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The Human Leukemia Inhibitory Factor Project

Summer Internship Repport

Forewords and Acknowledgments

The RIKEN Center of Developmental Biology (http://cdb.riken.jp/) is a Japanese research institute located in Kobe. Initially established as a Private research foundation (1917), RIKEN became an independent institution in 2003. RIKEN is composed of 7 majors institutes, including the Kobe's CDB. The CDB is funded at 85% by the Japanese government, and its main goal to use and develop Stem cell and Developmental Biology in order to get a better understanding of various human diseases (translational research). This institute is composed of a core of 6 research labs (including Nishikawa's lab) and around 15 smaller "creative research promoted labs" (http://cdb.riken.jp/en/02_research/0201_core00.html).
Riken CDB is also a part of the "Kobe biomedical cluster" including several Japanese and international pharmacological companies (research departements), Bioinformatic center, hospitals but also the news the RIKEN Center for Molecular Imaging Science and future RIKEN Next-Generation Supercomputer R&D Center. The goal of this cluster is to promote interactions between basic researchers, medical world and pharmaceutical industry (http://www.biobridge-kansai.com/about/kansai.php#kobe).

I had the chance to perform my internship into the "laboratory for Stem Cell Biology", leaded by Prof. Shin-Ichi Nishikawa.

This laboratory is droved by the stem cell research. Indeed the name is "Laboratory for Stem cell research" (http://www.cdb.riken.go.jp/scb/). According to that a lot of different topics are studied in this lab. Indeed, 17 researchers are working there with 7 technicians on many different topics. For example, one of the topics is to try to induce the production of a neuroblastoma from mouse embryonic stem cells. And that in order to understand the mechanism behind the formation of such tumor, but also to create a reliable model to find some therapies. Others topic are also studied, like the study of the role of Runx1 or Etv2, major genes involved into the emergence of hematopoietic linage. Another team is studying the effect of different type of cell culture media on the pluripotency of stem cells. Also, the study of epigenetic change into the hES cells during the differentiation, or the bioinformatics study of micro-array analysis of stem cells. So this lab is, in my point of view, quite atypical because of the number of topics studied by individual researchers.

In this laboratory, I could have worked on a lot of different topics, and I was asked to find a project, or to follow someone's project. Because of my readings, the "naive state of human stem cells", recently described by Austin Smith's lab (Nichols & Smith, 2009) explaining in part why the behavior of hES cells differs from the one of mES cells seemed to be a really interesting and challenging topic, but this studies require the use of human LIF, which is still very expensive, that's why I proposed to Shin-ichi Nishikawa to start my project by producing and purifying recombinant tagged hLIF.

Indeed, this project was more technical than strictly academic, but during the short time I had, my decision was to learn as much technics as possible, taking advantage of the expertise of the researchers present in the lab and also the abundance of available equipment, more than trying to start a project that will never been finished or to just follow someone's project. I justify it by the fact that during my previous internships, I followed two different researchers and that Dr Nishikawa offered wanted to give me the chance to work in an independent manner. Of course, this internship allowed me to learn a lot of different technics and knowledge, but I believe that the most important, was to acquire some autonomy in research,to be able, to think by myself, to choose the next step, to know when to stop and what to focus on.

For that I want to thanks especially Shinichi Nishikawa which gave me the chance to affirm myself but also Julien Bouissac for its daily help and who were there to help me focusing, and also all the lab's member who help me manipulating, and teach me a lot about the scientific world.

Introduction

Stem cell biology is a one of the field of science with a true promising future. In that field, mouse embryonic stem cells (mES) were widely used and numerous results were achieved over the years. But, until now human embryonic stem cells (hES) were less studied, mainly due, for researchers, to administrative issue underlying an ethical issue. The Induced Pluripotent Stem Cells (iPS) discovered by Prof. Yamanaka's lab (Takahashi et al. 2006), which prevent any ethical issue, as their production doesn't involve any human embryo, inverted this phenomena. Indeed, the iPS cells come from adult differentiated cells in which transgenic reprogramming factors are expressed. Nevertheless, hES, or hiPS are not similar to mES cells, even if both have the ability to give rise to the three main germs layers.

Recently two distinct murine totipotent cells types have been derived from the mouse embryo. The first, named mES cells is the "classical" one, and is derived from the inner cell mass (ICM) of the blastocyst stage (Evans et al. 1981). The second one is derived from the post implantation epiblast of Murine embryos (Tesar et al., 2007)(Brons et al., 2007) so called mouse epiblast stem cells (mEpiSC). These two cell lines are distinct molecularly and epigenetically and represent therefore two different states of pluripotency, later called *naive* and *primed* (Nichols & Smith, 2009). After injection in mouse blastocyst, the *naive* mES cells contribute to the embryonic cell mass and give rise to chimeric mice, female cells don't have undergone the X chromosome inactivation, and the cells maintain easily their pluripotency under normal cellular culture conditions. On the other hand, the *primed* mES cells almost don't contribute to embryonic and chimeric mice, female cells have undergone the X chromosome inactivation, and the cells differentiate more easily to form a teratoma under normal cellular culture conditions. The morphology and molecular dependence is also different, the *naive* cells, grow in dome shape colonies, can by passage as single cells, and are LIF/Stat3 pathway dependent and destabilized by bFGF and TGFβ/Activin pathway, whereas *primed* cells form flattened colonies, can't be passage as single cells, are bFGF and TGFβ/Activin pathway dependent, and destabilized by LIF/Stat3 pathway.

The hES are not identical as *primed* mES, but they share a lot of similarities: protein and mRNA expression patterns, epigenetic state, culture conditions, etc (Thomson et al., 1998)(Tesar et al., 2007)(Brons et al., 2007). It has then been shown that the primed state of hES and hiPS can be reversed to a "*naive like*" state in certain culture conditions (Hanna et al., 2010). According to that article, the human LIF (hLIF), activator of the Stat3 pathway is required to reverse the primed cells.

When I started my internship, it appeared that the recombinant hLIF was first very expensive and moreover very hard to find. Now, a lot of biotechnologies companies started to produced it and sell it which underlies an high demand from researchers and a probable major role of this protein in the future of human stem cell. Furthermore, for the mES, some transgenic cell lines expressing constitutively the mouse LIF exist, like the SNL (McMahon & Bradley, 1990). Moreover, hLIF has been described as a ligand of mouse LIF Receptor with almost the same affinity, also allowing its use in mES culture, while the murine LIF is inefficient in hES cells. (Huyton et al., 2007)

From this observation, the idea was to create a recombinant FLAG-tagged hLIF protein secreted by mammalians cells. An application would be to create a hLIF expressing feeder cells cell-line. In order to do so, the coding sequence (CDS) had to be obtained, the FLAG-tag to be added, the sequence to be inserted into a proper vector, a retrovirus to be produced and then a stable cell line to be derived. This is what it will be discussed in the following repport.

Result and Discussion

The human LIF, and the pCR4-TOPO-hLIF

The hLIF gene was purchased to OpenBiosytem (HLIF-CDS, Clone ID : 7939580 Available at: http://www.ncbi.nlm.nih.gov/nuccore/62739946). The cDNA, from the IMAGE gene bank, was inserted into the pCR4-TOPO backbone (Invitrogen) (Supplementary Fig 1). Nevertheless, it is always highly recommended to check by restriction, PCR, and/or sequencing analysis, that the provided plasmid contains the good cDNA.

According to the plasmid map (Supplementary Fig 1), the hLIF cDNA was flanked by two EcoRI site, and the expected fragments were 4kb, and 725bp. Furthermore, to verify the size, expected (4,7kb) of the plasmid, a NotI single cut was performed. But after the restriction/electrophoresis, it appeared that the size was around 6.5 kb and that the 2 fragments were about 4kb, and 2.5 kb (Fig 1a). The first hypothesis to explain this contradictory result was that we didn't obtain either the good plasmid or the good cDNA. Thus in order to be certain, the presence of hLIF was checked, 2 sets or primers were ordered using the Primer Bank Database (Wang & Seed, 2003) targeting two different small portion of the hLIF CDS (135bp and 208bp). First, the two restriction products were extracted from the gel using the Quiaquick Gel Extraction Kit (Quiagen) and the PCR was performed. The result was positive with no unspecific band for the hLIF gene (Fig 1b).

At that time, the presence of hLIF was confirmed, but as another control and to possibly explain the 2kb band, a second set of primers targeting this time the whole cDNA and CDS was designed using PrimerBlast, the result expected were 545bp for the CDS and 720pb for the cDNA. The CDS PCR was positive even if a ~2kb extra band appeared and regarding the cDNA three bands appeared ~2.1kb, ~1.5kb, and ~700kb (Fig 1c). According to those results, a probable hypothesis explaining the three band and the "non specific" 2kb amplification would be a triple integrations of the cDNA into the plasmid. Indeed, according to the Invitrogen pCR4-TOPO data sheet and IMAGE data bank, only one restriction site (TTAA) was used to integrate the DNA fragment inside the backbone allowing multiples integrations. Moreover, the size of the three bands, ~1x700bp, ~2x700bp (1400bp) and ~3x700bp (2100bp) is also in favor of this hypothesis. To confirm this hypothesis, a restriction using a hLIF unique restriction site or an extensive sequencing analysis should be done but because of a lack of time, and also because the presence was confirmed this experiment wasn't performed.

The construction of the DNA sequence

In order to add the FLAG-tag to the LIF protein without disrupting the protein, a structural study of the hLIF protein and it's interaction with his receptor (LIFR), a protein from the IL-6 receptor family interacting with gp130, showed that, neither the N-terminal end (Nter) or the C-terminal end (Cter) of the protein was interacting directly with the receptor (fig 2a) (Huyton et al. 2007). Nevertheless, the hLIF protein sequence contain in Nter a signaling peptide allowing the protein to be normally secreted, thus disrupting this part by adding the FLAG-tag could disrupted the cellular transport and exocytosis of this protein. However, this structural study doesn't allow any definitive conclusions and it was therefore decided to test both Nter and Cter integration of the FLAG-tag.

But the FLAG-tag sequence integration to the 5' or 3' of the CDS DNA wasn't the only modification (fig 2b). First, the main goal was to achieve a stable cell line producing tagged and functional hLIF, thus the construct needed to be integrated into a retroviral vector plasmid. Because of its high efficiency in mammalian cells infection, MSCV virus was chosen. The MSCV-IRES-eGFP (MIE) plasmid (Supplementary Fig 2) was used, because of the already integrated reporter gene eGFP and the Multi Cloning Site (MCS) EcoRI/ XhoI/ HpaI/ BamHI located just before the Internal Ribosome Entry Site sequence (IRES). Indeed, this pattern allows the monitoring of hLIF expression using the eGFP expression without disrupting both proteins. Moreover, the DNA construct needed to be flanked by the same restriction sites as in the MCS. In order to avoid any multiples integrations, inverted integration and also self-circularization during the ligation, two different sites were chosen: EcoRI and BamHI. Both because of their high restriction/ligation efficiency in the same buffer/temperature conditions. Also, a six-A nucleotide terminal sequence was added after the restriction site to allow the enzyme to cut properly. Then and in order to achieve a higher protein expression, the Khozac sequence (Khozac Sequence. Available at: http://en.wikipedia.org/wiki/Kozak_consensus_sequence), a recognition site of the Eukaryotic ribosome, was added before the hLIF CDS. Finally, a glycine was inserted between the FLAG-tag and the CDS to reduce the chance of possible mis-folding. Indeed, the glycine, because of its chemical structure, is the only amino acid allowing every angle between two part of an amino-acid chain. The final constructs was then as shown in Fig 2b.

In order to create that construct, many classical molecular biology methods were possible, the restriction/ligation system, the Multisite Gateway System (Invitrogen), or DNA polymerase and the PCR system with special primers (Fig 2c) as a DNA writer. The latest, because of its simplicity and accuracy, was chosen. However, as all techniques, some controls were needed. First the polymerase needed to be accurate, with proofreading activity to minimize the risks of mutation in the final sequence.

But even with such polymerase, because any DNA amplification has a mutation probability and because of the primers length, a sequencing of the result was required. The sequencing also allowed us to check that the expected 3' and 5' sequences were added to the CDS, and that no coding mutation was present in the final product. But, this sequencing is just a control of the efficiency of the PCR reaction. Indeed, only the major product appear in the sequencing analysis, however it's impossible to know which molecule will be integrated into the final vector. Thus, another sequencing of the final plasmid is needed.

The difficulty was to design the primers, indeed they had to be specific, containing the DNA construct and with an appropriate melting temperature (Tm). It was decided to select a 20bp recognition sequence of the CDS in addition to the restriction sites, Khozac, Glycine and FLAG-tag Sequence. Moreover, three taq-polymerase were tested: the KODPlus-taq, the LA-taq and the Ex-taq (respectively from Toyobo, Takara and Takara companies), but only the LA-taq polymerase gave significant result (data not shown). Indeed, because of the hypothetic triple integration of the cDNA, the major product was a 2kb band and almost no 735bp as expected (data not shown). In order to solve this issue, different template concentration and different melting temperature were tested (See materials and methods : PCRs) a 735bp band was finally obtained (Fig 2d). Indeed, the PCR reaction is multi factorial, and many things can make it work or not.

The next step was to control the correct integration of the DNA sequence into the construct. To do so, a sequencing analysis (see M.M. : Sequencing analysis) was performed using a double primer crossover method, mainly because the major part of interest was the 3'/5' extremity. Indeed, the sequencing analysis using the Big Dye Terminator kit (Applied Biosystems) and the GeneAmp PCR System 9700 doesn't allow any correct spectral analysis of the first 20-50bp. Two primers targeting the hLIF CDS spaced out of 135bp were used, one reading in the sense strand, the other one the anti-sense strand. After analysis, the two results were aligned using Geneious V4.8 software (Drummond et al. 2010) and that way the complete sequencing was achieved. The major PCR product had the new sequence inserted, and no mutations were detected (data not shown).

Next, the restriction/ligation into pMIE had to be achieved. Indeed, first a PCR product is a linear double stranded DNA which is not usable like that, secondly, to amplify this DNA construct the integration into a plasmid and the use of bacteria is the safest way. To do so, a double restriction using EcoRI and BamHI was performed overnight on pMIE, to ensure a complete digestion, and 1h on the two FLAG-hLIF construct. Then, the restricted DNA was purified with the PCR Purification Kit Quiaquick (Quiagen). First, in order to remove the restriction enzyme and buffer, but also to remove the small 40bp/9bp DNA fragments resulting from the restriction. Indeed, those small fragments would have interfered with the ligation reaction, resulting in wrong ligation or self-circularization of the plasmid. All those, reduce the efficiency and can lead to false-positive clone during the cloning procedure and then have to be avoided.

The two linear DNA fragment were mixed using a 1:3 molar ration (respectively MIE:FLAG-hLIF), and the DNA Ligation Mix (Takara) added (See M.M. : Digestion & Ligation (cloning)). Then DH5alpha E.coli bacteria were transformed and cultured (see M.M. : Bacterial Transformation and DNA purification). 1h ligation reaction was not enough (0 clone) so an overnight ligation was performed (>50 clone). Then, 10 clones were tested for hLIF by PCR (Cter-FLAG 1/10, Nter-FLAG 2/10 positives clones) and the hLIF positive clones were sequenced to check for possible mutations or wrong integration. Indeed, as said previously, it's impossible to know which molecule is integrated. The Cter-FLAG clone had a silent mutation (A18→G), and the Nter clones had a non-silent mutation (Pro9→Ser), but because this mutation was on the signaling peptide, it was decided to continue the experiment with it (Supplementary Fig 3).

Expression of the protein into 293T cells

pMIE was chosen not only because it's a retrovirus vector but also because it's an expression vector. Thus, the next step was to test the expression of hLIF and eGFP on 293T cells. To do so, a lipotransfection using FugeneHD (Roche) was performed (See M.M.: Lipotransfection).

First, in order to achieve the higher transfection efficiency, a yield assay was performed, using only the MIE plasmid. This assay consisted in a range of different transfection conditions (time of FugeneHD + DNA mix incubation and FugeneHD:DNA ratio). 293T cells (80% confluence) were transfected, and 48h after, a Flow cytometry (Fluorescent Activated Cell Sorting : FACS) analysis was performed to measure the transfection yield. The result was that the condition 8:2 incubation time 15 minute achieved 70% efficiency. Indeed, the lipotransfection condition changes a lot regarding mainly the cell type and the DNA size.

Secondly, the two FLAG-hLIF-MIE (Nter and Cter) were transfected using those conditions. 48H after, 1/10 of the cells were harvested to run a FACS analysis, 5/10 for a Western Blot (See Materials ans Methods : Western Blot), 4/10 for a mRNA extraction (See M.M. : mRNA extraction) followed by a RT-qPCR (See M.M. : RT-qPCR). Moreover, the medium was harvested and concentrate using a Amicon ultra YM-10 (Millipore) to be analyzed as well as the cells proteins in the western blot.

FACS analysis showed a good efficiency (between 36-40%) even if not as good as in the previous lipotransfection tests (Fig 3a). The mRNA analysis showed a higher level of hLIF compared to eGFP in the FLAG-hLIF-MIE conditions, only GFP in the MIE condition (Fig 3b).

The Western Blot showed, as expected, that the cytoplasmic overexpressed eGFP was present in the cells but not in the medium, and interestingly, only a very few amount of FLAG-hLIF could be detected in the cellular fraction (Fig 3c) in the two hLIF constructed. This intracellular –FLAG-hLIF is likely associated with Golgi apparatus, before exocytosis. Indeed, after looking at the medium the hLIF protein could be detected in the Cter-FLAG-hLIF condition, but only a few amount in the Nter-FLAG-hLIF and nothing in the MIE negative control condition (Fig 3c).

First, we can conclude that hLIF is produced and correctly secreted by 293T cells in the Cter FLAG-tag. Secondly, almost no hLIF can be detected in the Nter condition, but actually it's not necessarily true, because the antibodies used to detected hLIF targeted the FLAG-tag (no anti-hLIF was available). And, because the Nter-FLAG-tag is located before the signaling peptide, we can only conclude that no FLAG-tag was detected. Indeed, the signaling peptide might be cut by a protease as it's often the case in eukaryotic cells and then Nter hLIF become totally invisible in the supernatant. A first element in favor of this theory is that the Xray crystal doesn't contain the signaling peptide. Another one, is that a small amount of FLAG-tag can be detected in the medium fraction allowing us to thing that a few amount remain uncut. Another hypothesis is that the mutation and/or the Nter-FLAG-tag disrupted the normal folding or export of the protein and this protein is degraded by the proteasome as a misfold protein. But this is not the point of this study.

Functional hLIF assay

The next step was to test the functionality of hLIF. As previously described (Huyton et al., 2007) hLIF can be used on mES. It is also known that LIF is an activator of the JAK/Stat3 pathway (Niwa, Burdon, Chambers, & Smith, 1998) and more recently that after 24h LIF depletion, numerous Jak/Stat3 downstream genes are down-regulated, and the sudden exposition to LIF rapidly and directly (in less than one hour) up-regulate transiently the expression of Socs3 (a.k.a “STAT-induced STAT inhibitor 3”), one of the downstream genes of the Jak/Stat3 pathway (Niwa et al. 2009).

As a first functional test, we assayed the ability of FLAG-hLIF to activate the downstream Jak/Stat3 pathway, via its binding to the GP130-LIFR receptor. To do that, we followed the protocol recently described by Niwa et al. (Niwa et al. 2009). EB5 mES cells were cultured for 48h in presence of commercial ESGRO LIF (See M.M. mES culture conditions & hLIF functionality assay), then the cells were cultured in absence of LIF, in order to downregulate Jak/Stat3 pathway. 24h after the LIF starvation, the medium was changed for fresh mES medium containing either no LIF (negative control), ESGRO LIF (positive control and comertial recombinante murine LIF) or recombinant Cter-FLAG-hLIF. 1 h later, total cells RNA were collected and Socs3 expression was assessed by RT-qPCR. As expected, the recombinant Cter-FLAG-hLIF activates with the same efficiency the expression of Socs3 (Fig 4a), and so on the Jak/Stat3 pathway, than in the positive control,ESGRO LIF. Moreover, a dose effect is visible on the Socs3 activation under the different Cter-FLAG-hLIF dilutions (Fig 4a).

Even if this test shown that the recombinant hLIF is likely to bind GP130/LIF-R and so to have the same effect that the normal LIF on the Jak/Stat3 pathway. It has been described that LIF plays also other roles, notably for the maintenance of the mES cells pluripotency (Niwa et al. 2009).

To confirm that the recombinant hLIF is able to maintain mES self-renewal and pluripotency, a colony formation assay was performed (See M.M. : hLIF functionality assay). EB5 mES cells were cultured in subclonal conditions using the recombinant Cter-FLAG-hLIF in the medium while ESGRO LIF and no LIF condition were used as positive and negative controls, respectively.

The cells were first seeded and cultured 48h with normal ESGRO LIF, and then this one was changed for the Cter-FLAG-hLIF. After 10 days of subclonal culture (including 8 days of recombinant hLIF treatment) (Supplementary Fig 4), a phosphatase alkaline staining , and a mRNA extraction followed by a RT-qPCR was performed (Fig 4). The pluripotency-associated genes Oct3/4, Nanog, Klf2, Sox2, were assayed, but also some differentiation-associated markers: Gata6 as a primitive endoderm marker, Cdx2 and HandI as trophoectoderm markers, Dlx2 as ectoderm marker and Sox7 as an endoderm marker (Fig 4c)

Alcaline phosphatase assay shown us that the Cter-FLAG-hLIF–treated mES cells are still expressing this marker of undifferentiated ES cells and ES cells colonies shown a similar compact morphology than in the ESGRO LIF treated condition while the cells treated without LIF (negative control) shown differentiated morphology and a disappearance of AP staining. Moreover, the cells morphology seems to show a dose response with more differentiated cells as the concentration is lowering (Fig 4b).

On the molecular level, the cells treated using our recombinant hLIF shown a similar pattern of expression than the control ES cells for the pluripotency markers (Oct3/4, Nanog, Sox2 Klf4) while the level of expression of these genes is slightly downregulated in non-treated (and so differentiated) ES cells colonies. On the other hand, primitive endoderm (Gata6) and trophoectoderm (Cdx2 and HandI) gene expression were significantly up regulated in the differentiating conditions (w/o Lif or with diluted Lif). In the same time no significant change are observed on the endoderm marker sox7, but because of the random differentiation it doesn't mean anything. It would then be interesting to redo the experiment (Fig 4c).

To conclude, all those experiments shown that the produced hLIF is fully functional, able to maintain the pluripotency and self-renewal of mES cells. However, it is true that supplementary experiment should be needed to be sure of the produced hLIF efficiency, for example culture of mES during 4-5 passages and then redo a RT-qPCR.

Achieving a stable cell line

In order to achieve a stable cell line, a retrovirus random integration system was used. The virus was produced in a 293T cell line, cotransfected with pCter-FLAG-hLIF-MIE (inactivated virus), pGag-Pol and pEnv (vectors coding for the allowing the production of an active virus by the co-transfected cells) in a molar ratio 2:1:1 (See M.M. : Retrovirus) (Fig 5a).

The supernatant of the transfected cells, presumably containing viral particles was directly put into fresh 293T cells and after 3 passages, the cells should be sorted using the eGFP as a reporter gene for the correct integration of the gene. Then the cells should be passaged and sorted that way every 48h-72h.

But, the absence of 293T eGFP-positive cells after infection showed us that the virus production probably failed, likely because of an ineffective initial lipotransfection. Indeed, no eGFP positive cells can be harvested from the infected cells while few eGFP positive cells were observed in the initially transfected 293-T cells. So, the co-transfection probably failed and the observed eGFP cells were probably transfected only by the pCter-FLAG-hLIF-MIE vector, and so were unable to produce active virus. The usual things to check is the plasmid ratio, and also the FugeneHD:DNA ratio. Therefore, because of a lack of time, this experiment will be performed by other lab members.

Human ES and naive state of pluripotency

Finally, the main goal of this project was to use the hLIF on hiPS/hES to culture them into the naive state as described previously (Hanna et al., 2010). hiPS were cultured in normal condition during two month (See M.M. : hiPS culture condition) (Supplementary Fig 5), but because of a lack of time, this last project was not performed. Still, some transfection experiments were done on hiPS. According to previous study, a new lipotransfection technique using the Matrigel and a reverse-transfection (M-RT) increase the efficiency of the transfection (Villa-diaz et al., 2009). hiPS were then cultured on Matrigel (See M.M. : hiPS lipotransfection) and transfected using the two classical technique forward-transfection (FT) which consist in adding the DNA+lipotransfectant on adherent cells around 50-80% of confluency and reverse-transfection (RT) which consist in passaging the cells and adding the mix in the same time and also the new one M-RT which consist in putting the mix into the matrigel and growing the cells into it. The cells were transfected with the expression plasmid MIE. 48H after the cells were harvested and analyzed by FACS. It appears that the M-RT transfection was slightly more efficient than the RT when the normal FT didn't show any result .

Conclusion and perspective

To conclude, even if unfortunately this project is not fully achieved, tagged hLIF was successfully produced and functional.

Some technical concerns still have to be solved. The preliminary functional experiments described here were using hLIF concentrated from the conditioned medium gave expected results, but the main issue of this technique is that doing so also concentrate the knock out recombinant serum (KSR), the proteins, and the entire medium components. This can be an issue to obtain some reproducible results in further experiment. Also, the 293T cells are known to produce some cytokine, which can interfere with those experiments, notably with false positive result.

Indeed, biology is already a multi-factorial science, and reducing the parameters is the only way to understand a phenomena. That's in order to avoid this issue that it was decided to insert the FLAG-tag which will be used as a tool for the purification. It is planned to use Sepharose beads coated with antibody αFLAG-tag and then stack into a column. Using different salt concentration/pH buffers the hLIF can then be purified. Thus, the eluate should be concentrated again and the protein concentration measured, for example using the Bradford method or to be more accurate an Elisa assay.

Using this purified hLIF, others controls experiments will be performed, notably the usual LIF functionality assay which consists of culturing M1 cells (Leukemia cell line) in different concentration of LIF. Indeed those cells are LIF dependent and under subclonal culture condition, the number of colonies formed is used to measure the hLIF efficiency and so titrate (determine the number of units) reflecting LIF activity either than LIF concentration.

Of course, producing and purifying the hLIF was interesting but the main goal would be to try to culture hiPS in naive condition (Hanna et al. 2010) with this new hLIF in order first to achieve a stable naive hiPS cell line. Therefore, there is still a lot of issue, first, this naive state is still unclear, and all the trial to perform such experiment leads to a pseudo-naive state, unstable, in which the cells couldn't be passage more than 3-5 times before they differentiate.

A better understanding of the gene regulation network of pluripotency, and also of the two distinct state, primed and naive, will probably help to achieve a stable mES like hES cells.

Indeed, the actual hiPS state doesn't have the same potential as the mES, notably because of the lack of an easy cloning ability (even if the use of rock inhibitor now allows it, as it as been recently described (Ohgushi et al., 2010; Watanabe et al., 2007).

Finaly, the hiPS allowed the scientific academical community to bypass some administrative problems related to the work on hES cells and could so be considered as a great revolution in human stem cell research. Moreover, a lot of different commercial companies are now working more easily on hES via hIPS cells (which are less ethically and marketingly controversial) allowing us to expect exponential number of discoveries and then therapies in the future.

Materials and Methods (M.M.)

Cellular Culture

1. 293T cells culture condition

293T cells were cultured on Gelatin coated dishes in DMEM + 10%FBS (DF10). The cells were passaged when confluent at 80% (every 2 days (ratio 1:10) or every 4 days with a 1:30 ratio).

2. Murine Embryonic Stem (mES) cells culture condition

We used EB5 mES cells, which is a subline derived from E14tg2a ES cells (Niwa et al., 2000). This cell line was generated by integrating an IRES-BSD-pA vector into the Oct3/4 allele and so carries the blasticidin S-resistant selection marker gene driven by the Oct3/4 promoter.

This cell line is a powerfull tool to avoid mES cells spontaneous differentiation as only the ES cells expressing Oct3/4 (and so in undifferentiated state) will survive in presence of Blasticidin S..

The EB5 mES cells were cultured on 0,1% gelatin coated dishes, in mES cells medium [G-MEM (Gibco), 1M Sodium Pyruvate (Sigma), 2M L-Glutamine (Invitrogen), 1x Non Essential Amino Acid (NEAA, Sigma), 0.1M 2-MercaptoEthanol (2-ME), 10% Knockout™ serum replacement (KSR, Invitrogen), 1% Fetal Calf Serum (FCS, Gibco)], supplemented by LIF (ESGRO, Millipore, 1000 units/ml). Medium was changed daily.

The cells were passaged every 3-4 days. For dissociation, cells were washed (PBS) and incubated for 3 mn using 0.05% Trypsin-EDTA solution (Gibco, 3 minutes) and gently dissociated to a single cell suspension. Cells were counted (Thomas counting cell) and plated in gelatin-coated dishes at a concentration of 15 000 cells/cm².

3. Human induced Pluripotent Stem (hiPS) cells culture condition

Human iPS 4 factors (hIPS 4F, previously established in the lab) were cultured on a Mitomycine C-treated SNL layer (explain SNL, or simply say MEF), in hES cells medium [DMEM-F12 (Gibco), 1x NEAA, 20% KSR, 0.1M 2-ME], complemented with bFGF (5ng/ml). The cells were passaged every 5-7 days with a 1:4 ratio, using a gentle differentiation technique involving the Collagenase instead of the Trypsin. Indeed, hiPS, like hES cells can't be passaged as single cells and should be kept as small aggregates to avoid cell to cell contact dependent mortality. Nevertheless, recently, a Rho Kinase inhibitor (ROCK inhibitor, Y-27632) was discovered, allowing in some conditions single cell passage and thus cloning of hES/hiPS cells. (Sasai, Nature 2007)

DNA construction

1. FLAG Primer design

In order to clone the hLIF CDS from a commercial available clone to the vector of interest (MSCV), we designed primers including in 5'a hLIF binding site of 20mer (homologous sequence, including the endogenous ATG), the Khozac sequence, the FLAG Tag sequence with a Glycine bridge and the restriction sites EcoRI and BamH1 with a terminating sequence AAAAAA. According to the 3D structure study, two construction were designed : Nter-FLAG-Tag and Cter-FLAG-Tag proteins. The restriction site, EcoRI and BamHI, were chosen to fit in the Multi Cloning Site(MCS) of MSCV-IRES-eGFP retrovirus plasmid (Supplementary Fig 2).

2. Polymerase Chain Reaction (PCR) and PCR cloning

The FLAG-hLIF PCR amplification was performed using an high fidelity Taq Polymerase (with proof reading activity, LA Taq, Takara), following the manufacturer instructions, in a total volume of 50µl.

PCR optimization was performed by gradient PCR, testing a gradient of annealing temperatures. The optimal PCR program was

- 5mn of denaturation at 96°C,
- 25 cycles [98°C(denaturation, 10s) – 63°C(annealing, 30s) – 72°C(elongation, 60s)]
- 72°C (final elongation step, 3mn).

The others PCRs were performed using ExTaqHS (Takara), following manufacturer's protocol in a total volume of 50 ul, using 100 ng of template and the following PCR program:

- 4 mn of initial denaturation at 96°C (4mn)
- 30-35 cycles [98°C(denaturation, 10s) – Tmprimers -5°C(generally 55°C, annealing, 30s) – 72°C (elongation, 30-60s dependent of the size of the amplicon)]
- 72°C (final elongation step, 3mn).

3. Bacterial Transformation and DNA purification

The DH5α (Heat Shock competent bacteria) strain (Escherichia Coli) was used for bacterial transformation, following manufacturer recommendation.

Briefly, 50ng of plasmidic DNA was added to 50µl of DH5α, then incubate on ice for 30mn, heat shocked at 42°C for 30sec, and recovered for 1h in 1ml of SOC medium. 50-200µl were then plated on selective LB agar plates containing the appropriate antibiotic (100µg/ml of Ampicillin).

After 12-16h incubation (37°C), 6 clones were selected and cultured on 2-4ml LB + Ampicillin (100µg/ml) for 12-16h. 2ml was then harvested to do a plasmid extraction (using Invitrogen's MiniPreps and following the manufacturer protocol). Depending on the experiment, the plasmid was confirmed by restriction enzyme mapping, PCR, and/or sequencing.

When larger amount of plasmid was necessary, 100 ul of the confirmed clone was inoculated in 100 ml of LB medium containing the appropriated antibiotic.

After 12-16h of incubation (37°C), MidiPrep (Qiagen) following the manufacturer protocol.

4. Sequencing

The Big Dye Terminator kit (Applied Biosystem) and the GeneAmp PCR 9700 was used. This reaction is a Dye-Terminator reaction consisting in the incorporation of ddNTP tagged with a fluorescent dye. Then the result of incorporation is separate on an acrylamid gel and read (Dye-Terminator 2010).

The Big Dye reaction was done in 10µl, then an ethanol DNA precipitation was done to purify the product. Then it was resuspended into 25µl of Hi-Dye and put into the sequencing machine.

All sequencing analyses were performed in duplicate, allowing the automatic correction of reading error.

5. Digestion & Ligation (cloning)

The PCR product and the MIE destination vector were digested with EcoRI and BamHI (Takara) into 1xBuffer K, 1h, 37°C. After confirming the digestion efficiency by electrophoresis (??), the PCR product has been purified using direct PCR Purification, while linear plasmid has been purified after gel extraction (using QiaQuick kit (Qiagen)).

The ligation was performed using the Takara's Ligation Kit using a 1:3 molar ratio (corresponding to 30ng MIE :11ng PCR product). The mix was incubated at 16°C overnight.

The ligated circular MIE-hLIF plasmid has been used to transform bacteria (see M.M. : Bacterial Transformation and plasmidic DNA purification).

hLIF expression assay

1. Lipotransfection

FugeneHD (Roche) was used to transfect the 293T cells. The 293T max yield assay was first performed to assess the best plasmid ratio (8:2), and incubation time (15 min), which leaded to 70% of efficiency. All the other transfections were carried out using this two parameters and following the manufacturer protocol.

2. FACS Analysis & sorting

The analysis was performed on a FACS Canto (BD), using the eGFP expressed by the cells, and Propidium Iodide (PI) as a negative marker for living cells. FACS sorting was performed using a FACS Aria II (BD) system.

3. RNA Extraction

The total RNA from the cells was extracted using the RNA extraction kit Rneasy (Qiagen), following the manufacturer protocol. DNA digestion has been omitted in the majority of the experiments as we were using mRNA- specific primers for quantitative PCR. DNAse I treatment has been performed only to access hLIF overexpression efficiency (as the CDS of hLIF was overexpressed, genomic of plasmid DNA can interfere with qPCR assay). RNA quality and quantity were assessed by spectrophotometry (NanoDrop system).

4. Reverse Transcription (RT)

The RT was performed from 5 ug of total RNA using the SuperScript III kit (Invitrogen) and Oligo dT primers, as described in the manufacturer protocol.

5. Quantitative PCR (qPCR)

The qPCR was performed using the SYBR GREEN PCR Master Mix (Applied Biosystems), with the 7500 Real Time PCR System machine (Applied Biosystems). Sequence of the majority of the primers we used during this study were extracted from the Harvard Medical School open-access database PrimerBank (Spandidos, Wang, Wang, & Seed, 2010). When validated primer sequence was not available or when we needed specific primers, we used NCBI's PrimerBlast algorithm to design them (PrimerBlast).

6. Western Blot

The Western Blot assay was performed using a 15% Acrylamid gel for the SDS-PAGE electrophoresis, and PDVF membrane (Millipore) for the wet transfert of proteins. The antibodies used for the staining of the membrane were used with a 1/1000 ratio :

- αFLAG : (Sigma) M2 αFLAG mouse igG
- αGFP : (Invitrogen) αGFP rabbit igG
- αMouse : (Sigma) αMouse-HRP goat igG
- αRabbit : (Sigma) αRabbit-HRP goat igG.

The membrane was blocked with 5% non fatty milk (Gibco). The first antibody was incubate for 16h, 4°C, on 2.5% non fatty milk, and the secondary antibody was incubated for 1h, RT, on 2.5% non fatty milk also. The pictures were taken using the LAS-300 Mini (FujiFilm), exposition time : 1min.

hLIF production and functionality

1. hLIF functionality assay

Two different assay were performed.

Subclonal assay: EB5 cells were seeded on gelatin at subclonal density (200 cell/well (in 12 well plates), i.e. 55 cells/cm²) then cultured 48h in presence of commercial LIF (ESGRO). After PBS washing and 2h incubation in mES cells medium without LIF, the cells were cultured with a the normal medium containing either no LIF (negative control) commercial LIF (positive control), 4 dilution of FLAG-hLIF (1x, 10x, 100x, 1000x dilutions) for 8 days. Then, half of the cells were harvested to run a RT-qPCR targeting the pluripotency and differentiation marker genes. In the same time the Phosphatase Alkaline assay (Sigma) was performed on the colonies following the Sigma protocol.

Soc3 assay: Following the condition described recently by Niwa et al. (2010), EB5 cells were seeded at normal density (15000 cells/cm²) and cultured for 48h with commercial ESGRO LIF.
After PBS washing, the cells were cultured for 24h in mES medium without LIF. The medium was then changed as in the subclonal assay (No LIF, ESGRO LIF, different concentrations of FLAG-hLIF). 1h after, the cells were harvested for mRNA extraction, Reverse Transcription, and qPCR using primers targetting Socs3.

2. Production of inactivated retrovirus

The retrovirus was produced into 293T cells by co-transfection of 3 plasmid : pGag-Pol, pEnv, and pFLAG-hLIF-MIE using a molar ratio 1:1:2 in P3-safety culture room condition.
After 24h, the medium was changed. At 48h and 72h, the medium was harvested and stored on ice. Then 70% confluent 293T cells were infected by the virus using directly the harvested medium. Every 48H the cells were passaged. Then after 3 passage, the cells were sorted using the FACS Aria to sort the GFP positive cells.

The cotransfection assay allow the production of inactivated virus particles, indeed, in the vector plasmid the gag-pol and env gene are not present. Thus, during the virus production, the cells produced the corect enzymes, but the virus particle doesn't contain the replication genes and therefore can only insert the targeted gene.

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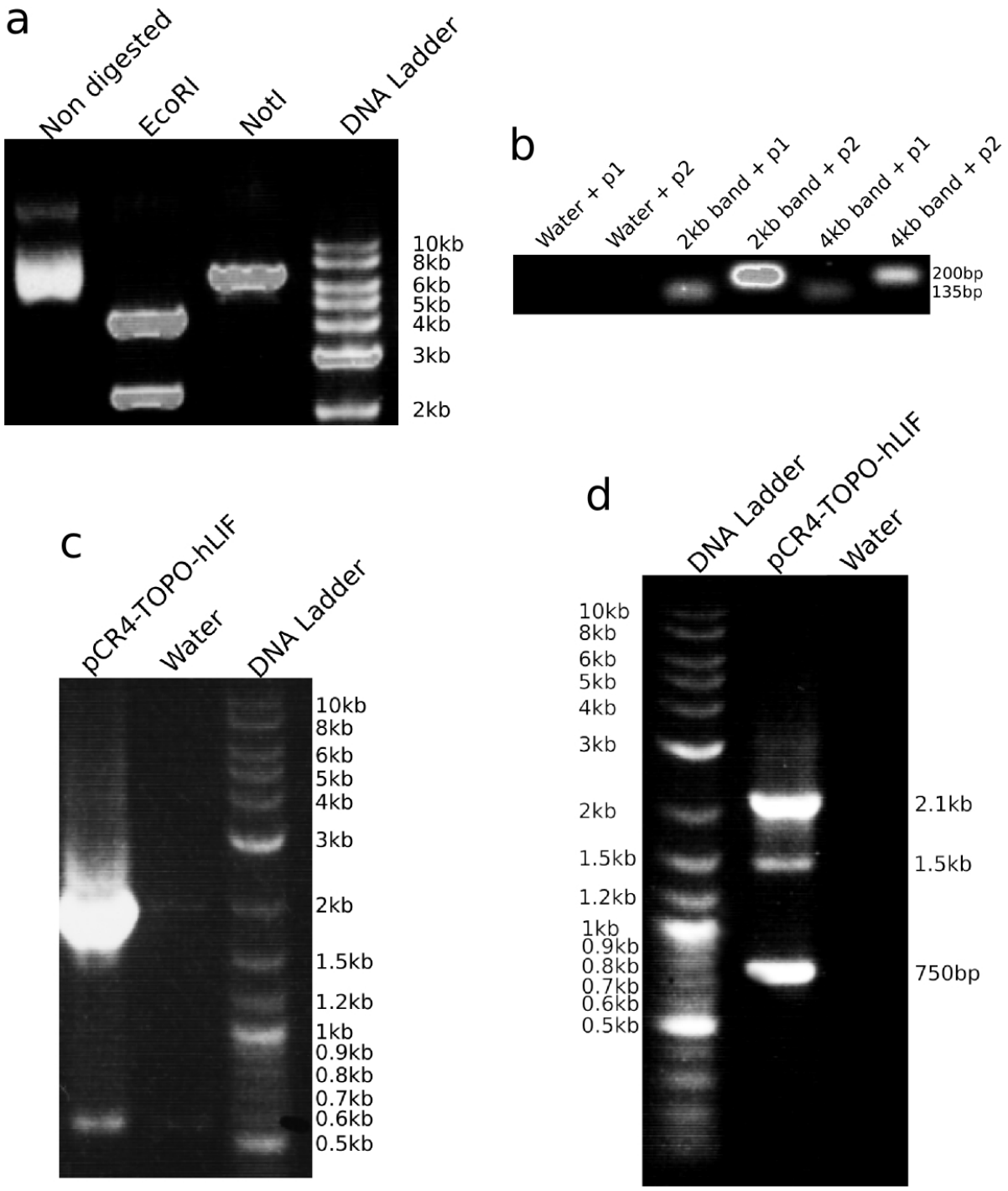


Figure 1 : Human LIF control experiments.
a: Quality control by enzyme restriction of pCR4-TOPO-hLIF. the reaction was performed using 1Unit/µg of DNA of EcoRI and NotI.
b: pCR4-TOPO-hLIF PCR with EXtaq (Takara) using 2 diferent pairs of primer targeting the human LIF CDS (p1 and p2).
c: pCR4-TOPO-hLIF PCR with EXtaq using primers targeting the whole hLIF CDS.
d: pCR4-TOPO-hLIF PCR with EXtaq using primers targeting the whole hLIF cDNA.

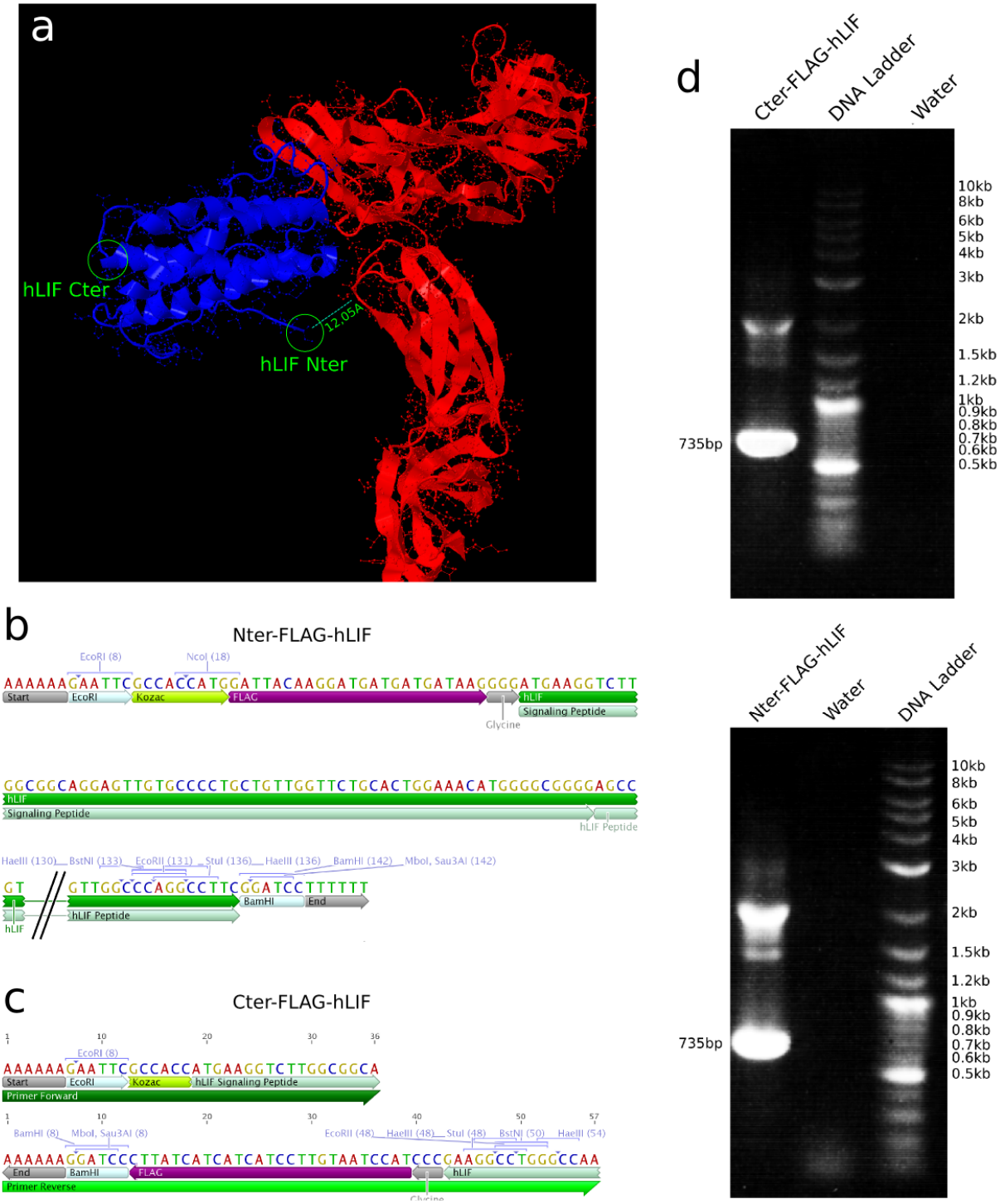
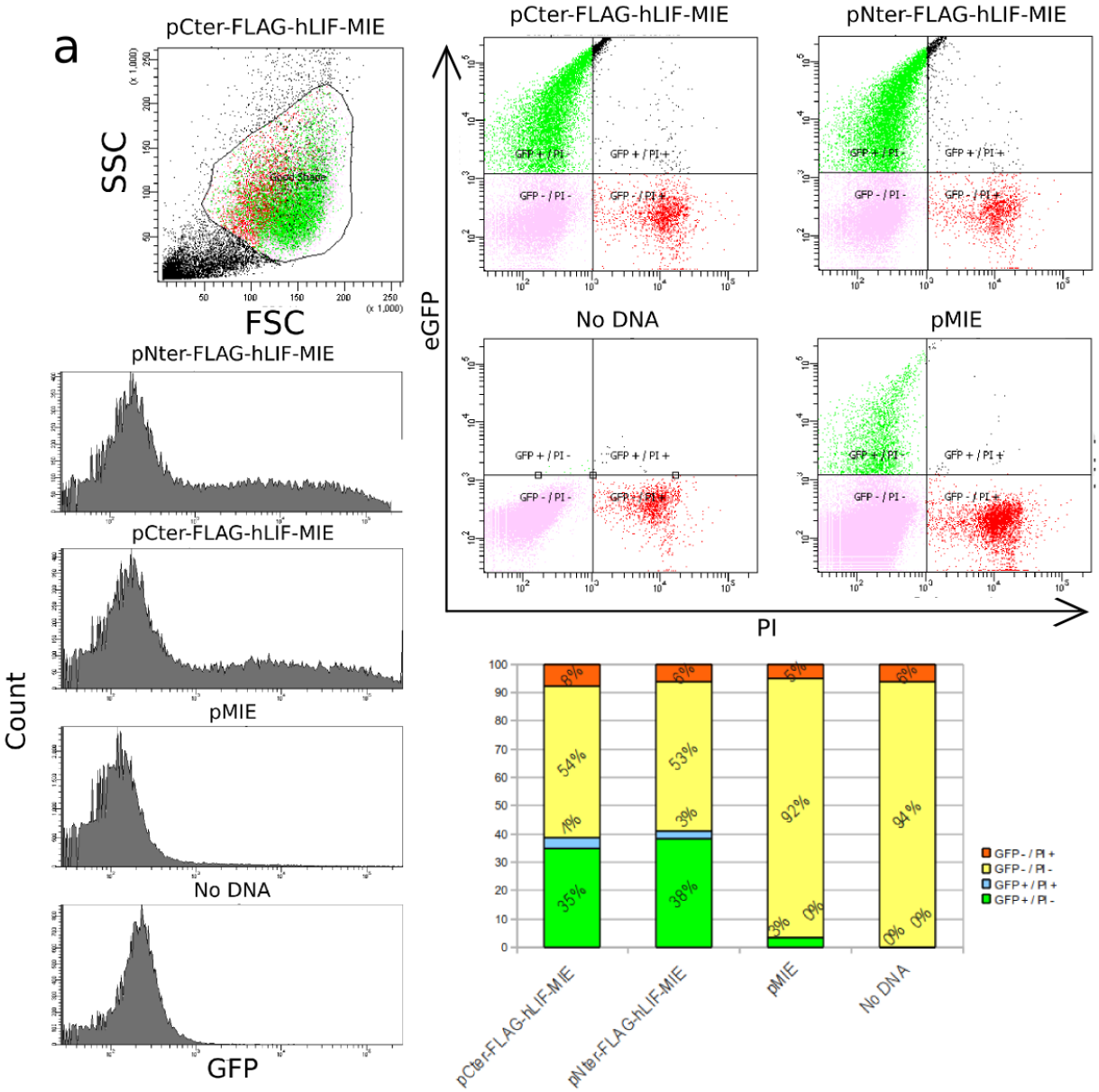
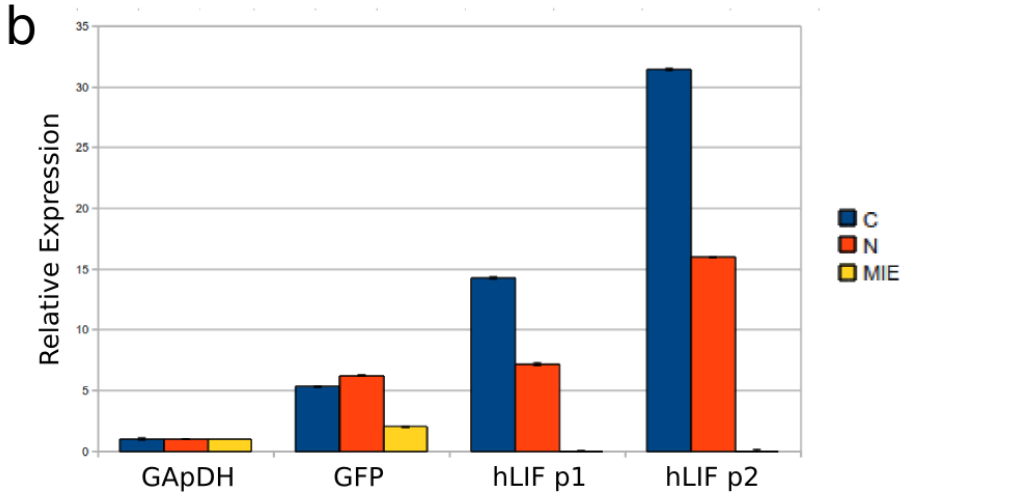


Figure 2 : DNA construction of the Flag-tagged human LIF
a: X-ray crystallography of hLIF with his receptor LIFR. The two extremity, Nter and Cter, are highlighted to show that there isn't any direct interaction between them and LIFR.
b: DNA sequence of the N-ter-FLAG-tag hLIF.
c: DNA sequence of the Cter-FLAG-hLIF sets of primers used to add the desired DNA sequence to the hLIF CDS.
d: pCR4-TOPO-hLIF PCR with EXtaq using the specials two sets of primers targeting the whole hLIF CDS. In both case, the expected 735bp amplicon is visible but also a 1.5kb and 2kb amplicon.


a
pCter-FLAG-hLIF-MIE
SSC
FSC
pNter-FLAG-hLIF-MIE
pCter-FLAG-hLIF-MIE
pMIE
No DNA
Count
GFP
eGFP
PI
GFP + / PI -
GFP + / PI +
GFP - / PI -
GFP - / PI +
54%
1%
35%
53%
3%
38%
92%
3%
0%
94%
0%
0%
GFP - / PI +
GFP - / PI -
GFP + / PI +
GFP + / PI -


b
Relative Expression
GAPDH
GFP
hLIF p1
hLIF p2
C
N
MIE

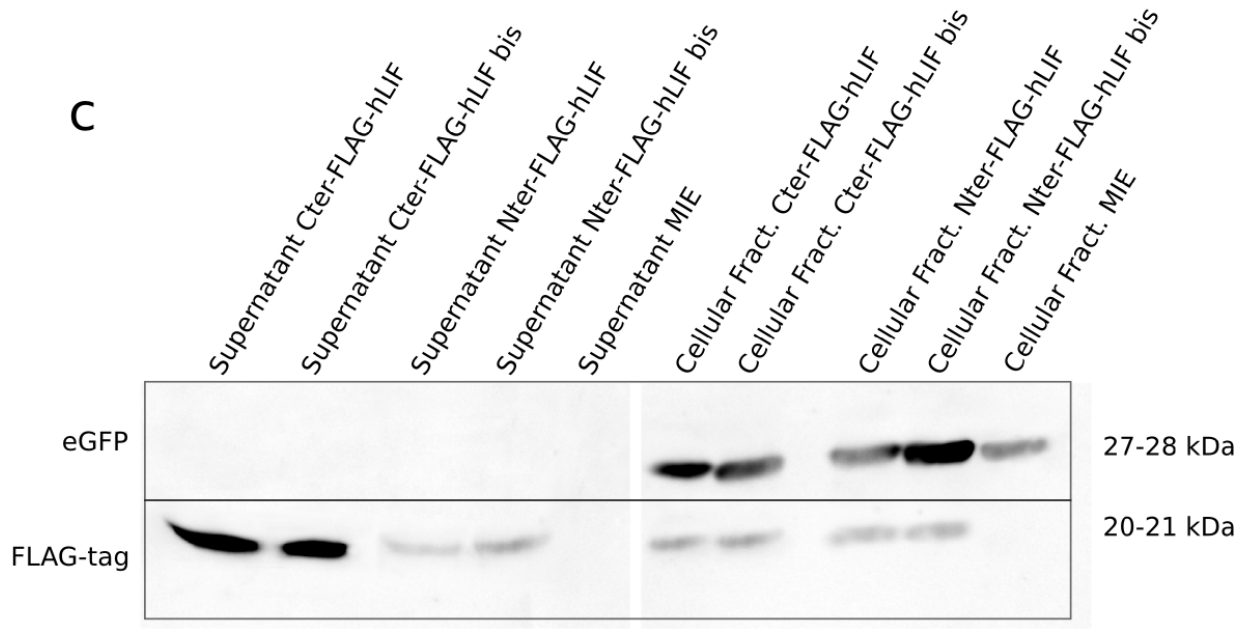


Figure 3 : human LIF expression assay
a: FACS Analysis of 293T cells transfected with FugeneHD. GFP is the repporter gene eGFP, PI is a marker for the liveability of the cells. The pMIE result are low due to a wrong DNA quantitiy during the transfection. 3 distincts population appear the dead cells, the GFP positive cells and the GFP negative cells.
b: qPCR analysis. Relative expression of eGFP and hLIF in 293T transfected cells leveled by the GApDH expression. The Cter construct seems to have a higher expression than the Nter, but in both case the expression of hLIF is really strong compare to the GApDH and the GFP.
c: Western Blot, targeting the FLAGtag and eGFP, of the culture medium (Supernatant) and the cellular fraction of transfected 293T cells. The cells where harvested 48h after the transfection. It appear clearly that the FLAG tag is detected in the supernatant, as well as the GFP in the cellular fraction. The lower result into the MIE plasmid is due to a wrong DNA amount during the transfection. Also a weak FLAG signal is detected in the cellular fraction as well as in the N-ter construct.

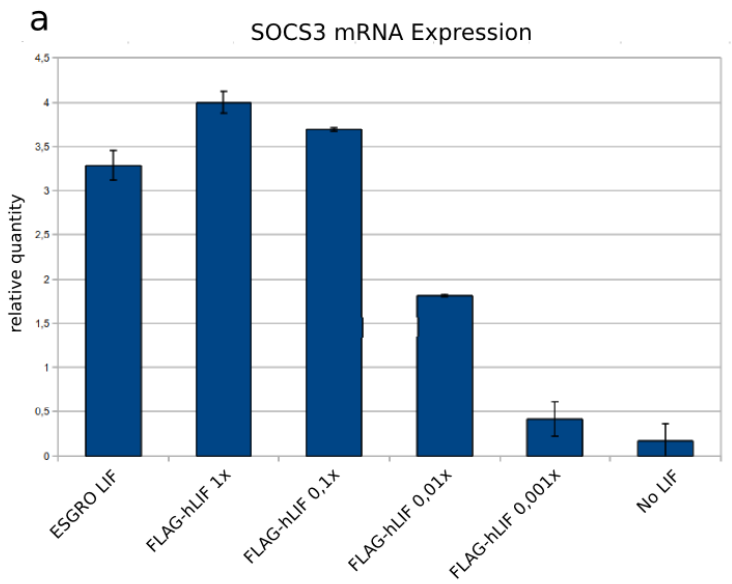
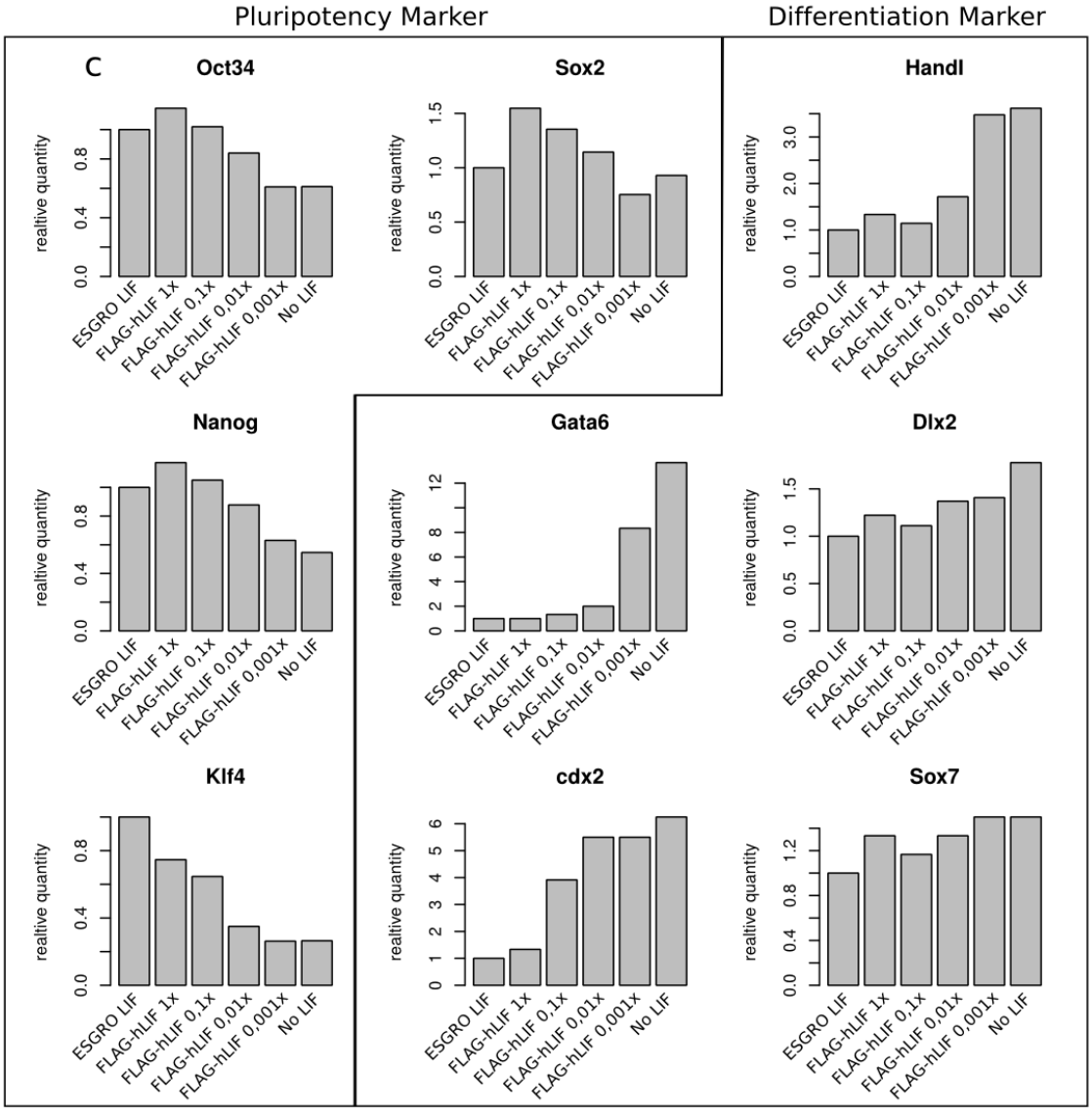
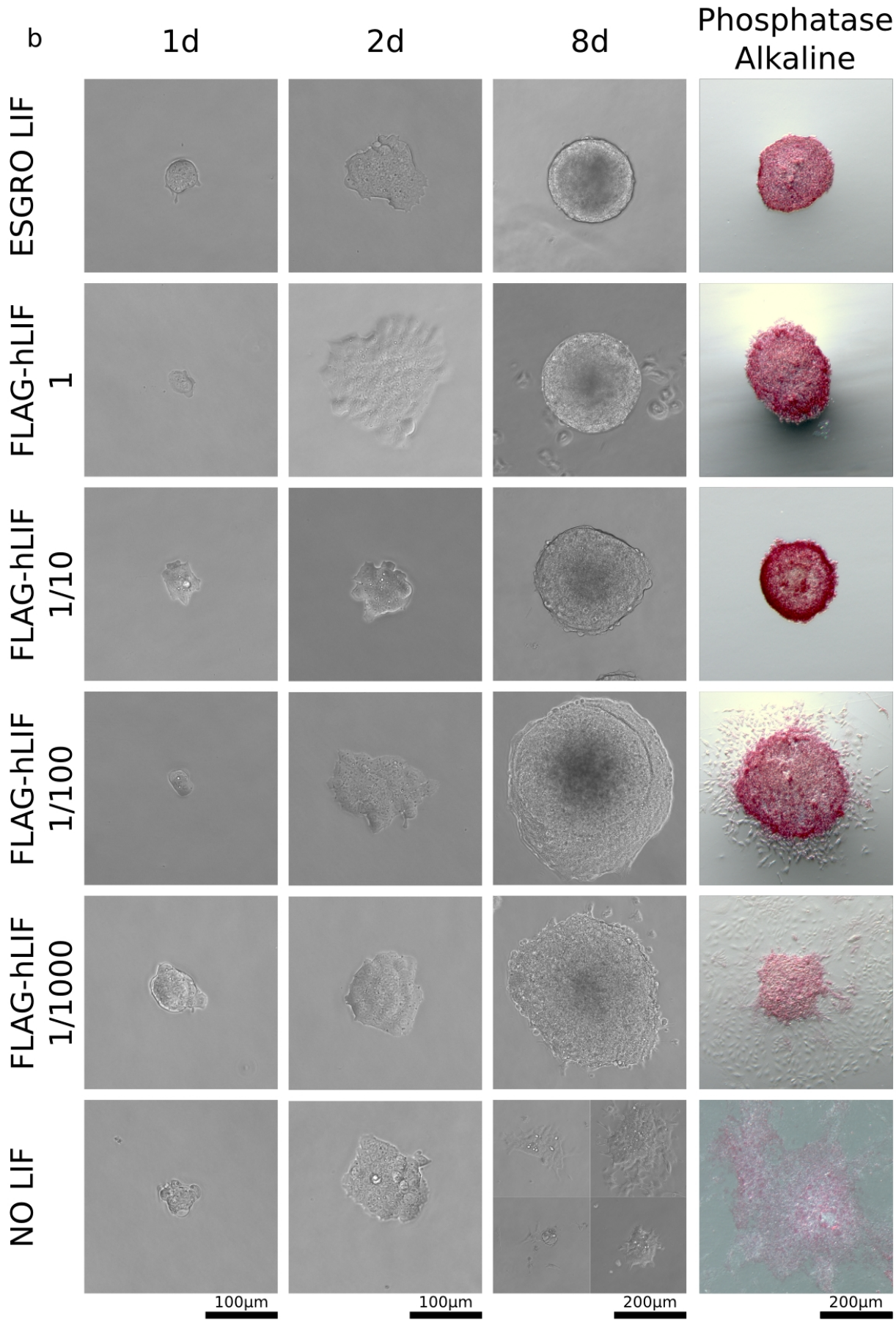


Figure 4 : Functionality assay
a: qPCR analyis. Relative expression level of Socs3 mRNA into EB5 mES cells after 24H LIF starvation followed by one hour LIF recovery.
b: Pictures of EB5 mES cells grown in subcloning conditions at differents days and pictures of those cells after a phosphatase alkalines staining. It is clear that the staining diminish in correlation with the LIF concentration.
c: RT–qPCR analysis of the expression of various cell lineage marker genes in ESGRO Lif control and hLIF at different dillution. Gene expression were normalized by GApDH and Ubiquitin gene expression levels and the expression level in ESGRO LIF-trated cells was set as 1.




b
1d
2d
8d
Phosphatase Alkaline
ESGRO LIF
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FLAG-hLIF 1/10
FLAG-hLIF 1/100
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NO LIF
100µm
100µm
200µm
200µm

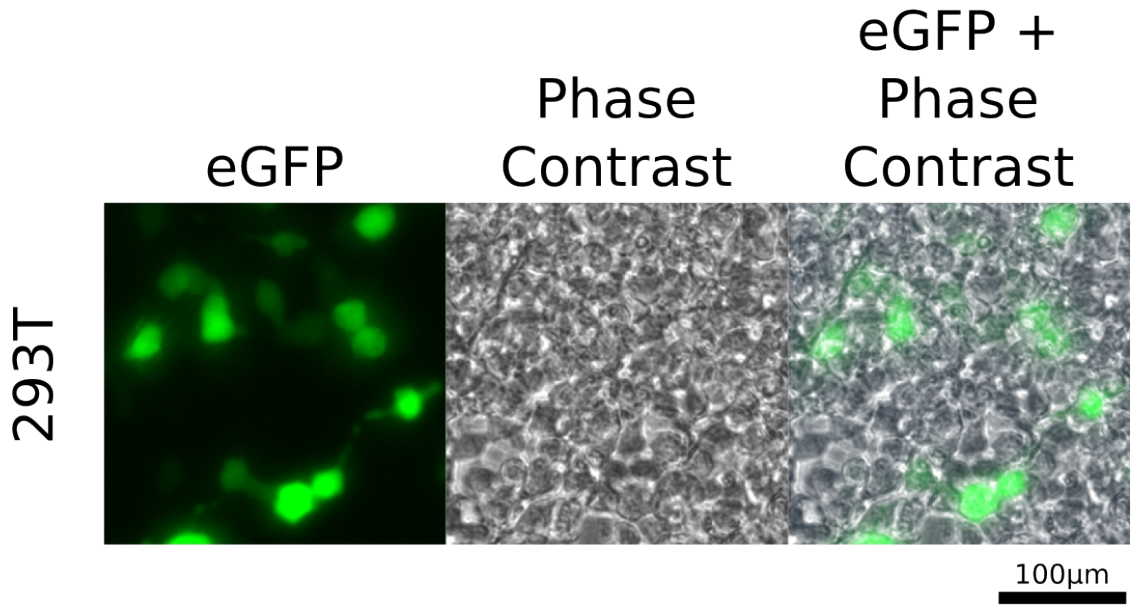

Figure 5 : Epifluoerescent microscopy of 293T cells, transfected by pCter-FLAG-hLIF-MIE.