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Differentiation of Human Embryonic Stem Cells Occurs through Symmetric Cell Division

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ABSTRACT

Embryonic (ES) stem cells can be expanded indefinitely, yet retain the ability to form all cell types of the body. Here we report that human ES cells differentiate exclusively by symmetric cell division in each of four distinct differentiation conditions examined. This suggests that, in some respects, ES cells more closely resemble precursor or transit amplifying cells rather than adult stem cells.
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INTRODUCTION

The role of adult stem cells is to sustain an established repertoire of cell types in essentially steady-state numbers over the life of the organism [1]. Depending on environmental demands and intrinsic factors, these stem cells can differentiate symmetrically or asymmetrically [1, 2]. The properties of human embryonic stem (ES) cells reflect their origin from pluripotent cells of the early embryo, a period during which the cellular machinery is geared toward the rapid expansion of cells and the diversification of cell types. Human ES cells are capable of unlimited self-renewal, while, at the same time, they maintain the potential to differentiate into derivatives of all three germ layers and into cells of extraembryonic lineages [3, 4]. In the intact mammalian embryo, first the blastomeres of the cleavage stage embryo, then the inner cell mass cells [5], then the primitive ectoderm cells, and finally the primordial germ cells are capable of differentiating into all cells of the adult body [6]. However, although each of these embryonic cell types is pluripotent, they all persist for only a limited number of cell divisions in the intact embryo and subsequently differentiate into other cell types with more restricted developmental potential [6, 7]. Thus, in vivo, pluripotent cells of the early embryo act as precursor cells, but when placed into an artificial environment in vitro, they can give rise to ES cells or embryonic germ cells that can self-renew without apparent limit.

Adult tissues with a high turnover rate (such as blood, skin, and intestinal epithelium) are maintained by tissue-specific stem cells, but the stem cells themselves rarely divide [1, 8, 9]. Maintenance of these stem cells depends on infrequent divisions, which are often asymmetric [10]. That is, one daughter cell remains a stem cell, while the other daughter cell enters a transient amplifying population whose rapid proliferation and ultimate differentiation sustain the tissue. In tissues that require a restoration of the tissue-specific stem cell pool, however, cell divisions may be predominantly symmetric [2].

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Materials and Methods

Cell Culture

H1.1 human ES cells were cultured as described [4]. Briefly, these cells were cultured in human ES cell medium, consisting of 80% Dulbecco's modified Eagle's medium (no pyruvate, high glucose formulation; Invitrogen, Carlsbad, CA, http://www.invitrogen.com)/F12 supplemented with 20% Gibco Knockout Serum Replacement, 1 mM glutamine, 0.1 mM β-mercaptoethanol (Sigma Chemical Corp., St. Louis, http://www.sigma-aldrich.com), 1% nonessential amino acid stock (Gibco, Carlsbad, CA, http://www.invitrogen.com), and 4 ng/ml human basic fibroblast growth factor (Invitrogen). ES cells were cultured under feeder-free conditions using medium conditioned overnight on dense murine embryonic fibroblasts [11]. Culture flasks were precoated with matrigel (Becton, Dickinson Labware, Bedford, MA, http://www.bdbioscience.com). Differentiation was induced using 100 ng/ml bone morphogenic protein 4 (BMP4) (R&D Systems, Minneapolis, http://www.rndsystems.com) or 10 μM retinoic acid (RA) (Sigma) in unconditioned medium.

Time Lapse Studies

For time lapse study and imaging, human ES cells were stimulated as described above and visualized under a fluorescent microscope (Leica IMBR). Phase contrast and enhanced green fluorescent protein (eGFP) images were acquired every 10 minutes (at 5-second exposure) for 5 days. To reduce background fluorescence, a Knockout Serum Replacement composition without riboflavin was used. The medium was changed daily. Mean fluorescence per cell was measured using the IPlab software package (Scanalytics, Fairfax, VA, http://www.scanalytics.com). To determine eGFP fluorescence, the shape of individual cells was determined and a region of interest (ROI) was defined. ROIs were transferred into the acquired fluorescence image, and the measure function was applied. Three sequential images were used to determine eGFP fluorescence intensity for individual cells.

Results and Discussion

To determine whether human ES cells differentiate symmetrically or asymmetrically, we monitored small ES cell colonies in three different cell culture conditions that promote differentiation (unconditioned medium, treatment with RA and treatment with BMP4) and we examined background differentiation in conditioned medium that is routinely used to culture undifferentiated ES cells. These differentiation conditions have discrete mechanisms of inducing differentiation and distinct outcomes. Unconditioned medium appears to act by deprivation of self-renewal factors provided by fibroblasts and gives rise to a mixed population of cells [3]; RA acts by direct stimulation of nuclear retinoic receptors and predominantly gives rise to

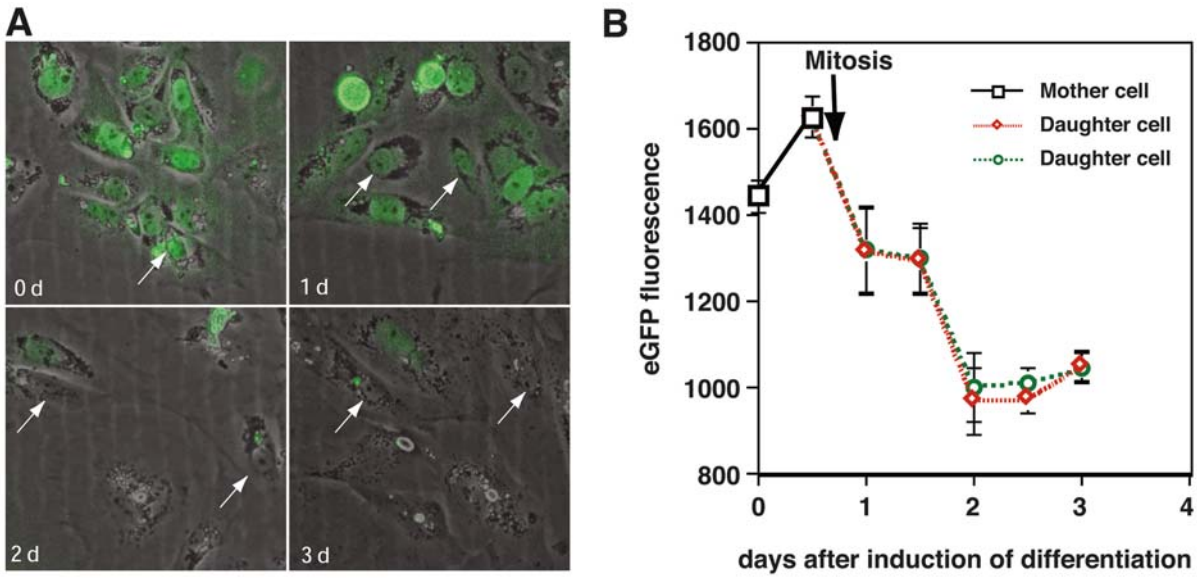
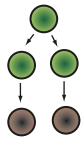
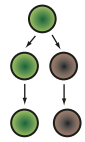
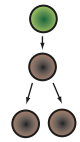


Figure 1. Human ES cells differentiate symmetrically. **(A):** Extracts from time-lapse analysis showing differentiation of one human ES cell colony after RA treatment. A representative cell and its two daughter cells are marked with arrows; green shows eGFP, reflecting the internal level of Oct4 in individual cells. Following cell division, both daughter cells downregulate eGFP and, therefore, Oct4 in the same way. **(B):** Mean eGFP fluorescence in one human ES cell after induction of differentiation with RA. The eGFP signal has been followed over time. The time point of cell division is marked. Error bar, standard error of mean. Abbreviations: eGFP, enhanced green fluorescent protein; ES, embryonic stem; RA, retinoic acid.

Table 1. Number of cells undergoing self-renewal or differentiation under the four cell culture conditions determined by time-lapse studies of Oct4-eGFP knock-in human embryonic stem cell lines

Culture condition	Self-renewal 	Symmetric differentiation 	Asymmetric differentiation 	Differentiation without cell division 	Apoptosis 	Total number of cells analyzed
Conditioned medium	45 (76%)	6 (10%)	0 (0%)	3 (5%)	5 (8%)	59 (100%)
Unconditioned medium	6 (11%)	30 (55%)	0 (0%)	5 (9%)	14 (25%)	55 (100%)
Retinoic acid	2 (3%)	39 (68%)	0 (0%)	6 (11%)	10 (18%)	57 (100%)
BMP4	5 (8%)	37 (61%)	0 (0%)	9 (15%)	11 (16%)	61 (100%)

Cells that did not divide or changed eGFP status were excluded from the analysis. Note the complete absence of asymmetric differentiation under all investigated conditions.
Abbreviations: BMP4, bone morphogenic protein 4; eGFP, enhanced green fluorescent protein.

an epithelium that expresses endoderm markers [12]; and BMP4 acts by stimulation of cellular surface receptors and gives rise to trophoblast cells [13].

We tracked the differentiation state of human ES cells using an Oct4-eGFP knock-in cell line [14]. Oct4 is a central regulator of pluripotency [15] and is expressed exclusively in the pluripotent cells of the embryo. We used time-lapse videomicroscopy over 5 days to track phase-contrast images and corresponding eGFP expression levels in individual cells as an indicator of Oct4 expression. Figure 1A depicts an undifferentiated, $Oct4^+$, human ES cells (arrow) undergoing a cell division in which both daughter cells show synchronous downregulation of eGFP, and therefore Oct4. Differentiation was also indicated by the change in morphology observed in the corresponding phase-contrast images. In total, we tracked the fate of 60 individual cells for 5 days under each of the four conditions (Fig. 1B; Table 1).

Most cells we observed downregulated Oct4 in both daughter cells following cell division. However, during the period monitored, there were also cells that did not divide, cells that underwent apoptosis, and cells that divided after downregulating Oct4. The downregulation of Oct4 prior to cell division surprised us, as we anticipated that a change in differentiation status would require cell division. No cell observed underwent asymmetric division. This behavior was independent of the stimuli used to induce differentiation and is in contrast with asymmetric cell division in adult stem cells. It is important to note that symmetric differentiation in human ES cells was not caused exclusively by strong inducers of differentiation. For example, in fibroblast-conditioned medium that supports undifferentiated ES cell growth, we monitored cells that underwent spontaneous differentiation. Although we predicted that both symmetric and asymmetric differentiation would occur under these conditions, we found that even this spontaneous background differentiation was always symmetric. Clearly, under some circumstances, the fate of daughter cells could be asymmetric. For example, if we had physically separated two undifferentiated daughter cells and applied a differentiation signal to one and not the other, we would expect only one to differentiate. In the conditions we tested, however, all daughter cell pairs from an observed cell division had an equivalent Oct4 status. One limitation of our study is the assumption that Oct4 expression is always strictly correlated with pluripotency since exceptions have been reported. For instance, it has been reported that under certain circumstances differentiating ES cells show a transient burst of Oct4 expression prior to downregulation. Thus it is conceivable that some of the symmetric self-renewal divisions were in reality asymmetric cell divisions with one daughter cell remaining pluripotent and the other daughter cell transiently upregulating Oct4 expression during differentiation.

Asymmetric cell division has an important role in the diversification of cell types in the early mammalian embryo—for example, in the initial differentiation of the trophectoderm. However, neither the inner cell mass nor the trophectoderm is equivalent to the blastomeres from which they are derived, and thus there is no maintenance of a stem cell. Because early embryonic cells are not main-

tained as tissue-sustaining stem cells throughout the life of the organism, it is not unreasonable to expect that the machinery that controls their proliferation and differentiation is distinct from the machinery that controls adult stem cells. In this regard, our results suggest that in some respects ES cells could resemble transient amplifying cell populations derived from adult stem cells more than they resemble adult stem cells themselves.

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