2.
11. Cormier, J.T., et al., *Expansion of undifferentiated murine embryonic stem cells as aggregates in suspension culture bioreactors.* Tissue Eng, 2006. **12**(11): p. 3233-45.
12. Gearing, D.P., et al., *Production of leukaemia inhibitory factor in Escherichia coli by a novel procedure and its use in maintaining embryonic stem cells in culture.* BioTechnology, 1989. **7**: p. 1157-61.
13. Samal, B.B., et al., *High level expression of human leukemia inhibitory factor (LIF) from a synthetic gene in Escherichia coli and the physical and biological characterization of the protein.* Biochim Biophys Acta, 1995. **1260**(1): p. 27-34.
14. Takashi, Y., *Increase of solubility of foreign proteins in Escherichia coli by coproduction of the bacterial thioredoxin* J Biol Chem, 1995. **270**: p. 25328-31.
15. LaVallie, E.R., et al., *A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm.* Bio/Technology 1993. **11**(2): p. 187-93.
16. Stewart, E., F. Aslund, and J. Beckwith, *Disulfide bond formation in the Escherichia coli cytoplasm: an in vivo role reversal for the thioredoxins.* Embo J,

1998. **17**(19): p. 5543-50.
17. Kokpinar, O., et al., *Innovative modular membrane adsorber system for high-throughput downstream screening for protein purification.* Biotechnol Prog, 2006. **22**(4): p. 1215-9.
18. Suck, K., et al., *Fast and efficient protein purification using membrane adsorber systems.* J Biotechnol, 2006. **121**(3): p. 361-7.
19. Tomaszewska-Grasa, J., et al., *Quantitative determination of titin and nebulin in poultry meat by SDS-PAGE with an internal standard.* Meat Science, 2002. **62**: p. 61-66.
20. van den Berg, S., et al., *Improved solubility of TEV protease by directed evolution.* J Biotechnol, 2006. **121**(3): p. 291-8.

7 Figures

Figure 1 A Vector map of pET32b-trx-his-tev-hLIF. B Schematic representation of the resulting fusion protein with a molecular weight of 34 kDa. N: amino terminus of the polypeptide, trx: thioredoxin, his: His-tag (6 x His), tev: protease cleavage site recognized by TEV protease, hLIF: human Leukemia Inhibitory Factor, C: carboxy terminus of the polypeptide.

Figure 2 Process flow chart for hLIF. The major steps of the production and purification process are depicted.

Figure 3 A Protein expression in recombinant E. coli BL21 (DE3)-pET32b-trx-his-tev-hLIF. Soluble cell protein was loaded before IPTG induction (BI), two and eight hours after induction respectively. B Summary of the hLIF purification process. Lane 1: hLIF fusion protein after affinity chromatography, lane 2: products after proteolytic cleavage: thioredoxin and hLIF, lane 3: hLIF after ion exchange chromatography, lanes M: molecular weight marker Fermentas SM0671.

Figure 4 Growth characteristics of murine ES cells Brachyury. A Proliferation and viability of the cells in Erlenmeyer flask cultures. B Glucose and lactate concentrations in the culture media.

Figure 5 Measurement of apoptosis via Annexin-V assay in cultures of Brachyury ES cells. Analysis of cells cultured (A) in the presence of the control LIF (ESGRO, Millipore), in the purified hLIF (B) and without LIF (C) are shown.

Figure 6 SSEA-1 expression of Brachyury ES cells. Expression levels of cells cultured (A) in the presence of the control LIF (ESGRO, Millipore), in the purified hLIF (B) and without LIF (C) are shown. Filled histograms: stained cells with anti SSEA-1 FITC antibody. Unfilled histograms: cells stained with matched isotype control antibody. Living cells were gated according to propidium iodide exclusion.

Figure 7 Expression levels of Oct-3/4 and SSEA-1 in cultures of iPS YM100 cells. Expression levels of cells cultured (A) in the presence of the control LIF (ESGRO, Millipore), in the purified hLIF (B) and without LIF (C) are shown. Cells were stained with anti- SSEA-1 PE antibody. Oct-3/4 expression was quantified via the GFP fluorescence level. Living cells were gated according to propidium iodide exclusion.