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Stem Cells

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Hematopoietic Development from Human Embryonic Stem Cells

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The most common human cell-based therapy applied today is hematopoietic stem cell (HSC) transplantation. HSCs can be defined by two essential properties: self-renewal and multilineage hematopoietic differentiation. These combined HSC properties allow them to differentiate into all blood cell types (multilineage) in a sustained manner for the lifetime of the animal, which requires their ability to make cellular copies of themselves (self-renewal). These features can be tested by transplantation from donor to recipient and provide a functional basis to define and identify HSCs. Currently, human bone marrow (BM), mobilized peripheral blood, and umbilical cord blood (CB) represent the major sources of transplantable HSCs, but their availability for use is limited by both quantity and compatibility. Although increasing evidence suggests that somatic HSCs can be expanded to meet current needs, their *in vivo* potential is concomitantly compromised after *ex vivo* culture. Pluripotent human embryonic stem cells (hESCs) may provide an alternative. hESCs possess indefinite proliferative capacity *in vitro*, and have been shown to differentiate into the hematopoietic cell fate, giving rise to erythroid, myeloid, and lymphoid lineages using a variety of differentiation procedures. In most cases, hESC-derived hematopoietic cells show similar clonogenic progenitor capacity and primitive phenotype to somatic sources of hematopoietic progenitors, but possess limited *in vivo* repopulating capacity when transplanted into immunodeficient mice. Although this suggests HSC function can be derived from hESCs, the efficiency and quality of these cells must be characterized using surrogate models for potential clinical applications.

Here, we review current progress in studies to derive primitive hematopoietic cells from human embryonic stem cell (hESC) lines and define the barriers to generating clinically applicable progenitors or hematopoietic stem cells (HSCs) from hESCs that include quality and quantity of hematopoietic cells as well as control of differentiation progression that affects overall efficiency of hematopoietic output from hESCs. Finally, we outline the potentially powerful use of hESC-derived hematopoietic cells as a model system to better understand somatic HSC biology and transplantation.

Origin and Pluirpotent Nature of hESC Lines

Human ESCs, similar to nonhuman primate and mouse (m)ESCs, are derived from the inner cell mass (ICM) of the human blastocyst.[1-7] By immunosurgical isolation of the ICM and culture under appropriate conditions,[8] ICM cells give rise to hESC lines that proliferate *in vitro*, and maintain a normal karyotype and high telomerase activity. Essentially, the ICM is composed of as few as 50 cells, and once removed from the blastocyst, is cultured in plastic tissue culture dishes in the presence of specific growth factors and/or fibroblast cell types that act as feeders to induce proliferation and outgrowth of cells within the ICM. Cells arising from the ICM proliferate and give rise to individual colonies once passaged by physical and enzymatic digestion into newly set-up dishes with similar growth factors. These colonies collectively define an individual hESC line, since these cells can be propagated upon serial passage, expanded, and the aliquots cyroperserved. As such, each hESC line is normally derived from a single blastocyst, and is therefore genetically unique to other hESC lines available. The ICM isolated from blastocyst-stage embryos has the capacity to develop into primitive ectoderm and into the three embryonic germ layers of definitive ectoderm, mesoderm, and endoderm during gastrulation that give rise

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to all types of somatic cells. It is believed that hESC lines possess similar developmental capacity to that of the ICM, and there are several *in vitro* and *in vivo* assays to measure this potential, termed "pluripotency." For example, hESCs can aggregate together to form structures known as human embryoid bodies (hEBs) if detached from the culture plates upon withdrawal of basic fibroblast growth factor (bFGF) or addition of fetal calf serum (FCS). Similar to embryo development, hEBs can develop into structures of endoderm, ectoderm, and mesoderm.[9,10] Upon injection into immunodeficient mice, hESCs generate teratomas composed of multiple tissue types,[1,11,12] suggestive of their pluripotent potential. *In vitro*, hESCs are capable of differentiating into multiple tissue types, including hematopoietic cells,[13-18] comprising erythroid, myeloid, and lymphoid lineages,[16,19-26] and therefore may provide alternative sources of human hematopoietic cells for transplantation.

Current Progress in Derivation of Hematopoietic Cells from hESC Lines

Differentiation methodologies of hESCs are largely adapted from methodologies used for mESC differentiation and include two main approaches: coculture on supportive stromal cell layers[16,21,27-32] or formation of EBs.[19,20,22,26,33-37] Combination[38,39] and side-by-side comparison[28] of both strategies have also been used.

Despite the different procedures applied in studying hematopoietic development from hESCs, different groups have achieved considerable common outcomes. First, different groups independently found that hematopoietic development from hESCs displays a specific pattern of timing after EB development or coculture, and that hematopoietic cells develop in clusters, as opposed to single independent cellular differentiation events occurring randomly within EBs or on stromal cocutlure.[16,19,21,22,24,26] Second, during early hESC differentiation, hematopoietic cells are derived from $CD45^-$ precursors that co-express CD31 and CD34 surface markers.[16,19,21,24,26] Two groups have identified an immature endothelial population as being responsible for giving rise to hematopoiesis from hESCs.[26,33] Through clonal experiments,[26] Wang et al demonstrated that a rare resident population within hEBs was able to generate both the endothelial and hematopoietic lineages, suggesting that this population contains cells with "hemangioblastic" properties. These combined findings recapitulate observations from human embryos,[40-42] further illustrating that hESCs can be applied as a model for studies of early human development.

Although the validity of ESCs as a model for adult hematopoiesis is controversial because long-term engrafting HSCs have been difficult to demonstrate from mESCs, preliminary data from our group,[37] and others[29,32] suggest that hESC-derived hematopoietic cells have HSC properties using immunodeficient mouse recipients. However, the ability to generate fully functional hESC-derived HSCs capable of long-term multilineage reconstitution of mouse models and ultimately patients remains a challenge and will depend upon further understanding of intrinsic gene regulation and extrinsic environmental cues.

Quantity and Quality of Hematopoietic Cells from hESC Lines

Prior to transplantation, evaluation of somatic human hematopoietic cells is based on both phenotypic properties such as the number of blood cells expressing putative HSC markers that include CD34, and functional properties using *in vitro* colony-forming unit (CFU) assays to measure multilineage hematopoietic capacity arising from progenitors. In the absence of defined HSC assays based on human transplantation and reconstitution, these *in vitro* assays serve as surrogate indices of *in vivo* function.[43,44] Based on the current understanding of human hematopoietic development and potential clinical applications, CFUs and cell-surface markers, including the pan-leukocyte marker CD45,[45,46] serve to evaluate hematopoietic potential of hESC line treatment under a variety of differentiation regimes.

The principle of using hESCs as a cell replacement source is deceptively simple, and fundamentally revolves around the obstacle, or our lack of understanding, of human developmental biology to differentiate these cells in a controlled and precise manner. Previous work using mESCs has demonstrated that bone morphogenic protein-4 (BMP-4) represents a strong hematopoietic-inducing factor. Capitalizing on previous efforts to support adult hematopoietic cells in culture with stem cell factor (SCF) and FLT-3L, these factors have been combined with BMP-4 to culture hEBs that allow robust hematopoietic differentiation.[47-49] These conditions work consistently on several hESC lines, and represent the optimal conditions defined by our laboratory. It's important to note that the magnitude (exact percentage of hematopoietic cells or progenitors detected by CFU assay) varies among hESC lines. Consistently, all hESC lines we have tested demonstrate the emergence of hematopoietic cells at day 10 of EB treatment,[50] suggesting that precursor populations within the EB must exist that possess hemogenic properties. Detailed investigation and phenotypic analysis of cells comprising EBs prior to day 10 of hematopoietic emergence indicated that cells with endothelial phenotype developed under these conditions, and ultimately gave rise to mature hematopoietic cells.[50] Prospective and clonal isolation experiments demonstrated that cells with endothelial phenotype and endothelial potential were exclusively responsible for hematopoietic progenitors derived from hESCs.[50] We had defined these cells as $CD45^-$ to denote the absence of hematopoietic marker expression and expression of cell-surface PECAM-1, VE-cadherin, and Flk-1 as $CD45^-$ PFV cells. $CD45^-$ PFV cells could be differentiated into hematopoietic lineages whereas no other cell in the EB without this phenotype pos-

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sessed hematopoietic potential. Since endothelial lineages could also be derived from clonal starts of CD45⁻PFV cells, this unique population seems to possess hemangioblastic properties.[50] This series of studies provides biological insights into hESC differentiation towards hematopoiesis and, more important, provided a phenotypic and timed road map of hematopoietic development from hESC lines.

The ability to isolate CD45⁻ PFV cells and differentiate them to hematopoietic progenitors provided an approach to engineer and transplant a focused population of hematopoietic cells from hESC cultures.[37,48] This allowed additional studies in which we examined the *in vivo* repopulating potential of resulting hematopoietic progenitors derived from CD45⁻ PFV cells. Initially, primitive CD34⁺CD45⁺ cells derived from hESCs were transplanted into nonobese diabetic severe combined immunodeficiency (NOD-SCID) mice by both intravenous and interfemoral injection.[37] Although intravenous introduction did not lead to any detectable reconstitution after more than 6 weeks in NOD-SCID mice, interfemoral injection allowed multilineage human engraftment to be detected up to 10 weeks in several NOD-SCID recipients. This provided initial observations to suggest that human SCID repopulating cells (SRCs) can be derived from hESCs similar to adult sources of umbilical cord blood (CB) or bone marrow (BM).[37] However, upon further characterization of hESC derived SRCs, we observed a limited proliferative and migratory capacity, compared with adult sources of SRCs. Molecular profiling of ESC-derived SRCs to adult sources (both purified for the CD34⁺CD38⁻CD45⁺ phenotype) provided surprising differences in both homing molecules associated with HSCs and expression of HOX gene clusters known to be important in the self renewal of adult HSCs.[37] Taken together, these initial studies were interpreted by our lab as an inability to activate essential genetic programs in hESCs to derive bona fide somatic-like HSCs. We believe this inability leads to the atypical *in vivo* behavior of hESC-derived SRCs. More globally, these studies in the hematopoietic system that use rigorous *in vivo* assays underscore the importance of both molecular and functional comparisons between cell lineages (of any kind) derived from hESCs to their adult/somatic counterparts.

Controlling Hematopoietic Differentiation from Pluripotent hESC Lines

Maintenance of undifferentiated ESCs, prospective isolation of committed precursors, and subsequent differentiation into a specified lineage have proven to be difficult in the human system. Compared with mESC work, studies towards the differentiation of hESC-derived hematopoietic cells have raised new issues. Although hESCs are competent for spontaneous development into all cell types,[13-18] the cellular and molecular mechanisms that control this process are very poorly understood and yield heterogeneous outcomes. Awareness of these difficulties and variations will assist us to understand and interpret consistent and meaningful observations that elucidate the underlying biology at play. Acknowledging and then reducing these variations will eventually depend upon deciphering the fundamental biology of hESCs, which may segregate into many categories, two of which are discussed below.

Differences between human and mouse ESCs

Significant differences between hESCs and mESCs have been documented in terms of growth factor response, surface marker expression, and culture dynamics/procedures applied to the maintenance and differentiation of these cells.[1,14,16,26,51-55] Although we have benefited from the knowledge accumulated from mESC work over two decades, increasing evidence suggests that attempting to directly adapt these procedures to the study of hESCs will not provide immediate insights into hESC biology. For instance, leukemia inhibitory factor (LIF) and BMP-4 have been well defined as two crucial growth factors for mESC self-renewal.[56,57] However, hESCs lack the LIF receptor, and maintenance of undifferentiated hESCs is independent of LIF or BMP-4. In sharp contrast, BMP-4 drives hESC differentiation,[58] and bFGF, a neuron differentiation factor for mESCs,[59] plays a crucial role in sustaining hESCs in undifferentiated conditions.[58,60,61] Human ESCs express stage-specific embryonic antigen (SSEA)–3, SSEA-4, tumor rejection antigen (Tra)–1-60 and Tra-1-81, while mESCs lack these surface markers and express SSEA-1.[51,53,62] Unlike mESCs, clonal differentiation analysis of hESCs has proven difficult, since hESCs seemingly prefer passage and differentiation as groups of cells or clusters (personal unpublished observations). Although suboptimal culture conditions for hESCs may account for these shortcomings compared with mESCs, the inability to use chimeric assays to define the most robust culture conditions for hESCs, combined with the inability to apply signaling pathways shown to support mESC self-renewal, further debilitates progress in understanding hESC biology.

Differences between human and mESCs in hematopoietic specification have also been documented.[14,16,24,26,51,54,63] Surface markers that have been successfully applied to identify early hematopoietic progeny during mESC development seem unsuitable for hESC differentiation, since many of the homologous markers are expressed on cells within the hESC cultures prior to differentiation.[24,26] For example, Flk-1⁺ cells only emerge after mESC differentiation,[64] giving rise to either endothelial or hematopoietic lineages.[64-67] However, the frequencies of Flk-1⁺ cells in undifferentiated hESCs and differentiated hEBs are similar, suggesting that Flk-1 is not an appropriate marker for early hematopoietic progeny of hESCs.[26] CD105 (endoglin) is an accessory receptor for members of the TGF-β family, and is expressed during the progression from the Flk-1⁺CD45⁻ to the Flk-1⁻CD45⁺ stage during hematopoietic development of mESCs.[66] Again, CD105 shows simi-

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lar levels of expression on undifferentiated hESCs and differentiated hEB cells.[26] Currently, CD41 antigen has been demonstrated as a reliable marker for early hematopoiesis in mESCs.[68-70] However, very few cells expressed CD41 during hESC hematopoietic development.[26] The comparisons and observations between mouse and human ESC differentiation illustrate important cellular differences of mouse and human specification towards the hematopoietic lineages.

Variations in studies of hESC-derived hematopoietic cells

We propose that variations in studies of hESC-derived hematopoietic cells may result from, but are not restricted to, the following factors, alone or in combination: (1) different hESC lines; (2) different passage number; (3) differences in culture conditions to maintain hESCs (e.g., culture on MEFs versus feeder-free systems); (4) differences in differentiation protocols (e.g., EB versus coculture); and (5) growth factor source, concentrations, and stability.[2,6,16,19-24,71] Changes of culture conditions such as different sources and batches of growth factors, serum replacement, mouse embryonic fibroblasts (MEFs), MEF–conditioned media (CM), matrigel, enzymes for cell dissociation, cell density, and unknown reasons have shown variable influence on undifferentiated markers of hESCs (Oct3/4, SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81) and colony morphology, subsequently affecting hematopoietic differentiation (unpublished observations). In addition to these variables, hESC lines demonstrate genomic instability and nonpredictable differentiation after long-term growth,[72,73] and most likely represent much more heterogeneous cell populations than their murine counterparts. Therefore, verification of the "measurable" undifferentiated status of hESC cultures prior to hematopoietic differentiation studies is critical. The reduction of these variations and identification of others will depend upon further studies of hESC biology and the timely sharing of these observations during the early phases of enterprise.

Thus far, most differentiation regimes of hESCs produce variable numbers of hematopoietic cells. However, few have demonstrated that these protocols direct the cell fate of pluripotent cells to the hematopoietic fate, as opposed to creating conditions permissive to spontaneous differentiation and/or subsequently expanding spontaneously differentiating cells. The differences in the cell biology being regulated are likely to affect both the quantity and quality of hESC-derived hematopoietic cells. Unfortunately, current studies have yet to quantitate input and output of hematopoietic cells generated using both phenotypic and functional assays, making it difficult to know whether directed differentiation using specific growth factor or methods has been achieved. New experimental approaches of hematopoietic differentiation and optimization of methods will be required to convincingly demonstrate hematopoietic specification and compare methodologies.

Human ESCs as Models for Somatic HSC Biology

Immortal hESCs can differentiate into multilineage hematopoietic and immune cells. Similar to somatic counterparts, hESC-derived hematopoietic cells have similar *in vitro* clonogenic capacity and cell-surface phenotype. Preliminary studies indicate that hESC-derived hematopoietic cells have HSC properties. By further understanding intrinsic and extrinsic factors affecting development of hESC-derived hematopoietic cells, hESCs may become potent sources for hematopoietic cell replacement therapy, and may also assist in understanding abnormal hematopoietic development arising during emergence of human leukemias. However, hESCs hold immense potential in examining the human HSC niche and signaling pathways controlling self-renewal and cell-cycle machinery of human HSCs, and in understanding the molecular events that initiate human leukemogenesis.

Unlike the murine system, the study of human HSCs is limited by human-to-animal xenotransplantation assays where regulation and effects of the immune system are not acknowledged, nor are issues of xeno-incompatability. Therefore, many of these functional *in vivo* assays provide unproven surrogates of clinical transplantation. The ability to engineer mouse development in both the embryonic and adult stages provides an incredible tool to determine the necessity and sufficiency of specific pathways and/or transcription factors thought to modulate HSC biology. This depth of engineering is not applicable to somatic sources of human HSCs due to technical difficulties and infrequencies of being able to "knockout" or "knock-in" specific genes or reporters. The lack of these genetic tools combined with inherent variances in sample-to-sample harvests of progenitors and HSCs make experimental approaches to better understand human HSC biology extremely difficult. Some of these may be achievable in the hESC system. Several groups have demonstrated the ability to create transgenic lines using lentiviral systems, or to knock-out genes and introduce promoters into the undifferentiated hESCs. hESCs engineered for this purpose can be selected, purified, and compared between genetically dissimilar hESC lines to evaluate their role in hematopoietic differentiation and/or subsequent hematopoietic function of several of the blood lineages, including stages of progenitor self-renewal. Although this does not provide a direct means to use hESCs in the clinic, the hESC lines and the information derived from their engineering can provide invaluable insights into how somatic HSCs may be regulated for improved transplantation, engraftment, and *in vivo* function.

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