

Attenuation of extrinsic signaling reveals the importance of matrix remodeling on maintenance of embryonic stem cell self-renewal

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The role of extrinsic factors in maintaining self-renewal of embryonic stem cells (ESCs) has been extensively studied since the cells' isolation, but the necessity for cell-secreted factors in self-renewal has remained undefined to date. Although it is generally accepted that addition of leukemia inhibitory factor (LIF) together with either serum or bone morphogenetic protein 4 (BMP4) is sufficient to maintain mouse ESCs (mESCs) in a self-renewing state, this does not preclude the possibility that autocrine factors are also required. Here we make use of a microfluidic perfusion device that is able to globally diminish diffusible autocrine signaling by applying continuous media flow to deplete cell-secreted factors. We demonstrate mESC culture for several days under continuous microfluidic perfusion and show that cell-secreted factors are removed and can be recovered downstream. We find that perturbing cell-secreted signaling causes mESCs to exit their stable self-renewing state in defined conditions that normally support self-renewal and to exhibit properties characteristic of epiblast cells. This state change is not due to the presence of the known autocrine differentiation inducer fibroblast growth factor 4, but, remarkably, it can be prevented by global remodeling of the extracellular matrix (ECM). We also find that cell-secreted matrix remodeling proteins are removed under perfusion and that inhibition of extracellular matrix remodeling causes mESCs to differentiate. Taken together, our data indicate that LIF and BMP4 are not sufficient to maintain self-renewal and that cell-secreted factors are necessary to continuously remodel the ECM and thereby prevent differentiation, revealing a previously undescribed level of mESC regulation through the use of microfluidic perfusion technology.

It has long been known that cell-secreted signals are required for cellular processes such as growth, survival, differentiation, metastasis, and apoptosis (1–5). However, the precise contributions of autocrine and/or paracrine signals to a particular process are often difficult to determine. When the cell-secreted factors and/or receptors are known, one can use chemical or genetic inhibition of target molecules, derivation of knockout cell lines, or overexpression of candidate molecules and receptors to study autocrine/paracrine processes. However, when the cell-secreted factors are unknown, one is typically limited to varying cell density and looking for density-dependent phenotypes. Because autocrine loops can be self-sufficient even at clonal density (6), these methods are incomplete.

Pluripotent stem cells isolated from the developing blastocyst are well-suited for the study of cell-secreted signaling, because extrinsic signals generated by the embryo are essential for proper development (7, 8), and autocrine and paracrine signals are likewise important in stem cell self-renewal (9), growth (3), and differentiation (1, 10). Mouse embryonic stem cells (mESCs) are pluripotent cells derived from the inner cell mass of preimplantation blastocysts (11, 12), whereas mouse epiblast stem cells (mEpiSCs) are isolated from the postimplantation epiblast (13, 14). Critically, these stem cells retain many features of the embryonic cells from which they are derived, including responsiveness

to autocrine and paracrine signals. Thus, understanding the autocrine and paracrine signaling pathways involved in pluripotency and fate specification is crucial for enhancing our comprehension of early embryonic fate choices and for exploiting the therapeutic potential of these cells.

Autocrine factors involved in mESC self-renewal and differentiation include leukemia inhibitory factor (LIF), which mESCs secrete and respond to in an autocrine fashion (15, 16), and fibroblast growth factor 4 (FGF4), which signals through ERK1/2 to initiate a program of differentiation (1, 17). EpiSCs, on the other hand, secrete and respond to Nodal to maintain self-renewal (18), whereas autocrine Activin/Nodal has been implicated in mESC growth but not self-renewal (2). Activin also acts in an autocrine manner for maintenance of self-renewal in human ESCs, in cooperation with autocrine-acting FGF2 (19, 20). The autocrine-acting self-renewal proteins LIF and Activin/Nodal are added exogenously in mESC and mEpiSC culture media, respectively, because the levels of cell-secreted factors are not sufficient to maintain self-renewal in bulk culture.

To date, no cell-secreted factors have been shown to be necessary for maintenance of self-renewal other than those that are saturated in culture by exogenous addition. This could be because no others exist, or it could be due to the fact that even in completely defined medium, cells have fully active autocrine/paracrine signal production and uptake. Whereas the ESC state has been identified as a ground state that can be maintained by blocking signaling through ERK1/2 and glycogen synthase kinase 3 (21), it is possible that cell-secreted factors are also acting to maintain this state. To gain further insight into the role that cell-secreted signals play in the maintenance of the ESC state, we have made use of a microfluidic system in which cells can be cultured under continuous media perfusion. In these conditions, cell-secreted diffusible molecules can be removed by flow, establishing culture conditions in which signaling pathways are not obscured by cell-secreted signals. With the ability to modulate mESC cell-secreted signaling, we show that this signaling is necessary to maintain self-renewal of mESCs. Upon down-regulation of cell-secreted signaling, mESCs undergo a transition into an epiblast-like state that the presence of LIF and bone morphogenetic protein 4 (BMP4) is not sufficient to halt. Finally, we show that perfusion removes the extracellular matrix (ECM)-remodeling protein matrix metalloproteinase 2 (MMP2) and that intact ECM is required for the state change to epiblast-like cells. These results suggest that the cues emanating from the ECM are primarily prodifferentiation, extending beyond those that act

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through the ERK1/2 signaling pathway. Together, our results indicate that defined media with LIF and BMP4 is not sufficient to maintain mESC self-renewal in the absence of cell-secreted factors and that these factors specifically act to remodel ECM, thus preventing an ECM-mediated state change into an epiblast-like state.

Results

Cell Culture Under Perfusion with Depletion of Soluble Factors. To remove cell-secreted proteins from the culture medium, we made use of a microfluidic perfusion device (Fig. 1A and Fig. S1A–D) made from transparent, biocompatible polydimethylsiloxane, a material commonly used for microfluidic cell culture (22) that we have previously shown is suitable for culture of mESCs (23). Polystyrene tissue culture slides coated with gelatin were used as the device culture substrate, so that their substrate is identical to that of cells grown in conventional polystyrene tissue culture dishes (referred to as static culture throughout the text). Additionally, we provide serum-free self-renewal medium (N2B27 with added LIF and BMP4) to cells in the microfluidic devices and to cells in static culture, so that the medium is the same in both conditions.

Central to the use of microfluidic perfusion for our purposes is that the flow convects away diffusible secreted factors. To design and operate a system such that fluid flow will remove molecules without causing shear-related stress to the cells, we consider the three molecular transport mechanisms that act on secreted

molecules, namely convection, diffusion, and reaction (i.e., ligand binding to receptor). For molecules to be removed, convection must dominate over reaction and diffusion. To compare the importance of the different transport mechanisms, we make use of established nondimensional parameters (*SI Discussion, Fluid Transport Qualitative Model*). We find that the Peclet number Pe , which determines the balance between convection and diffusion, is ≈ 37 in our system, where $Pe > 1$ indicates a convection-dominated regime. To assess the relative contributions of convection and reaction, we examine the ratio of the Peclet number and the Damkohler number Da , the latter of which compares rates of reaction vs. diffusion. The resulting Pe/Da ratio compares convection to reaction and is $\approx 1,500$, indicating that convection dominates over reaction as well. Together, these calculations suggest that secreted proteins that detach from the cell surface will be convected away and not recaptured. Using these transport parameters, the shear at the culture surface is ≈ 0.007 dynes/cm², two orders of magnitude lower than what is considered low fluid shear stress for cells (24), and much lower than shears present in bioreactors in which ESCs can be grown indefinitely without any effects on self-renewal properties (25, 26). In addition, our chamber height (250 μ m) was chosen to be substantially higher than the colony heights (55 μ m) to minimize the effects of cell or colony height or morphology on flow patterns (*SI Discussion, Effects of Colonies on Fluid Flow*).

The theoretical predictions regarding removal of secreted factors were experimentally verified by collecting VEGF, which is known to be secreted by mESCs (27). We were indeed able to measure secreted VEGF (Fig. S2A), and interestingly, found that cells under perfusion showed an almost 10-fold higher amount of VEGF collected after 30 h of culture than those in static, in both differentiation (N2B27) and self-renewal (N2B27+LIF+BMP4) medium. The increased VEGF collected from cells under perfusion is consistent with autocrine systems in which the binding of ligand to receptor is blocked (28). In these systems, in which secreted ligand can be recaptured by its receptor, blocking that capture [via blocking antibody/small molecule (29), or in this case, by flow] causes more ligand per cell to be delivered into the bulk media and recovered. These results verify removal and downstream recovery of secreted molecules in this system.

During culture of mESCs under microfluidic perfusion in serum-free self-renewal conditions for up to 3 d, we found that cells grew with normal morphology and proliferation rates (Figs. S1C and 2B) and that expression of the early differentiation markers Brachyury and FGF5 was not altered compared with static self-renewal cultures (Fig. S2C), whereas those markers did increase in static differentiation conditions (N2B27 alone; Fig. S2C; all primers listed in Table S1). Day-two perfusion cells subsequently differentiated using embryoid bodies (Fig. S2D) showed similar expression kinetics for markers from all three lineages [Brachyury for mesoderm, Alpha-fetoprotein (AFP) for endoderm, Sox1 and Nestin for ectoderm] compared with cells cultured in static conditions (Fig. 1B), indicating that acute perfusion does not markedly alter differentiation potential. Nanog levels decreased by day 3 under perfusion compared with static, although they did not decrease to levels seen in static differentiation conditions (Fig. S2C). Thus, we show that diffusible signaling can be minimized in this system and that cells under perfusion predominantly resemble self-renewing mESCs.

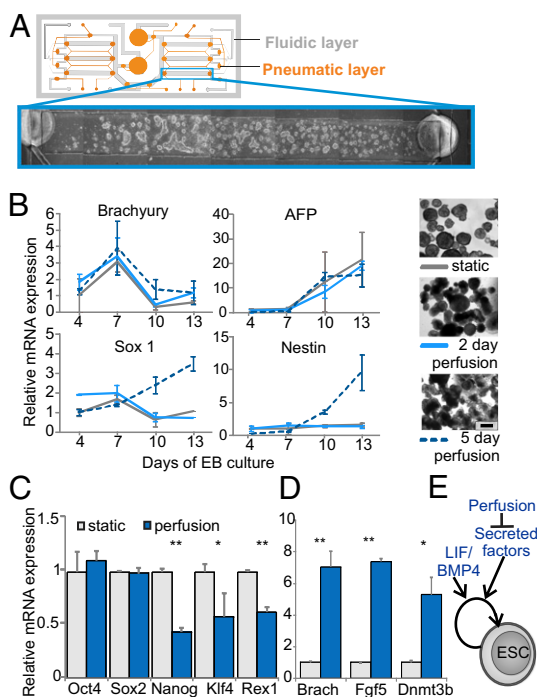


Fig. 1. ESCs exit their stable state under long-term perfusion. (A) Schematic of the microfluidic perfusion device and image of one chamber with growing mESCs. (B) Embryoid body (EB) time course mRNA expression of embryoid bodies made from cells previously grown in static culture (gray solid line) or perfusion culture for 2 d (blue solid line), or perfusion culture for 5 d (blue dashed line). Images show representative embryoid bodies on day 5 formed from cells grown under the conditions indicated. (Scale bar, 800 μ m.) (C and D) mRNA expression levels of self-renewal (C) or differentiation (D) markers after 5 d in static or perfusion culture. (E) Model indicating that perfusion blocks secreted factors that in turn act to maintain the self-renewing mESC state along with LIF/BMP4. $***P < 0.001$, $*P < 0.05$ for pairwise comparisons; all data represent averages of at least three independent experiments, and error bars represent SD.

Cells Under Perfusion Transition out of the Stable ESC State. Upon continued culture under perfusion, growth stagnated such that by day 5, less substrate surface area was covered by cells and colony size was smaller, and differentiated-looking cells were more numerous (Figs. S1D and S2B and E). When we examined the expression of key pluripotency genes in cells that were cultured for 5 d under perfusion in the presence of LIF and BMP4, we found that Klf4 and Rex1 were down-regulated, along with

a further down-regulation of Nanog levels (Fig. 1C), and that Brachyury and FGF5, which had been unaffected after 3 d of culture (Fig. S2C), were dramatically up-regulated, along with the differentiation marker Dnmt3b (Fig. 1D). Oct4 and Sox2 mRNA levels did not change (Fig. 1C), indicating that the cells still expressed some elements of the core stem cell transcription network. A similar expression pattern was seen between static and perfusion cultures with cells grown in static N2B27+2i/LIF media (Fig. S2F), indicating that the perfusion phenotype is not a result of activation or block of specific signaling pathways. The differentiation potential of cells grown under perfusion in the presence of LIF and BMP4 was also altered: embryoid bodies formed with slightly abnormal morphology (Fig. 1B) and had increased expression of the ectoderm differentiation markers Sox1 and Nestin compared with cells grown in static conditions (Fig. 1B). We did not observe any apparent heterogeneity in Oct4 protein levels or cell number along the length of the chamber (Fig. S3A and B), suggesting that observed differences in phenotype are not simply due to spatially varying differentiation in the flow field. Additionally, it is unlikely that the phenotypic changes observed under perfusion were due to selection of a specific cell population, because there was no massive cell death during the culture period, the low shear rates present in our system are >1,000-fold below those known to cause ESC detachment (26), and we recovered ≤ 600 cells released from all chambers over 5 d, which is <2% of the number of cells present in the chambers on day 5 (Fig. S3C).

To ensure that the changes observed under perfusion were indeed due to removal of secreted factors, several control perfusions were performed. Increasing the LIF concentration fivefold did not restore Nanog levels (Fig. S3D), suggesting that local concentration effects due to the perfusion transport environment are not the cause of the observed changes, whereas cells grown in the presence of cell-conditioned serum-containing media under perfusion did see restoration of Nanog to levels seen in static serum-containing culture (Fig. S3E). Cells grown in a micro-device using a small-volume recirculating-loop system in which cells were fed with the media collected under perfusion had marker expression similar to that in cells grown in static cultures, as did cells grown using defined feeding intervals (Fig. S3F, recirc loop perf and pulse perf, respectively). Thus, approximating the soluble microenvironment of static culture by allowing cells to condition the media generates a phenotype similar to the static phenotype, but in a system that includes microculture and shear. Conversely, cells grown at a fourfold lower perfusion rate (and thus fourfold lower shear) did not show any substantial differences compared with cells grown at our normal perfusion rate (Fig. S3F, perf 25 μ l/h). Thus, lowering the shear but maintaining a convection-dominated microenvironment does not substantially change phenotype, consistent with transport rather than shear being dominant. Together, these results further indicate that neither the microculture itself nor shear are artifactually altering the observed phenotype.

These results indicate that depleting cell-secreted signals does not allow mESCs to maintain their self-renewal program even in the presence of LIF and BMP4 (Fig. 1E), a finding that motivated further study into the nature of the cells that arise out of an environment with minimal cell-secreted signaling and the mechanism behind this transition.

Down-Regulation of Cell-Secreted Signals Drives ESCs Toward an Epiblast-Like Cell State. Because mouse epiblast cells and the related EpiSCs express Oct4 and Sox2, have low levels of Klf4 and Rex1 and high levels of Brachyury and FGF5, and are pluripotent (13, 14, 30), we examined whether cells under perfusion, which have a comparable expression pattern, were similar to epiblast cells. Using a quantitative RT-PCR array, we analyzed expression of relevant markers over time in static and perfused

self-renewal cultures (Fig. S4 and Table S2). Among the most highly altered genes in cells grown under perfusion, we found many postimplantation markers indicative of epiblast (Fig. 2A), including FGF5 (31), Brachyury (T) (14), Lefty1 (30), and Dnmt3b (32), which increased relative to static, and the ESC markers Gbx2 and Cd9, which decreased (14, 33). Further growth (7 d) under perfusion resulted in increased expression of epiblast markers, including Eomes, Sox17, Lefty1, and Gata6 (14) (Fig. S5A), suggesting entrance into a stable epiblast-like state. We found that cells grown under perfusion for 5 d were fairly homogeneous in terms of self-renewal markers, because flow cytometry histograms for Oct4, Sox2, and Nanog were primarily unimodal (Fig. S5B). To further examine whether the cells grown under perfusion are undergoing nonspecific differentiation, we compared gene expression in perfused cells with expression in cells undergoing undirected differentiation in embryoid body culture and with expression in mESCs cultured in conditions that induce an EpiSC-like state [culture in the presence of Activin and FGF2 (34)] from days 3 to 7 of culture. We observed higher expression of genes associated with mesoderm differentiation in embryoid bodies compared with either EpiSC-like cells or cells grown under perfusion, where these genes were expressed at low levels at both day 5 and day 7 (Fig. S5C). This signifies a lack of indiscriminate differentiation in cells with minimal cell-secreted signaling and instead indicates a more directed differentiation pathway, providing further evidence for exit from the ESC state toward a state that closely resembles an epiblast-like state.

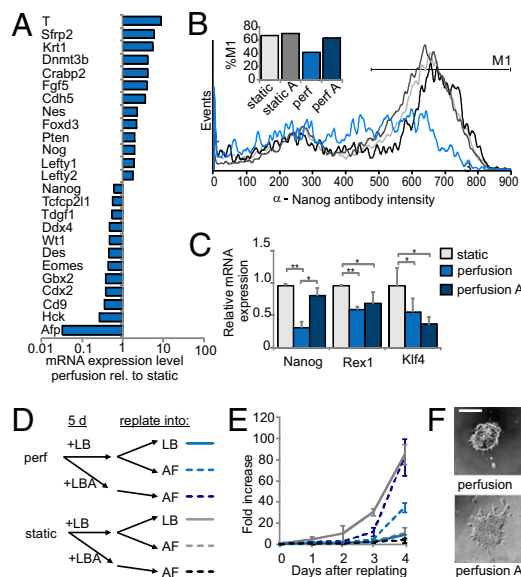


Fig. 2. An epiblast-like state is attained upon cell-secreted factor removal. (A) mRNA expression levels of markers from a quantitative PCR array that changed expression more than twofold on day 5 under perfusion compared with day 5 in static conditions. (B) Flow cytometry histogram depicting Nanog protein levels in the presence or absence of Activin (A). *Inset:* Percentage of cells in the M1 range. (C) Self-renewal marker expression for cells cultured for 5 d in N2B27+LB in the presence or absence of Activin (A). (D) Schematic explaining experiment depicted in E, in which cells were grown in static or perfusion in N2B27+LB with or without Activin (A) for 5 d, then replated into either ESC (LB, solid lines) or EpiSC [Activin + FGF2 (AF), dotted lines] static culture conditions for 4 d. (E) Fold increase in growth of cells from conditions in D upon replating. (F) Images of representative colonies from replating into EpiSC medium, replated from indicated conditions, taken on day 3 after replating. (Scale bar, 200 μ m.) ** $P < 0.001$, * $P < 0.05$ for pairwise comparisons; all data represent averages of at least three independent experiments, and error bars represent SD.

This transition that occurs under perfusion is surprising given that LIF and BMP4 are still present in the culture medium, because their presence has previously been shown to be sufficient to maintain mESC self-renewal (35). Indeed, we found that mild induction of the EpiSC-like state using Activin and FGF2 did not alter marker expression in static culture when in the presence of LIF and BMP4 (Fig. S5D). Thus, in conventional culture systems, ESCs are able to withstand state change cues as long as LIF and BMP4 are present, whereas these molecules do not have the same effect under perfusion, indicating a lack of sufficiency.

One notable difference seen in cells under perfusion compared with EpiSCs is lower Nanog expression levels. Although Nanog is required for the maintenance of pluripotency in mESCs (36) and EpiSCs (18), the upstream regulation occurs by different processes. In the ESC state, LIF activates Stat3 to up-regulate Klf4 and Nanog and inhibit expression of FGF5 (37). In EpiSCs, however, Stat3 is still responsive to LIF, but cells are not dependent on that pathway for self-renewal (14, 38); instead, Activin is required to up-regulate Nanog through Smad2/3 signaling (18). We found that cells cultured in perfusion maintained the ability to activate Stat3 by Y705 phosphorylation in response to LIF (Fig. S5E) but without up-regulation of downstream self-renewal genes (Fig. 1C). However, addition of Activin under perfusion causes cells to up-regulate Nanog protein and mRNA levels (Fig. 2B and C), indicating a shift from an LIF-dependent ESC state to an Activin-responsive epiblast-like cell state. Activin supplementation under perfusion did not up-regulate Rex1 and Klf4 (Fig. 2C), other downstream targets of Stat3, implying that Activin supplementation does not revert perfusion cultures back to an ESC state, nor does it broadly alter marker expression in static cultures (Fig. S5F).

To test whether addition of Activin under perfusion is able to stabilize a self-renewing state that is more epiblast-like than ESC-like, we replated cells after culture with or without added Activin into either ESC or EpiSC static self-renewal medium (Fig. 2D). Only cells grown under perfusion with added Activin

were able to be replated and grown, and these cells grew best in EpiSC conditions and showed EpiSC-like colony morphology (Fig. 2E and F). The same did not hold true for cells that had been grown in static with added Activin; instead, cells grown in static were only able to proliferate after being replated in ESC medium (Fig. 2E), indicating that culture in the microdevice primes cells to be receptive to Activin supplementation for maintenance of self-renewal. Cells grown under perfusion were also unable to replate and grow in N2B27+2i/LIF minimal self-renewal media (Fig. S5G), another characteristic that these cells share with epiblast cells (34). Together, our results demonstrate that a lack of diffusible signaling causes mESCs to leave their stable self-renewing state and enter a more epiblast-like state that is characterized by the expected marker expression profiles as well as the appropriate downstream signaling responses and state stabilization resulting from addition of Activin. However, because several self-renewing multi- or pluripotent epiblast-like states have been identified (14, 39, 40), the precise identity of the cells with reduced soluble signaling is not known.

Extracellular Matrix Remodeling Prevents the Transition out of the mESC State. Spontaneous differentiation of ESCs is often attributed to cell-secreted factors, the primary example being autocrine/paracrine FGF4 signaling through the ERK1/2 pathway (1, 17). Although cell-secreted FGF4 is likely removed under perfusion, we sought to determine whether ERK signaling was still active under perfusion and thereby mediating the observed changes, because LIF is known to activate ERK in a context-dependent manner (41). Adding the potent MAP kinase/ERK kinase (MEK) inhibitor PD0325901 (PD03) under perfusion was effective at inhibiting active ERK (Fig. 3A), but it did not cause an up-regulation of self-renewal genes under perfusion, whereas it was effective in up-regulating these genes in static cultures (Fig. 3B), as previously reported (21). In addition, PD03 did not decrease differentiation marker expression to the level of static controls (Fig. S6A). The fact that inhibition of downstream

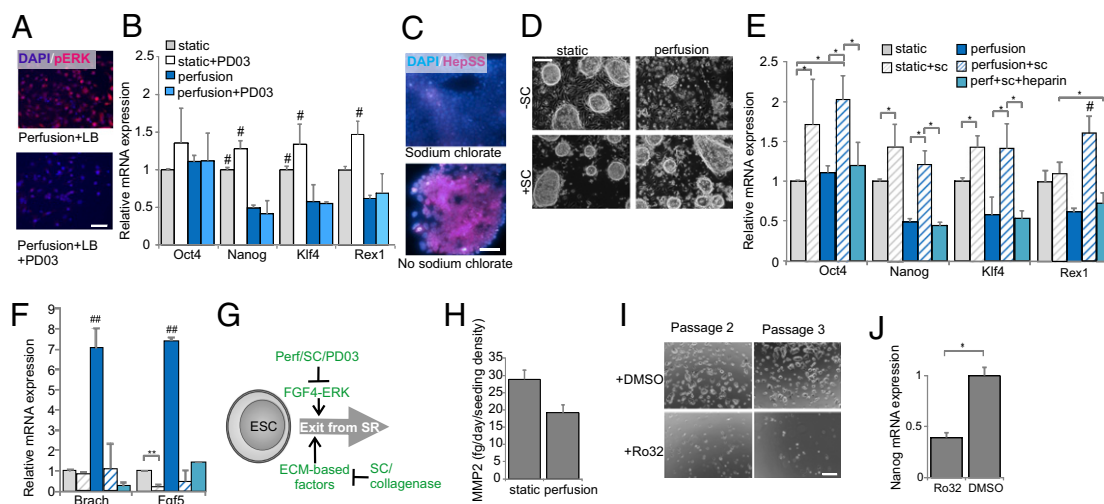


Fig. 3. Globally disrupting the ECM blocks state change. (A) Fluorescent images of cells grown in perfused culture in N2B27+LB with and without the MEK inhibitor PD0325901 (PD03), stained for phosphorylated ERK1/2 (red) and counterstained with DAPI (blue). (Scale bar, 100 μ m.) (B) Expression levels of self-renewal markers after growth in static or perfusion self-renewal culture in the presence or absence of PD03. (C) Immunofluorescence staining for sulfated heparan after 5 d of growth in the presence or absence of sodium chloride. (Scale bar, 100 μ m.) (D) Representative morphology of cells grown in static or perfusion self-renewal with or without sodium chloride (+/-SC). (Scale bar, 200 μ m.) (E and F) mRNA expression levels of self-renewal (E) and differentiation (F) markers in the presence and absence of sodium chloride with or without the addition of soluble heparin in static or perfusion self-renewal. (G) Model depicting two modes of exit from self-renewal, the lower of which was revealed on the basis of perfusion experiments. (H) Secreted protein levels of MMP2 in static and perfusion culture over 5 d, analyzed by ELISA. (I) Cells grown with or without the MMP inhibitor Ro32-3555 for multiple passages. (Scale bar, 400 μ m.) (J) Relative Nanog mRNA expression levels of cells grown for 5 d in Ro32-3555 or DMSO. ** $P < 0.001$, * $P < 0.05$; # $P < 0.05$, ### $P < 0.001$ for all pairwise comparisons; all data represent averages of at least three independent experiments, and error bars represent SD.

pathways does not affect the cells under perfusion further confirms an upstream removal of cell-secreted signals and shows that the transition out of the ESC state seen under perfusion is not a result of signaling through the MEK/ERK pathway.

Because the ECM has been implicated in contributing to spontaneous differentiation by binding of cell-secreted factors (42), we sought to assess the contribution of the ECM in the phenotype seen under perfusion. We initially used sodium chlorate, a sulfation inhibitor that blocks the ability of most proteoglycans to act as protein tethers or reservoirs within the ECM (43). Sodium chlorate is able to remove sulfated heparan chains (Fig. 3C), and, similar to previous reports, disruption of heparan sulfation in static culture decreased spontaneous differentiation, up-regulated Nanog, and decreased FGF5 (Fig. 3D–F). Intriguingly, under perfusion, addition of sodium chlorate enhanced mESC-like morphology, up-regulated self-renewal markers, and down-regulated differentiation markers compared with cells grown under perfusion without sodium chlorate (Fig. 3D–F). Further, adding soluble heparin along with sodium chlorate reduced self-renewal markers to the levels seen under baseline perfusion conditions (Fig. 3E), confirming that the phenotypic changes caused by sodium chlorate were due to the loss of the heparan sulfate binding function. Adding low concentrations of collagenase to disrupt the ECM produced a phenotype similar to that resulting from the addition of sodium chlorate (Fig. S6B). Thus, broadly disrupting the ECM by either small molecule or enzyme addition under perfusion allows ESCs to maintain their self-renewing state, whereas functional recovery of ECM protein binding causes cells under perfusion to exit this state, indicating the importance of ECM disruption in mESC self-renewal. This implies that ECM-based factors are responsible for an exit from self-renewal, whereas ECM disruption acts to remove these factors (Fig. 3G).

The MMP family of proteins includes endogenously secreted molecules responsible for remodeling and curating the ECM (44). Because the exit from the ESC state seen under perfusion is related to the presence of the intact ECM, it is possible that a removal of MMPs under perfusion is responsible. Indeed, MMPs are known to be secreted by mESCs (27), and we are able to recover MMP2 from perfused cells (Fig. 3H), illustrating that it is being removed by flow. Relative levels of MMP2 protein from static and perfusion were comparable to transcript levels (Fig. S6C). To assess the necessity for MMPs in static culture, we examined whether blocking endogenous remodeling proteins in static culture would also affect ESC self-renewal. We found that ESCs cannot be cultured for multiple passages in conditions under which MMPs are inhibited by the MMP inhibitor Ro32-3555. After two passages in the presence of this inhibitor, cells grew poorly and differentiated (Fig. 3I) and were no longer viable by passage 3. Cells that were seeded in self-renewal media with Ro32-3555 added after attachment showed a decrease in Nanog expression levels after 5 d (Fig. 3J), indicating that the inhibitor is not merely causing an exit from self-renewal by altering attachment or growth. Together, these results indicate that matrix remodeling is critical in maintaining the mESC state and that removal of cell-secreted factors means that matrix remodeling cannot occur properly, thus inducing exit from the self-renewing state.

Discussion

By using microfluidic perfusion to continuously remove mESC cell-secreted factors, we show that a global reduction of cell-secreted signals drives cells out of self-renewal and toward a defined lineage that closely resembles the epiblast state. Strikingly, this occurs even in the presence of LIF and BMP4, which had previously been shown to be sufficient to maintain self-renewal in static culture. We further show that this result is due to the continued presence of the intact ECM, the constant remodeling of which is necessary to retain mESC self-renewal.

Combining these results, a model arises in which, in addition to the known pro-self-renewal LIF/BMP4 signals and the pro-differentiation FGF4-ERK autocrine stimulus, there also exist ECM-bound factors that initiate an exit from mESC self-renewal. In normal cultures, matrix remodeling is constantly occurring to remove these prodifferentiation factors. However, under perfusion, the secreted factors that are responsible for remodeling are removed such that ECM turnover does not occur and mESCs are cued by the ECM-bound prodifferentiation factors to exit their naïve self-renewing state (Fig. 4). These results emphasize the power of microfluidic perfusion in uncovering previously unknown roles for cell-secreted signals. This robust method can be broadly applied to other cell types to test hypotheses based on the effects of cell-secreted signals or the roles and contributions of ECM-based signals.

It is currently thought that mESCs grown in self-renewing culture conditions exist with some level of heterogeneity due to spontaneous conversion between a naïve ESC state and a more primed epiblast-like state (45, 46). In serum-free N2B27+LIF+BMP4 media, this interconversion is thought to be a result of the opposing actions of LIF and BMP4 signaling vs. autocrine/paracrine FGF4-ERK signaling (47). Thus, blocking FGF4-ERK signaling in static cultures pushes cells toward the naïve ESC state, as does inhibiting heparan sulfation, because sulfation is necessary for FGF4 signaling, consistent with our results. However, if FGF4 was the primary cell-secreted stimulus acting in ESCs, one would expect global down-regulation of cell-secreted signaling to have an effect similar to ERK inhibition, maintaining ESCs in a more naïve state. However, surprisingly, we find that minimal cell-secreted signaling causes ESCs to instead be pushed in the opposite direction, toward a more primed epiblast-like state.

Critically, this transition out of the ESC state occurs in the presence of LIF and BMP4 or 2i/LIF, indicating a lack of sufficiency for these self-renewal factors, and thus a requirement for an additional pathway involved in ESC maintenance. This pathway pushes ESCs away from their naïve state and is normally blocked in static culture by cell-secreted factors. By showing that this pathway is blocked under perfusion by broadly disrupting the ECM, we implicate matrix remodeling as a crucially important process in removing differentiation-inducing proteins from the ESC microenvironment to maintain self-renewal and show that the presence of LIF and BMP4 and a matrix remodeler is sufficient for this maintenance.

An important class of endogenous cell-secreted ECM remodelers is the MMP family, and indeed, their inhibition in static culture causes mESC differentiation. Although various components of the ECM have been shown to play a role in enhancing or inhibiting ESC self-renewal (42, 48–50), we demonstrate the importance of ECM remodeling on maintenance of

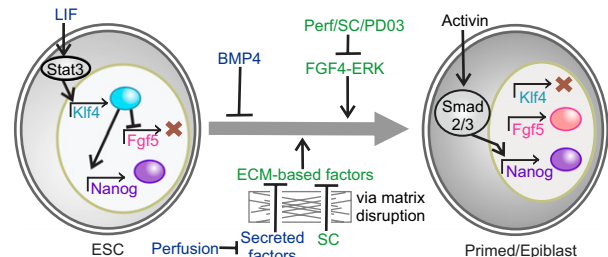


Fig. 4. Model depicting the signaling processes involved in maintenance of self-renewal in mESCs and exit from that state. Transitions between the stable LIF-Stat3-dependent naïve ESC state (Left) and the Activin-dependent primed epiblast-like state (Right) are dependent on a balance between LIF/BMP4 vs. FGF4-ERK signaling. Signals emanating from the ECM, which are obscured in static culture by matrix remodeling but uncovered by perfusion, stimulate the transition to a more primed epiblast-like cell state.

self-renewal. Thus, our evidence points to the presence of an important but previously undescribed pathway that is sufficient to maintain ESC self-renewal in conjunction with LIF and BMP4 in the absence of other cell-secreted signals.

Materials and Methods

Cell Culture. Mouse ESCs [CCE, ABJ1 (51), and Sox2-GFP lines] were routinely cultured in medium consisting of DMEM supplemented with 15% defined FBS (HyClone), 4 mM L-glutamine, 1 mM nonessential amino acids, 1× penicillin-streptomycin, 100 μM β-mercaptoethanol (Sigma), and 10 ng/mL LIF (ESGRO, Chemicon). All cell culture reagents were from Invitrogen unless otherwise noted. Cells were grown at 37 °C in a humidified incubator with 7.5% CO₂. For experiments, cells were plated at a density of 1.25 × 10⁴ cells/cm² into gelatin-coated wells or seeded into a gelatin-coated perfusion device. For serum-free culture, N2B27 medium with 10 ng/mL LIF and 10 ng/

mL BMP-4 (R&D Systems) was used (35). For 2i/LIF culture, CHIR99021, PD0325901, and LIF were added to N2B27 media.

Perfusion Culture Device. The microdevice consists of six 1.25 mm × 13 mm × 250 μm (width × length × height) chambers, with two separate media inputs and dual addressability. ESCs were loaded into the device, which was then placed in a humidified incubator, and perfusion was initiated once the cells were firmly attached to the surface (≈24 h). Perfusion was run continuously at 0.1 mL/h, unless otherwise noted.

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- Kunath T, et al. (2007) FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development* 134:2895–2902.
- Ogawa K, et al. (2007) Activin-Nodal signaling is involved in propagation of mouse embryonic stem cells. *J Cell Sci* 120:55–65.
- Mittal N, Voldman J (2011) Nonmitogenic survival-enhancing autocrine factors including cyclophilin A contribute to density-dependent mouse embryonic stem cell growth. *Stem Cell Res (Amst)* 6:168–176.
- Sporn MB, Roberts AB (1985) Autocrine growth factors and cancer. *Nature* 313:745–747.
- Dhein J, Walczak H, Bäuml C, Debatin KM, Krammer PH (1995) Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature* 373:438–441.
- van Zoelen EJJ, et al. (1989) Production of insulin-like growth factors, platelet-derived growth factor, and transforming growth factors and their role in the density-dependent growth regulation of a differentiated embryonal carcinoma cell line. *Endocrinology* 124:2029–2041.
- Feldman B, Poueymirov W, Papaioannou VE, DeChiara TM, Goldfarb M (1995) Requirement of FGF-4 for postimplantation mouse development. *Science* 267:246–249.
- Sun X, Meyers EN, Lewandoski M, Martin GR (1999) Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev* 13:1834–1846.
- Bendall SC, et al. (2007) IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. *Nature* 448:1015–1021.
- Peerani R, et al. (2007) Niche-mediated control of human embryonic stem cell self-renewal and differentiation. *EMBO J* 26:4744–4755.
- Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154–156.
- Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 78:7634–7638.
- Brons IGM, et al. (2007) Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 448:191–195.
- Tesar PJ, et al. (2007) New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448:196–199.
- Davey RE, Onishi K, Mahdavi A, Zandstra PW (2007) LIF-mediated control of embryonic stem cell self-renewal emerges due to an autoregulatory loop. *FASEB J* 21:2020–2032.
- Zandstra PW, Le HV, Daley GQ, Griffith LG, Lauffenburger DA (2000) Leukemia inhibitory factor (LIF) concentration modulates embryonic stem cell self-renewal and differentiation independently of proliferation. *Biotechnol Bioeng* 69:607–617.
- Stavridis MP, Lunn JS, Collins BJ, Storey KG (2007) A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification. *Development* 134:2889–2894.
- Greber B, et al. (2010) Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. *Cell Stem Cell* 6:215–226.
- Greber B, Lehrach H, Adjaye J (2007) Fibroblast growth factor 2 modulates transforming growth factor β signaling in mouse embryonic fibroblasts and human ESCs (hESCs) to support hESC self-renewal. *Stem Cells* 25:455–464.
- Dvorak P, et al. (2005) Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. *Stem Cells* 23:1200–1211.
- Ying Q-L, et al. (2008) The ground state of embryonic stem cell self-renewal. *Nature* 453:519–523.
- Meyvantsson I, Beebe DJ (2008) Cell culture models in microfluidic systems. *Annu Rev Anal Chem (Palo Alto Calif)* 1:423–449.
- Kim L, Vahey MD, Lee HY, Voldman J (2006) Microfluidic arrays for logarithmically perfused embryonic stem cell culture. *Lab Chip* 6:394–406.
- Grabowski EF, Lam FP (1995) Endothelial cell function, including tissue factor expression, under flow conditions. *Thromb Haemost* 74:123–128.
- Cormier JT, zur Nieden NI, Rancourt DE, Kallos MS (2006) Expansion of undifferentiated murine embryonic stem cells as aggregates in suspension culture bioreactors. *Tissue Eng* 12:3233–3245.
- Fok EYL, Zandstra PW (2005) Shear-controlled single-step mouse embryonic stem cell expansion and embryoid body-based differentiation. *Stem Cells* 23:1333–1342.
- Guo Y, Graham-Evans B, Broxmeyer HE (2006) Murine embryonic stem cells secrete cytokines/growth modulators that enhance cell survival/anti-apoptosis and stimulate colony formation of murine hematopoietic progenitor cells. *Stem Cells* 24:850–856.
- DeWitt AE, Dong JY, Wiley HS, Lauffenburger DA (2001) Quantitative analysis of the EGF receptor autocrine system reveals cryptic regulation of cell response by ligand capture. *J Cell Sci* 114:2301–2313.
- Lauffenburger DA, Oehrtman GT, Walker L, Wiley HS (1998) Real-time quantitative measurement of autocrine ligand binding indicates that autocrine loops are spatially localized. *Proc Natl Acad Sci USA* 95:15368–15373.
- Bao S, et al. (2009) Epigenetic reversion of post-implantation epiblast to pluripotent embryonic stem cells. *Nature* 461:1292–1295.
- Hayashi K, Lopes SM, Tang F, Surani MA (2008) Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. *Cell Stem Cell* 3:391–401.
- Hirasawa R, Sasaki H (2009) Dynamic transition of Dnmt3b expression in mouse pre- and early post-implantation embryos. *Gene Expr Patterns* 9:27–30.
- Oka M, et al. (2002) CD9 is associated with leukemia inhibitory factor-mediated maintenance of embryonic stem cells. *Mol Biol Cell* 13:1274–1281.
- Guo G, et al. (2009) Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development* 136:1063–1069.
- Ying QL, Nichols J, Chambers I, Smith A (2003) BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 115:281–292.
- Mitsui K, et al. (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113:631–642.
- Jiang J, et al. (2008) A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat Cell Biol* 10:353–360.
- Hanna J, et al. (2010) Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proc Natl Acad Sci USA* 107:9222–9227.
- Chou YF, et al. (2008) The growth factor environment defines distinct pluripotent ground states in novel blastocyst-derived stem cells. *Cell* 135:449–461.
- Rathjen J, et al. (1999) Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. *J Cell Sci* 112:601–612.
- Niwa H, Ogawa K, Shimosato D, Adachi K (2009) A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature* 460:118–122.
- Lanner F, et al. (2009) Heparan sulfation dependent FGF signalling maintains embryonic stem cells primed for differentiation in a heterogeneous state. *Stem Cells* 28:191–200.
- Baeuerle PA, Huttner WB (1986) Chlorate—a potent inhibitor of protein sulfation in intact cells. *Biochem Biophys Res Commun* 141:870–877.
- Matrisian LM (1990) Metalloproteinases and their inhibitors in matrix remodeling. *Trends Genet* 6:121–125.
- Chambers I, et al. (2007) Nanog safeguards pluripotency and mediates germline development. *Nature* 450:1230–1234.
- Enver T, Pera M, Peterson C, Andrews PW (2009) Stem cell states, fates, and the rules of attraction. *Cell Stem Cell* 4:387–397.
- Lanner F, Rossant J (2010) The role of FGF/Erk signaling in pluripotent cells. *Development* 137:3351–3360.
- Chowdhury F, et al. (2010) Soft substrates promote homogeneous self-renewal of embryonic stem cells via downregulating cell-matrix tractions. *PLoS ONE* 5:e15655.
- Domogatskaya A, Rodin S, Boutaud A, Tryggvason K (2008) Laminin-511 but not -332, -111, or -411 enables mouse embryonic stem cell self-renewal in vitro. *Stem Cells* 26:2800–2809.
- Hayashi Y, et al. (2007) Integrins regulate mouse embryonic stem cell self-renewal. *Stem Cells* 25:3005–3015.
- Bortvin A, Goodheart M, Liao M, Page DC (2004) Dppa3 / Pgc7 / stella is a maternal factor and is not required for germ cell specification in mice. *BMC Dev Biol* 4:2.