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Expression profiling in transgenic FVB/N embryonic stem cells overexpressing STAT3.

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Abstract

The transcription factor STAT3 is a downstream target of the LIF signalling cascade. LIF signalling or activation is sufficient to maintain embryonic stem (ES) cells in an undifferentiated pluripotent state. To further investigate the importance of STAT3 in maintaining the pluripotency of ES cells we have generated transgenic FVB mice expressing a conditional tamoxifen dependent STAT3-MER fusion protein. Transgenic blastocysts have yielded pluripotent germ line competent ES cells at high frequency when established in a tamoxifen-containing medium in the absence of LIF. The expression profiling of tamoxifen induced transgenic FVB ES cell lines when compared with wild type cells has revealed a set of genes that were markedly up- or down-regulated. The expression of four of the up-regulated genes (Hexokinase II, Lefty2, Pramel7, PP1rs15B) was shown to be restricted to the inner cell mass (ICM) of blastocysts. These differentially expressed genes represent potential candidates for the maintenance of pluripotency of ES cells. We finally overexpressed two of the identified genes, Pem and Pramel7, in embryonic stem cells and demonstrated that their overexpression is sufficient for maintaining the expression of markers of the undifferentiated, pluripotent state and typical morphology of pluripotent ES cells in absence of LIF.

Background

ES cell lines that keep their pluripotency after transfection and selection procedures are essential for the introduction of selected targeted mutations into the germ-line of mice. Pluripotent ES cells are established *in vitro* from the inner cell mass (ICM) cells of explanted blastocyst-stage embryos [1-3]. Murine ES cells are maintained in a pluripotent state by co-culturing with mitotically inactivated feeder cells, such as embryonic fibroblasts, and/or the addition of leukaemia inhibitory factor (LIF: [4, 5]. These ES cells can be maintained indefinitely in the presence of LIF, and express markers of the undifferentiated, pluripotent state, including the POU-domain transcription factor POU5F1 (OCT-4), a factor that is essential for the development of the ICM (reviewed by [6]; [7]. Upon removal of LIF, the cells rapidly lose self-renewal capacity and differentiate into a variety of cell types. The pathway by which LIF signalling acts to promote ES cell self-renewal has been well studied (reviewed by [8]. LIF belongs to the Interleukin-6 family of cytokines and the members of this family have diverse effects on a variety of cell types [9]. The shared usage of signal transducers (i.e. gp130) in the multichain cytokine receptor complexes clearly explains the functional redundancies of these cytokines (reviewed by [10].

LIF signals via heterodimerization of the two class I cytokine receptors, the low affinity LIF receptor (LIFR) and the common subunit, gp130. The cytoplasmic domain of gp130 contains several tyrosinase residues that are phosphorylated by associated JAK (Janus kinase) kinases after ligand-stimulated dimerization. Four of these phosphorylated tyrosines have been identified as putative interaction sites with the SH2 (Src homology 2) domain of the transcription factor STAT3 (signal transducer and activator of transcription; [11]. Stimulation of gp130 signalling in ES cells also phosphorylates SHP-2 (SH2-domain-containing tyrosine phosphatase) and leads to activation of the mitogen-activated protein (MAP) kinases ERK1 and ERK2 [12]. Inhibition of the SHP-2/RAS/ERK pathway promotes self-renewal over differentiation and treatment of mouse ES cells with the MAPK-inhibitor PD098059 [13] was shown to enhance self-renewal [14].

Matsuda et al. (1999) have shown that activation of the STAT3 transcription factor is sufficient to maintain an undifferentiated state of mouse ES cells in the absence of LIF [15]: A transgene-construct encoding an inducible fusion protein (STAT3-MER) was introduced into ES cells. The construct generated to express the fusion protein contains the entire coding region of STAT3 and the mutated ligand-binding domain of the estrogen receptor. The fusion protein is only active upon binding of the synthetic ligand 4-hydroxytamoxifen (OHT) but not endogenous estrogen. ES cells expressing the STAT3-MER fusion protein maintained their undifferentiated state in the presence

of OHT and in the absence of LIF [15]. If *in vitro* STAT3 activation is sufficient for stem cells maintenance, the *in vivo* relevance of the LIF pathway is not yet entirely clear. LIF expression can be detected in the TE of the blastocyst whereas LIF receptor is expressed in the ICM. However, neither LIF mutants [16] nor mutants of the receptors LIFR [17, 18] and gp130 [19] result in any defects in the development of the ICM or early epiblast. Recent evidence suggests that the LIF pathway is necessary for survival of the mouse epiblast during diapause [20].

Recent studies have begun to identify key players involved in the intracellular signal transduction pathways regulating stem cell renewal and proliferation. Several transcription factors including the POU5F1 have been shown to be essential to maintain pluripotency in ICM, but until recently none of them had been shown to function independently of the LIF pathway. The newly identified homeobox transcription factor Nanog, directs pluripotency in mouse ICM and mouse ES cells and functions independently from LIF dependent STAT3 activation [21, 22]. Nanog is detected in the ICM and early germ cells, as well as in the ES and embryonic carcinoma (EC) cell lines derived from these stages [21]. Overexpression of Nanog relieves mouse ES cells cultured without feeder cells in the presence of serum from dependence on LIF stimulation for self-renewal whereas nanog-deficient mouse ES cells loose pluripotency and differentiate into extra embryonic endoderm lineages [22].

ES cell lines exhibit different degrees of LIF dependency as has been demonstrated in STAT3 gene targeting experiments by Raz et al. [23]. ES cells heterozygous for a Stat3 mutation could only be established from E14 cells (129P2/OlaHsd; [24]. Targeted clones from other cell lines were invariably trisomic for chromosome 11 that carries the Stat3 locus, and retained normal levels of activated STAT3.

In our study we have evaluated whether overexpression of active STAT3 supports the survival and derivation of pluripotent ES cells in the FVB/N mouse strain. To this extend we have generated FVB/N transgenic mice overexpressing a tamoxifen inducible STAT3. The inbred mouse strain FVB/N has been chosen since it is widely used for the generation of transgenic animals [25], however to this day only one germline competent ES cell line has been reported [26].

Our data confirm that overexpression of STAT3 in the ICM of the blastocyst clearly supports establishment of ES cells in the FVB/N mouse strain. Furthermore, using germline proven ES cells we performed a microarray-analysis comparing WT and STAT3 overexpressing FVB/N ES cells and identified a pool of genes that are differentially expressed. In situ hybridization analysis of part of these genes showed expression in the central part of the morula and in the ICM of the blastocyst

suggesting a potential role of these candidate genes in maintenance of pluripotency. In order to confirm their potential role in maintaining pluripotency, two of the identified genes, Pem and Pramel7 were overexpressed in ES cells. We cultivated the ES cells in absence of LIF and observed that they maintained the typical morphology of pluripotent stem cells and expressed the characteristic pluripotency related markers SSEA-1 and Oct4. This clearly demonstrates that these two genes are involved in maintenance of pluripotency.

Methods

Generation, Identification, and Maintenance of Transgenic Mice

The pCAGmusstat3ER plasmid containing the full length sequence of murine STAT3 cDNA fused to the ligand-binding domain of mouse estrogen receptor under the control of the chicken β-actin promoter [15] was propagated in *Escherichia coli* DH5α and the minigene was excised with NotI. The fragments were purified from a 1% agarose gel with a Qiaquick extraction kit (Qiagen, Basel, Switzerland) and processed as described. Nuclear injections into fertilized FVB/N oocytes were carried out by conventional methods [27, 28]. Transgenic founder were identified by PCR using the β-actin promoter specific primer ggbactfor2: (5'-GGG TTC GGC TTC TGG CGT G-3') and a STAT3 specific primer mmSTAT3back2: (5'-CCA AGG TGC CAG GAA CTG CCG-3'). Two primers specific for the TAG-1 gene (TAG82B: 5'-ACA CGA AGT GAC GCC CAT CCG T-3'; TAG83F: 5'-GGA GGA GAG AGA CCC CGT GAA A-3') were used as a positive control for both wild-type and transgenic mice. ggbactfor2 and mmSTAT3back2 generate a 397bp band whereas TAG82B and TAG83F generate a 300bp product.

Cell culture

ES Cell Medium for FVB/N ES cells: KSR-KDMEM (Invitrogen) with 1000 units per ml human LIF, KSR-KDMEM w/o LIF and KSR-KDMEM w/o LIF with 1 μM 4-hydroxytamoxifen (OHT, Sigma).

ES Cell Medium for E14 ES cells: G-MEM (Sigma) containing 100 mM sodium pyruvate (GIBCO), 10%FBS, 50 mM β-mercaptoethanol and containing 10^7 U/ml ESGRO murine LIF (Chemikon Int.).

Embryonic feeder fibroblasts (MEF) were derived from explanted day 14 fetuses of CD-1-M-TKneo strain mice [29]. Prior to co-culturing with ES cells, confluent layers of MEF cells were treated with mitomycin C (10 μg/μl) for 2.5 hrs and extensively washed. Growth arrested fibroblasts were used as feeder cells for up to one week.

Embryo recovery, embryo culture, isolation of ES cell lines
Donor females of strains FVB/N (RCC Füllinsdorf, Switzerland and Harlan Horst
Postbus, Netherlands) were induced to ovulate by an injection i.p. of 5 I.U. PMSG
(Folligon, Intervet), followed 46 h later by an i.p. injection of 5 I.U. HCG (Chorulon,
Intervet). Subsequently, donor females were mated with STAT3-MER transgenic
male mice. Morulae were flushed from the uterotubal junction 3 days after mating
and cultured overnight in M16 medium (Sigma). Fully expanded blastocysts were
transferred onto MEF in KSR-KDMEM, KSR-KDMEM w/o LIF with 1 μM OHT or
KSR-KDMEM w/o LIF and cultured at 37°C in an atmosphere of 10 % CO_2 in air for
6-7 days without media changes. Full-grown ICMs were picked from the outgrown TE
and transferred into droplets of trypsin-EDTA solution (Invitrogen) by a mouth-
controlled glass-capillary. The ICMs were dissociated mechanically into groups of
cells and these aggregates reseeded onto embryonic feeder fibroblasts. 3-4 days
later, compact stem cell colonies could be identified. Single colonies were
dissociated as described above and reseeded. Subsequently, non-differentiating
clonal lines were partly frozen, partly passaged onto 6 cm plates, and splitted after 2-
3 generations for further characterization.

Karyotype and Sex Determination of ES cell lines
For chromosome counts, ES cells were pre-treated for 3 hrs with colcemide (Sigma,
0.05 μg/ml) and metaphase spreads were prepared according to Triman et al.
(1975). Sex determination was carried out using primers specific for the smcy and
smcx gene. The following primers were used: SMC4-1: 5'-CTG AAG CCT TTG GCT
TTG AGC AAG CTA C-3'; SMCX-1: 5'-CAA AGA ATT TGG CAG CGG TTT CCC T-
3'. Female cell lines exhibit a single band of 341 bp whereas male cell lines exhibit 2
bands of 341 and 312bp respectively.

Generation of injection chimeras
C57BL/6 host embryos were recovered at the morula stage from the oviducts of
hormonally treated females and cultured overnight (see above). Blastocysts were
transferred into drops of M16 medium (Sigma) and ES cells into drops of HEPES-
buffered ES-medium. About 10–15 ES-cells were injected into each blastocyst. After
a recovery period of about 2 hrs, injected blastocysts were transferred into the
uterine horns of pseudopregnant NMRI foster mothers (Harlan, England). Chimeric

offspring were identified by the absence of coat color pigmentation. Chimeric males were set up to breed at the age of about 8 weeks.

Western Blotting

ES cell cultures were homogenized with RIPA buffer (50 mM Tris-Cl pH 7.4, 1% NP-40, 0.25% Sodium Deoxycholate, 150 mM NaCl). Protein concentration was determined with BCA-Method (Pierce). Samples were subjected to SDS–PAGE and blotted onto PVDF membranes (Millipore, Volketswil, Switzerland) at 100 V for 1–2 h at 4°C. Immunodetection and chemiluminescent visualization were performed as recommended by the supplier of the chemiluminescence blotting kit (Roche Diagnostics, Rotkreuz, Switzerland). Anti STAT3 antibody (C-20) was purchase from Santa Cruz Biotechnology, anti-phospho (Y705) was purchased from New England Biolabs.

Immunohistochemistry and alkaline phosphatase staining

For alkaline phosphatase staining the cells were washed with CMF-PBS and fixed in 4% paraformaldehyde in PBS. The cells were washed with PBT (PBS with 0.1% triton X) and incubated in alkaline phosphatase buffer (100 mM Tris-Cl pH9.5, 50 mM $MgCl_2$, 100 mM NaCl) containing the AP-substrates nitrotetrazolium-blue and X-phosphate (Roche Diagnostics, Rotkreuz, Switzerland).

For immunohistochemistry the cells were washed in PBS and fixed with 4% paraformaldehyde in PBS. Cells were washed with PBT and incubated with the primary antibodies diluted in PBT containing 2% (v/v) horse serum. Secondary fluorescence labelled antibodies were used for detection. Anti OCT-4 (N-19) antibodies were purchased by Santa Cruz Biotechnology, SSEA-1 (Mouse mAb) by Chemicon International and Alexa Fluor 488 anti mouse and Alexa Fluor 594 anti rabbit secondary antibodies were purchased by Molecular Probes.

cRNA labelling and Hybridization of microarrays

cDNA synthesis 1st round of amplification. RNA was combined with $1\mu g$ of T7-dT primer (5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-(dT)24-3') in a total volume of 12 μl and incubated at 70°C for 10 min. The reaction was placed on ice and the following reagents added in a total volume of 20 μl: first strand buffer (1x), DTT (10 mM), dNTP mix (500 μM), and Superscript III (200 units, Invitrogen) and incubated at 42°C for 2 h. Second strand synthesis was performed by adding the following reagents in a total volume of 150 μl: second strand buffer (1x), dNTP mix (200 μM), *E. coli* DNA ligase (10 units), *E. coli* DNA polymerase (40 units),

RNase H (2 units), and incubated at 16°C for 2 h. T4 DNA polymerase (10 units) was then added to fill in the ends of the cDNA and incubated at 16°C for an additional 15 min. Following phenol:chloroform extraction and ethanol precipitation, the cDNA was resuspended in RNase-free water. Transcription was performed using Ambion's (Austin, USA) MEGAscript reagents. A 20 μl reaction containing cDNA, NTP mix (7.5 mM), reaction buffer (1x), and 2 μl enzyme mix was incubated at 37°C for 4 h and the primary cRNA was purified using the RNeasy kit (Qiagen, Chatsworth, CA) per the manufacturer's specifications. Two hundred nanograms of the purified cRNA were then carried forward in the secondary amplification. Samples with less than 200 ng cRNA were concentrated to a 5 μl volume and the entire sample used for the next round of amplification.

cDNA synthesis 2nd round of amplification. First-strand cDNA was synthesized by incubating the above cRNA with 1 μg of random primers (Invitrogen, Carlsbad, CA) at 70°C for 10 min. The reaction was then placed on ice and the following reagents added in a 20-μL reaction: first-strand buffer (1x), DTT (10 mM), dNTP mix (500 μM), and Superscript III (200 units). The reaction was incubated at 42°C for 2 h. RNase H (2 units) was added to the reaction and incubated at 37°C for 20 min and then heat-inactivated. The cDNA was combined with 1 μg of the T7-dT primer and incubated at 70°C for 10 min. The reaction was then placed on ice and the following reagents added for a final volume of 150 μl: second-strand buffer (1x), dNTP mix (200 μM), *E. coli* DNA Polymerase (40 units), and then incubated at 16°C for 2 h. T4 DNA polymerase (10 units) was added to fill in the ends of the cDNA and incubated at 16°C for an additional 15 min. The cDNA was purified with phenol:chloroform and precipitated with ethanol.

Biotin secondary IVT. The secondary transcription reaction was performed using the Enzo BioArray HighYield RNA kit (Affymetrix, Santa Clara, CA). The secondary cDNA was incubated at 37°C for 4 h in a 40 μl reaction as indicated by in the manufacturer recommendations and the labeled cRNA purified using the RNeasy kit.

Array Hybridizations and Analysis

The biotin-labeled cRNA was fragmented in 40 mM Tris-acetate buffer pH 8.1 containing 100 mM potassium acetate and 30 mM Magnesium acetate. 15 μg of labelled cRNA was mixed with the appropriate buffers and hybridized to a mouse U74Av2 (Affymetrix) for 16 h at 45°C. Computer analysis of the resulting data was performed using the DChip software package [30]. Samples were run on triplicate microarrays and the resulting data combined into subsets and compared using

DChip. A total of 25 genes were identified as showing changes in gene expression using a value of 1.5 for the lower bound of the 95% confidence interval for the fold change as a cutoff.

Real-Time Quantitative PCR (Q-PCR)

The total RNA from cultured ES cells was obtained using Qiagen© Rneasy mini-kit and reverse transcribed with oligo-dT primers (Invitrogen) and SuperscriptIII (Invitrogen). Quantitative Real time experiments were performed with the SyberGreen technology using the Quantitec (Qiagen) and an ABI Prism 7700 cycler. For quantitation of gene expression comparative Ct-method was used after normalization with beta-actin. The following primers were used:

βactin_fwd:	5'-cat cca ggc tgt gct gtc cct gta tgc-3'
βactin_bwd:	5'-gat ctt cat ggt gct agg agc cag agc-3'
Ebaf_fwd:	5'-aca gcg cgg atg tgg agg aga tgg-3'
Ebaf_bwd:	5'-atc ctc acg gac tct cag cca ttc a-3'
Eif2s2_fwd:	5'-tac atc gtc aac cca aac atc tcc ttg c-3'
Eif2s2_bwd:	5'-ggc acg gag ctg tgc tcg ctt-3'
Dppa3_fwd:	5'-agg gtc cgc act ttg ttg tcg gtg c-3'
Dppa3_bwd:	5'-gct cct aat tct tcc cga ttt tcg cat-3'
NDP5211_fwd:	5'-cat gag cag cta cag agg aag ca-3'
NDP5211_bwd:	5'-gtg cct cag att cac tgt gta gct aa-3'
pramel6_fwd:	5'-cag gaa gac gag tgg caa agc acg t-3'
pramel6_bwd:	5'-agc cct gga atc tca tag cct aca tc-3'
pramel7_fwd:	5'-gag gag aag cag aac atc agc aag a-3'
pramel7_bwd:	5'-ctc tta gag gcg tga cat cta ggt t-3'
stat3_fwd:	5'-ggc aag ggc ttc tcc ttc tg -3'
stat3_bwd	5'-agc tgc tgc ttg ttg gtg tat gg -3'
socs3_fwd:	5'-caa cat ctg ggc gca gcg cga gc-3'
socs3_bwd:	5'-cgc tgg tcc gag ctg tcg cgg ga-3'
Trim41_fwd:	5'-tga gcc gca tgt ttt gtc agg ctg c-3'
Trim41_bwd:	5'-c aca ctt cgc gct gga cta gga gct-3'

Quantitative RT-PCR for each gene was done in triplicates and the values were normalized to the corresponding amounts of β-actin RNAs. Each Ct value used for the calculation was the mean of results obtained from triplicates and PCR were

performed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland).

In situ hybridization

Template for riboprobe synthesis were obtained by amplification through RT-PCR. Briefly, total RNA was isolated from FVB/STAT3-MER ES cells cultivated in presence of 4OHT and RT was performed with oligo-dT primers. 300-400bp long fragments containing part of the ORF of the genes of interest were amplified by PCR and cloned in a pCRII® TOPO® dual promoter vector (Invitrogen). DNA templates for riboprobe synthesis were digested with appropriate enzymes to provide fragments for either sense or antisense orientation of the PCR product respect to the vector. Labelled riboprobes were synthesized by SP6 or T7 RNA polymerases by incorporation of digoxygenin-labelled UTPs.

The embryos were transferred in a micro pore insert (12µm) sitting in a well of a 4 well plate and fixed with freshly prepared 4% PFA/PBS, washed twice in PBT and dehydrated once in 25%, 50%, 75% and twice in 100% methanol/PBT. The dehydration was followed by rehydration in the reverse order of the MeOH/PBT series 75%, 50%, 25% for 5min each. The embryos permeabilized in RIPA buffer and refixed in 4% PFA/0.2% glutaraldehyde. After prehybridisation in hybridisation solution for ≥2 hours at 70°C the embryos were incubated in hybridisation solution containing 1.6 µg/ml of the corresponding riboprobe. As a control for the specificity of the labeling, in each hybridization experiment control embryos were hybridized with an equal concentration of a sense probe transcribed from the same template as the antisense probe. After high and low stringency washes hybridized riboprobes were detected using an AP-coupled anti-digoxigenin antibodies (Roche Diagnostics, Rotkreuz, Switzerland) and the AP-substrate BM-Purple (Roche Diagnostics, Rotkreuz, Switzerland). The staining reaction was stopped by rinsing in 2mM EDTA/PBT. Embryos were subsequently post-fixed in 4% PFA/0.1% glutaraldehyde in PBT and cleared in a glycerol:PBT 1:1 solution.

Overexpression of Pramel7, Pem and Nanog

The full length open reading frames (ORF) of Pramel7 and Pem were amplified by RT-PCR and sequenced. The full length cDNAs were cloned in the pfloxedNanog expression vector [21] by exchanging the Nanog cDNA with either the one of Pem or Pramel7. The expression vectors were completely sequenced and finally electroporated in E14 ES cells. After selection with puromycin single colonies were isolated and expression levels were determined by real time PCR. The clones with the strongest overexpression were chosen for further experiments.

Results

Generation of transgenic mice overexpressing STAT3-MER

We overexpressed a fusion protein composed of the entire coding region of mouse STAT3 and the modified ligand-binding domain (G525R) of mouse estrogen receptor [15]. The modified ligand-binding domain binds the synthetic steroid ligand 4-hydroxytamoxifen (4OHT) but not 17β-estradiol [31]. The expression of the transgene is driven by a chicken β-actin promoter and therefore is expected to express ubiquitously. After injection of the construct into the pronucleus of fertilized FVB/N eggs we obtained seven positive founder animals. When crossed with wild-type FVB/N partners, six of them showed germ line transmission. Multiple tissue analysis was performed by western blot in order to define animals exhibiting ubiquitous expression (data not shown). Two lines (Tg741 and Tg743) expressing large amounts of STAT3-MER were selected for further experiments. Both transgenic lines contain a single integration of the transgenic cluster and were maintained in a hemizygous state by breeding with WT FVB/N mice.

Overexpression of inducible active STAT3-MER enables the establishment of germline competent ES cells from FVB/N blastocysts

We were able to establish both wild type and transgenic ES cell lines. However, wild-type ES cells were only obtained when LIF was present in the medium (Table 1). When using OHT-supplemented Medium without LIF 50-73 % of the embryos from both Tg741 and Tg743 transgenic lines yielded ES cell lines, all of them being transgenic (Table 1). Because the mice used were hemizygous for the transgene it is fair to assume that we were able to derive ES cells from virtually all the transgenic embryos. These results strongly indicate a supportive effect of active STAT3 on the maintenance of pluripotent ES cells. In order to confirm the pluripotency of these ES cells karyotypically normal male cells from both transgenic lines (Tg741 and Tg743) have been injected into C57BL/6 host blastocysts. Chimeric males were identified by the absence of eye (pink) and coat (albino) pigmentation and mated to wildtype FVB/N females. Germline transmission of the FVB/N ES cell genome resulted in albino offspring. To confirm that overexpression of active STAT3 supports the survival and derivation of pluripotent ES cells also in the F1 generation, transgenic germline F1 offspring from the line Tg741 were mated to wildtype animals. Blastocyst stage embryos were isolated and cultivated as previously described, if cultivated in

presence of 4OHT stem cell lines could be established from 44% of the embryos, all lines being transgenic (Table1).

Characterization of the newly established FVB/N ES cells overexpressing STAT3-MER

The expression level of STAT3-MER in the ES clones obtained from the line 743, was tested by western blot (see Fig. 1). Furthermore, upon LIF stimulation STAT3 is phosphorylated on the tyrosine residue (Y705), dimerizes and can bind DNA [32, 33]. In order to test if 4OHT is able to induce STAT3-MER phosphorylation FVB/N ES cells expressing STAT3-MER were stimulated either with LIF or 4OHT for 10 minutes up to 24 hours. Cell extracts were separated by SDS-PAGE, blotted and probed with anti-STAT3 and anti-phospho (Y705) antibodies (Fig. 1). LIF stimulation induced tyrosine phosphorylation of both endogenous STAT3 and STA3-MER. As previously observed [15], endogenous STAT3 was dephosphorylated rapidly whereas dephosphorylation kinetics of STAT3-MER were slower. A dose dependence could be also observed, in ES cells derived from the Tg743 line, that are expressing higher STAT3-MER amounts, dephosphorylation was slower compared to the cells derived from the lower expressing Tg747 line (data not shown). In Tg743 derived ES cells stimulation with 4OHT resulted in a strong tyrosine phosphorylation of STAT3-MER, only a limited phosphorylation could be detected for endogenous STAT3. During the 24 hours of induction with either LIF or 4OHT expression of Oct4 was confirmed (data not shown).

ES cells overexpressing STAT3-MER express the typical ES-cell markers

ES cells, as well as cells of the ICM of mouse blastocysts, express a panel of surface markers that are used to characterize undifferentiated, pluripotent embryonic cells. The expression of the transcriptional factor OCT-4 and the surface marker SSEA-1 was tested by immunohistochemistry; wild type as well as the transgenic ES cells expressed both markers (see Fig. 2). Nanog expression was tested by semi-quantitative RT-PCR, also in this case the cells were expressing nanog but no expression differences between WT and STA3TER overexpressing cells could be detected (Fig. 1B). In all cases the expression was restricted to the ES cells and no signal could be detected in the inactivated fibroblast used as feeder cells.

Microarray analysis

In order to identify which genes are regulated trough overexpression of STAT3 we compare WT FVB cells cultivated in presence of LIF with transgenic FVB cells

overexpressing STAT3-MER cultivated in presence of 4OHT. Total RNA was isolated and an expression analysis was performed by hybridizing Affymetrix chips containing probes covering the complete mouse transcriptome. Genes showing expression changes higher then 1.5fold were considered. We first could confirm the overexpression of STAT3-MER beeing the ration of the transgenic line 743 40 times higher than the WT. We furthermore could identify a set of 25 genes differentially regulated, 11 were upregulated (Table 2) whereas 14 were downregulated (Table 3).

In situ Hybridization

Nine out of the 25 identified genes were considered as candidates to have a function in determination and maintenance of pluripotency in ES cells. With these genes *in situ* hybridization was performed in order to define in which regions of the preimplantation embryos these genes are expressed. The temporo-spatial expression was analyzed by whole mount *in situ* hybridisation of morulae and blastocysts. Four genes, Pramel7, Lefty2, Protein Phosphatase 1 regulatory subunit 15B and hexokinase II were expressed only in the central part of the morula and in the ICM of the blastocyst (Fig 3). The other five genes, Pramel 6, Eif2s2, Pem, Dppa3 and Skp2 were found to be expressed in all cells of the morula and blastocysts (Fig 3A), a more exact analysis of the preimplantation stages was performed for the Pramel7 gene (Fig 3B).

Overexpression of Pem and Pramel7 allows cultivation w/o LIF

For the overexpression the full length cDNAs of Pem and Pramel7 were inserted in the pfloxedNanog vector (see Chambers et al., 2003) instead of the cDNA of Nanog. The pfloxed vector was also electroporated in E14 cells as a positive control for the experiments. All electroporated cells were selected with puromycin and resistant colonies were picked and expanded. After testing for the presence of the vectors by PCR the positive clones were analyzed by real time PCR and the clones with the strongest expression were used for further experiments. In order to test for the capacity of maintaining pluripotency in absence of LIF, the cells were cultivated for 8 days without addition of LIF to the medium. After 8 days in culture IHC was performed in order to detect the expression of OCT-4 and SSEA-1 (Fig.4). E14 WT ES cells started after 4 days to differentiate and showed the typical flattened morphology of differentiating cells (data not shown), after 8 days the cells were completely differentiated and didn't express anymore OCT-4 and SSEA-1. Nanog overexpressing cells as expected maintained their pluripotent state also in absence of LIF. Both Pramel7 and Pem overexpressing clones showed a similar behaviour as

the Nanog overexpressing cells. The colonies maintained the typical round shaped morphology and expression of OCT-4 and SSEA-1 was present indicating that these two genes are able to maintain pluripotency also in absence of LIF.

Discussion

The data clearly support a necessary role for the gp130-JAK-STAT3 pathway in ES cell selfrenewal. Until to date the role of the STAT3 pathway in maintaining pluripotency in ES cell *in vitro* cultures was repeatedly demonstrated [15, 26]. Our work indicates that the activation of the STAT3 pathway during cultivation of blastocysts supports ICM outgrowth and clearly favors the establishment of new ES cell colonies in the FVB mouse strain. However, the *in vivo* relevance of the LIF pathway is not entirely clear. LIF is expressed in the TE of the blastocyst and the LIF receptor in the ICM, demonstrating the requirement of this pathway for pluripotent cell survival *in vivo*. However, neither LIF mutants [16] nor mutants of the receptors, LIFR [17, 18] and gp130 [19], cause any defects in the development of the ICM or early epiblast. Another key player in maintenance of pluripotency is the transcriptional factor OCT-4. The nature of the relationship between STAT3 and OCT-4 is not yet completely clear. OCT-4 has a fundamental role in maintaining pluripotency since ICM cells in OCT-4-deficient embryos are absent. The formation of a normal epiblast in STAT3-deficient embryos indicates that the function of STAT3 is dispensable and therefore, an interaction between OCT-4 and STAT3 is not obligatory *in vivo* [14]. Recent evidence suggests that the LIF pathway is necessary for survival of the mouse epiblast during implantation delay [20]: reviewed by [34], the so-called diapause. Diapause is a state of arrested development occurring in mated female mice that are lactating or have been experimentally ovariectomized. During diapause the embryos develop to the hatched blastocyst stage but do not implant until maternal estrogen is restored (reviewed by [35]. Normal embryos can resume development even after 3 to 4 weeks of implantational delay. In contrast, embryos lacking gp130 fail to recover after only a few days in diapause, due to an inability to maintain pluripotency of the epiblast. In addition, the efficiency of ES cell establishment is enhanced when using blastocysts in diapause [36, 37] further emphasizing that signaling through gp130 is essential for prolongation of the epiblast life span. Furthermore, the knockout studies in the gp130 pathway demonstrate that the normal transient phase of epiblast expansion in vivo does not rely on gp130 signaling. Evidence has been presented for a gp130-independent mechanism of supporting at least limited expansion of mouse ES cells [38].

In order to identify genes, which might be related to the STAT3 pathway we compared gene expression changes in FVB/N embryonic cells overexpressing activated STAT3 and cultivated in presence of 4OHT and absence of LIF compared with WT FVB cells cultivated in presence of LIF. A group of about 25 genes showed significant differential expression. These genes can be divided in different categories according to their function: The first group contains regulatory members of the STAT3 pathway that are involved in the regulation of downstream events of the JAK/STAT cascade (like SOCS-3, protein phosphatase-1 regulatory subunit 15B and Eif2s2), the second group of genes is involved in pluripotency maintenance and cell viability, whereas the third group contains genes involved in the regulation of the metabolism in ES cells.

In the first group we find SOCS-3; the members of suppressor of cytokine signalling (SOCS) family have been implicated in the negative regulation of several pathways, particularly the JAK/STAT pathway, and since this signalling pathway is responsible for their induction, they form part of a classical negative feedback circuit. At least three different modulating mechanisms have been demonstrated: through the SH2 domain they bind to phosphotyrosine on the target protein, leading to inhibition of signal transduction by N-terminal inactivation of JAK, by blocking access to STAT to the receptor sites, or by SOCS box-targeting bound proteins to proteosomal degradation. The transcriptional upregulation of SOCS-3, a member of the suppressor of cytokine signalling family, confirms that the functional overexpression of STAT3-MER induces the activation of the classical LIF-dependent negative feedback mechanism (for a review see [39]). Previously Duval et al. (2000) showed that expression of SOCS-3, but not SOCS-1 and SOCS-2, was stimulated in ES cells in presence of LIF. The author further demonstrated that, uncontrolled overexpression of SOCS-3 leads to repression of LIF-dependent transcription and severely reduces cell viability. This suggests that the disturbance of a well balanced SOCS protein content has adverse effects on cell survival [40]. Because the FVB ES cells overexpressing STAT3-MER are viable and pluripotent it is to assume that the SOCS-3 upregulation observed in presence of 4OHT is a modulatory reaction due to the overproduction of STAT3 in these cells, through this compensatory mechanism the cells are able to maintain a properly activated LIF signalling cascade. It is to believe that the upregulation of SOCS-3 is a direct transcriptional activation mediated through STAT3 because the promoters of both mouse and rat SOCS-3 genes contain putative STAT1/STAT3 binding elements, which are necessary and sufficient for LIF-dependent activation of the SOCS-3 promoter activity in reporter assays [41, 42]. Protein phosphatase 1 regulatory subunit 15B (PP1rs15B) is a constitutively

expressed inhibitory subunit of PP1, one of the major eukaryotic serine/threonine phosphatases. PP1rs15B dephosphorylates the α-subunit of eIF2 [43]. Under stressful conditions four different kinases phosphorylate eIF2α, which leads to a decrease of translational activity. PP1rs15B maintains basal low levels of eIF2α phosphorylation in unstressed cells and therefore inhibits the shut down of translational activity [43]. The microarray data confirm an upregulation of both PP1rs15B and eIF2s2 indicating that these proteins seem to be involved in the regulation of the STAT3 overexpression, whether these genes have a function in maintenance of pluripotency is still unclear.

The second group of interesting genes contains molecules involved in pluripotency and cell viability. Interestingly no changes in the regulation of the classical marker for pluripotent ES cells could be detected. Alkaline phosphatase, OCT-4, SSEA-1, and Nanog were correctly expressed in both wildtype and transgenic ES cell lines (see Fig 2), but the overexpression of STAT3 didn't induce any changes in the expression levels. Regarding the Nanog expression, our observations are in accordance with previous works describing that nanog can function independently of STAT3 and that nanog appears not to be a transcriptional target of gp130. Because, in the absence of gp130 stimulation, endogenous nanog expression is insufficient to sustain self-renewal, it is to assume that ES cells are normally propagated by collaboration between cytokine-activated STAT3 and nanog. Furthermore, even if increased Nanog concentrations render the LIF-R/gp130 signalling dispensable, maximal self-renewal can be obtained by combination of nanog overexpression and LIF stimulation [21].

Within the identified differentially expressed genes, genes of mainly unknown function are found that were previously correlated with pluripotency [44] or with embryo viability in somatic nuclei derived cloned blastocysts [45]. Dppa3, NDP52l1, Pramel6, Pramel7 were identified as a set of OCT-4 related genes that are not correctly reactivated in somatic nuclei derived cloned embryos. Mice lacking OCT-4 gene develop to the blastocyst stage but die after implantation, due to the lack of truly pluripotent ES cells. Similarly, cloned animals deriving from transfer of somatic nuclei into enucleated oocytes mainly die after implantation of the embryo. Bortvin et al. examined the hypothesis that the limited developmental potency of cloned embryos is a result of incomplete reactivation of genes that, like OCT-4 function specifically in pluripotent embryonic cells. To test this hypothesis the authors identified 10 candidate genes with an expression pattern similar to Oct4 and

compared their expression between normal and cloned preimplantation embryos [45].

Dppa3 (developmental pluripotency-associated 3; Stella; PGC7; Crg1) is preferentially expressed in primordial germ cells, oocytes and preimplantation embryos. In blastocysts, Dppa3 is expressed in TE and ICM and in the early postimplantation embryos Dppa3 expression disappears. The expression re-emerges when at day E7.5 the first primordial germ cells (PGCs) are built [46]. Dppa3 knockout mice are compromised in development; some embryos develop to the two or four cell stage, but fail to reach 8-cell stage [45, 47]. Dppa3 was proposed by Sato et al. to play a role in germ line specification in mice by preventing nascent germ cell populations from a somatic cell fate and by retaining their pluripotency [46]. Pramel6 and Pramel7 (preferentially expressed antigen in melanoma like 6 and 7) are prevalently expressed in preimplantation embryos and embryonic pluripotent cells [45]. Our results confirm these expression patterns and clearly show that whereas Pramel6 is typically expressed in all cells of the morula and the blastocyst, Pramel7 is expressed in the inner part of the morula and in the ICM of the blastocyst. The function of Pramel's in embryonic development is unknown, but interestingly, PRAME inhibits retinoic-acid induced differentiation in mouse embryonic carcinoma F9 cells [48]. Recently Kaji et al (2006) showed that Pramel6 and Pramel7 expression is mediated by Mbd3, a component of the nucleosome remodelling and histone deacetylation (NuRD) complex [49]. Mbd3 knockout ES cells do not appropriately repress transcription of Pramel6 and Pramel7, which are normally expressed in preimplantation embryos [45]. Kaji et al. proposed that the Mbd3/NuRD-mediated silencing of Pramel6 and Pramel7 in ES cells offers an epigenetic environment in which Mbd3/NuRD is not absolutely required but facilitates differentiation. Furthermore the authors describe that Mbd3 deficiency leads to down regulation of Dppa3 in ES cells. In general, expression pattern analysis corroborates the idea that Dppa3, Pramel6 and Pramel7 are collaborating in deciding the fate of ES cells. Overexpression of Pramel7 in ES cells, allows the maintenance of pluripotency, as shown by IHC with the typical pluripotency related markers OCT-4 and SSEA-1, indicating the importance of this protein in maintaining ES cells in a pluripotent state (Fig. 4).

Murine Pem is an X-linked homeobox-containing gene [50, 44], whose homeodomain shares important structural features with two other homeobox genes which are expressed in extra embryonic lineages and during spermatogenesis [51, 52]. The Pem protein is expressed in the late morula stage, in TE and ICM of blastocyst and after implantation in extra embryonic tissues, in the parietal and visceral endoderm,

but not in the primitive ectoderm derivatives. Pem is also expressed in ES cells, in primordial germ cells and in teratocarcinoma cell lines [53]. Fan et al. (1999) altered pem gene expression in ES cells for functional studies. Overexpression of Pem had no phenotype in ES cells, but blocked completely differentiation into the three primary cell lineages, when ES cells were cultured as embryoid bodies in suspension without LIF [54]. Two different models of action for Pem are possible. Fan et al. (1999) suggested that Pem first helps to maintain the undifferentiated cell state, and in a second step promotes a defined cell population of undifferentiated stem cells for differentiation into extra embryonic lineages [54]. Sasaki et al (1991) proposed an alternative in which Pem directs early differentiation to specific lineages, but does maintain actively the undifferentiated state [44].

NDP52l1 (Nuclear dot protein 52) was also identified from Bortvin et al. (2003) as a gene incorrectly reactivated after somatic cloning. The embryonic function is to date unclear but it is capable of forming dimers and contains contains leucine zipper motifs indicating a possible function in splicing processes. The last 50 aminoacids show strong homology to oocyte and gastrula zinc finger proteins, and especially to proteins containing a LIM domain [55]. The overexpression experiments confirm the previous observation indicating that the overexpression of Pem in ES cells allows the maintenance of pluripotency in absence of LIF (Fig. 4):

The observed overexpression of hexokinase II corroborates the importance of this isoform of hexokinase in embryo viability. Upon fertilization and over the next 4 to 5 days, the embryo undergoes significant changes in carbohydrate and amino acid consumption and utilization. The significance of enzyme isoforms and their relative changes in abundance in terms of embryo physiology and metabolism has not been fully determined, although it is evident that the isoforms of key enzymes do change during the pre- and periimplantation period. In all probability, glucose utilization during the preimplantation period is not regulated by transport across the plasma membrane or the absence of sufficient enzyme activity. Rather substrate availability (concentration) and the specific regulation of enzyme activity appear to control glucose utilization by the preimplantation embryo. Kinetic analysis of hexokinase in preimplantation mouse embryos indicates that there is a switch from isozyme I at the zygote to isozyme II at the blastocyst stage [56, 57]. Moreover, mice homozygous for hexokinase II deficiency die at approximately 7.5 days post-fertilization, indicating that hexokinase II is vital for mouse embryogenesis after implantation and before organogenesis [58]. Our observation that the concentration of hexokinase II is increased in cells overexpressing STAT3 confirms the importance of this isozyme for embryo viability and indicates that a correct energetic balance is extremely important

in the late stages of preimplantation and at the beginning of postimplantation in the embryos.

In summary the data presented indicates that overexpression of functional STAT3MER in FVB/N blastocysts sustains the establishment of germline competent ES cells in absence of LIF. Furthermore, gene expression analysis of these transgenic cells cultivated in presence of OHT showed that 25 genes were differentially expressed compared to WT cells cultivated in presence of LIF. By *in situ* hybridization analysis it was possible to identify four up-regulated genes (Hexokinase II, Lefty2, Pramel7, PP1rs15B) which expression was restricted to the ICM of the blastocysts. These genes represent potential candidates for maintenance of pluripotency of ES cells. Overexpression of two of the upregulated genes, Pem and Pramel7, in E14 cells and cultivation in absence of LIF demonstrated that these two genes are able to maintain the embryonic stem cells in a pluripotent state without addition of LIF to the culturing media. WT cells cultivate under the same conditions differentiated and lost expression of the pluripotency markers OCT-4 and SSEA-1. All together these data show that overexpression of STAT3-MER facilitates the establishment of germline competent embryonic stem cells by activating genes directly involved in maintenance of pluripotency and opens the possibility to establish germline competent ES cells from non-permissive mouse strains.

Acknowledgments

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Table 1: Establishment of FVB/N ES cells.

WT FVB/N or hemizygous FVB/N males carrying the STAT3MER transgene (lines 741 and 743) were mated with WT FVB/N females. Blastocysts were collected and cultivated either in presence of LIF or OHT. Outgrown ICM's were picked and cultivated till ES cell colonies were visible. Cells were tested for the presence of the transgene by PCR analysis. As expected it was possible to establish WT ES cells only in presence of LIF but not when OHT alone was added to the culture. Furthermore in presence of OHT all both transgenic lines 741 and 743 generated ES cell colonies that were carrying the transgene. Because theoretically only 50% of the blastocysts were expected to be transgene it is to assume that the establishment frequency was almost 100%. After generation of germline competent chimera with the newly established 743 cell line hemizygous transgenic F1 animals were generated and mated with WT FVB/N females. In this breeding the establishment efficiency in presence of OHT was similar to one observed in the parental blastocysts indicating that the STAT3 induced stabilization is kept also in the F1 generation.

Table 2: Upregulated genes.

Genes upregulated after STAT3MER activation through OHT. 11 genes were found to be significantly upregulated when compared with WT FVB/N cells cultivated in presence of LIF. Only genes with a 1.5x fold increase were considered as significant. The expression levels of selected genes were confirmed by real time PCR analysis.

Table 3: Downregulated genes.

Genes downregulated after STAT3MER activation through OHT. 14 genes were found to be significantly downregulated when compared with WT FVB/N cells cultivated in presence of LIF. Only genes with a 1.5x fold decrease were considered

as significant. The expression levels of selected genes were confirmed by real time PCR analysis.

Figure 1: <u>Characterization of the 743 transgenic ES cell line:</u>

A. Phosphorylation/dephosphorylation analysis of Tyr705 in endogenous and transgenic STAT3. After 24 hours of LIF and OHT deprivation cells were cultivated in presence of either LIF or OHT. No changes in the protein expression levels could be detected, but after 24 hrs the Tyr705 residue of both transgenic and WT STAT3 was completely dephosphorylated. 10 minutes after addition of LIF the tyr705 residue of both STAT3 wasphosphorylated whereas after addition of OHT complete phosphorylation was obtained only after 6 hrs. **B.** Dephosphorylation of Tyr705 was analyzed by eliminating LIF or OHT from respectively WT or 743 cells. After 24 hrs dephosphorylation of WT Tyr705 was almost complete whereas the dephosphorylation of STAT3MER occurred only after 46 hrs. **C.** Nanog expression was tested by semi quantitative RT-PCR. Both WT and 743 ES cell lines expressed Nanog but no difference in expression levels could be detected. D. After injection of 743 in C57BL/6 host blastocysts a 50-60% chimera was generated. Littermates from the crossing of the chimera with a WT FVB/N female generated white littermates, 50% of which were hemizygous for the transgene, indicating germline competence of the 743 cell line.

Figure 2: <u>Immunohistochemical analysis of WT, 741 and 743 ES cell lines</u>. All cell lines express the nuclear marker OCT-4, the surface marker SSEA-1 and alkaline phosphatase. The expression was restricted to ES cells. Mouse feeder cells were positive for DAPI staining (big nuclei) but negative for OCT-4, SSEA-1 and alkaline phosphatase.

Figure 3:

In situ hybridization of selected genes: **A.** Dppa3, Eif2s2, Pem and Pramel 6 exhibited a general expression in all cells of the morula and of the blastocyst. Expression of Hexokinase II, Lefty-2 and PP1s15B was restricted to the cells of the inner part of the morula and to the ICM in the blastocyst. Sense probes in the same concentration of the antisense probes were used as negative controls for the hybridization. **B.** In situ hybridization with a Pramel 7 antisense riboprobe of preimplantation embryos; bottom right negative control with sense riboprobe.

Figure 4:

Immunohistochemical analysis of WT E14 ES cells and E14 overexpressing, Nanog, Pem and Pramel7: IHC analysis with OCT-4 and SSEA-1 was performed after cultivation of the cells for 8 days without LIF. E14 ES cells differentiated under these conditions and lost the expression of OCT-4 and SSEA-1. Both Pem and Pramel7 overexpressing cells maintained their pluripotency. Nanog overexpressing cells, used as a control for the experiment, as expected maintained their pluripotency.
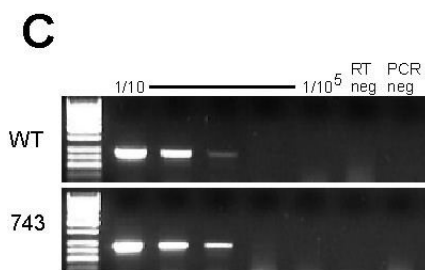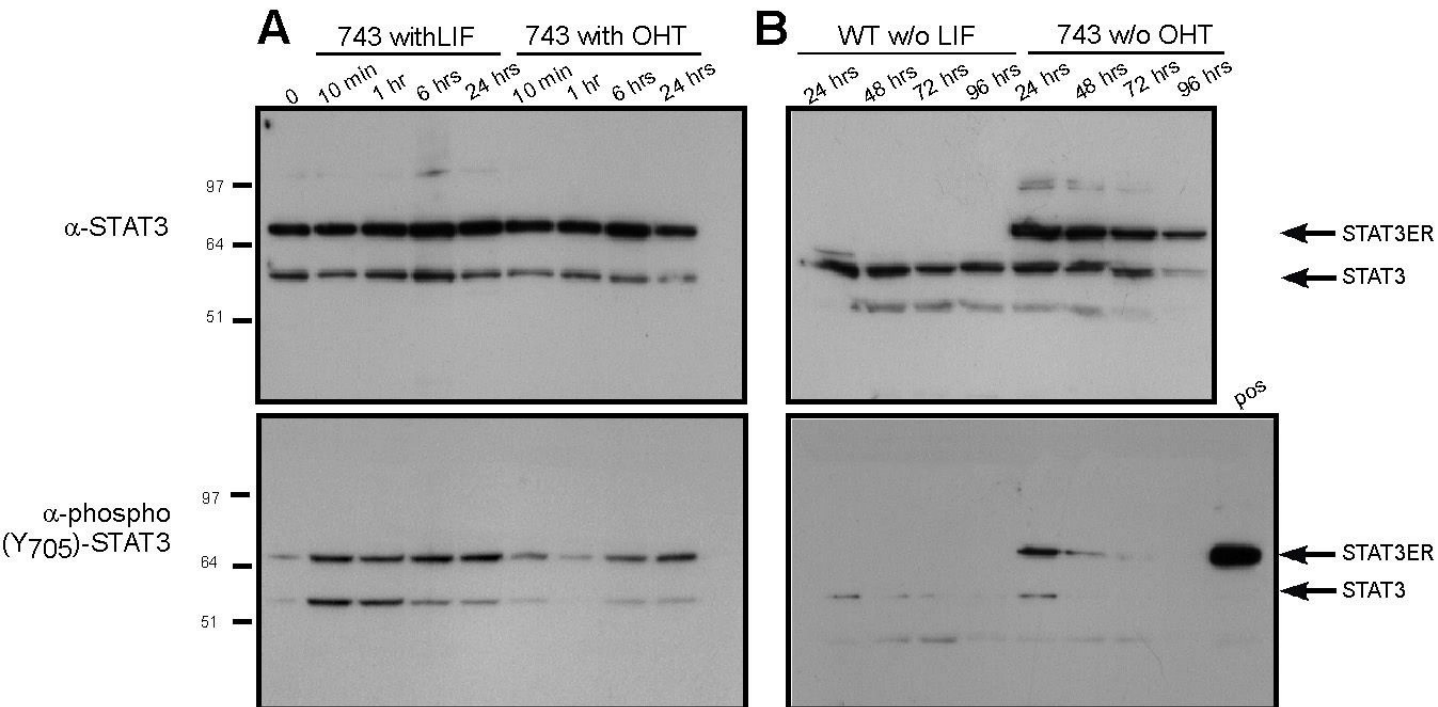
A

743 withLIF | 743 with OHT

0 | 10 min | 1 hr | 6 hrs | 24 hrs | 10 min | 1 hr | 6 hrs | 24 hrs

B

WT w/o LIF | 743 w/o OHT

24 hrs | 48 hrs | 72 hrs | 96 hrs | 24 hrs | 48 hrs | 72 hrs | 96 hrs

α-STAT3

97
64
51

← STAT3ER
← STAT3

α-phospho
(Y$_{705}$)-STAT3

97
64
51

pos

← STAT3ER
← STAT3

C

1/10 ———————— 1/10^5 | RT neg | PCR neg

WT

743

D

Figure 1

	FVB WT	FVB 741	FVB 743

Figure 2

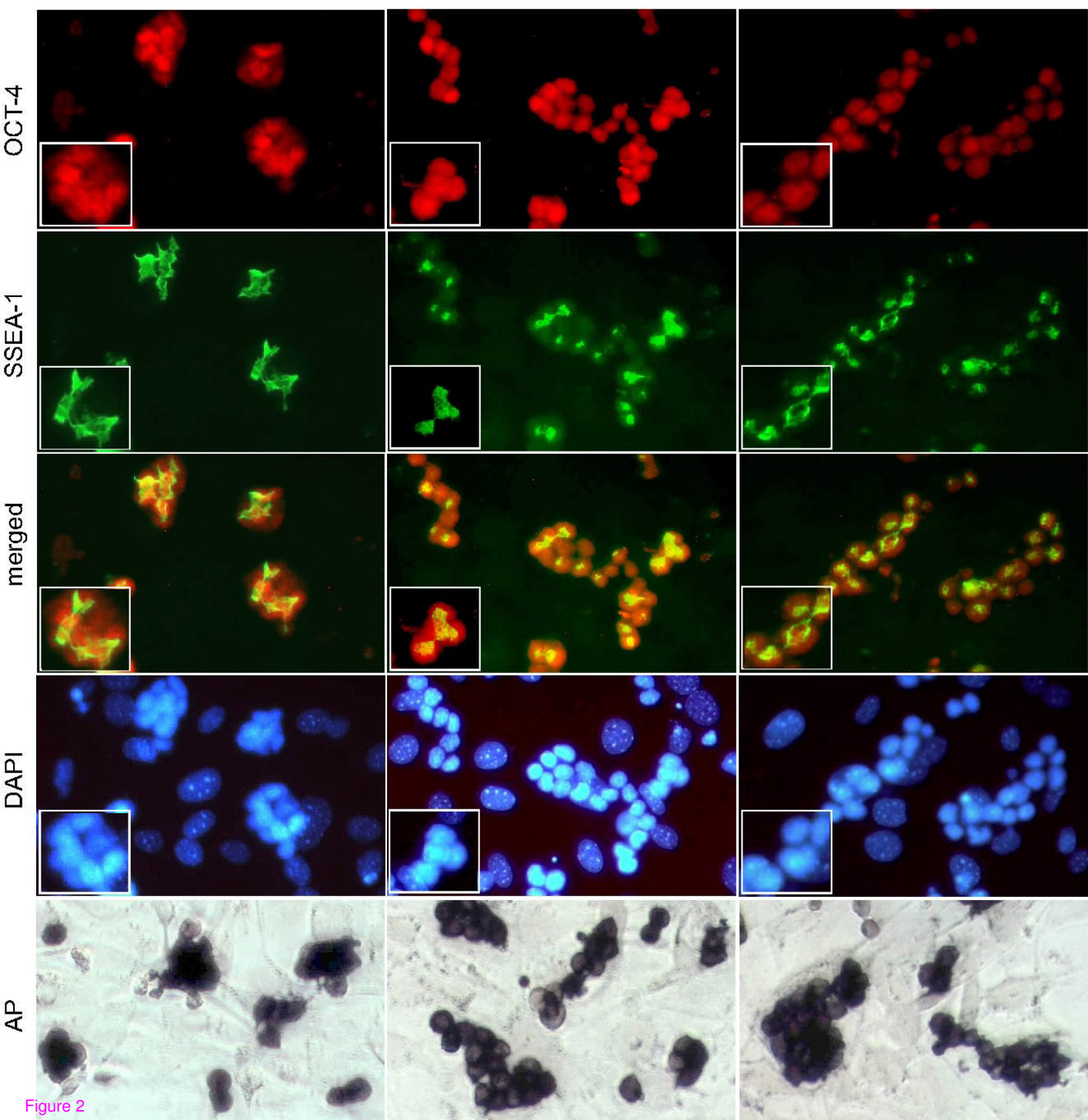**A**

Dppa3

Eif2s2

Hex II

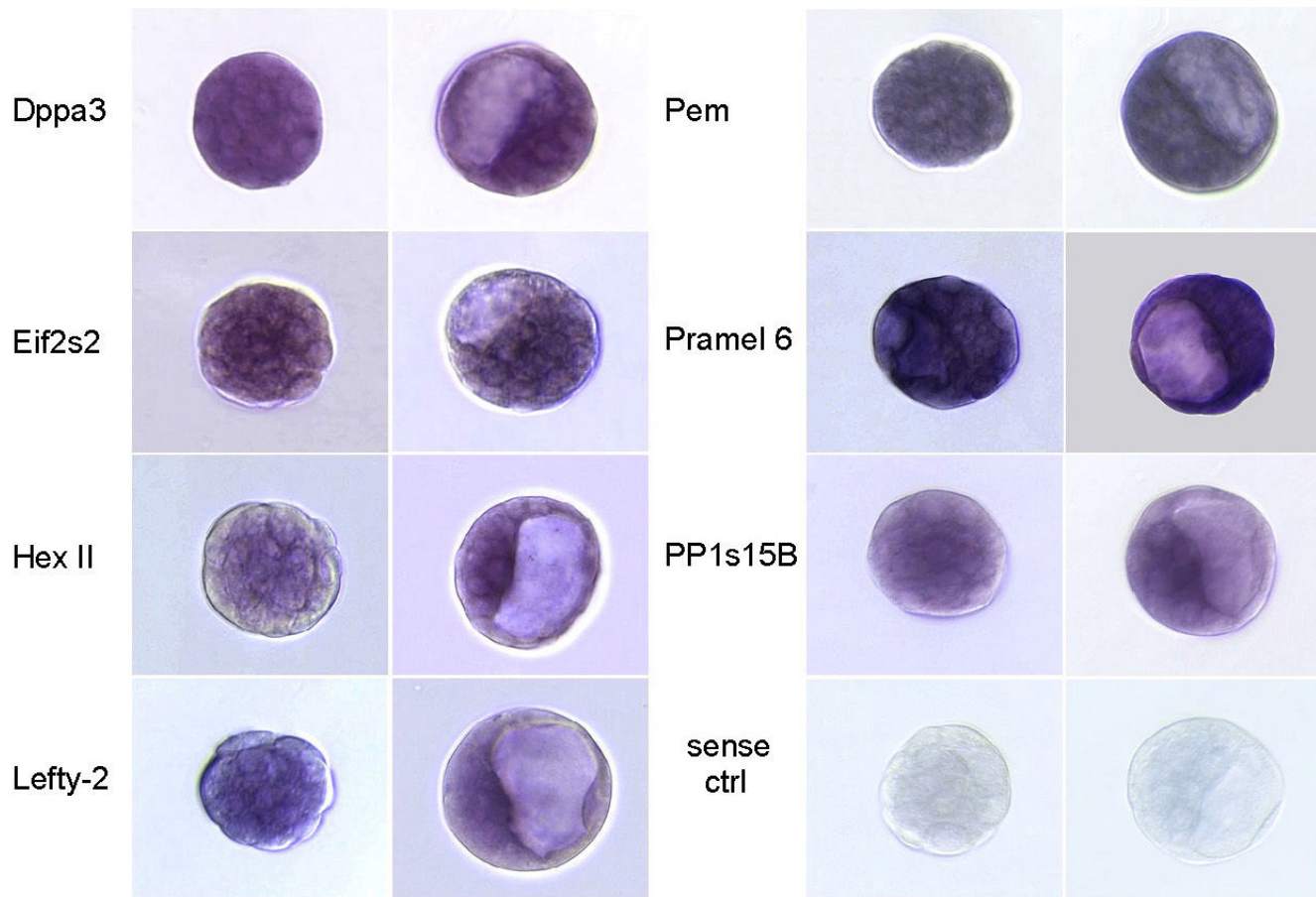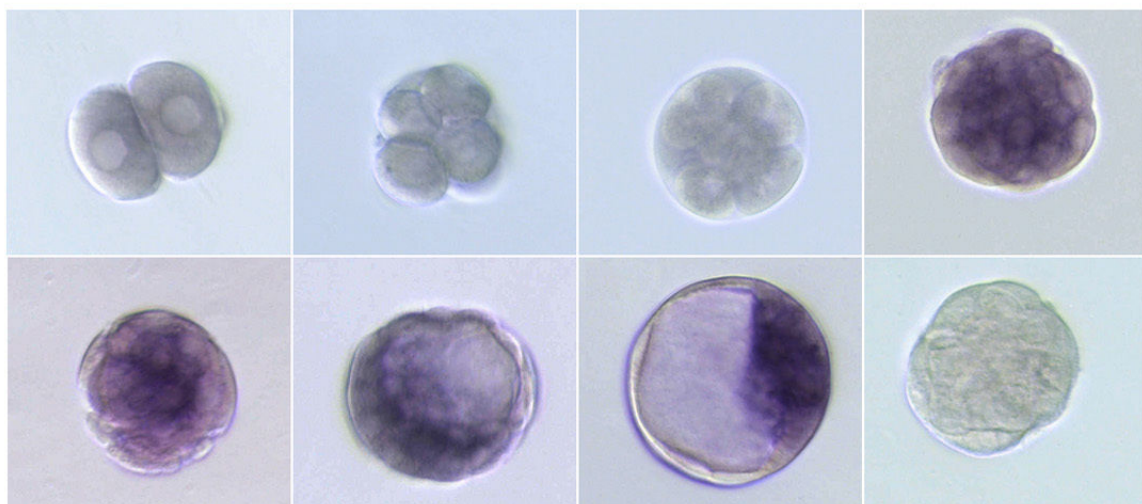Lefty-2

Pem

Pramel 6

PP1s15B

sense ctrl

B
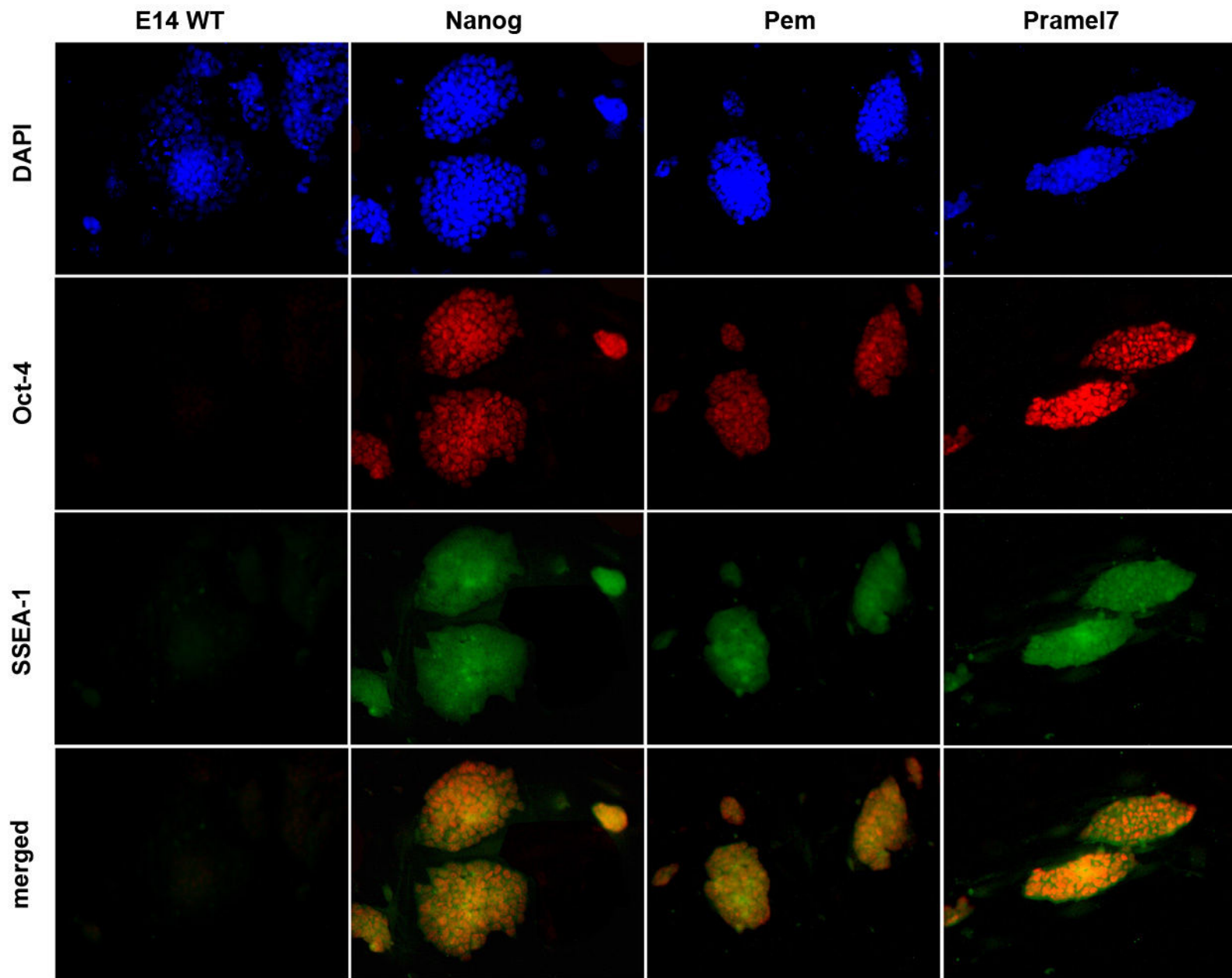
Figure 3

Figure 4

Table 1

Founder Line	No of embryos	Medium Supplements	No of ICM	No of stem cell colonies	Cell lines	Transgenic
WT	15	LIF	8	5	5	-
	15	OHT	4	0	-	-
741	28	OHT	23	10 (43 %)*	7	7 (100 %)[#]
743	28	OHT	14	10 (71 %)*	10	10 (100 %)[#]
741-F1	26	OHT	18	8 (44 %)*	8	8 (100 %)[#]

* percent colonies per picked ICM

[#] percent transgenic per cell lines

Figure 5

Table 2

probe set	Identifier	Acc. Number	Chr.	MM/PM fold change	PM fold change	Q-PCR fold change
98524_f_at	**ectodermal-neural cortex 1**	AI848479	13	1.87	1.91	
94375_at	**hexokinase 2**	Y11666	6	1.6	1.61	1.83
95545_at	**IGF-1A**: insulin-like growth factor 1A	X04480	10	2.34	1.6	
98059_s_at	**lamin A**:	D49733	3	1.58	1.47	
95562_at	**NDP52l1**	AA615127	11	2.66	2.08	2.59
101368_at	**Pem** :placentae and embryos oncofetal gene	M32484	X	1.68	1.82	
103470_at	**Pramel6**	AI596993	2	6.18	4.14	6.86
93245_at	**Pramel7**	AI645718	2	3.51	2.77	2.36
95609_at	**PP1**: Protein Phosphatase 1, regulatory subunit 15B	AA869927	1	1.6	1.5	
162206_f_at	**SOCS-3**: cytokine inducible SH2-containing protein 3	AV374868	11	4.56	4.4	4.13
101449_at	**Trim41**	AW120841	11	-	4.11	no changes

Figure 6

Table 3

probe set	gene	Identifier	Chr.	MM/PM fold change	PM fold change	Q-PCR fold change
97283_at	**DPPA3**/Stella/PGC7/compaction-associated protein 1 (Crg1)	AI606389	6	-2.16	-2.28	-3.56
133948_at	**Eif2c4**: Eukaryotic translation initiation factor 2C, 4	AI504948	17	-1.53	-1.46	
134566_at	**Lefty2**/ Ebaf: endometrial bleeding associated factor	AI643778	1	-4.37	-3.66	-6.11
103389_at	**lysine oxoglutarate reductase, saccharopine dehydrogenase**	AJ224761	6	-2.04	-1.71	
167617_r_at	**Mitsugumin 23**, similar to [Oryctolagus cuniculus]	AV224168	19	-1.89	-1.73	
166131_at	**NPC** derived proline rich protein 1	AA561632	1	-1.55	-1.46	
138014_at	**Nucleoporin 153**	AI846692	13	-1.55	-1.49	
168078_f_at	**phospholipase A2**, group IB, pancreas	AV073802	5	-2.24	-2.18	
167824_f_at	**Skp2**: S-phase kinase-associated protein 2 (p45)	AV299777	15	-1.74	-1.62	-1.73
100499_at	**syntaxin 3**	D29797	19	-2.09	-2.03	
166600_at	**T-STAR**/ etoile/ SML-2	AA189288	15	-1.66	-1.52	
93568_i_at 93569_f_at	**unknown**, embryonic gene	AI853444	14	-3.29	-2.94	
161004_at	**unknown EST**	AA250414	17	-2.06	-1.85	
167843_f_at	**unknown EST**	AV321065	14	-2.71	-1.81	

Figure 7