Human Oncostatin M is functionally equivalent to mouse LIF in supporting mouse ES cell pluripotency & germline competency

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ABSTRACT

Maintenance of pluripotency is essential for the use of mouse ES cells in transgenic and knockout studies. A variety of factors have been found to influence ES cell pluripotency, one of which is culture in the presence of Leukemia inhibitory factor (LIF). There are conflicting reports about the ability of Oncostatin M (OSM) to compensate for LIF in ES cell culture. Some have suggested that OSM is not as efficient as LIF in maintenance of ES cell pluripotency, while others have found them equivalent. In the present study, we demonstrate human OSM is able to maintain ES cell pluripotency in a similar manner to mouse LIF, including long-term (10+ passages) maintenance of pluripotency and the ability to differentiate *in vitro*, the ability to activate STAT3, and germline transmission competency. Our studies suggest that OSM may be an additional factor that plays a role in early embryonic development and that it may be used as an alternative to LIF in mouse ES cell culture.

Oncostatin M maintains undifferentiated ES cell morphology

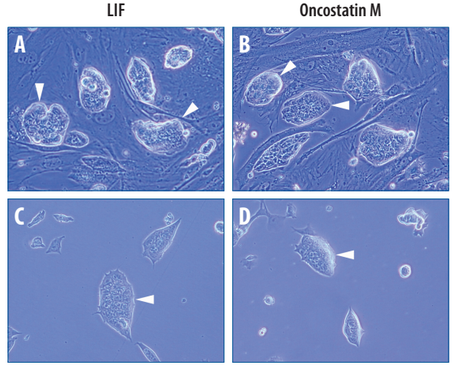

Figure 1: Cell morphology after long-term culture in LIF versus OSM. (A, C). Cells cultured in 10^3 U/mL LIF. (B, D). Cells cultured in 10 ng/mL OSM. Panels A and B are cells grown on irradiated MEF cells and panels C and D are cells grown on gelatin-coated plates. Images were taken at passage 6, however, cells maintained undifferentiated morphology in OSM medium through passage 25. Arrowheads indicate examples of ES cell colonies with undifferentiated morphology. All photos are at 200x.

MATERIALS & METHODS

Cell Culture: The CJ7 ES cell line was a kind gift of Tom Gridley. ES cells were maintained in high-glucose Dulbecco's minimal essential medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 4 mM glutamine, 10 μM 2-mercaptoethanol, 1% MEM non-essential amino acids, 50 IU of penicillin per mL, 50 μg of streptomycin per mL, and 10^3 U per mL LIF (ESGRO; Chemicon, Temecula CA) or 10 ng/mL of oncostatin M (R&D Systems, Catalog # 295-OM). Cells were grown on either irradiated mouse embryonic feeder cells (MEFs) or on 0.1% gelatin-coated plates. Cells were passaged every 2 days.

Embryoid bodies (EBs) were prepared by plating 10^6 cells on a 6-well tissue culture dish in DMEM supplemented with 10% FBS, 50 IU of penicillin per mL, 50 μg of streptomycin per mL, and 4 mM glutamine. Cell clumps were detached by flushing media over the dish. The clumps were transferred to a petri dish, and the media was changed daily. For differentiation, EBs were grown in suspension for 4-6 days and then plated onto 0.1% gelatin coated 24-well plates and allowed to attach and grow for an additional 7 days prior to performing immunocytochemistry or extracting RNA for RT-PCR.

RT-PCR: RNA was extracted from undifferentiated ES cells and differentiated EBs using the RNAeasy kit (Qiagen) according to manufacturer's instructions. 500 ng of RNA was used for reverse transcription with random hexamers using the Superscript First-Strand Synthesis System (Invitrogen) according to manufacturer's instructions. 2 μL cDNA was used as a template for amplification. All primers were annealed at 55°C and amplified for 35 cycles.

Immunocytochemistry: All primary antibodies (rtxh/mOct3/4; mxh/mSox2; gtxmNanog; mxβIIITubulin; mxhTroponinT; and gtxhHNF3β) used were from R&D Systems. Cells or EBs were plated onto coverslips in 24-well plates. Cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature and then washed 4 times in PBS containing 1% BSA (PBA). Cells were blocked with 10% donkey serum containing 0.1% Triton® X-100 in PBA for 45 minutes. Primary antibody was then added in block at 10 μg/mL for 3 hours, after which cells were washed 3 times in PBA, 5 minutes each wash. Cells were then incubated in NothernLights™ 557-conjugated secondary antibodies (R&D Systems) at 5 μg/mL for 1 hour, then washed as before.

STAT3 Activation Levels: Levels of activated STAT3 were assessed with the Active STAT3 DuoSet IC® Kit (R&D Systems). Nuclear extracts were made from undifferentiated ES cells grown either on or off MEFs according to kit instructions. Protein levels were quantitated by the Bradford assay. STAT3 activation levels were determined according to manufacturer's instructions and then normalized to overall protein levels.

Mouse Chimera Production: All procedures were approved by the University of Michigan Committee on Use and Care of Animals. Animal care was provided in accordance with the principles and procedures outlined in the National Research Council Guide for the Care and Use of Laboratory Animals. After five serial passages on MEFs in media containing either LIF or OSM, cells were trypsinized and 16 ES cells were microinjected into blastocysts. Blastocysts were collected from superovulated C57BL/6NCrl female mice (Charles River Laboratories, Wilmington, MA) mated with C57BL/6J X DBA/2J/F1 males (The Jackson Laboratory, Bar Harbor, ME). Microinjected blastocysts were transferred to pseudopregnant recipient females the day of injection using standard methods. Chimeras were bred to C57Bl/6J females (The Jackson Laboratory, Bar Harbor, ME) to test for germline transmission.

Markers of pluripotency are expressed equivalently in OSM versus LIF cultured cells

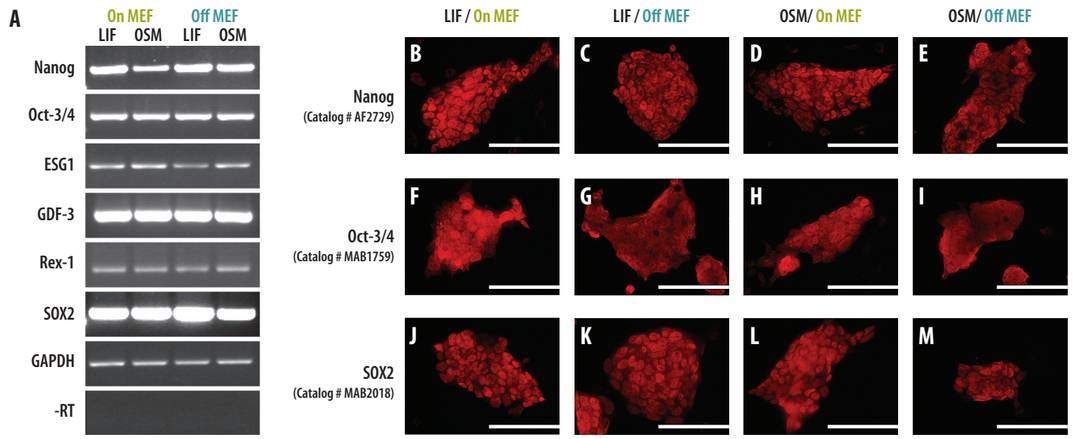

Figure 2: Pluripotency marker expression in cells grown in 10^3 U/mL LIF versus 10 ng/mL OSM. Cells were maintained for 16 passages in each condition, 11 of which were off feeders for the off MEF samples. (A). RT-PCR analysis of pluripotency markers using cDNA extracted from cells grown in either LIF or OSM and grown on or off MEFs. GAPDH levels were used as a cDNA loading control and samples without reverse transcriptase (-RT) were used as a negative control. (B-M). Immunocytochemistry for markers of pluripotency in cells grown either in LIF (B, C, F, G, J, and K) or OSM (D, E, H, I, L, and M) and grown on (B, F, J, D, H, and L) or off MEFs (C, G, K, E, I, and M). Antibody staining was performed using primary antibodies indicated under the respective marker and the appropriate NorthernLights™-557-conjugated secondary antibody. All photos are at 400x. Size bars equal 200 μm.

ES cells maintained in OSM retain capacity to differentiate into cell types from all three germ layers

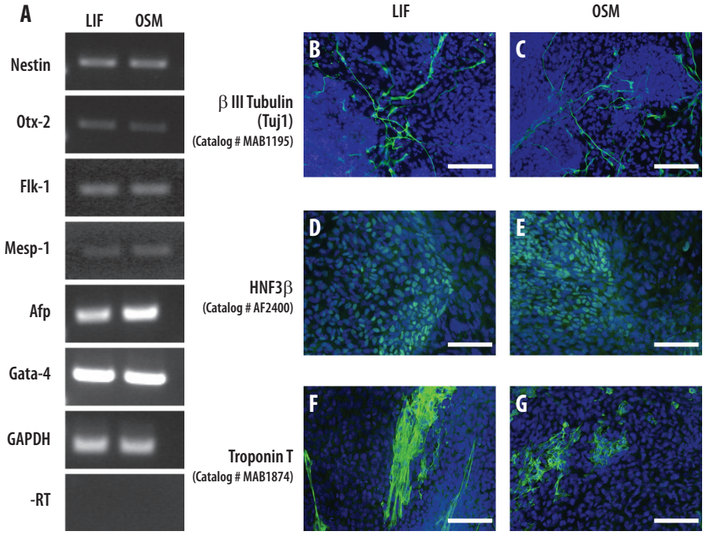

Figure 3: Differentiation of cells grown in 10^3 U/mL LIF versus 10 ng/mL OSM into all three germ layers. (A). RT-PCR analysis of differentiation markers using cDNA extracted from EBs made from cells grown in either LIF or OSM for a minimum of 17 passages. Markers for the three germ layer are ectoderm (Nestin and Otx-2), mesoderm (Flk-1 and Mesp-1), and endoderm (Afp and Gata-4). (B-G). Immunocytochemistry for markers of differentiation from: ectoderm (βIII Tubulin), endoderm (HNF3β), and mesoderm (Troponin T). Green indicates positive staining and blue is DAPI counterstain. Cells are all outgrowths of EBs made from cells grown in either LIF (B, D, and F) or OSM (C, E, and G). Antibody staining was performed using primary antibodies indicated under the respective marker and the appropriate NorthernLights™-557-conjugated secondary antibody. All photos are at 200x. Size bars equal 200 μm.

STAT3 activation levels of LIF versus OSM cultured cells

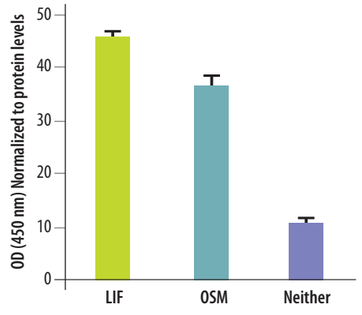

Figure 4: Levels of STAT3 activation as determined by binding to a STAT3-specific oligonucleotide in cells grown in 10^3 U/mL LIF versus 10 ng/mL OSM under feeder-free conditions. Each bar represents the average of three experiments performed at p13, p15, and p18. The bar for "neither" condition represents the average of two experiments done at p3 with cells grown without LIF or OSM. Error bars represent the confidence interval for each set of experiments. STAT3 activation levels in cells cultured in OSM or LIF are 71% and 77% higher, respectively, than cells cultured without OSM or LIF.

Oncostatin M maintains germline competency of ES cells

Culture Conditions	Blastocyst injected	Animals born	Total Chimeras	Male Chimeras (>50% Contribution)	Germline Chimeras
LIF	60	10	6	4	3
OSM	60	10	9	5	3

Table 1: Chimera formation and germline transmission arising from injection of CJ7 cells grown in 10^3 U/mL LIF versus 10 ng/mL OSM for 5 passages. CJ7 ES cells were injected into C57BL/6NCrl host blastocysts. Male chimeras with >50% chimerism were bred to C57BL/6J females to test for germline transmission.

SUMMARY

We sought to determine if a closely related cytokine to LIF, Oncostatin M (OSM), could mimic the activity of LIF in the maintenance of embryonic stem (ES) cell pluripotency. We demonstrate that ES cells grown in 10 ng/mL of OSM are able to maintain pluripotency in a manner similar to cells grown in LIF as judged by chimera contribution and germline transmission, expression of markers of pluripotency, and the ability to differentiate *in vitro*.

A slight difference was detected in activation levels of STAT3, a known downstream target of LIF, between ES cells cultured in OSM versus LIF (Figure 4). Since these cells are still able to maintain pluripotency, we hypothesize that this decrease is not significant enough to affect levels of signaling downstream of STAT3. This is further supported by the fact that Nanog RNA and protein levels are not detectably altered in cells grown in OSM off MEFs (Figure 2). Although it was previously thought that Nanog was in a pathway independent of STAT3, recent evidence has suggested Nanog is a direct target of STAT3 and LIF signaling (Suzuki, *et al.* 2006). Additionally, we find that when cells are cultured in the absence of OSM and LIF, their levels of STAT3 are reduced dramatically within 3 passages along with the loss of pluripotent cell morphology.

Both OSM and LIF are expressed in the uterus around the time of implantation, which in addition to the studies described here implicate both LIF and OSM as important factors for the development of the early embryo (Fouladi-Nashta, *et al.* 2005). Knockout mice lacking either LIF or OSM are born and survive to adulthood (Morikawa, *et al.* 2004; Fouladi-Nashta, *et al.* 2005). It is possible that these two cytokines are able to compensate for each other during early development of the embryo.

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