Nonmitogenic survival-enhancing autocrine factors including cyclophilin A contribute to density-dependent mouse embryonic stem cell growth

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Non-Mitogenic Survival-Enhancing Autocrine Factors Including Cyclophilin A Contribute to Density-Dependent mESC Growth

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Abstract
An improved understanding of the role of extracellular factors in controlling the embryonic stem cell (ESC) phenotype will aid the development of cell-based therapies. While the role of extracellular factors in controlling the pluripotency and differentiation of embryonic stem cells (ESCs) has been the subject of much investigation, the identity and role of extrinsic factors in modulating ESC growth under conditions supporting self-renewal remain largely unknown. We demonstrate that mouse ESC growth is density-dependent and that one of the mechanisms underlying this phenomenon is the action of survival-enhancing autocrine factors. Proteomic analysis of proteins secreted by mouse ESCs demonstrates significant levels of Cyclophilin A which increases the growth rate of mouse ESCs in a dose-dependent manner. Additionally, inhibition of the Cyclophilin A receptor – CD147 decreases the growth rate of mESCs. These findings identify Cyclophilin A as a novel survival-enhancing autocrine factor in mouse ESC cultures.

Keywords
Embryonic stem cells; autocrine signaling; growth; apoptosis; Cyclophilin A

Introduction
Embryonic stem cells (ESCs) are cells derived from the inner cell mass (ICM) of pre-implantation blastocysts with the ability to produce any cell type in the adult animal. While ESCs can differentiate to form various cell types, they can also divide to form two daughter ESCs via self-renewal. Although self-renewal encompasses both growth and maintenance of developmental potential, most research has focused on understanding the signaling and transcriptional networks involved in the latter, i.e., maintenance of the ESC pluripotent state [1]. In contrast, comparatively few studies have investigated pathways involved in ESC growth and death under conditions that have been shown to maintain pluripotency. Studies have shown that the addition of EGF, ANG-2, Activin, Nodal, LIF or BMP-4 proteins to...
mouse ESC (mESC) cultures results in improved growth [2–6]. However, these studies did not include testing for secretion of these proteins by ESCs, i.e., they did not attempt to fully demonstrate that these pathways are constitutively active in mouse ESC cultures and that the factors are acting in an autocrine fashion. SDF-1 has been shown to act as an autocrine survival factor for mESCs, but only in the absence of serum (i.e. during serum withdrawal) [7].

Because ESCs grow as colonies, juxtacrine signaling could also presumably play a growth-supportive role. Todorova et al. used pharmacological blockers of gap junctional communication and Connexin-43 small-interfering RNA to inhibit signaling via gap junctions in mESCs [8]. They demonstrated that gap junctional communication enhances either the growth rate or the attachment efficiency (or both) of mESCs.

Cell growth is intimately tied to progression through the cell cycle, and several studies have focused on understanding cell cycle control in mESCs. mESC growth in culture is characterized by a short cell cycle (11–16 hours), primarily owing to a reduction in the duration of G1 phase as compared to somatic cells [9]. This short G1 phase is due to a lack of the mitogen-dependent early G1 phase of the cell cycle, owing to constitutive Cyclin E–CDK2 activity [10]. This constitutive activity of Cyclin E-CDK2 in turn appears to be driven at least partially by a constitutively active Ras protein expressed specifically in ESCs, called ERas, which stimulates PI3K [11], which is upstream of Cyclin E [12]. Thus, it is unlikely that secreted factors will modulate the length of the G0/G1 phase of the cell cycle, as they do in several other cell types. However, secreted factors could affect growth by modulating the rate of apoptosis.

One approach to searching for growth-enhancing secreted molecules has been to investigate whether proteins secreted by mESCs influence the growth of other types of cells. Guo et al. have shown that mESC conditioned medium improves the survival of hematopoietic progenitor cells [13], while Singla et al. showed that mESC conditioned medium inhibits apoptosis in H9c2 cardiomyocyte cells in a PI3K-dependent manner [14]. They determined that one of the factors responsible for this effect was TIMP-1. Since these studies show that mESCs secrete factors with anti-apoptotic activity for other cell types, we wondered whether these (or other mESC-secreted) factors might have pro-survival and/or mitogenic effects on mESCs themselves. Indeed, although mESCs can be clonally propagated, it is commonly known that their proliferation is density dependent.

Here we demonstrate the existence of one or more autocrine survival factor(s) in mESC cultures. By performing LC/MS-MS analysis of mESC conditioned medium, we determined that Cystatin C and Cyclophilin A are two major constituents of the mESC secretome. We tested the effects of these proteins, as well as other proteins known to be secreted by mESCs (such as TIMP-1), and found that only Cyclophilin A improved the growth of mESCs. Additionally, antibody-mediated inhibition of the Cyclophilin receptor with a polyclonal antibody decreased the growth rate of mESCs. These results demonstrate that Cyclophilin A is an autocrine survival factors in mESC cultures.

Results

Growth and Colony-forming Efficiency of mESCs in Serum-containing Medium are Density-Dependent

We first examined whether mESC growth is density dependent, which is a simple indicator (with caveats, elaborated below) of the existence of autocrine growth-supportive factors in a culture [16]. Throughout, we differentiate between growth (which is the rate of change of cell number in the culture) and proliferation (travel through the cell cycle, i.e., mitosis); the
rate of cell growth is equal to the rate of cell proliferation minus the rate of cell death. We plated ABJ1 mESCs at various densities and measured the increase in cell number at the ends of day 1 (24 hours after plating) and day 2. We observed density-dependent growth during both time periods (Figure 1a, day 1, \( p = 5.7e-6 \), day 2, increasing region (250–2500 cells/cm\(^2\)), \( p = 0.003 \)). Separately, we determined that the lag phase for mESC culture is less than 12 hours (Fig. S1). Thus the density-dependent fold increase in cell number over day 2 is solely due to differences in the growth rate, and not due to variations in the length of the lag phase. We observed similar trends for a second independently derived cell line (Fig. S2, CCE mESCs). The average doubling time on day 2 for ABJ1 mESCs was \(~11\) hours, similar to that measured for D3 mESCs by Stead et al. [10]. The decrease in growth observed on day 2 for densities greater than 5000 cells/cm\(^2\) is likely due to a combination of nutrient depletion and the accumulation of metabolites (Fig. S3). To determine the contribution of density-dependent colony-forming efficiencies on the observed density dependence of growth, we measured the colony-forming efficiency of cells plated at two different densities using a microfluidic device that enabled the plating of single cells at defined densities (Supplemental Information). The colony-forming efficiencies we obtained (\(~40–60\%\)) are comparable to those measured by others [17]. Importantly, we found that the colony-forming efficiency was \(~15\%\) higher at the higher plating density (Figure 1b, \( p = 0.004 \); Fig. S2), in agreement with the fold growth data. These results suggest that an autocrine survival factor may exist in mESC cultures.

**mESC cultures contain one or more autocrine growth-supportive large molecules**

While a density-dependent growth increase is usually associated with the presence of an autocrine growth-supportive factor in the culture [16], such an effect could also be produced by the density-dependent depletion of a growth inhibitor from the medium. Since the contents of serum are not defined, it is not possible to rule out the existence of a growth-inhibiting molecule as one of its constituents. To distinguish between these two mechanisms, we used a complementary approach of using conditioned medium (CM) from culture in a serum-free medium to determine if cell-secreted molecules are growth supportive, along with assays of the constituents of the serum-free medium to determine if they are growth inhibitory. We focused on large molecules (>3 kDa) as they are the most common signaling molecules, and because we could dialyze the conditioned media with fresh media to equilibrate any changes in the small molecule fraction of the media (e.g., due to nutrient depletion).

As a first step, we grew cells in a serum-free medium formulation (N2B27 + LIF + BMP-4) developed by Ying et al. [15] specifically for expanding mESCs while maintaining them in a pluripotent state. We observed density-dependent growth in this medium as well (Fig. S4). Next, we collected conditioned medium from cultures, dialyzed it against fresh media using a 3 kDa membrane, and compared growth of cells in dialyzed CM against control medium that was treated identically except that it was never cultured with cells. We observed that the large-molecule fraction of CM (> 3 kDa) prepared using this medium was growth supportive on day 1 and day 2, resulting in a \(~15\%\) increase in growth (relative to control) on day 1, and a \(~25\%\) increase in growth on day 2 (Figure 2a).

The serum-free medium (N2B27) consists of a 1:1 mix of N2 medium and Neurobasal/B-27 medium, to which leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP-4) are added to prevent the differentiation of the mESCs. There are a total of 7 large molecules in the final medium – bovine serum albumin (BSA), transferrin, insulin, superoxide dismutase 1 (SOD-1), catalase, LIF, and BMP-4 [15,18] (Supplementary Information). Although these molecules have been shown to be growth supportive for several other cell types [5,6,19–21], we examined whether they might be growth inhibitory for mESCs. While the contents of B-27 are known [18], the relative amounts are proprietary.
(Invitrogen), precluding the ability to remove the protein components on an individual basis. Instead we removed the protein components of N2 (BSA, transferrin, and insulin) individually, and then removed the high MW components of the B-27 formulation entirely. When we removed the BSA or insulin from N2, we observed no significant change in the growth of mESCs over two days (in N2B27 + LIF + BMP-4, Figure 2b), indicating that unless there is a substantial decrease in the concentration of these proteins (these proteins are present in B-27 as well [18]), they are neither growth supportive nor inhibitory. When we removed transferrin from N2 we found that the growth rate of mESCs decreased by ~15% (p<0.05), demonstrating that transferrin is growth supportive for mESCs. When we removed the high MW components of B-27 altogether, there was an ~60% decrease in mESC growth (p<0.05) indicating that B-27, which contains BSA, transferrin, insulin, SOD-1, and catalase [18], is strongly growth supportive, perhaps due to the synergistic action of some of these proteins. Separately, we also examined the impact of catalase supplementation on the growth of mESCs in N2, and observed a growth-supportive effect (Fig. S5). Finally, although complete removal of LIF and BMP-4 from the medium would induce differentiation, we assayed whether reduction of LIF or BMP-4 concentrations by 50% would affect growth, which it did not. Other studies have shown that near-complete depletion of LIF [5] or BMP-4 [6] does lead to a reduction in mESC survival, indicating that they are growth-supportive as well. Together these results demonstrate that the large (>3 kDa) molecules present in N2B27 + LIF + BMP-4 have either no effect or a supportive effect on growth, and thus are not growth inhibitory.

To summarize, the large-molecule fraction of CM is growth supportive and there are no large growth-inhibitory molecules in the original medium. Thus, the growth supportive effect of the CM must be due to the production of a large growth-supportive factor by the cells used to condition the medium. This further implies that the density-dependent growth observed in Figure 1 is at least partially due to the presence of a >3 kDa growth-supportive autocrine molecule.

The autocrine growth factor(s) in mESC cultures is (are) not mitogenic, but improve(s) survival

The growth rate of a culture can be increased via two mechanisms – a reduction in the length of the cell cycle (“mitogenic activity”, i.e., increased proliferation rate), or a reduction in the rate of cell death. We wanted to determine whether the enhanced growth of mESCs at particular densities was due to increased mitogenic activity and/or improved survival. If a mitogenic factor was being produced by the cells, one would expect that the G1 phase would be shorter (because S and G2/M are typically fixed), and hence the proportion of cells in G1 would decrease. Using flow cytometry, we measured the proportion of cells in different phases of the cell cycle at two densities at the end of day 1, and observed no difference (Figure 3a). Addition of CM also did not affect the cell-cycle distribution (Figure S6). This indicates that the growth enhancement at higher densities is not due to increased mitogenic activity but is instead likely due to a decrease in the rate of cell death. The lack of mitogenic activity is perhaps not surprising, given that ESCs appear to lack the mitogen-dependent early G1 phase [10].

Since the colony-forming efficiency improves with increasing density (Figure 1b), this suggests that there is indeed improved survival at the higher densities. To investigate this further, we measured the fraction of dead cells in the culture at the end of day 2. We focused on day 2 because difference in the death rate on day 1 could also be due to differences in the attachment efficiency. We added either CM or control medium to cells on day 2; at the end of day 2 we stained cells using YO-PRO-1 Iodide which selectively stains dead cells. We observed that when grown in CM, the fraction of stained cells was only 62 ± 10% of the
corresponding fraction for cells grown in control medium (p=0.01, Figure 3b). This result confirms that mESC secreted autocrine factors promote survival.

Interestingly, we observed that most cells that stained positively for YO-PRO-1 exhibited an unattached, rounded morphology (142/143 cells or 99%, Fig. S7), and conversely 95% (142/149) of unattached cells stained positively for YO-PRO-1 iodide, indicating a compromised membrane. Similarly, 97% of unattached cells showed the presence of DNA nicks via TUNEL assay (Fig. S8). Thus death and loss of attachment seem to occur almost simultaneously in mESC cultures. This observation enables an alternate method for measuring the rate of cell death in these cultures – via counting of the fraction of unattached cells in the culture. We counted the fraction of cells that were detached at the ends of day 1 and day 2, at 1000 cells/cm$^2$ and 5000 cells/cm$^2$, the latter being the density that shows the highest growth on day 2 in serum-free conditions (Fig. S4, ABJ1 cells). From this, we calculated the fraction of cells that detached on day 2, and indeed found a density-dependent decrease in the fraction of these cells (Figure 3c). Additionally, cells grown in CM on day 2 showed a ~50% reduction in the percentage of unattached cells (Fig. 3d), again confirming that the autocrine factor(s) decrease the rate of cell death in mESC cultures.

Cyclophilin A is an autocrine growth factor in mESC cultures

We next sought to identify candidate autocrine factors that might be responsible for the observed growth supportive effects. Cystatin C, TIMP-1, osteopontin, and clusterin have been identified (via multiplexed ELISA) as proteins secreted from mESCs grown without LIF for two days [22] (at concentrations of > 1ng/ml), while TIMP-1 has also been found in the CM of mESCs grown with LIF [13]. Additionally, transcripts for osteopontin and clusterin have been detected in mESCs grown in medium containing LIF [23]. FGF-4 has been separately identified as being secreted by mESCs, but does not affect the survival or cycling rate of mESCs [24]. We examined the effect on mESC growth of supplementing the media with recombinant versions of each of these proteins at levels likely to be present in the culture and observed either no effect or a growth inhibitory effect (Supplemental Information).

To determine what other proteins might be secreted by mESCs, we performed a gel analysis of mESC conditioned medium followed by LC/MS-MS. We detected two bands in the CM lane of the gel that were not present in the control (Figure 4a, Supplemental Information). The lower-MW band was found to consist primarily of profilin-1 (UniProt MW 15 kDa), cystatin C (UniProt MW 15.5 kDa), and thioredoxin (UniProt MW 12kDa). The presence of profilin-1 is somewhat unexpected as it is an intracellular protein, although a study on proteins secreted by neurospheres found profilin-2, also an intracellular protein, in the CM [25]. A major component of the higher-MW band was found to be cyclophilin A (CypA), also known as peptidyl-prolyl isomerase A (Ppia).

We next sought to determine whether supplementing media with any of these factors would increase survival. As mentioned above, we found that cystatin C was not growth supportive for mESCs (Supplementary Information). CypA secretion has been shown to be induced by oxidative stress [26, 27], and CypA has been shown to protect various types of cells against cell death induced by such stress [26, 28]. Additionally, CypA$^{-/-}$ embryos show slightly reduced fertility [29] indicating that it may play a protective role in embryos, and CypA protein is expressed in mouse ESCs [30]. Given this information, we decided to investigate the effects of CypA on mESC growth. Based on the relative intensity of staining of the bands in the gel, we estimated the concentration of CypA in the CM at ~100 ng/ml. When we added recombinant CypA to N2B27+LIF+BMP-4 on day 2, we observed a 9% increase in growth at 100 ng/ml, and a significant 21% increase at 200 ng/ml for ABJ1 mESCs.

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This increased growth upon CypA addition was confirmed in an independently derived cell line (Figure S9b, D3 mESCs).

We next sought to block the cyclophilin A receptor. Yurchenko et al. have identified CD147 (also known as Basigin and/or Emmprin) as a receptor for CypA [31]. In particular, they determined that the proline 180 and glycine 181 residues in the extracellular domain of CD147 are critical for signaling induced by extracellular CypA. mESCs have been found to express the CypA receptor CD147 [32], with expression decreasing as the cells differentiate. We also detected the presence of the CD147 protein in mESCs (Figure S10). No monoclonal antibody against mouse CD147 exists that has been shown to specifically target only the CypA binding site on mouse CD147. However, when we added a polyclonal anti-CD147 antibody on day 2 to mESC cultures plated at 5000 cells/cm², we did observe a decrease in the growth rate of ABJ1 mESCs by ~20% relative to normal goat IgG (Figure 4c, p<0.05). The observed decrease in growth upon addition of the receptor antibody is comparable to the increase in growth produced by the action of mESC CM (Figure 2a). Similar results were obtained for D3 mESCs (Figure S9). Together, these results demonstrate that cyclophilin A is an autocrine survival factor in mESC cultures.

While previous studies have shown that CypA-CD147 signaling in other cell types can lead to activation of the Erk pathway [28,31], studies in mESCs have suggested that inhibition of the PI3k/Akt pathway, but not the Erk pathway leads to changes in cell cycle [12] and rate of apoptosis [33]. However, we did not detect activation or inhibition of Akt upon addition of CypA to mESC cultures did not lead to activation of Akt (Fig S11), suggesting that CypA may improve survival in mESCs via an Akt-independent mechanism.

### Discussion

Identification of the extrinsic factors required for growth of self-renewing mESCs is interesting both to complement efforts focused on understanding the extrinsic factors required for self-renewal [15], and because improving the growth of ESCs in culture would aid in their expansion for therapeutic purposes. That mESCs can be clonally propagated suggests that there may be no extrinsic factors required for their growth, although autocrine loops can operate at the single-cell level. The observation that mESC growth is density-dependent suggests that, even if not required, autocrine factors may promote the growth of mESCs.

One potential approach for identifying such factors would be to examine the in vivo compartment from which ESCs are derived, namely the inner cell mass (ICM). Indeed, within reasonable limits, mouse, bovine, and porcine embryos show improved survival at higher densities [34–36], suggesting the presence of soluble signals, which appear to act via the PI3-K pathway [37]. Additionally, ICM cells have been found to undergo apoptosis in vivo; ~10–20% of cells in the mouse and human ICM are undergoing apoptosis on Day 5 post-fertilization [38]. Again, the percent of dead cells has been shown to be inversely correlated with the total number of cells in the blastocyst, suggesting that anti-apoptotic factors may play a role in modulating this process. However, ESCs may not be identical to cells of the ICM [39].

We have used an alternate approach for identifying ESC growth/survival factors, which is to identify factors secreted directly from mESCs, in vitro. While two previous studies have examined the mESC secretome [13,22], they both used ELISA-based approaches, which are limited to assaying factors for which antibodies are available. Instead, we used mass spectrometry to identify secreted factors present in the media that could be candidate autocrine molecules. From this list of factors, we identified cyclophilin A as an autocrine...
ligand in mESC cultures. It is possible that other factors in addition to CypA act in an autocrine fashion to affect the growth of mESCs. The approach taken in this study helps guide the discovery of future factors. For instance, the lack of change in the cell cycle distribution across cell density or with CM (Figure 3, Figure S6) makes it likely that additional autocrine growth factors will also be survival factors, rather than mitogens.

Cyclophilin A was isolated in 1984 as a protein with high affinity for the immunosuppressive drug Cyclosporin A [40] (hence the name cyclo-philin A). Additionally, most cyclophilins possess peptidyl-prolyl-isomerase activity, i.e., they help in the folding of proteins [41]. While the above two functions are associated with intracellular CypA, subsequent studies found that CypA is also present extracellularly [42]. While CypA appears to be expressed ubiquitously [41], recently it has been found that CypA is overexpressed in several cancers [43–45], suggesting that it may play a role in carcinogenesis.

It has been shown that some of the actions of extracellular CypA are mediated through its binding to CD147 [31,43], which is also highly expressed in some cancers [46]. However, it is not clear whether the growth-enhancing effect of Cyclophilin A is mediated by CD147 alone. One group reported that a CD147 antibody blocked the pro-growth effect of CypA in human pancreatic cancer cells [43], but not in human vascular smooth muscle cells [47], suggesting that CypA may act through more than just CD147. With regard to its expression in mESCs, CD147 has been found to be expressed in the inner cell mass of mouse blastocysts [48], and it has been recently found to be expressed more strongly in mESCs than in their differentiated progeny [32].

Interestingly, CypA also appears to be produced by human embryonic stem cells (hESCs; [49], supplemental data), and the CD147 precursor protein was detected in the plasma membrane of hESCs via mass spectrometry [50], raising the possibility that CypA plays a protective role in hESC cultures as well. This may have useful implications for improving the cloning efficiency of hESCs, which can be as low as 1% [51]. Additionally, if CypA-CD147 signaling is important for the survival of hESCs and other human stem cells it may be important for the survival of human cancer stem cells as well.

Materials and methods

Cell culture and staining

ABJ1 (with Oct4-GFP reporter, kindly provided by George Daley’s lab), CCE, and D3 mESCs were cultured in TCPS dishes (Nunc) coated with 0.1% gelatin (Millipore) in a 37°C humidified environment with 7.5% CO₂. For maintenance of mESCs, we fed cells daily and passed every other day at 10,000 cells/cm². Serum-containing media consisted of DMEM (Invitrogen) supplemented with 15% ES-qualified fetal bovine serum (Invitrogen), 4 mM L-glutamine (Invitrogen), 1 mM non-essential amino acids (Invitrogen), 50 U/mL penicillin, 50 μg/mL streptomycin (Invitrogen), 100 μM β-mercaptoethanol (Sigma-Aldrich), and 1000 U/ml leukemia inhibitory factor (LIF, Millipore). Serum-free medium consisted of N2B27 supplemented with LIF and BMP-4 [15]. N2B27 is a 1:1 mixture of N2 medium and B27 medium: the N2 medium consists of 25 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 20nM sodium selenite, 100 μM putrescine (all from Sigma-Aldrich), and 50 μg/ml BSA (Invitrogen), in DMEM/F12 (Invitrogen); B27 medium was made by adding B27 supplement (Invitrogen) to Neurobasal medium (Invitrogen). N2B27 was supplemented with 1000 U/ml LIF (Millipore) and 10 ng/ml rhBMP-4 (R&D Systems). Cells in serum-free medium were dissociated from the surface with TrypLE Select (Invitrogen) instead of 0.25% trypsin-EDTA (used for serum-containing media). Cells were allowed to adapt to serum-free medium by culturing them for 10–15 passages in this medium prior to performing.
experiments in this medium. Experiments were performed between 3–7 passages after thawing cells.

Recombinant mouse (rm) Cystatin C, rmTIMP-1, rmOsteopontin and rmClusterin, rhFGF-4, and rhCyclophilin A were purchased from R&D Systems. Human Cyclophilin A (CypA) shares a 96% sequence similarity with mouse CypA, while human FGF-4 shares an 80% sequence similarity with mouse FGF-4. Goat anti-mouse CD147 polyclonal antibody and goat IgG control were purchased from Santa Cruz Biotechnology.

Staining with YO-PRO-1 Iodide (Invitrogen) was performed for 15 minutes at 37°C, at 2 μM final concentration. TUNEL assay reagents were purchased from Roche and staining was performed according to manufacturer’s protocols.

**Growth and colony-forming efficiency measurements for various densities**

Growth versus density experiments were performed in 12-well plates (Nunc, Delta surface), in which the wells were large enough to allow for uniform seeding. Cells were counted using a Coulter Counter (Beckman Z2). Measured cell numbers were adjusted by subtracting background counts from control media with trypsin/TryptLE but not containing cells. To allow for the accumulation of autocrine factors and to avoid a potential second lag phase associated with cells having to adapt to fresh medium, we did not feed cells during the 48 hour assay period.

Colony-forming efficiency was measured using a microfluidic device that allows plating of cells at defined densities (Supplemental Information). The initial number of cells and the number of colonies at the end of day 2 were counted using phase-contrast microscopy (Zeiss Axiovert 200). Data shown is from three experiments. As above, cells were not fed during the 48 hour assay period.

**Conditioned medium**

We prepared conditioned medium (CM) by collecting medium from a dish and filtering it through a 0.25 μm filter (with a syringe) to remove floating cellular debris. The control was incubated for the same length of time in a similar dish as the cell culture, and filtered in an identical manner. We then dialyzed equal amounts by weight of CM and control using a 3 kDa centrifugal filter unit (Millipore), replenished the > 3 kDa fraction with fresh < 3 kDa nutrient components, and again checked that both media had the same final weight. We also added LIF (1000 U/ml) and BMP-4 (10 ng/ml) to compensate for any potential depletion during culture, to ensure that cells would be maintained in a self-renewing state. For day 1 growth/death assessment, CM was collected at the end of day 1 from cells plated at 15000 cells/cm². For day 2 growth/death assessment, CM was collected at the end of day 2 from cells that were plated at 30000 cells/cm², and then fed at 24 h with fresh medium. The CM was applied to cells plated at 1000–2000 cells/cm². Experiments were performed three times, in triplicate each time. Data from different days were normalized using the control data.

**Cell cycle analysis**

Cells were detached using TrypLE (Invitrogen), permeabilized with 0.1% Triton X, and stained with 50 μg/ml Propidium Iodide (Sigma) in the presence of 500 μg/ml Ribonuclease A (Sigma). The samples were then analyzed using a FACScan cytometer (Becton Dickinson). The data obtained was analyzed using ModFit LT (Verity Software House) to determine the percentages of cells in the various phases of the cell cycle.
Statistical analysis of density-dependent growth

ANOVA was performed on fold growth data for day 1 to obtain the presented p-value. For comparison of growth for day 2, we used ANOVA on day 2 growth data for the first 3 densities shown i.e. 250, 1000, and 2250 cells/cm\(^2\), which corresponds to the increasing region of the growth curve for this period of time. Since cells at this density show no difference in fold growth at the end of day 1, it is enough to just compare the growth at the end of day 2 to make a statement about growth on day 2. Adjusting for the day 1 growth led to similar results (Fig. S2).

SDS-PAGE and mass spectrometric analyses

Cells were plated at 30,000 cells/cm\(^2\), fed on day 1, and CM was collected at the end of day 2. Control medium (RM) was similarly incubated, but without cells. 4 ml CM and RM were concentrated to approximately 100 μl using a 3 kDa centrifugal filter unit (Millipore), CM and RM were mixed 1:1 with Laemmli buffer (Bio-Rad), heated at 95°C for 5 minutes, and run on 12% or 18% polyacrylamide gels (Bio-Rad) at 15 mA. Gels were stained using a silver stain (SilverQuest, Invitrogen) for visualization. For mass spectrometric analyses, 15 ml CM and RM were concentrated using a 3 kDa centrifugal filter unit (Millipore) first, followed by drying in a SpeedVac to approximately 15 μl. Following SDS-PAGE, gels were stained using Coomassie Blue (SimplyBlue, Invitrogen). Appropriate bands were analyzed using LC/MS-MS at the Biopolymers Laboratory at the Koch Institute for Integrative Cancer Research (MIT).

Cell detachment analysis

Cell culture supernatant (including detached cells) was gently triturated once while in the well-plate and then counted using a Coulter Counter (Beckman Z2, 10–20 μM size range). To measure the disintegration rate of the unattached cells we incubated culture supernatant (containing unattached cells) for 24 hours and counted the number of cells at the beginning and end of the incubation. We found that detached cells disintegrated with a half-life of 10.5 ± 0.9 hours (Supplemental Information). Thus the number of cells that detached during day 2 was determined as: Number of cells detached at the end of day 2 (i.e. over days 1 and 2) – 0.21 × Number of cells detached at the end of day 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Density-dependent growth of mESCs. (a) Fold growth over day 1 and day 2, for ABJ1 mESCs plated at various densities. (b) Colony-forming efficiencies for ABJ1 mESCs plated at different densities, assessed at the end of day 2. Cells plated at 3000 cells/cm² have ~15% higher colony-forming efficiency than cells plated at 300 cells/cm² (p=0.004).
Figure 2.
Effects of secreted proteins and medium proteins on mESC growth. a. Fold growth over day 1 and day 2 for ABJ1 mESCs grown in conditioned medium versus control. CM increased proliferation by ~15% on day 1 (p=.02) and ~25% on day 2 (p=5e-4). b. Fold growth over day 2, relative to control, in media where different components were removed. Removal of BSA or insulin (Ins) from N2 resulted in no significant decrease in proliferation. Removal of transferrin from N2 (Trans), or of the high MW components of B-27 entirely, caused significant growth reduction (p=7e-4 and 1e-3 respectively). Reduction of LIF or BMP-4 concentrations by 50% resulted in no change in fold growth.
Figure 3.
a. Cell cycle distribution for cells plated at 1000 cells/cm$^2$ and 10,000 cells/cm$^2$. b. The percentage of YO-PRO-1 positive (dead) cells in the culture at the end of day 2 for cells grown in conditioned medium (CM) versus control ($p=0.01$). c. The fraction of cells that detached on day 2, for two plating densities ($p=0.01$). d. The fraction of unattached cells at the end of day 2 for cells grown in conditioned medium (CM) versus control ($p=2e-5$).
Figure 4.
a. Image of a portion of a silver-stained gel resulting from SDS-PAGE of medium conditioned by ABJ1 cells and control (serum-free) medium. The arrows indicate the location of two additional bands observed in the ABJ1 CM lane. b. Fold growth on day 2 of mESCs cultured with different concentrations of cyclophilin A (added on day 2), showing that growth increases in a concentration-dependent manner (p=0.03) c. Fold growth on day 2 of mESCs after addition of no antibody, goat IgG, or a polyclonal antibody to CD147 (2 μg/ml), showing significantly decreased growth upon addition of the CD147 Ab (p(CD147, normal IgG) = 4e-6, p(CD147, PBS) = 2e-8).