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Differential responses to retinoic acid and endocrine disruptor compounds of subpopulations within human embryonic stem cell lines ☆

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ABSTRACT

The heterogeneous nature of stem cells is an important issue in both research and therapeutic use in terms of directing cell lineage differentiation pathways, as well as self-renewal properties. Using flow cytometry we have identified two distinct subpopulations by size, large and small, within cultures of human embryonic stem (hES) cell lines. These two cell populations respond differentially to retinoic acid (RA) differentiation and several endocrine disruptor compounds (EDC). The large cell population responds to retinoic acid differentiation with greater than a 50% reduction in cell number and loss of Oct-4 expression, whereas the number of the small cell population does not change and Oct-4 protein expression is maintained. In addition, four estrogenic compounds altered SSEA-3 expression differentially between the two cell subpopulations changing their ratios relative to each other. Both populations express stem cell markers Oct-4, Nanog, Tra-1–60, Tra-1–80 and SSEA-4, but express low levels of differentiation markers common to the three germ layers. Cloning studies indicate that both populations can revive the parental population. Furthermore, whole genome microarray identified approximately 400 genes with significantly different expression between the two populations ($p < 0.01$). We propose the differential response to RA in these populations is due to differential gene expression of Notch signaling members, CoupTF1 and CoupTF2, chromatin remodeling and histone modifying genes that render the small population resistant to RA differentiation. The findings that hES cells exist as heterogeneous populations with distinct responses to differentiation signals and environmental stimuli will be relevant for their use for drug discovery and disease therapy.

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1. Introduction

Human embryonic stem cells (hESC) are derived from a small group of cells from the inner cell mass of human blastocysts. They are defined as being pluripotent and thus are able to self-renew and contain the capacity to develop into all three primordial germ cell layers (Thomson et al., 1998). They can be maintained in vitro as immortal pluripotent cells, but are responsive to various differentiation signals, such as growth factors and retinoic acid (RA) (Hu and Zhang, 2010; Niederreither and Dolle, 2008; Strickland and Mahdavi, 1978). Treatment with RA causes hESC cells to differentiate predominately into neural progenitors with the characteristics of Pax6-positive radial glial cells (Kayama et al., 2009). Human stem cell lines H1, H9 and BGN1 express stem cell markers characteristic of a puripotent stem cell (Oct-4, Nanog, SSea-4, Tra-1–60 and Tra-1–81), have a normal karyotype of either XY or XX and form teratomas with all three germ layers present when injected into Beige-Scid mice (Jiang et al., 2010; Lecina et al., 2010; Meng et al., 2010).

While many of the hundreds of hESC derived worldwide share many similarities, it is also clear that they exhibit multiple differences that may reflect their different genetic backgrounds, environmental exposures, methods of derivation and culture conditions (Allegrucci et al., 2007; Rao, 2008). Indeed, individual cell lines, while sharing many characteristic surface makers,

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including glycolipid antigens and keratin sulphate antigens as well as pluripotency factors, are not identical with respect to gene expression (Canham et al., 2010; Sharov et al., 2011; Tavakoli et al., 2009). As an example, a broad range of surface antigens including but not exclusive of CD133, SSEA1, CD117 and CD135 were observed on some but not all hESCs in culture, raising the possibility of subpopulations within individual cell lines (Nagano et al., 2008). Elegant FACS sorting experiments using CD133 and CD135 markers in combination with fluorescently tagged hESCs (King et al., 2009) provided more recently strong support for the view that existing stem cell lines are indeed heterogeneous. These studies demonstrated the existence of distinct subpopulations differentially expressing surface makers and consistent with the concept that not all the cells within a hESC line are pluripotent, but may have the propensity to differentiate towards a particular lineage depending on endogenous and exogenous signals (King et al., 2009).

There are clear and compelling molecular mechanisms by which chromatin architecture, microRNAs, DNA methylation, histone modifications, and ATP-dependent chromatin remodeling complexes may play a role in pluripotency and differentiation (Card et al., 2008; Hawkins et al., 2010; Meshorer and Misteli, 2006; Young, 2011). Indeed, it is now well established that the mammalian SWI/SNF complex is essential for embryonic stem cell self-renewal and pluripotency in mice (Gao et al., 2008; Ho et al., 2009; Yan et al., 2008). Similarly, differences in histone modifications, imprinted genes, and DNA methylation are providing insights into the fundamental characteristics of hESCs (Hawkins et al., 2010; Lengner et al., 2010; Sharov et al., 2011). Finally, it is clear that the context of stem cell derivation, the in vivo niche, and the subsequent culture conditions and attendant signaling programs are critical to understanding pluripotency (Harb et al., 2008; Stewart et al., 2008). The plethora of studies undertaken to characterize human ES cells assume that clonal hESCs are stable homogenous populations. We have examined the possibility that hESCs exist as heterogeneous populations of pluripotent stem cells distinguishable by physical characteristics and response to differentiation agents, which can alter their distribution.

We show that hESCs are heterogeneous populations that show distinct characteristics in size, cell cycle profile, gene expression, and responsiveness to retinoic acid differentiation and several endocrine disruptor compounds. The two populations express the nuclear stem cell markers, Oct-4 and nanog, and differentially express stem cell surface markers including Tra-1–60 and Tra-1–80. Furthermore, whole human genome microarray analysis indicates roughly 400 genes and ESTs that were significantly differentially expressed ($p < 0.01$) between the two subpopulations. The two populations expressing different gene profile signatures may account for their differential response to RA and upon exposure to endocrine disrupting chemicals, a class of compounds important for human health and development. Finally, differential responses to these compounds is evident in the gene expression differences between the two populations for notch signaling, CoupTF1 and CoupTF2, components of the SWI/SNF complex and histone-modifying enzymes.

2. Material and methods

2.1. Culture of hES cells and in vitro differentiation and endocrine disruptor treatment of hES cells

H1 and H9 hES cells (WA01 and WA09, WiCell Research Institute, Madison, WI) and BGN1 hES cells (NIH BG01, hESBGN-01, BresaGen, Inc., Athens, GA) were cultured on gelatinized (0.2%) 6-well plates plated with irradiated (8000 rad) mouse embryonic feeder cells (CF-1, Chemicon International, Phillipsburg, NJ) in media containing DMEM F-12 (Invitrogen, Carlsbad, CA) supplemented with 20% Knockout Serum Replacement (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol, 1% nonessential amino acids (Invitrogen), and 4 ng/mL human βFGF (Invitrogen) at 37 °C and 5% CO_2. Cells were maintained in fresh media every day and passaged harvesting with collagenase (Gibco, Grand Island, NY), split weekly 1:6 and replated in gelatinized (0.1%) 6 well plates. H1 and H9 hES cells were used between passage 39 and passage 49, and BGN1 hES cells between passage 35 and passage 45. H1 and BGN1 cells were also cultured on matrigel plates with mTeSR™1 media (STEMCELL Technologies, WiCell Research Institute, Madison, WI).

Retinoic acid (RA), a standard method for differentiating stem cells, was used to differentiate both H1 and BGN1 hES cells. Three biological replicates of H1 and BGN1 hES cells were treated for 6 or 12 days with retinoic acid (1 μM, Sigma-Aldrich, St. Louis, MO) and then sorted to obtain subpopulations. Three biological replicates of H1 and H9 hES cells were also treated for 2 days with 4 endocrine disruptor compounds; 75 nM tamoxifen (Sigma-Aldrich), 75 nM kaempferol (Ivychem), 75 nM kepone (Cerilliant) or 37.5 nM apigenin (LKT Laboratories, Inc.). Equivalent volumes or concentrations of DMSO (Caledon Laboratories, Georgetown, Canada), ETOH (Warner-Graham Company, Cockeysville, MD) or 10 nM β-estradiol (Sigma-Aldrich) were used as control. After two days of treatment, cells were trypsinized, washed with 3% BSA/PBS, hybridized with FITC Rat anti-SSEA-3 antibody (BD Pharmingen) for 30 min, then washed twice with 3% BSA/PBS, filtered and separated on the flow cytometer as described below. All cells tested negative for mycoplasma.

2.2. Purification of subpopulations of ES cells by flow cytometry

Undifferentiated and RA treated ES cells were isolated by trypsin (Gibco). Cells were initially stained with propidium iodide (PI; 10 μg/ml, final) just prior to examination to remove any dead or dying cells from the analysis. Stained cells were examined on a FACSVantage SE flow cytometer equipped with digital electronics (Becton Dickinson, San Jose, CA). All samples were excited at 488 nm and cells were analyzed on the forward-scatter versus PI dot plot where gates were set to isolate the viable larger (high forward scatter) cells from the smaller (lower forward scatter) cells for further analysis. Sorted cells were collected by centrifugation at 3000 rpm for 25 min. Ten thousand cells were examined per sample for analysis using BD FACSDiVa software. Cells treated with endocrine disruptor compounds were gated for large and small populations and then examined for percent of positive FITC cells.

2.3. Cell imaging by flow cytometry

H1 cells were stained with DRAQ5 (Biostatus Limited, Leicestershire, United Kingdom) at a final concentration of 5 μM for 30 min at room temperature. Propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 1 μg/ml immediately prior to analysis. Cells were analyzed using an ImageStream^X flow cytometer (Amnis Corporation, Seattle, WA, USA). Signals from PI and DRAQ5 were detected in channels 4 and 5, respectively, while brightfield and side-scatter were detected in channels 1 and 6, respectively. Data was collected by INSPIRE acquisition software using 488 nm (PI) and 633 nm (DRAQ5) lasers with appropriate compensation controls and a 40 × objective with extended depth of field settings. Data from a minimum of 3000 cells were collected and analyzed using IDEAS software. DRAQ5 positive, PI negative cells were focused, and then examined on a Brightfield Area versus Aspect Ratio dot plot. Gates were drawn