Embryonic Stem Cells Cultured in Microfluidic Chambers Take Control of Their Fate by Producing Endogenous Signals Including LIF

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ABSTRACT

It is important to understand the role played by endogenous signals in shaping stem cell fate decisions to develop better culture systems and to improve understanding of development processes. In this study, we describe the behavior of mouse embryonic stem cells (mESCs) inside microfluidic chambers (microchambers) operated under conditions of minimal perfusion. mESCs inside microchambers formed colonies and expressed markers of pluripotency in the absence of feeders or pluripotency-inducing signals such as leukemia inhibitory factor (LIF), while mESCs in standard cultureware differentiated rapidly. In a series of experiments, we demonstrate that remarkable differences in stem cell phenotype are due to endogenous production of LIF and other growth factors brought upon by cultivation in confines of a microchamber in the absence of perfusion (dilution). At the protein level, mESCs produced ~140 times more LIF inside microchambers than under standard culture conditions. In addition, we demonstrate that pluripotent phenotype of stem cells could be degraded by increasing the height (volume) of the microchamber. Furthermore, we show that inhibition of LIF in microchambers, via the JAK/STAT3 pathway, leads to preferential differentiation into mesoderm that is driven by bone morphogenetic protein (BMP)-4. Collectively, we demonstrate for the first time that it is possible to design a cell culture system where stem cell fate is controlled solely by the endogenous signals. Our study may help shift the paradigm of stem cell cultivation away from relying on expensive exogenous molecules such as growth factors and toward designing culture chambers for harnessing endogenous signals. STEM CELLS 2016; 00:000—000

SIGNIFICANCE STATEMENT

Cell secreted molecules play an essential role in modulating self-renewal and differentiation of embryonic stem cells (ESCs). To date, ESCs have not been thought capable of secreting enough growth factors to maintain their phenotype. We investigated the role of endogenous leukemia inhibitory factor in controlling self-renewal in mouse ESCs confined to small volumes and found remarkable differences compared to cells in standard cultureware. Our study demonstrates that in a small volume endogenous signaling is sufficient to control stem cell fate, and can be selectively manipulated to direct differentiation. This may help shift the stem cell culture paradigm away from relying on expensive exogenous signals and toward harnessing of endogenous signals through optimal design of the cell culture platform.

INTRODUCTION

Embryonic stem cells (ESCs) are derived from the epiblast of the preimplantation embryo, which expands encapsulated by the trophectoderm and primitive endoderm in the developing blastocyst [1–4]. In vivo, secreted signals accumulate in the local microenvironment and shape stem cell fate decisions. In vitro, stem cells are typically cultured in large volumes of media where secreted signals become rapidly diluted and do not reach threshold concentrations required to drive fate decisions. As a consequence, conventional mouse embryonic stem cell (mESC) cultures either use mouse embryonic fibroblast (MEF) feeder cells to secrete desired inductive signals or rely on addition of these signals exogenously into culture media [3–5]. While a number of reports have shown that ESCs are capable of producing pluripotency-inducing signals in vitro, including leukemia inhibitory factor (LIF) and

bone morphogenic protein (BMP)-4 [5, 6], no report, to the best of our knowledge, demonstrated that endogenous signals alone are sufficient to shape phenotype of ESCs.

Microfluidics and surface micropatterning are becoming increasingly popular as means of gaining more precise control over the stem cell niche and cellular microenvironment [7, 8]. In mESCs, loss of function and signaling modification studies using controlled microfluidic perfusion have elucidated the importance of endogenous extracellular signaling in maintaining "stemness" [9–11]. Other recent studies employed surface micropatterning for control of mESC cell density/colony size to study the role of autocrine signaling in the maintenance and acquisition of naïve pluripotency [12, 13]. Furthermore, modulation of endogenous signaling has led to novel differentiation methods for generation of adipocytes, as well as endothelial and cardiac lineages [14–16]. These previous studies have pointed to the importance of autocrine and paracrine signals [8, 10, 14, 17]. However, the vast majority of signaling pathways require media supplementation with supraphysiological growth factor concentrations for maintained activation in vitro. In contrast, our work detailed below demonstrates that the phenotype of mESCs may be defined solely by the endogenous signals harnessed through the appropriate design of culture microchambers.

This study began with an observation that mESCs cultured in microfluidic devices without perfusion, in the absence of feeders or LIF, formed colonies and expressed markers of pluripotency, while stem cells cultured in 12-well plates at the same density and in the same media differentiated. We hypothesized that the environment inside microchambers was conducive to production and accumulation of endogenous pluripotency signals. Given its central role in maintenance of pluripotency of mESCs, we focused on LIF signaling and demonstrated that stem cells were producing ∼140 times more LIF inside microchambers compared to standard large volume culture dishes. Interfering with LIF signaling using inhibitors or neutralizing antibodies caused mESCs to exit pluripotency state and begin differentiating. It should be noted that confinement to microchambers resulted in mESCs upregulating production of multiple other growth factors (GF) in addition to LIF. For example, blocking LIF brought BMP4 signaling to a position of prominence, which favored selective differentiation of mESCs toward mesoderm. Importantly, such germ layer-specific differentiation was not observed when inhibiting LIF under standard culture conditions. Our results suggest that the geometry of the cell culture chamber, its height and cell-to-volume ratio, play a critical role in local accumulation of endogenous factors and triggering of autocrine/paracrine signals. These findings are highly significant as they may help shift the paradigm of stem cell cultivation away from reliance on expensive exogenous signals (e.g., recombinant growth factors) and toward harnessing cells' own endogenous signals.

MATERIALS AND METHODS

Cell Culture

Mouse ES-D3 [D3](ATCC CRL-1934, Manassas, VA, http://www.atcc.org) were propagated on growth-arrested mouse embryonic fibroblasts (CF-1 MEF, GlobalStem, Gaithersburg, MD, https://www.mti-globalstem.com) in ES FBS medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% ES FBS, 2 mM L-glutamine, 1 mM MEM NEAA, 100 nM 2-mercaptoethanol, 1×penicillin-streptomycin, and 10^3 U/ml LIF (ESGRO, Millipore, Billerica, MA, http://www.emdmillipore.com). mir-290-mCherry/mir-302-eGFP (dual-reporter) ESCs were expanded in 2iL media consisting of DMEM supplemented with 15% ES FBS, 2 mM L-glutamine, 1 mM MEM NEAA, 100 nM 2-mercaptoethanol, 1×penicillin-streptomycin, and 10^3 U/ml LIF (ESGRO, Millipore), 1 µM PD0325901 (Stemcell Technologies, Vancouver, Canada, http://www.stemcell.com), 3 µM CHIR99021 (Stemgent, Lexington, MA, https://www.stemgent.com). Undifferentiated ESCs were kept at 37 °C, 5% CO_2, and 90–95% humidity, with medium exchanged daily, and passaged every 2 to 3 days using trypsin-EDTA. Under experimental conditions, cells were plated into 0.1% gelatin (Millipore)-coated wells or seeded into the microfluidic chambers precoated with 0.1% gelatin. For serum-free cultures, chemically defined medium contained a 1:1 mixture of Neurobasal and DMEM/F12 supplemented with 0.5×N2, 1×B27, 2 mM Glutamax, 1 mM MEM NEAA, 0.5 mM sodium pyruvate, 1×penicillin-streptomycin, and 100 nM 2-mercaptoethanol. All cell culture reagents were from Gibco, Life Technologies (Grand Island, NY, https://www.thermofisher.com/us/en/home/brands/gibco.html) unless otherwise mentioned.

Mouse ESC Differentiation

Prior to differentiation, cells were cultured inside microfluidic chambers or in 12-well tissue culture plates precoated with 0.1% gelatin for 24 hours in ES FBS media. To initiate differentiation, cells were switched to differentiation media containing DMEM supplemented with 15% ES FBS (Invitrogen), 2 mM L-glutamine, 1 mM MEM NEAA, 100 nM 2-mercaptoethanol, and 1×penicillin-streptomycin, and 1 µM Jak1 inhibitor (J1I; Calbiochem, Millipore, Billerica, MA, http://www.emdmillipore.com) for 5 days. Five micromolar of dorsomorphin (an inhibitor of BMP-mediated Smad1/5/8 phosphorylation, Stemgent) was utilized where indicated. From day 5 through day 10 cells were cultured in 1% ES FBS, 2 mM L-glutamine, 1 mM MEM NEAA, 100 nM 2-mercaptoethanol, and 1×penicillin-streptomycin. Fresh media was exchanged daily throughout all experiments.

LIF Detection and Ligand Trapping

mESCs were seeded in microfluidic devices and allowed to expand in ES FBS media for 24 hours. Subsequently, cells were cultured in media without LIF or containing a LIF blocking antibody (500 ng/ml; R&D Systems, Minneapolis, MN, https://www.rndsystems.com) for 48 hours with daily media exchange. Samples were then harvested for mRNA isolation and PCR analysis. Cells in LIF-free media were dissociated and counted, while media supernatant was stored at −20 °C. LIF levels in the cell culture supernatant were detected using the Quantikine Mouse LIF Immunoassay (R&D Systems).

Additional Materials and Methods

Device fabrication and microfluidic culture, RNA isolation, quantitative reverse transcription polymerase chain reaction (qRT-PCR), microarray, on-chip immunostaining and live cell imaging, and modeling of oxygen and nutrients in microfluidic chambers are further detailed in Supporting Information Materials and Methods.

Statistical Analysis

Experiments were repeated at least two to three times with duplicate samples for each condition. Data from representative
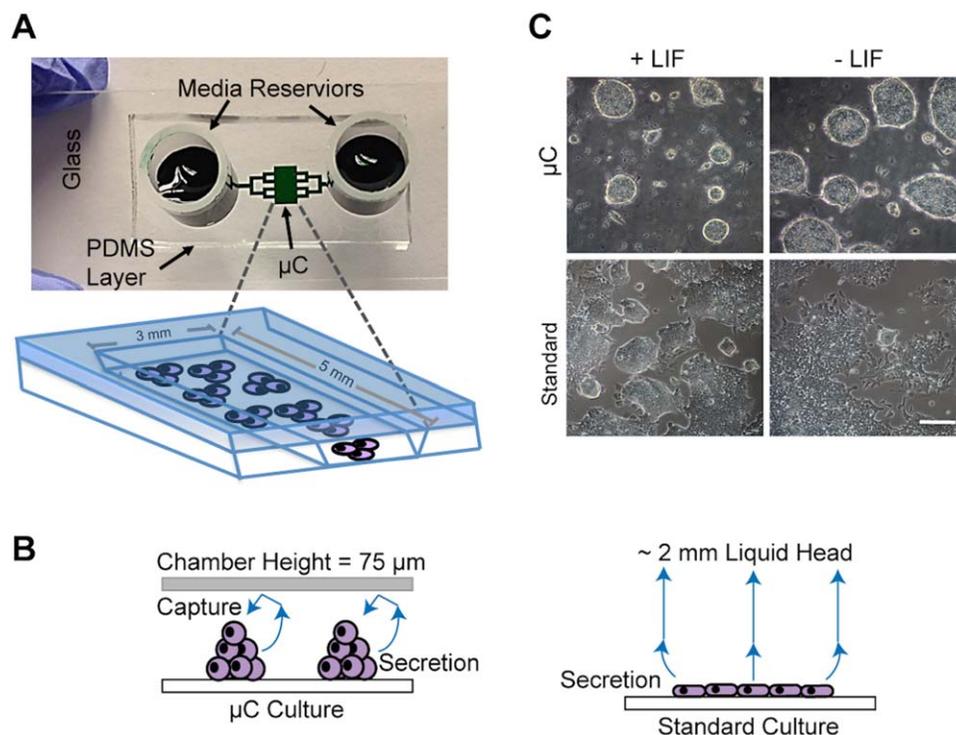
Figure 1. Microfluidic chamber culture system. Mouse embryonic stem cells (mESCs) were cultured for 3 days in ES FBS media. **(A)**: Fabricated PDMS device with the chamber filled with food dye, attached to a schematic showing mESC colonies within the chamber. Cloning cylinders atop the inlet and outlet of the microchamber serve as media reservoirs. **(B)**: Hypothetical illustration showing mESCs maintain pluripotent phenotype (dome-shaped colony) in small volumes but differentiate (scattered phenotype) in large volumes. **(C)**: Brightfield images of mESCs in the presence and absence of exogenous LIF. The difference in phenotype is connected to the accumulation of endogenous signals in small volumes. Scale bar: 100 μm. Abbreviations: LIF, leukemia inhibitory factor; PDMS, Polydimethylsiloxane; μC, microfluidic chamber.

experiments are presented. All error bars represent standard deviation from the mean.

RESULTS

Cultivation of mESCs in Microchambers

In an effort to develop easy-to-handle microfluidic devices, we eliminated tubing and pumping such that our devices may be more accurately described as microchambers for cell cultivation. In contrast to standard macroscale cultures where secreted factors diffuse away and become diluted, confinement of cells inside microchambers favored retention and accumulation of secreted factors (Fig. 1A). In a typical experiment, microchambers were 75 μm in height whereas liquid head in 12-well plates employed for macroscale cultures was ∼ 2 mm (Fig. 1B). Given that seeding density was the same for both conditions (∼20,000 cells per square centimeter), the number of cells in a given volume (or cell-to-volume ratio) was ∼27-fold higher in the case of microchambers, once again ensuring accumulation of secreted factors. The dimensions (height, width, and length) of our microfluidic channel were 5 mm × 3 mm × 75 μm, creating a functioning volume of ∼1 μL, which allowed sufficient growth of colonies (∼55 μm height).

First, we wanted to consider the possibility that factors other than autocrine signals contributed to mESC phenotype enhancement in microchambers. Given that low oxygen tension and glucose concentration are known to affect stem cell pheno-

type [18–20], these parameters were evaluated for stem cells cultured in microchambers. Let us first consider glucose concentration. Taking into account reported glucose consumption rate of mESCs [18], we modeled levels of glucose available to stem cells over the 24-hour time period inside microchambers versus standard culture plates (see Supporting Information Table 1 for modeling parameters). Our model set up in COMSOL, also included an experimental observation of the existence of slow back and forth flow inside the microchambers for 24 hours after media exchange. Modeling results presented in Supporting Information Figure S1, demonstrate that, while stem cells inside microchambers experience somewhat lower glucose levels compared to standard culture dishes (∼25% lower), these levels were not outside of the normal glucose reported to be between 100 mg/dL and 450 mg/dL for mESCs. Therefore, stem cells were not deprived of glucose in our microfluidic cultures. Let us shift our attention to oxygen tension. When considering oxygen tension one should note that oxygen diffusivity through polydimethylsiloxane (PDMS) (material comprising the roof of our microchamber) is much higher than through the aqueous media (7.9×10^{-5} cm^2/s vs. 2.8×10^{-5} cm^2/s), meaning that stem cells cultured in 75-μm tall microchambers are much better oxygenated than cells in standard culture dishes with ∼2 mm height of media. To quantify the differences in oxygen tension, we constructed, in COMSOL, a diffusion-consumption model that included stem cell oxygen consumption rate [19], cell density, and appropriate dimensions for both culture systems. The modeling (see Supporting Information Table 2 for model parameters)
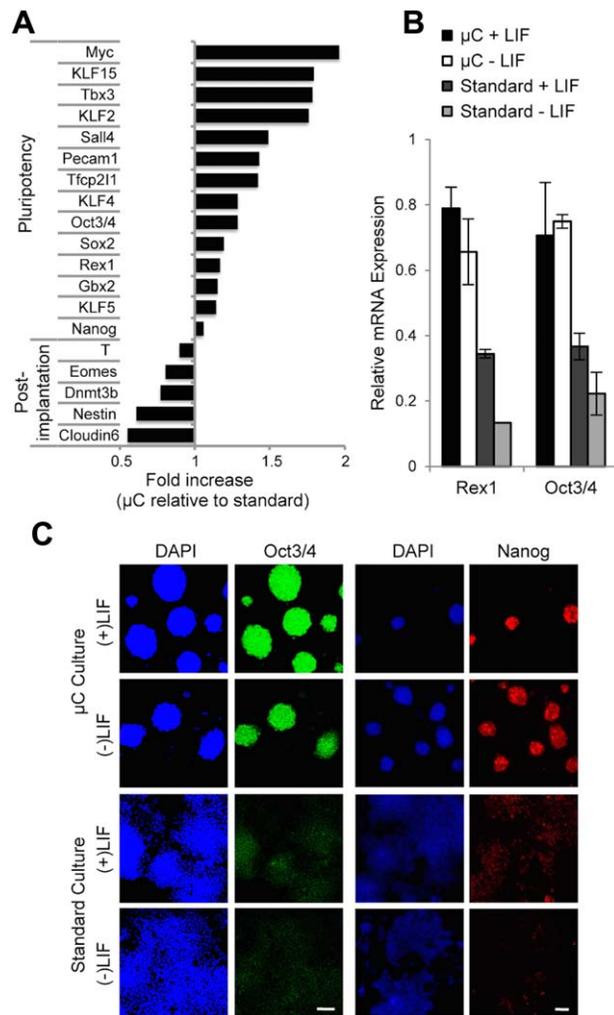
Figure 2. Microchamber culture of mouse embryonic stem cells (mESCs) facilitates self-renewal in the absence of feeder cells and exogenous growth factors. mESCs were cultured for 3 days in ES FBS media. **(A)**: mRNA expression levels of naïve and primed pluripotency markers analyzed by gene chip array. Fold increase is relative to values obtained for mESCs in standard tissue culture. **(B)**: Relative mRNA expression level of pluripotency markers in mESCs cultured in standard tissue culture wells and microfluidic devices by qRT-PCR. Expression relative to mESCs harvested prior to seeding. **(C)**: Immunofluorescent images of cells in standard tissue culture wells and microchambers, stained for Oct3/4 (green) or Nanog (red) and counterstained with DAPI (blue). Scale bar: 100 μm. Abbreviations: LIF, leukemia inhibitory factor; μC, microchamber; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

confirmed that stem cells in microchambers were in fact well oxygenated (p_{O_2} = 141 mmHg in μCs vs. 121 mmHg in standard culture wells) and thus did not experience hypoxic conditions known to induce phenotype enhancement. The above considerations pointed to the fact that neither glucose nor oxygen levels were outside of the ordinary in our microchambers [19] and, therefore, were unlikely to affect stem cell phenotype in a major way. In light of this, autocrine signals remained as the most likely reason for enhancement of stem cell phenotype.

In the next set of experiments, we focused on characterizing phenotype of stem cells cultured in microfluidic chambers. When removed from feeders and maintained in gelatin-

coated microchambers in 15 percent serum containing (ES FBS) media, mESCs formed compact dome-shaped colonies indicative of pluripotency (Fig. 1C). Intriguingly, this distinctly contrasted culture in standard 12-well plates where cells over equivalent time periods in the same media exhibited a scattering phenotype indicative of differentiation. Microarray analysis was employed to characterize behavior of mESCs in microchambers (Fig. 2A). We found the expression of many pluripotency markers, including Myc, KLF4, Oct3/4, Sox2, Nanog, and Rex1 increased relative to standard tissue culture condition. Similarly, the expression of postimplantation markers, including Eomes, Dnmt3b, nestin, cloudin6, and brachyury (T) decreased in cells cultured for 3 days inside the microchambers. The microarray data were validated by qRT-PCR and immunofluorescence staining for core pluripotency transcription factors. Expression of Rex1 and Oct3/4 confirmed the observed phenotype, and was significantly higher in cells cultured in microchambers, compared to cells maintained under standard culture conditions (Fig. 2B, 2C). Also, formation of compact cell colonies and the level of expression of Oct3/4 was uniform throughout the length of the microchamber (data not shown). Most strikingly, there were minimal differences in phenotype of mESCs cultured in FBS media with or without exogenous LIF. The maintenance of uniform dome-shaped colonies in the absence of exogenous GFs suggested that mESCs cultured inside microchambers may have produced endogenous pluripotency-inducing signals (Fig. 1B).

Confirming this observation, the expression of pluripotency markers Oct3/4, Nanog, and Rex1 did not show a change with addition of exogenous LIF (Fig. 2B, 2C). In contrast, under standard culture conditions, both Oct3/4 and Rex1 decreased significantly in both the presence and absence of exogenous LIF. While expression of pluripotency markers was increased in the presence of LIF under standard culture conditions it should be noted that LIF alone was not sufficient to induce optimal pluripotency. This is consistent with the dependence of D3 mESCs on feeder cells in combination with LIF to maintain pluripotency in standard tissue culture wells. These data underscored a fundamental difference in maintaining pluripotency of mESCs in bulk culture versus our microfluidic system. It should also be noted that viability of mESCs was not compromised by cultivation in microchambers (Supporting Information Fig. S2).

We reasoned that if stem cell phenotype were shaped by the endogenous signals then it could be modulated by varying the microchamber volume. To test this hypothesis, we fabricated a series of microdevices with identical lateral dimensions but with height varying from 75 μm to 2 mm. The volume of the culture chamber ranged from 1 μl for shallow chambers to 30 μl for taller chambers. As highlighted in Figure 3, stem cell morphology and expression of pluripotency markers (Oct3/4 and Nanog) decreased in titratable manner as a function of increasing microchamber volume. We hypothesized that increasing the microchamber volume had the effect of diluting endogenous factors and that in turn led stem cell phenotype to change. It is worth noting that microfluidic devices with varying chamber heights offer the advantage of diluting cell-secreted factors without changing the flow rates or shear stresses experienced by the cells. These devices were designed so as to connect media reservoirs to cell culture chambers via long and narrow channels with high fluid
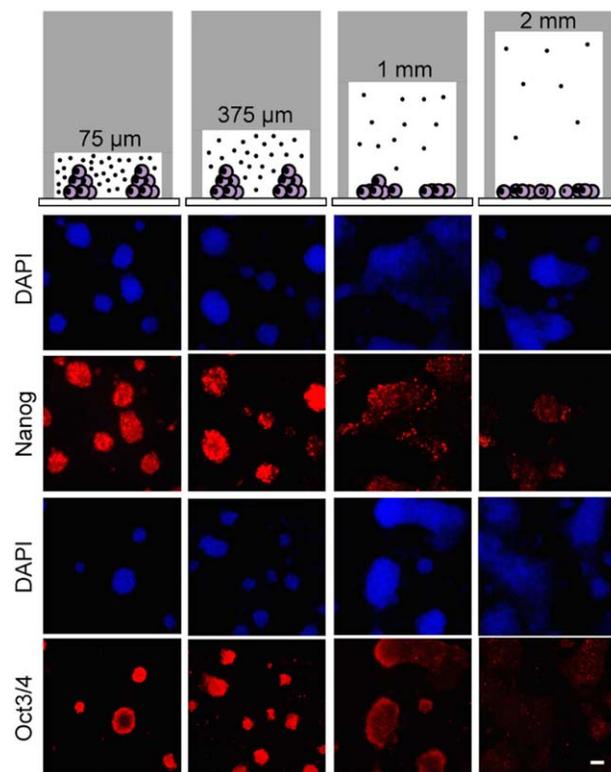
Figure 3. Pluripotency marker expression after manipulation of microchamber geometry (height). Microchamber height was scaled to increase local volume and deplete concentrations of cell-secreted factors. Mouse embryonic stem cells cultured inside microfluidic chambers for 3 days showed decreased pluripotency and increased scattering phenotype with increasing chamber height. Please note that cartoon of microchambers is stylized. In real devices only the channel height was varied while the thickness of the roof was kept constant. Scale bar: 100 μm.

resistance. Because the resistance was dominated by these narrow channels, the volume of the cell culture chamber had minimal effect on total fluid resistance of the microfluidic circuit. For example, the total fluid resistance within the microdevice decreases only 3.2% (250 Pa s/mm^3 to 241 Pa s/mm^3) as the height of cell culture chamber increases from 75 μm to 1 mm. Given that flow rate is directly proportional to fluid resistance; flow and perfusion inside the microfluidic circuit were expected to remain unchanged as the volume of culture chamber increased. Bead tracing experiments supported our theoretical predictions, and demonstrated a flow rate of approximately 0.3 μl/h. Therefore, changes in stem cell phenotype were likely affected by dilution of cell-secreted signals and not by the changing perfusion rates or shear stresses inside microchambers of varying volumes. In the next set of experiments, we focused on specific endogenous signals shaping pluripotent phenotype of mESCs.

Local Signals Accumulate in Microchambers to Maintain Pluripotency of mESC

ESCs and epiblast stem cells (EpiSCs) are both pluripotent stem cell lines derived from the blastocyst. However, in vitro they represent slightly different states of development. Naïve ESCs represent the preimplantation blastocyst, while EpiSCs resem-

ble the slightly more developed (differentiated) postimplantation epiblast [21]. Although these cell types share some transcription factor circuitry, including expression of Oct4, their gene expression patterns and signals controlling their self-renewal are distinct. Naïve mESCs depend on LIF and BMP4, or simultaneous inhibition of Erk1/2 and Gsk3 in combination with LIF. The cytokines Activin A and basic fibroblast growth factor induce and maintain EpiSCs [21, 22]. In microchambers, the distinct dome-shaped stem cell morphology was consistent with morphology of naïve mESCs. Indeed, hierarchical clustering of differentially expressed genes demonstrated that microchamber culture results in a pattern of gene expression comparable to that of naïve mESCs [23] (Supporting Information Fig. S3; Supporting Information Table S3). Therefore, we hypothesized that endogenous signals required for maintenance of naïve pluripotency were produced by these cells.

First, to eliminate the possibility that unknown serum components affected stem cell phenotype preferentially inside microchambers, we employed a chemically defined serum-free media. Under defined conditions, cells once again showed increased colony formation and expression of pluripotency markers (Supporting Information Fig. S4A–S4C). These data indicated that phenotype enhancement by microchamber culture occurred irrespective of media composition and highlighted the importance of endogenous cell-produced factors.

Given its central role in maintaining pluripotency of mESCs, we chose to focus on LIF signaling inside microchambers. Microarray and qRT-PCR analysis revealed that LIF ligand gene expression of mESCs was higher in microchambers compared to standard culture dishes (Fig. 4A, 4B). At the same time EpiSC and differentiation inducing factors such as fibroblast growth factors (FGF) and vascular endothelial growth factor (VEGF) were downregulated relative to standard tissue culture condition (Fig. 4A). ELISA revealed that mESCs in microchambers produced 140 times more LIF compared to stem cells in 12-well plates. Cell number was 120,000 to 150,000 per device, resulting in production rate of 53 to 63 pg of LIF in each microfluidic chamber over 24 hours (Fig. 4C). As multiple other gp130 ligands, including OSM, CNTF, and CT-1, are also expressed in mESCs (Fig. 4A) and able to maintain their self-renewal [24–26], we used a neutralizing antibody to target the LIF ligand exclusively. Expression of KLF4, a marker of naïve pluripotency and direct JAK/STAT3 target gene [21] was diminished after 48 hours in the presence of a LIF blocking antibody (Fig. 4D), confirming the dominant role of endogenous LIF in microchambers. These data support the hypothesis that microchambers enable maintenance of mESC pluripotency by increasing the local concentration of LIF and, in turn triggering autocrine LIF signaling.

LIF and other interleukin (IL)-6 family ligands activate the JAK/STAT pathway in a positive autoregulatory loop, and a threshold ligand concentration is required to maintain expression of pathway components [6, 27]. Thus, to better understand the role of LIF ligands produced in microchambers, we investigated signaling through JAK/STAT pathway. First, we stained for activated STAT3 after 3 days of culture, in both the presence and absence of exogenous LIF (Fig. 4E). Levels of STAT3 activation increased significantly under microfluidic conditions, with and without exogenous LIF, and were significantly lower under standard conditions even in the presence of exogenous LIF. However, the apparent inability of exogenous LIF to rescue STAT3 activation on day 3 of culture is not
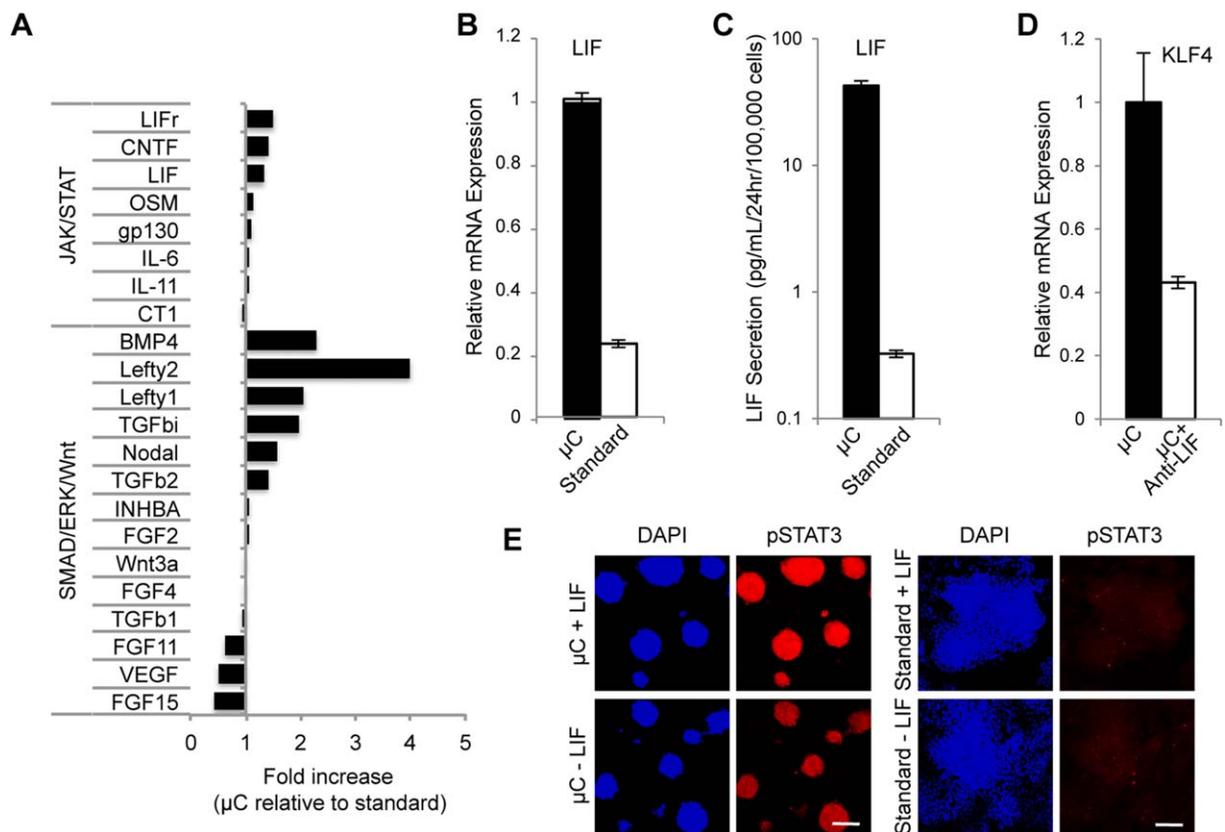
Figure 4. Retention of pluripotency in microfluidic devices is a result of enhanced LIF/STAT3 signaling. **(A)**: mRNA expression levels of endogenous ligands and receptors from microarray data. Fold increase is relative to values obtained for mouse embryonic stem cells (mESCs) cultured for 3 days in standard tissue culture with ES FBS media. **(B)**: Expression of endogenous LIF in mESCs cultured for 3 days in standard tissue culture wells and microchambers without exogenous LIF supplementation. **(C)**: Secretion of LIF from mESCs cultured in the absence of exogenous LIF (ELISA results are represented as mean ± SD, $n = 3$; $p < .05$). **(D)**: Expression of pluripotency marker and direct LIF/STAT3 target gene KLF4, with or without a LIF blocking antibody. **(E)**: Immunofluorescent staining of phospho-STAT3 (Y705), in the presence and absence of exogenous LIF. Scale bar: 100 μm. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; KLF4, Kruppel-like factor 4; LIF, leukemia inhibitory factor; pSTAT3, phosphorylated (Y705) signal transducer and activator of transcription 3; STAT3, signal transducer and activator of transcription 3.

unexpected as differentiating mESCs rapidly become unresponsive to LIF [12, 13].

Although LIF is the dominant signaling molecule inducing pluripotency in mESCs, it is not the only inductive signal. For example, BMP4 signaling through SMAD1/5 has been shown to play a role in maintaining pluripotency and facilitating JAK/STAT signaling [5, 13, 28]. In concordance with these previous reports, mESCs cultured inside microfluidic chambers for 3 days showed ∼ 2.5-fold increase in BMP4 expression compared to cells in 12-well plates (Fig. 4A). In addition, analysis of phosphorylated Smad1/5 confirmed enhanced BMP4 signaling (Supporting Information Fig. S5). This result suggested that endogenous BMP4 is likely to cooperate with LIF ligands to maintain pluripotency. Furthermore, as will be demonstrated later in this study, upregulated expression of BMP4 in microchambers may be leveraged to drive mesodermal differentiation of mESCs.

Employing a Novel mESC Cell Line to Investigate Naïve Versus Epiblast Phenotype Inside Microchambers

In the next set of experiments, we sought to first eliminate the possibility that enhanced pluripotency in microchambers was a cell line-specific artifact and second to employ an alternative strategy for characterizing naïve versus epiblast pluripotency of mESCs. To achieve this, we utilized a dual reporter mESC cell line derived from mice with mCherry (miR-290) and eGFP (miR-302) reporters incorporated at the endogenous miRNA loci [29]. In mESCs, the miR-290 cluster is associated with naïve pluripotency, whereas the miR-302 cluster is expressed upon differentiation into EpiSCs [30, 31]. The use of this stem cell line offered an exciting opportunity to monitor pluripotency and early differentiation of mESCs using green and red fluorescence proteins.

In accordance with our goal to assess naïve pluripotency in mESCs, we expanded the reporter stem cell line in 2iL media (CHIR99021, PD0325901, and LIF)—conditions known to maintain naïve pluripotency of mESCs [21, 22]. Following removal from 2iL media and cultivation in microchambers for 3 days, reporter stem cells once again formed dome-shaped colonies, while cells cultured under standard culture conditions scattered and differentiated rapidly (Fig. 5A). Confirming this observation, stem cells in microchambers retained expression of miR-290—indicative of naïve pluripotency while cells under standard cultures began expressing EpiSC marker miR-302 in the absence of 2iL. Immunofluorescence protein
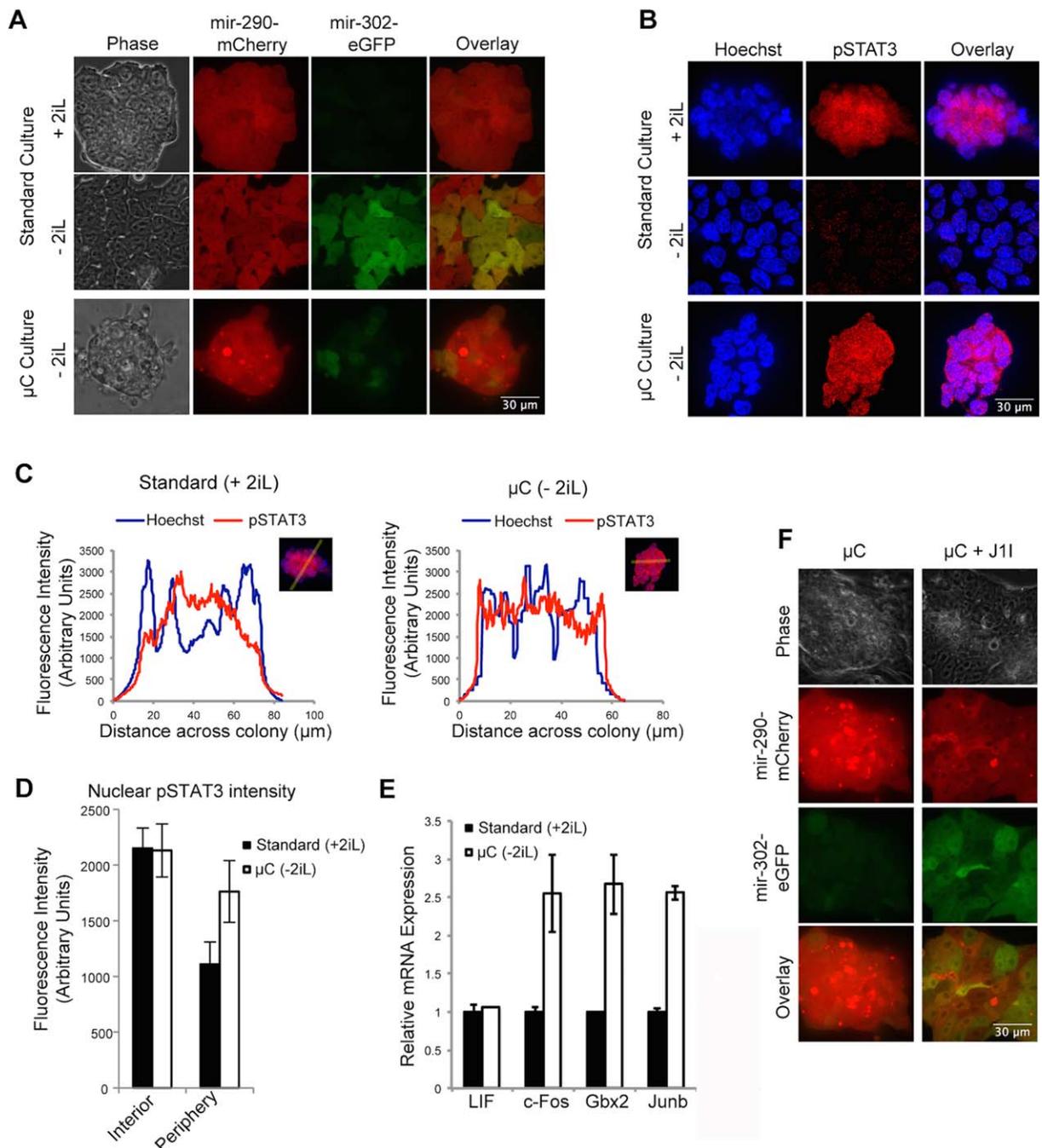
Figure 5. Mouse embryonic stem cells (mESCs) inside μC maintain naïve pluripotency and JAK/STAT pathway activation in the absence of 2iL (CHIR99021, PD0325901, and LIF). mESCs were expanded inside μCs for 3 days without 2iL and compared to cells cultured in standard tissue culture plates in the presence and absence of 2iL. **(A)**: Fluorescence images of mCherry and eGFP indicate miRNA expression profiles in 2iL or differentiation conditions (mCherry+/eGFP−, naïve pluripotency; mCherry+/eGFP+, postimplantation epiblast-like state). **(B)**: Immunofluorescent staining of phospho-STAT3 (Y705) in dual reporter mESCs after 48 hours of culture. **(C)**: Line scan of fluorescence intensity of phospho-STAT3 across the mESC colonies was analyzed by image J software. **(D)**: Graph showing average nuclear intensity of pSTAT3 from cells located in the center or periphery of hemispherical cell structures. Cells were chosen at random from five independent colonies (Data represents mean ± SD, $n = 10$; *p(interior) = .84, *p(periphery) = .00001). **(E)**: Relative mRNA expression of LIF target genes in D3 mESCs cultured under standard and μC conditions. **(F)**: When exposed to a Jak inhibitor (J1I) dual reporter mESCs in μCs differentiated rapidly as determined by morphology and coexpression of *mir-290 and mir-302* on day 3 of culture. Scale bar: 30 μm. Abbreviations: LIF, leukemia inhibitory factor; pSTAT3, phosphorylated (Y705) signal transducer and activator of transcription 3; μC, microchambers.

analysis corroborated reporter fluorescence results, indicating that Oct3/4 expression was high in microchambers in the absence of 2iL (Supporting Information Fig. S6).

Next, we analyzed phosphorylation of STAT3 in dual reporter mESCs maintained in the standard cultures in the presence or absence of 2iL and in microchambers without 2iL.

Significantly, the levels of STAT3 phosphorylation were similar for stem cells cultured in microchambers without 2iL and for standard cultures with 2iL. STAT3 phosphorylation was diminished in standard cultures without 2iL compared to standard cultures with 2iL (Fig. 5B). We also noticed that STAT3 activation in stem cells cultured in 2iL in large volumes (standard cultures) was higher in the center than on the periphery of a colony whereas in microfluidic chambers STAT3 activation was uniform (Fig. 5C, 5D). Such patterning of STAT3 phosphorylation in standard culture may suggest that ligands triggering JAK/STAT signaling are present at higher concentration in the center than in the periphery of colonies. In microchambers on the other hand, mESCs are exposed to higher and likely more uniform concentrations of endogenous ligands, resulting in more uniform activation of STAT3 and presumably more homogeneous population of pluripotent stem cells. To further address these potential differences, we analyzed both expression of the LIF ligand and LIF/STAT3 target genes relative to standard 2iL conditions. Gene expression analysis confirmed our observation of enhanced STAT3 phosphorylation patterns in microchambers. Expression of the LIF ligand was consistent between 2iL and microchamber conditions, and LIF/STAT3 target genes c-Fos, Jun-b, and Gbx2 were upregulated in microchambers (Fig. 5E). While some reports have shown that expression of LIF may be elevated during differentiation [32], our results as well as reports by others [6] demonstrate that threshold concentrations of LIF are required for maintenance of pluripotency. It would appear that endogenous LIF reaches such threshold concentrations in our microchambers, thereby enhancing pluripotent phenotype of stem cells.

In the next set of experiments, we interfered with JAK/STAT3 signaling to confirm its importance in confinement-induced pluripotency of mESCs. An inhibitor of Janus Kinase 1 (J1I) was added to the culture medium, and cell phenotype was assessed after 3 days of culture in microchambers. As a first indication of differentiation, mESCs showed flattened colony morphology that reflects an exit from pluripotency (Fig. 5F). Furthermore, analysis of miR-290-mCherry and miR-302-eGFP confirmed the observed phenotype, as dual reporter mESC transitioned from a naïve state (mCherry+, eGFP−) to an EpiSC-like state (mCherry+, eGFP+). Staining for activated STAT3 also confirmed decreased pathway activation in the presence of J1I; however, Oct3/4 expression did not show a significant decrease when signaling through STAT3 was blocked for 48 hours (Supporting Information Fig. S7, Fig. 6A). In contrast, Rex1, a marker of naïve pluripotency, decreased 10-fold in mESCs treated with J1I inside microchambers, while FGF5, a marker of EpiSC state, increased by a factor of 3 (Fig. 6A). Our observations were consistent with previous studies that showed EpiSCs to maintain Oct3/4, downregulate Rex1, and enhance FGF5 expression [21]. Thus, it appears that disruption of LIF/STAT3 signaling in our microchambers leads to induction of a differentiation program that begins with a transition from a naïve into an EpiSC-like cell state.

Taken together, our results indicate that cultivation in microchambers enhances endogenous signaling and facilitates mESC pluripotency in the absence of exogenous signals. Importantly, these effects were observed in two different mESC cell lines and, in addition to standard molecular biology approaches, were observed using reporter genes. Our results are significant in demonstrating that given correct design of the culture system mESCs are capable of producing endogenous signals to maintain pluripotency.

Blocking JAK/STAT Pathway Induces Mesoderm Lineage Specification via BMP4 Signaling

We noted earlier in this study that mESCs inside microchambers exhibited upregulated expression of BMP4 (Fig. 4A). Given the role of BMP4 as an inductive signal for both pluripotency maintenance and differentiation of stem cells, we wanted to explore the possibility that, upon inhibition of LIF/JAK/STAT3 signaling, endogenous BMP4 may promote differentiation of mESCs. As detailed in Figures 5F and 6A, inhibition of JAK/STAT3 signaling caused mESC to loose naïve pluripotency and to begin differentiation toward an EpiSC-like state. Further phenotype characterization of mESCs exposed to J1I inside microchambers revealed very interesting temporal dynamics of BMP4 expression. It decreased 25-fold at day 3 as stem cells differentiated toward epiblast state but then increased 70-fold (compared to epiblast state) by day 5 (Fig. 6B). Expression of EpiSC marker FGF5 and mesoderm marker Brachyury mirrored this transition. FGF5 expression drastically increased on day 3, after introduction of J1I, while brachyury remained low. On day 5, brachyury showed a significant increase in expression whereas FGF5 expression was diminished (Fig. 6B). The dynamics of BMP4 expression observed by us are supported by evidence from developmental biology that points to the inhibitory role of BMP4 in the conversion of naïve ESCs into EpiSCs, and stimulatory role in the conversion of EpiSCs into mesoderm and endoderm lineages [33, 34].

At day 5 of, mESCs cultured in a microchamber in the presence of J1I differentiated, forming a monolayer, whereas stem cells in the absence of this inhibitor maintained compact colonies indicative of pluripotency (Fig. 6C). Characterization of phenotype and linage commitment confirmed that mESCs cultured in the presence of J1I inside microchambers lost expression of pluripotency (Oct3/4) but upregulated expression of BMP4 and brachyury 10-fold and 3-fold, respectively, in comparison with cells in standard tissue culture wells (Fig. 6D). Importantly, inhibition of JAK/STAT3 signaling inside microchambers selectively upregulated expression of mesoderm (brachyury) over ectoderm (Sox1) and endoderm (FoxA2). In contrast, mESCs treated with J1I in standard tissue culture platform did not show preferential lineage-specific differentiation (Fig. 6C).

To further demonstrate the possibility of differentiating stem cells inside microchambers, we developed a protocol whereby J1I was removed following induction of differentiation at day 5 and stem cells were allowed to mature for an additional 5 days in low-serum media (Fig. 6E). Analysis of mesoderm markers Nkx2.5, Cdx2, and Flk1 on day 10 of differentiation confirmed maturation into this germ layer (Fig. 6F, 6G). For control experiments, stem cells were exposed on days 3–5 to dorsomorphin, a selective inhibitor of type 1 ALK receptors through which BMP4 signals [35]. Cells treated with dorsomorphin showed diminished expression of mesoderm markers at day 10 of differentiation and confirmed the role of endogenous BMP4 signaling in driving the program of mesoderm differentiation inside microchambers (Fig. 6F, 6G). The results described in this section highlight the fact that milieu of endogenous signals accumulating inside microchambers may be manipulated to direct stem cell differentiation (Fig. 6H).
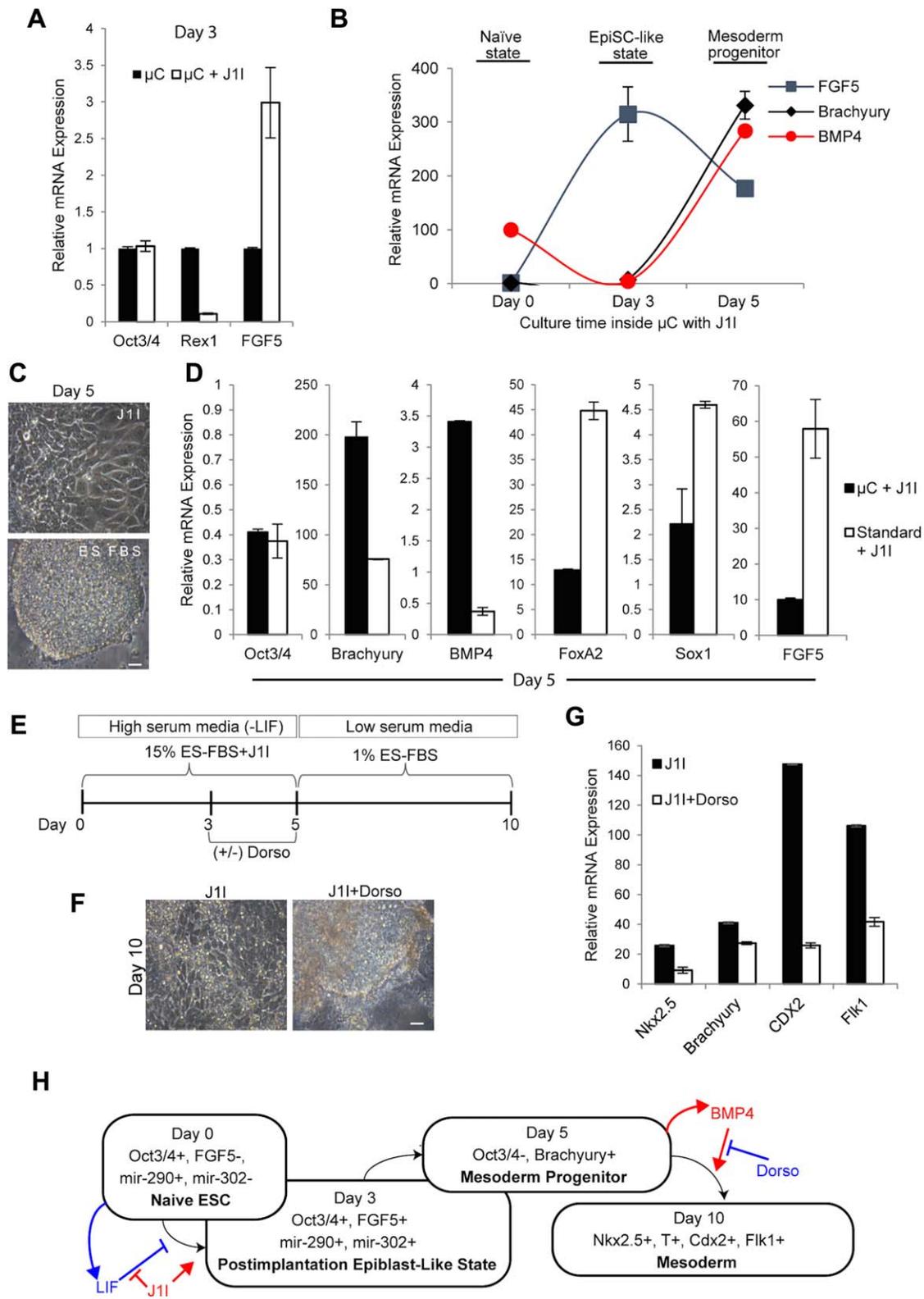
Figure 6. Subsequent BMP4 upregulation, following inhibition of gp130 ligand signaling, results in sequential differentiation of mouse embryonic stem cells (mESCs) into mesoderm. **(A)**: Expression of naïve and primed pluripotency markers after 3 days of culture in microfluidic devices, with or without a Jak1 inhibitor (J1I). **(B)**: BMP4 expression following addition of a Jak1 inhibitor (J1I) reflects stage dependent expression as cells enter and exit EpiSC-like pluripotency. FGF5 and Brachyury were used as epiblast and mesoderm-lineage specific markers, respectively. **(C, F)**: Brightfield image of cells after 5 and 10 days of differentiation in microfluidic devices. **(D)**: Expression of BMP4, along with pluripotency (Oct3/4) and three germ layer markers (mesoderm: Brachyury, endoderm: FoxA2, and ectoderm: Sox1), after 5 days of culture under the indicated conditions. mESCs cultured in tissue culture plate under the same media conditions to that of the microchamber was used as standard. **(E)**: Diagram describing media conditions that mESCs were exposed to over 10 days of differentiation. **(G)**: Expression of mesoderm specific markers after 10 days of differentiation in microfluidic devices. J1I was used to induce differentiation for first 5 days, while Dorsomorphin (Dorso) was used from 3 to 5 days of differentiation to block BMP4 signal pathway. **(H)**: Schematic diagram showing the role of LIF in maintaining self-renewal and BMP4 in mesoderm differentiation. J1I acted to switch the fate of mESCs from their pluripotent state into a differentiation program. Scale bar: 50 μm. Abbreviations: BMP4, bone morphogenetic protein-4; EpiSC, epiblast stem cell; ESC, embryonic stem cell; LIF, leukemia inhibitory factor.
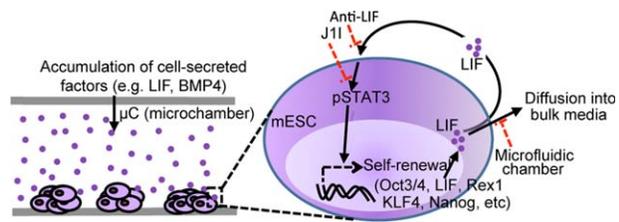
Figure 7. Model depicting enhanced mESC pluripotency in microfluidic chambers. Endogenous LIF and other growth factors, including BMP4, are present at high local concentration in microchambers and effectively signal to ESCs, whereas in the large volume of a standard culture dish secreted factors diffuse away from the culture surface rapidly and do not achieve threshold concentrations required for phenotype enhancement. Abbreviations: BMP4, bone morphogenetic protein-4; LIF, leukemia inhibitory factor; mESC, mouse embryonic stem cell.

DISCUSSION

There has been significant interest in employing microfluidic devices for cultivation of cells in general and stem cells in particular [36–40]. In comparison to macroscale cultures, microfluidic cell cultures enable improved miniaturization/multiplexing, allow to minimize reagents and cells, and offer precise control over extracellular microenvironment (e.g., gradient generation). These advantages are more tangible and obvious. Our study highlights a less explored difference between standard culture dishes and microfluidic channels or microchambers. In the former case, cells are bathed in a large volume of media that serves as a sink for secreted signals whereas in the latter scenario cells are cultured in a small volume that helps with accumulation of secreted signals. The results presented here demonstrate that stem cells cultured in small volumes in the absence of perfusion upregulate production of endogenous signals that achieve high enough levels to shape stem cell phenotype and to render exogenous signals unnecessary. While stem cells have previously been cultured in confines of microfluidic chambers, these previous studies focused on perfusion which has the effect of diluting endogenous signals through convection [8, 9, 11]. The observations made in the present study have been hinted upon by Beebe and coworkers who focused on potential differences between macroscale and microscale cultures under diffusion-controlled regime [41–43]. However, these previous studies did not focus on the behavior of pluripotent stem cells confined to small volumes.

As described in Figure 7, our findings suggest that by designing stem cell culture systems of correct geometry it may be possible to harness production of endogenous factors such as LIF and BMP4 that shape pluripotent phenotype of stem cells in the absence of exogenous signals or feeder cells. This finding is highly significant given the high cost of recombinant GFs and other media supplements required for stem cell maintenance and differentiation protocols.

Another way to utilize microchamber-based cultures is for stem cell differentiation. The concept of using small molecule inhibitors to interfere with signaling pathways and drive stem cell differentiation has considerable precedent in the literature. For example, a widely used differentiation protocol relies on two inhibitors of Smad signaling, Noggin and SB431542, to achieve high efficiency neural conversion from human ESCs

[44]. Other reports describe manipulating Wnt signaling to guide human ESC differentiation into cardiomyocytes [16, 45]. However, the fact that stem cells inside microchambers are bathed in a milieu containing high local concentrations of signaling molecules offers new opportunity for manipulating signaling pathways and directing stem cell differentiation. As an example, we demonstrated that inhibition of JAK/STAT3 pathway with a small molecule caused BMP4-driven mesoderm differentiation of mESCs inside microchambers. When implemented in macroscale cultures the same protocol did not result in specification of mesoderm. We attribute selective differentiation inside microchambers to BMP4 accumulating and reaching threshold concentrations required for driving stem cell fate selection.

CONCLUSION

This study proposes that endogenous signaling can be harnessed by miniaturizing cell culture chambers to allow for maintenance of pluripotency in mESCs in the absence of exogenous growth factors or feeder cells. To date, ESCs have not been thought capable of secreting enough growth factors to maintain their phenotype. Our work demonstrates that in a small volume, endogenous signaling is sufficient to control stem cell fate, and can be selectively manipulated to direct differentiation. Specifically, we demonstrated that mESCs cultured in microchambers produced 140 times more LIF than mESCs cultivated under standard (large volume) conditions. Impairing LIF signaling either by increasing the microchamber volume or by using LIF neutralizing antibodies or by inhibiting the JAK/STAT pathway resulted in mESCs exiting self-renewal and entering differentiation program. In light of recent reports connecting LIF signaling with naïve pluripotency of human ESCs [46–52], the concepts of upregulated LIF signaling in small volumes may be applied in the future to hESCs. From a practical standpoint, our findings may help shift the stem cell culture paradigm away from relying on expensive exogenous signals and toward harnessing of endogenous signals through optimal design of the cell culture platform.

AUTHOR CONTRIBUTIONS

J.G., A.H.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; P.G., Y.G., E.F.: collection and/or assembly of data, data analysis and interpretation; K.J.S., S.D.: data analysis and interpretation; A.R.: conception and design, collection and/or assembly

of data, data analysis and interpretation, manuscript writing, final approval of manuscript. J.G. and A.H. contributed equally to this article.

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