Stress Forces First Lineage Differentiation of Mouse Embryonic Stem Cells; Validation of a High-Throughput Screen for Toxicant Stress

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Mouse Embryonic Stem Cells (mESCs) are unique in their self-renewal and pluripotency. Hypothetically, mESCs model gestational stress effects or stresses of in vitro fertilization/assisted reproductive technologies or drug/environmental exposures that endanger embryos. Testing mESCs stress responses should diminish and expedite in vivo embryo screening. Transgenic mESCs for green fluorescent protein (GFP) reporters of differentiation use the promoter for platelet-derived growth factor receptor (Pdgfr) α driving GFP expression to monitor hyperosmotic stress-forced mESC proliferation decrease (stunting), and differentiation increase that further stunts mESC population growth. In differentiating mESCs Pdgfra marks the first-lineage extraembryonic primitive endoderm (ExEndo). Hyperosmotic stress forces mESC differentiation gain (Pdgfra-GFP) in monolayer or three-dimensional embryoid bodies. Despite culture with potency-maintaining leukemia inhibitory factor (LIF), stress forces ExEndo as assayed using microplate readers and validated by coexpression of Pdgfra-GFP, Disabled 2 (Dab2), and laminin by immunofluorescence and GFP protein and Dab2 by immunoblot. In agreement with previous reports, Rex1 and Oct4 loss was inversely proportional to increased Pdgfra-GFP mESC after treatment with high hyperosmotic sorbitol despite LIF. The increase in subpopulations of Pdgfra-GFP⁺ cells>background at ~23% was similar to the previously reported ~25% increase in Rex1-red fluorescent protein (RFP)-negative subpopulation at matched high sorbitol doses. By microplate reader, there is a ~7–11-fold increase in GFP at a high nonmorbid and a morbid dose despite LIF, compared with LIF alone. By flow cytometry (FACS), the subpopulation of Pdgfra-GFP⁺ cells>background increases ~8–16-fold at these doses. Taken together, the microplate, FACS, immunoblot, and immunofluorescence data suggest that retinoic acid or hyperosmotic stress forces dose-dependent differentiation whether LIF is present or not and this is negatively correlated with and possibly compensates for stress-forced diminished ESC population expansion and potency loss.

Keywords: high throughput screens, embryonic stem cells, hyperosmotic stress, transcription factors, potency, compensatory differentiation

Introduction

PROLIFERATION AND DIFFERENTIATION of the embryonic stem cell (ESC) lineage is highly regulated during embryonic development. Many stimuli adversely affect embryogenesis by altering the balance of the potency and differentiation of stem cells [1,2]. Normal differentiation

occurs in cultured ESC when leukemia inhibitory factor (LIF) is removed and extraembryonic endoderm (ExEndo), and then the three germ layers, endoderm, mesoderm, and ectoderm, arise to emulate gastrulation in vivo [2,3].

Increasing stress exposures result in miscarriage or pre- and/or postnatal disease. Animal models are used to study stress-related embryonic abnormalities, but in Developmental

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Toxicology, “reduce, refine, and replace” are the 3Rs of expediting and lowering costs of testing. Thus, ESCs are needed to reduce animal use, and assays that rapidly monitor potency and differentiation in response to toxicological stress should reduce animal testing.

Hyperosmotic stress forces nuclear transcription factor loss in two-cell embryos and the ESC and the placental trophoblast stem cell (TSC) lineage in the blastocyst [4–8]. Moreover, stress forces potency transcription factor loss in nontransgenic ESCs and transgenic Rex1 promoter-red fluorescent protein (RFP) reporter ESCs [9–11], despite LIF and this should force differentiation to first-lineage ExEndo despite LIF [9].

Hyperosmotic stress was used to clone the first stress enzymes in yeast [12], test for stress effects in mammalian oocytes [13,14], embryos [7,15–17], TSCs [18,19], ESCs [9], and validate Rex1-RFP reporter ESCs [10]. Fluorescent promoter reporters are used to directly monitor the potency factor or differentiation factor activity levels in viable stable transgenic ESC.

Normally the embryo is in an exponential growth phase during the blastocyst formation and resulting in ESC and TSC lineages. For both cultured ESCs and TSCs, stress forces decreased growth and potency and increased differentiation to first lineage, despite the presence of conditions that should maintain potency and growth [2]. A previous study showed that stress forces potency loss of Rex1 promoter-RFP reporter ESCs' increased potency loss in 28% for cells exposed to high morbid stress [10]. ESC's respond to the stress forced diminished growth by differentiating early resulting in insufficient stem cells and possibly contributes to miscarriage [9–11]. This high-throughput screen (HTS) was validated by four corroborating assays [10] and a reduce-to-practice study using 22 pharmaceuticals and pesticides showed that potency loss gave reliable half maximal inhibitory concentrations (IC50)s [11].

Platelet-derived growth factor receptor (Pdgfr)-green fluorescent protein (GFP), precisely reports ESC differentiation to first-lineage ExEndo in vivo or in cultured ESCs that normally expresses the Pdgfra gene after LIF removal or retinoic acid (RA) addition [20,21]. But, these previous reports did not study stress-forced differentiation. Under stress conditions, the Pdgfra-GFP hypothetically reports ESC differentiation to first-lineage ExEndo that normally expresses the Pdgfra gene [20,21]. We test this hypothesis in this study, using a microplate reader to detect GFP in HTS mode, and corroborated by immunofluorescence, immunoblot, and flow cytometry (FACS).

Materials and Methods

Materials

Mouse 129S4-derived AK7 ESCs that express transgenic fluorescent protein (GFP) that report Pdgfra and ExEndo expression was a kind gift from Dr. Anna-Katerina Hadjantonakis (Sloan-Kettering Institute, New York, NY). This cell line is heterozygous for a knockin H2B-GFP fusion gene replacing the first two immunoglobulin domains of the Pdgfra ligand-binding domain, enabling GFP expression to report ESC differentiation to ExEndo [22]. The parental 129S4/SvJae ESC (J1 mouse strain) was purchased from

ATCC (Manassas, VA) [23]. Dulbecco's modified Eagle's medium (DMEM) was from HyClone (Logan, UT). Gibco™ glutamine supplement solution and sodium pyruvate were from Life Technologies (Grand Island, NY). ESC-qualified EmbryoMax fetal bovine serum, 0.1% gelatin solution, and ESGRO™ Mouse LIF medium supplement were from EMD Millipore (Billerica, MA).

Phosphate-buffered saline (PBS) ± calcium and magnesium were from HyClone Laboratories (Logan, Utah). Basement membrane matrix (Matrigel) and Alexa 633-conjugated donkey anti-rabbit antibody were from BD Biosciences (Bedford, MA). Rabbit anti-Rex1 antibody (AB2814) was from Abcam (Cambridge, MA). Mouse anti-GFP antibody (SC9996), mouse anti-Oct3/4 antibody (SC5279), rabbit anti-laminin antibody (SC5583), and rabbit anti-Dab2 antibody (SC13982) were from Santa Cruz Biotechnology (Santa Cruz, CA). We note that SC5279 detects the pluripotency-maintaining isoform of Oct4 [24]. Mouse monoclonal anti-tubulin antibody (T4026) was from Sigma-Aldrich (St Louis, MO) and horseradish peroxidase (HRP)-conjugated second antibodies were from Cell Signaling Technology (Danvers, MA).

The 7-Aminoactinomycin D (7-AAD), Pierce RIPA lysis buffer, protease inhibitor cocktail, and Pierce BCA protein assay reagent were from Thermo Scientific (Rockford, IL). ECL chemiluminescence reagent was from GE Healthcare Bio-Sciences (Pittsburgh, PA). MEM nonessential amino acid solution, sorbitol, 2-mercaptoethanol, and other chemicals were from Sigma (St. Louis, MO).

ESC culture and stimulation

Mouse transgenic and parental ESCs were cultured in the absence of feeder cells in DMEM supplemented with 15% mouse ESC (mESC)-screened fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 1,000 U/mL murine LIF on 0.1% gelatin-coated tissue culture plastics at 37°C in humidified air with 5% CO₂ as done previously [10,11,25]. ESCs were passaged at 20% and cultured overnight to acclimate and avoid passage stress, reach 40% confluence at Tzero (Fig. 1A), and then were ready for stress stimulation. Osmotic stress was used by supplementing the culture media with different concentrations of sorbitol as described previously [9,10]. ESCs were cultured in medium after removal of LIF and addition of 1 μM RA as the positive control for differentiation to ExEndo as done previously [20,26].

In three-dimensional culture, the cells were loaded onto Matrigel and cultured with ESC medium supplemented with 2% Matrigel as done previously [27,28]. The Matrigel layer was made by loading 40 μL of Matrigel onto a 12-mm coverslip that was placed into 24-well plates with 0.5 mL media/well. ESCs were cultured overnight to adapt to Matrigel invasion and then stressed with different sorbitol concentrations for 4 days during spheroid formation.

Fluorescent plate reading as a HTS for stress induced ESC differentiation

mESCs were cultured on black 96-well plates overnight to reach 40% confluence. The cells were treated with sorbitol in LIF⁺ media with different sorbitol concentrations for 3 days. The cells were rinsed with PBS + Ca + Mg, then

FIG. 1. (A) The protocol for the biological experiments to test dose- and time-dependent stress responses of Pdgfra-GFP and the assays used to do so. (B) Stress-mediated ESC differentiation was monitored by the Pdgfra reporter GFP in two-dimensional cultured cells. Pdgfra-GFP cells were cultured on coverslips for 3 days with or without incremental concentrations of sorbitol as the stressor. Normal cultured ESC (LIF⁺) is the negative control LIF. (C) Stress-mediated ESC differentiation was monitored by Pdgfra reporter GFP in three-dimensional cultured cells. Pdgfra-GFP cells were cultured on Matrigel for 1 day to adapt and then for 3 days with or without stress. Normal cultured ESC (LIF⁺) is the negative control for differentiation and positive control for potency. Stimuli for 3 days were LIF removal (LIF⁻ for normal differentiation), 1 μM RA and LIF removal for the normal differentiation favoring extraembryonic endoderm, or 200 mM sorbitol are the stressors. After the culture, the cells formed spheroidal structures (shown here are the GFP *green* fluorescent images (Pdgfra reporter GFP) superimposed on the phase contrast images of the spheroidal structures. GFP, green fluorescent protein; (Pdgfr)a, platelet-derived growth factor receptor; ESC, embryonic stem cell; LIF, leukemia inhibitory factor; RA, retinoic acid.

measured for GFP fluorescence using a Synergy H1 Multi-mode microplate reader (BioTek, Winooski, VT). The excitation and detection wavelengths for GFP were 469 and 525 nm, respectively. After GFP reading, the cells were stained with 5 μM of Hoechst 33342 for 30 min in a 37°C incubator. The cells were rinsed and measured for Hoechst-stained nuclei using excitation and detection wavelengths at 392 and 440 nm, respectively.

The optimal stimulation period was tested with a time course, where ESCs were treated with sorbitol concentrations (200, 250, 300, and 350 mM) for 1–4 days. The results showed that on day 3, GFP fluorescence expression level reached a high plateau (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). This enables comparison with Rex1-RFP ESCs, which were optimized for a 3-day exposure [10]. Considering that the average half-life of wild-type GFP is ~26 h [29], we chose 3 days as the treatment time in this study. If stress

completely turns off the Rex1 promoter, residual RFP would be ~50% at T24 h, ~25% at T12 h, and ~12.5% at 72 h.

The loss rate of RFP requires 72 h to provide an ~87.5% Rex1-RFP decrease. For Pdgfra-GFP, 250 mM sorbitol causes a ~2-fold decrease in GFP from 3–4 days, whereas 200 mM sorbitol increases GFP ~50%. This suggests that stress at 250 mM sorbitol is either turning off the Pdgfra promoter or causing morbidity from 3 to 4 days. Thus, a 3-day exposure was chosen.

To validate the Hoechst staining, protein concentrations in cell lysates were measured. ESCs were cultured on 6-well plates and stimulated as described above. The cells were trypsinized, resuspended in PBS in 96-well plates, and assayed for GFP by microplate reader. After reading GFP, the cells were lysed with 50 μL of lysis buffer (1% NP-40, 0.5% TX-100, 100 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, and pH 7.5) and assayed for total protein concentrations by Pierce BCA protein assay following the manufacturer's instructions.

The GFP fluorescence readings from live cells were normalized/total protein concentration. The ratio of GFP/protein represents the average ESC differentiation to ExEndo.

Western blot

ESCs were rinsed with PBS, lysed with RIPA buffer supplemented with protease inhibitors, and sonicated. Total protein concentration was determined using Pierce BCA assay. Lysates was mixed with Laemmli sample buffer, boiled for 5 min, and equal protein amounts were loaded onto 5%–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels for electrophoresis. The protein bands were transferred onto a nitrocellulose membrane. Immunofluorescent signals were detected using ECL reagent with X-ray films and quantified using ImageJ 1.48v software (National Institutes of Health, Bethesda, MD) and protein bands were quantified.

Flow cytometric analysis of mESCs expressing differentiation reporter GFP

FACS was used to detect cell subpopulations corresponding to fluorescence detected by microplate reader and suggesting mean ExEndo differentiation. ESCs were cultured in 6-well plates to reach 40% confluence. The cells were treated with stressors as described in immunoblots above. ESCs were trypsinized, resuspended, and stained with the cell viability dye 7-AAD at 4 μ g/mL for 30 min at 0°C.

FACS results were analyzed using FlowJo 7.6.2 software (FlowJo, LLC., Ashland, Oregon). The raw data were processed to identify and subtract debris, aggregates, and dead cells. The live cells were then analyzed for fluorescence and for granularity. Live ESCs were first measured for geometric mean of fluorescence of total cells to give mean GFP intensity. Live ESC subpopulation sizes were quantitated for their green fluorescent intensity. By microscopic observation, we noticed that GFP levels are heterogeneous in cellular intensity but are in dark, intermediate, and the rare groups of bright cells (Supplementary Fig. S2). Micrographs were quantitated using SimplePCI DNN module (Hamamatsu Corp., Sewickley, PA) (Fig. 5).

Microscopic and micrographic observation and quantification of dark, bright, and intermediate fluorescent subpopulation sizes of ESCs stimulated under control and several hyperosmotic levels in Pdgfra-transgenic and the parental J1 cells, specified two fluorescence intensity gates, 1,000 and 5,000. These were used to separate the three cell subpopulations. Group sizes were counted and reported as the percentage of total live cells.

Granularity is a controversial yet often-observed index of cell differentiation [22,30]. In this study, we measured the side scatter area A, (SSC-A), values in the total live cells and in the three cell subpopulations. Geometric mean of the SSC-A values show the mean granularity of the cells in each group (Fig. 5, panel D–F).

Statistics

Data from at least three independent biological experiments were analyzed using Microsoft Excel and presented as mean $\pm$ standard error of the mean. Statistical analysis was done by Student's *t*-test. Geometric mean analysis

was used in the fluorescent FACS study; the brightness of the fluorescent labeled cells range from low to high over a logarithmic range of fluorescence axis. This feature of fluorescent FACS fits this criterion of geometric mean quantification, where higher reliability and repeatability were achieved than arithmetic mean quantification [31].

Results

To obtain the best regulation of the transgenic reporter, we chose ESCs that had a GFP reporter knocked into the Pdgfra structural gene and regulated by its normal promoter–enhancer. Randomly integrated transgenes may also be inactivated more readily [32]. GFP regulated by the wild-type Pdgfra promoter is activated in ExEndo cells, which are the first differentiated lineage from ESC lineage cells from the inner cell mass (ICM) of the mouse blastocyst [22]. The H2B gene was fused to the N-terminal of GFP, allowing the GFP nuclear expression. We use an experimental design (Fig. 1A) to test the hypothesis that Pdgfra-GFP ESC reports ESC differentiation to first-lineage ExEndo using a microplate reader to detect GFP in HTS mode, and corroborated by immunofluorescence, immunoblot, and FACS.

Normal culture with LIF present maintains Pdgfra-GFP ESCs potency and few green cells were found (Fig. 1B). Withdrawal of LIF with the addition of the ExEndo inducer RA [20,26], or addition of hyperosmotic stress despite LIF, induced GFP-positive cells that report Pdgfra⁺ ExEndo. LIF⁺ maintained Pdgfra-GFP ESCs in a nonexpressing state. Interestingly, in large ESC epithelia, GFP-expressing cells arose in groups of contiguous or nearly contiguous cells in small areas.

Embryoid bodies are used to emulate early peri-implantation interaction of outer ExEndo epithelia and inner pluripotent and differentiating cell types before gastrulation and also lineages arising at gastrulation due to interaction of the two lineages [33,34]. LIF suppressed Pdgfra-GFP in ESCs in embryoid bodies, whereas LIF removal with or without RA increased GFP expression and sorbitol overrode LIF and increased Pdgfra-GFP (Fig. 1C). Thus, in monolayer or embryoid bodies, LIF removal or RA without LIF induced differentiation, but hyperosmotic sorbitol overrode stemness conditions of LIF to force differentiation.

When Pdgfra-GFP were first tested (Fig. 2A), the microplate reader was used to sensitively determine fluorescence of cells, but protein level detection used to normalize GFP/cell number was less sensitive. Due to insensitive protein measurements, reporter ESCs were trypsinized after a 3-day treatment, transferred from the 6- to 96-well plate and after centrifugation, GFP was quantitated by microplate reader. Then the cells were lysed and protein concentration determination was performed. The 3-day exposure was optimized based on several criteria as discussed in Materials and Methods and as shown in Supplementary Fig. S1. To simplify the assay, Pdgfra-GFP cells were stressed in 96-well plates and tested on the microplate reader in the linear GFP intensity range and a highly sensitive Hoechst assay of nuclei done immediately after GFP testing at Tfinal. Side-by-side comparisons of Hoechst and protein normalization of Rex1-RFP showed that the two gave similar results but that Hoechst was more sensitive [11]. As shown in Fig. 2B, the stress-mediated ESC differentiation is dose dependent

when normalized to protein, similar to that observed from Fig. 2A when normalized to Hoechst.

Hyperosmotic stress significantly promoted ESC differentiation Pdgfra^+ status in a dose-dependent manner. Withdrawal of LIF and treatment with RA also significantly promoted differentiation. Microplate reading results are consistent with the microscopic observation and shows that the assays are accurate and sensitive for testing stress-forced, cultured ESC differentiation. Fig. 2A, B translated low sensitivity to high-sensitivity automatable assays for HTS GFP^+ - and total cell signal, but used a dosimetry of 2/3rd dilutions from stock that did not enable comparison with previously published Rex1-RFP sorbitol dose-response [10].

To compare doses used previously [9] in the Rex1-RFP validation [10], we used the GFP/Hoechst protocol in Fig. 2B, but added sorbitol doses at the high sorbitol dose range. Stress may generate autofluorescence in the RFP and Hoechst emission spectra, GFP and blue autofluorescence from parental ESCs exposed to the same sorbitol doses were subtracted from the Pdgfra -GFP signal to achieve a net signal, normalized to cell number. In this study, as with Rex1-RFP decreases, highest significant increases in Pdgfra -GFP were detected at 300–350 mM sorbitol (two-way t -Test, $P < 0.05$). Sorbitol at 400–600 mM was largely apoptogenic, but had the highest net Pdgfra -GFP/cell.

Immunoblot data support dose-dependent increase in ESC differentiation (Fig. 3), accompanied by stemness decrease with stress [10]. Similar dosimetry by microplate reader for GFP showed that RA and sorbitol doses above 250 mM increased Pdgfra -GFP the most as measured by immunoblots for GFP (Fig. 3A). Dab2 is an early ExEndo marker that is increased in stressed ESCs [9] and had a similar dose-dependent increase by immunoblot (Fig. 3B) compared with GFP tested by the microplate reader or immunoblot (Fig. 2A–C). Where GFP and Dab2 were high, after RA treatment and LIF removal, or with higher sorbitol doses despite LIF, Rex1 protein (Fig. 3C) and Oct4 protein (Fig. 3D) were lowest. As previously shown [9,10], Rex1 loss was greater and more stable than Oct4 loss after three days of RA or stress treatments [24].

To corroborate that Pdgfra -GFP is expressed in two ExEndo lineage markers, they were used to test for cellular coexpression with Dab2 and Laminin (Fig. 4). Generally, there was coexpression of cell autonomous markers of ExEndo markers, Dab2 and Pdgfra -GFP, in hyperosmotically stressed ESCs despite LIF (Fig. 4A). Interestingly, ESC differentiation by LIF removal without stress causes differentiation of coexpressing cells of the first-lineage ExEndo. In contrast to stress-forced differentiation, putative later ExEndo lineage cells that express Pdgfra but not Dab2 were induced. Laminin is a secreted molecule of the basement membrane and is expressed near GFP-positive cells (Fig. 4B).

It is possible that some remodeling removes laminin or GFP^+ cells may migrate away from laminin after secretion.

FIG. 2. (A) Fluorescence plate reading of GFP in live ESCs cultured on 6-well plates reports stress-mediated differentiation in a dose-dependent manner. Mouse ESCs were plated and cultured on 6-well plates. Then, the cells were treated with incremental concentrations of sorbitol for 3 days. The cells were trypsinized, harvested, washed, transferred to 96-well plates, and subjected for GFP green fluorescence reading. Then, the cells were lysed and subjected for protein concentration determination. Three types of controls were included: (1) normal culture (LIF^+); (2) LIF removal for normal differentiation (LIF^-); or (3) LIF removal with 1 μM RA for normal differentiation with a known morphogen. Green fluorescence per protein concentration (arbitrary unit) is plotted here. Asterisk (*) shows significance of the bars compared with the LIF^+ control $n = 3$. From triplicate biological experiments were tested for significant differences with two-way t -tests; $P < 0.05$. (B) Fluorescence plate reading of GFP in live ESCs cultured on 96-well plates reports stress-mediated differentiation in a dose-dependent manner. Mouse ESCs were plated and cultured on 96-well plates. Then, the cells were treated with incremental concentrations of sorbitol for 3 days. GFP green and Hoechst-stained blue fluorescence were determined on a plate reader as described in the Materials and Method and GFP intensity was normalized to Hoechst intensity to obtain average brightness per cell and this was normalized to LIF^+ (which was set to 100). Three types of controls were included: (1) normal culture (LIF^+); (2) LIF removal for normal differentiation (LIF^-); (3) LIF removal with 1 μM RA for normal differentiation with a known morphogen. Asterisk (*) shows significance of the bars compared with the LIF^+ control. Triplicate biological experiments were tested for significant differences with two-way t -tests; $P < 0.05$.

FIG. 3. GFP reporter level correlates with loss of Rex1 and Oct4 potency markers and gain of Dab2 differentiation marker. GFP protein assay by western blot. Pdgfra cells were stressed with incremental concentrations of sorbitol (mM) for 3 days as per Pdgfra-GFP culture before microplate reader assay in Fig. 2A–C. Cells were lysed, size fractionated by SDS-PAGE, transferred to blots, developed using specific antibodies as noted for each blot and graph above, HRP, and substrate, followed by exposure to film and quantitation of band density by ImageJ software. Treatment doses are in the X-axis, normalized band densities of protein of interest/tubulin loading control, and then normalized to the LIF⁺ control which was set to 1. Three types of controls were included: (1) normal culture (LIF⁺); (2) LIF removal for normal differentiation (LIF⁻); (3) or LIF removal with 1 μ M RA for normal differentiation with a known morphogen. Asterisk (*) shows significance of the bars compared with the LIF⁺ control $n = 3$. Triplicate biological experiments were tested for significant differences with two-way t -tests; $P < 0.05$. HRP, horseradish peroxidase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

As noted before, the Pdgfra-GFP reporter cells and their Dab2 or laminin markers tended to be expressed in small near-continuous groups, not dispersed in a salt and pepper distribution throughout the epithelium.

The measurement of reporter GFP fluorescent intensity by FACS revealed more details by gradation of fluorescent intensity. The geometric mean of the green fluorescence by FACS from the total live cells largely verified the fluorescent plate readings (Fig. 5A, B). Furthermore, the fluorescent measurement by FACS gave details about the contributions of fluorescence from the three subpopulations of cells (bright green, intermediate green, and dark cells) (Fig. 5C).

Although the bright and intermediate subpopulations of cells contributed to the increase of fluorescent intensity in proportion to the increase of sorbitol concentration, the contribution from the bright cell subpopulation increased sig-

nificantly only at the high sorbitol concentrations (>200 mM), whereas the contribution from the intermediate cell subpopulation increased significantly at sorbitol doses as low as 50 mM (one-way t -test, $P < 0.05$). These data suggest that the intermediate cell subpopulation plays a more important role in the magnitude of total green fluorescence (Fig. 5B, C).

Cellular granularity measured by FACS correlated with the reporter GFP fluorescent intensity by FACS. Cellular granularity is another important but controversial parameter and reporter of cellular differentiation or differentiation potential [35]. Side scatter measures cellular granularity and has reported to decrease with pluripotent stem cell (PSC) differentiation, but is associated with higher apoptosis and not uniquely a marker for stress-forced differentiation. Granularity analysis showed a similar trend as the change in total cellular fluorescence (Fig. 5D, B). Further analysis of

FIG. 4. Colocalization of Dab2 or laminin and Pdgfra reporter GFP in stressed ESCs. Pdgfra-GFP cells were cultured on coverslips for 3 days with or without stressor as in Fig. 2 and 3. Normal cultured ESC (LIF⁺, *left column*) is the control. Withdrawal of LIF (LIF⁻ and incremental concentrations of sorbitol at 100, 200, 250, and 300 mM) were the stress treatments in columns from *left to right* as indicated. After culture, the cells were fixed and immunostained for Dab2 (**A**) or laminin (**B**). Plotted here are GFP images (**A, B**; green, *top row*), Dab2 immunofluorescence (**A**, magenta, *middle row*) or laminin immunofluorescence (**B**, magenta, *middle row*), and DAPI-stained nuclei (**A, B**; blue, *bottom row*).

the three cell subpopulations (ie, bright, intermediate, and dark) revealed more details of granularity trends that were similar to the fluorescence outcomes (Fig. 5E).

The increase of granularity was proportional to increased sorbitol concentration, the significant increase of granularity in the bright and intermediate portions took place only in middle-to-high range of sorbitol (>200 mM), and the magnitude of granularity increase was within ~10% (Fig. 5E).

In contrast, the granularity change in the dark cell subpopulation was more significant from as low as 100 mM of sorbitol concentration and after LIF removal. The magnitude of granularity increase in response to the sorbitol dose in the dark cell portion was also much higher (~600%) than that of the bright and intermediate subpopulations (Fig. 5E). The extent of granularity in the dark cell subpopulation was always less (<50%) than the granularity in the bright and

intermediate portions. The granularity in the bright and intermediate portions was at a high level that reached a plateau at the high end of sorbitol concentrations.

Similar to GFP dose-response and the microplate reader dose-response, FACS showed RA differentiation control and dose-dependent hyperosmotic sorbitol override of LIF increases Pdgfra-GFP intermediate and bright subpopulation sizes. Bright groups increase from less than 0.01% in the LIF potency control to 0.05% in the RA differentiation control, 0.3 at 300 mM, and 9% at 350 mM sorbitol with LIF. This is much smaller than the intermediate subpopulation sizes of 1.5% in LIF potency control, ~23% in the RA differentiation control, at 300 mM sorbitol, and 28% at 350 mM sorbitol. Altogether, the intermediate and bright groups are 23.3% of all cells, which is similar to the 24.4% of Rex1-RFP cells, which lose all RFP at 300 mM sorbitol and are dark as nontransgenic ESCs at this dose [10].

Cell growth rate did not significantly change in the absence of LIF, in the presence of RA or in the presence of low-to-moderate concentrations of sorbitol through 200 mM (Fig. 6). Morbid doses at 300–350 mM are defined by having fewer cells at $T_{\text{final}} < T_{\text{zero}}$; with only 5%–5.9% of cells remaining at $T_{\text{final}}/T_{\text{zero}}$. An interesting submorbid dose 250 mM sorbitol, there are ~16-fold increases in intermediate Pdgfra-GFP⁺ and ~6-fold increases in bright Pdgfra-GFP⁺ cells (Fig. 5C). Thus, 250 mM would be a stunting dose that has increased differentiation. The doubling time of mESCs in culture dishes is 16–20 h. The starting confluence of cells for stress treatment in this experiment was ~35%.

Correspondingly, we chose 3 days as the standard time of stress treatment. Under normal culture (LIF⁺ control), cells reached 100% confluence before day 3. Measurement of blue fluorescence from Hoechst-stained cells ended up with result that is in proportion of the confluences between the day 0 and the day 3 LIF⁺ control (Fig. 6).

In contrast, cells under low-to-moderate concentrations of sorbitol or in the absence of LIF or in the presence of RA slowly reached 100% confluence by day 3 (Fig. 6, LIF⁻, RA, and 50–200 mM concentration range of sorbitol). However, the cell growth under high concentrations of sorbitol was significantly retarded. This retardation of cell growth is in proportion of sorbitol concentration at 250–350 mM.

Discussion

We report in this study that dose-dependent hyperosmotic stress forces decreased proliferation and stemness, and increased differentiation to first-lineage ExEndo by overriding culture conditions that should maintain stemness and proliferation. Pdgfra-GFP ESCs report these effects by mean fluorescence are assayed by the microplate reader. This supports the use of Pdgfra-GFP ESCs in an HTS.

Stress dose-dependent Pdgfra-GFP reports of forced differentiation are corroborated by immunoblot, immunofluorescence, and FACS. For immunoblot, similar dose-dependent increases in GFP and ExEndo marker Dab2 were observed compared with those from the microplate reader. For immunofluorescence stress-forced increase in ExEndo markers, laminin and Dab2, colocalized with GFP⁺ viable reporter cells. For FACS, bright and especially intermediate bright GFP⁺ cell subpopulation sizes increased in a dose-dependent manner. Taken together the HTS microplate reader assay is supported by three assays, suggesting that dose-dependent stress effects for many stressors should be testable in this HTS.

Stress-forced differentiation increase is complemented by stemness decrease

Differentiation is accompanied and requires decrease in stemness [1,2]. In agreement with this, immunoblots of Pdgfra-GFP ESC lysates showed that when differentiation markers GFP and Dab2 increased, stemness markers, oct4 and Rex1, decreased. In agreement with past studies, decreases in Rex1 protein were greater and occurred at lower stress doses than Oct4 protein, suggesting it is a more sensitive stress reporter that can report at nonmorbid stress doses [9–11]. But the dose-dependent, stress-forced Pdgfra-GFP increases reported in this study also are consistent with reports of stress-forced decreases in Rex1-RFP reported previously [10,11]. Consistent with the increase of total Pdgfra-GFP, bright and intermediate from 1.5% to 23.3% at 0 to 300 mM sorbitol reported in this study, we previously reported that Rex1-RFP increased from 8.6% to 24.4% at the same doses [10].

This suggests that Pdgfra intermediate and bright cells arise within stress-induced Rex1 dark population, and at every dose, the Rex1 dark subpopulation is >Pdgfra intermediate and bright subpopulations. This needs to be established experimentally. Although some Rex1-RFP dark cells could be fibroblast growth factor (FGF)5 extraembryonic “epistemic cell” marker positive, we previously showed that 24 h of hyperosmotic stress increases endogenous LRP2 and Dab2 but suppresses FGF5 [9]. Unpublished data from wild-type ESC embryoid bodies also show stress-induced decreased FGF5 and increases in ExEndo markers, Dab2 and LRP2 mRNA transcripts, at 24–48 h (unpublished data).

This suggests that stress favors first lineage over stemness (compensation for decreased proliferation) and first lineage over later lineages (aka prioritized differentiation) as theorized as a generic stress response to hyperosmotic, hypoxic, and other stresses [2,4,36–38]. Stress-forced compensatory and prioritized differentiation has been reported in ESCs, and TSCs, and effects in TSCs involve greater cell subpopulations [2].

Reversibility is established in TSCs, and can now be tested in stressed ESCs

Hypothetically, the intermediate group may be only partially or reversibly committed to first-lineage differentiation. This is suggested by the reversibility of Rex1⁻/Oct4⁺ and Rex1⁺/Oct4⁺ ESCs after FACS isolation and culture observed

FIG. 5. Hyperosmotic stress and RA increase reporter GFP expression and cellular granularity in FACS assay. Pdgfra-GFP ESCs were treated for 3 days with the indicated stimuli. Cells were harvested by trypsinization, stained with 7-AAD (to identify and exclude nonviable cells) and assayed for FACS. The gated live cells were analyzed for *green* fluorescence (A–C) and for granularity (D–F). (A); representative GFP histograms from the total live cells in one of three replicates were graphed for four orders of magnitude in the X-axis (*green* fluorescence intensity) and cell number in the Y-axis. The two X-axis gates, 1,000 and 4,000, are used to separate the bright, intermediate, and dark cell portions (see Materials and Methods section for rationale for gating). (B, C) Cells were analyzed for geometric mean of the *green* fluorescence from the total cells (B) or from each of the three portions (C). (D, E) Cells were analyzed for granularity (SSC-A) from the total cells (D) or from each of the three portions (E). (F) Representative granularity histograms from the total live cells in one of three replicates. Triplicate experiments were averaged with errors bars indicating the SEM and graphed in bar charts to show *dark* (black), intermediate (blue), and bright Pdgfra-GFP (*green*) Figure 5 can be viewed in greater detail online at www.liebertpub.com/scd cell numbers. Triplicate biological experiments were tested for significant differences compared with LIF⁺ control using Student's *t*-test. *, significant difference from total live cells. a, b, and c, significance from *dark*, intermediate, or *bright* portion of cells, respectively ($n=3$, $P<0.05$). SEM, standard error of the mean. FACS, flow cytometry; 7-AAD, 7-aminoactinomycin D. Figure 5 can be viewed in greater detail online at www.liebertpub.com/scd

under unstressed conditions with LIF [39]. These Rex1⁻ and Rex1⁺ cells became redistributed into identical heterogeneous pluripotent populations after 14 days of LIF culture. But, significant Rex1⁺ cell subpopulations were detected by day 1 and were substantial by day 5 in the Rex1⁻ sorted

cells. However, TSCs under hyperosmotic stress at 400 mM sorbitol [8] at day 2 of culture or at 0.5% O₂ [40] become irreversibly differentiated by day 4 of culture. These irreversibly differentiated TSC lineage cells brightly express first lineage marker placental lactogen (PL)1 protein after

FIG. 6. Comparison of cell growth rates under different stress conditions in plate reading assay of Hoechst-stained cells. Mouse ESCs were plated and cultured on 96-well plates and allowed to incubate overnight to reach ~35% confluence and maintain stable growth condition. One plate of cells was stopped from culture for the day 0 measurement. Other plates of cells were treated with incremental concentrations of sorbitol for 3 days. Three types of controls were included: (1) normal culture (LIF⁺); (2) LIF removal for normal differentiation (LIF⁻); (3) or LIF removal with 1 μ M RA for normal differentiation with a known morphology. Hoechst-stained blue fluorescence was measured using a plate reader as described in the Materials and Methods section. Shown here are averages and SEM from three replicates. a, significant difference compared with day 0. b, significant difference compared with LIF⁺ control ($n = 3$, $P < 0.05$).

exposures that would cause irreversible differentiation. Moreover, once stress-forced differentiation of TSCs occurs, stemness markers do not return and first lineage markers are not lost despite reculture in stemness media. The irreversibility of stressed TSC differentiation occurs over the same time period in stemness culture when unstressed ESCs are plastic in regaining stemness. It was previously shown that hyperosmotic stress forces ESCs to express Dab2 and LRP2 [9], members of the lipid uptake complex that arises in first differentiated lineage, primitive endoderm from E3.5 to E4.5 in the mouse blastocyst. The ICM of the blastocyst is Rex1⁺ but becomes differentiated to Rex1⁻ primitive endoderm. However, when primitive ExEndo markers arise, the embryo is plastic for commitment [41]. Thus, normal and stress-forced differentiation of ICM or cultured ESC to ExEndo may be reversible. But we anticipate that differentiation of intermediate bright Pdgfra-GFP cells is reversible and bright Pdgfra-GFP⁺ cells is irreversible, and this should be tested.

Nutrient acquisition by first differentiated lineage is a hypothetical adaptation to stress

Dab2 is first expressed at E4.5 in late blastocyst and continues to be expressed in visceral endoderm apposite the embryonic and extraembryonic ectoderm, which necessarily mediates embryo survival [42]. Both primitive and visceral endoderm express villin, absorb, and transport nutrients by a mechanism similar to small intestine, which also ramifies cell apical surface using villin [43]. Knockout of transcription factors required for ExEndo function, such as hepatocyte nuclear factor, lead to death of the embryonic ectoderm before gastrulation due to insufficient nutrients or other factors from the endoderm.

These events may not all occur in a monolayer of stress-forced differentiation of ESCs to primitive endoderm, but

stress-forced differentiation to ExEndo occurs in embryoid bodies, suggesting this may occur in the endoderm adjacent to the ICM (Slater et al., unpublished results). There are many markers of ExEndo that distinguish these cells from later-arising definitive embryonic endoderm [44]. Thus, it should be possible to test whether stress forces differentiation to first ExEndo lineages and later ExEndo and definitive endoderm lineages have fewer cells.

Stress-forced differentiation of ESCs affects smaller subpopulations than in TSCs and reports different outcomes

Although there is much testing of huiPSC and huESC with monogenic mutations that mediate human disease [45], testing for general stress responses affecting gestational embryogenesis is not done. We propose using HTS of ESCs and TSCs to test for stimuli that stunt stem cell growth and force compensatory differentiation, as a test for gestational growth stunting, miscarriage, and teratogenesis. Miscarriage testing has the clearest rationale for HTS TSCs testing, since trophoblasts produce a measurable pregnancy hormone—human chorionic gonadotropin beta subunit (hCG-beta) or PL1 (in mouse). Stressors leading to fewer cells and less beta hCG levels result in miscarriage.

These hormones maintain luteal progesterone and consequent implantation site nutrition for the exponentially growing embryo [2,4]. Hyperosmotic stress at 300 mM forces a maximal increase of the subpopulation of Rex1-RFP- ESCs at 24.4%. In this study, we show that bright Pdgfra-GFP, at this sorbitol dose (ie, 300 mM), is only 0.3%, but intermediate and bright together is 23.3%. Nonmorbid 250 mM also has similar subpopulation sizes of Rex1-RFP-cells and Pdgfra-GFP⁺ cells. For TSCs, hyperosmotic and hypoxic stresses increase differentiation, by morphology and PL1 expression to >50% bright cells [8,40,46–48].

TSC undergoes a higher level of differentiation at a similar stress level. TSCs become irreversibly differentiated at higher exposures of hyperosmotic or hypoxic stress [8,40]. Hypothetically TSCs best predict miscarriage, but ESC lineage cells in the ICM and embryonic ectoderm support TSCs through necessary signaling of FGF4 [49], and thus may report miscarriage. Moreover, submorbidly stressed ESCs may retain pluripotency and sustain epigenetic errors that propagate through germ cells into the next generation. This hypothesis is supported by studies on loss of imprinting, as during mouse embryo in vitro fertilization culture is different in different media [50], but is proportional to the amount of stress (eg, stress-activated protein kinase/SAPK activity) and to loss of growth in the same media [51]. However, loss of imprinting occurs at higher levels in the TSCs [52]. Thus, ESC and TSC HTSs may have unique and shared means to report stresses that can affect pre- and postnatal pathology.

Pdgfra-GFP⁺ bright cells arise in near contiguous groups leaving the predominate group of cells in a Pdgfra-GDP intermediate or dark state. It is not clear how this near contiguous differentiation occurs. It is unlikely that stress forces differentiation of a founder cell in the lineage and that cell proliferates. Fairly a high number of cells arise at higher sorbitol doses where proliferation is nil. A second hypothesis is that daughter cells from an earlier set of division have restricted in pluripotency to be primed to the primitive ExEndo. A third hypothesis is that differentiating cells recruit nearby cells to differentiate. It is suggested that human ESCs in microprinted culture wells self-pattern differentiation by growth factors [53,54]. This hypothesis can be tested by time-lapse micrography and trial inhibition with siRNA to growth factors.

Priming ESC lineage cells in culture or in the blastocyst generally means a decrease of pluripotency and progression to a state of epistemic cell status [55,56]. If priming includes restricting potency and this leads to irreversibility or limiting number or size of later lineages, this could be teratogenic. This needs to be tested in embryoid bodies and during peri-implantation stress in vivo.

Finally, the immunoblot data suggest that increased GFP or DAB2 is not an agonal response as has been reported for other stresses [57]. Although high exposures of many stressors act through general pathways that induce a large number of outcomes in agonal doses, our data suggest that Rex1 and Oct4 are lost at high stress doses and that GFP and DAB2 undergo diminishing or negative marginal changes at high stress doses. This is similar to first-lineage induction in TSC, where HAND1 increases through 400 mM sorbitol but decreases at higher, agonal dose [48]. Thus, in ESC and TSC, stress-forced stemness loss continues at highest doses, and stress-forced differentiation increases at submorbid-to-morbid doses, but decreases at highest agonal stress. Stemness and differentiation assays should report true outcomes through high-dose ranges as is required for HTSs.

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Author Disclosure Statement

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