



MINI-REVIEW

PLURIPOTENT STEM CELLS

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Pluripotent Stem Cells

Few areas of biology currently garner more attention than the study of human pluripotent stem cells (hPSC). This interest has arisen because of their potential to form the basis of cellular therapies for diseases affecting organ systems with limited regenerative capacity, to provide enhanced systems for drug screening and toxicity testing as well as to gain insight into early human development obviating the need for human embryos. There are currently two major methods for generating cells with pluripotent properties. The first involves isolating the inner cell mass from an early human blastocyst and culturing the resulting cells in appropriate culture conditions (see below) to generate human embryonic stem cells (hESC).¹ The second involves artificially expressing a defined number of factors in somatic cell types, which, with the appropriate culture conditions, causes the cells to be reprogrammed into induced pluripotent stem cells (iPSC).²⁻⁴

Generation of Induced Pluripotent Stem Cells

Much effort has been dedicated to understanding the transcriptional state of undifferentiated pluripotent stem cells, and many of the findings in hESCs have been shown to also be relevant to hiPSCs. For example, Oct-3/4,⁵ Sox2,⁶ and Nanog^{7,8} have been shown to be central to the specification of pluripotent stem cell identity due to their unique expression patterns and their essential roles in early development. These efforts, along with others, enabled the discovery of defined factors for the reprogramming of somatic cells. The specific molecular process by which somatic cells are reprogrammed into pluripotent stem cells is largely unknown and much research is currently focused on elucidating the mechanisms by which this takes place. The process is also very inefficient and slow and current research focuses on understanding the role of several variables in the process of reprogramming: (1) choice of factors used to reprogram the cells (2) the methods used to deliver these factors; (3) the choice of target cell type; (4) the parameters of factor expression, such

as timing and levels; and (5) the culture conditions used to derive iPSCs. In addition to this, methods to identify and characterize truly reprogrammed pluripotent cells are also important.⁹

The original cocktail of factors, Oct4, Sox2, c-myc and Klf4, continue to be the major factors that are used for reprogramming. Originally, delivery of the factors was achieved through the use of viruses that integrated into the genome. However, concerns over the clinical use of these cells and the potential for insertional mutagenesis have led to the exploration of non-integrating methods of factor delivery including transient transfection,¹⁰ non-integrating viral approaches¹¹ and protein transduction.¹² Other recent methods such as the use of polycistronic minicircles¹³ and synthetic RNA¹⁴ have also been shown to be successful. Particularly exciting are recent research efforts to identify small molecules that replace some of these factors by either modifying genome methylation patterns or inhibiting key signalling pathways (Table 1). The ultimate goal of this research is to define stepwise protocols by which cells can be fully reprogrammed solely by chemical means.

There is also a close interplay between the number of factors needed to reprogram cells and the starting cell type. For example, neural stem cells can be reprogrammed with one factor alone because of the endogenous expression of the other reprogramming factors.¹⁵ Different cell types also appear to have different reprogramming efficiencies. For example, in mice, stomach and liver cells appear to be reprogrammed more efficiently and completely than fibroblasts.¹⁶ Similarly, the reprogramming of human adipocytes is ~20-fold more efficient than fibroblasts and have the added advantage of being a readily available source of cells.^{17,18} Novel methods to identify reprogrammed cells have also been explored. Recently, it was discovered that the degree to which cells were reprogrammed could be elucidated by the expression pattern of several cell surface markers.¹⁶



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A common phenomenon observed during reprogramming is the emergence of partially reprogrammed colonies which are usually associated with continued expression of the reprogramming factors. These cells exhibit a range of phenotypes but often fail at certain crucial tests of fully pluripotent cells.¹⁹ A recent observation by Chan et al.²⁰ showed that while overall reprogramming efficiency was lower in feeder-independent conditions, the only types of cells that emerged were fully reprogrammed cells. This indicates the importance of culture conditions in the process of reprogramming.

While early studies relied on the many similarities between embryonic and induced pluripotent stem cells, it was noted early on that molecular differences between the two cell types existed. However, to date, no consistent differences have been shown at the molecular level,^{21,22,23} indicating that variances may be a result of experimental conditions. Several reports have noted retained gene expression from the parental cell types²⁴ and have also shown that the epigenetic state is predictive of the original somatic cell type.²⁵

Several studies have recently shown that cells can be reprogrammed directly into other cell lineages without the generation of a stable pluripotent state through a process of lineage switching.^{26,27,28} Although still preliminary, these studies provide an intriguing alternative method of generating differentiated cell types that does not rely upon pluripotent stem cells.

Culture Conditions for hPSCs

Initial methods to culture hESCs were modeled on techniques originally developed to culture mouse ESCs.^{30,31} These techniques involved the culture on a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) in medium supplemented with 20% fetal bovine serum (FBS). In these conditions, hESC lines could be propagated indefinitely with retention of their pluripotent properties.¹ From the initial development of these culture conditions, it was realized that the continued use of feeders and animal-derived components in hESC cultures would hinder the development of clinical applications due to: a) the presence of immunogenic material; b) the risk of transmitting animal virus or prion material; and c) difficulty with quality control of these undefined components.

Subsequently, improvements to these procedures have largely focused on removing the undefined and non-human components. Several groups have developed culture conditions for hESCs that are, to various degrees, serum- and MEF-free. It was discovered

that serum could be replaced with Knock-Out Serum Replacement, a commercially available serum substitute that exhibits considerably less batch-to-batch variability compared to serum, but which is still not fully defined.³² Xu et al. reported a culture system that utilized BD Matrigel™ as a culture matrix and MEF-conditioned medium (consisting of animal component-containing serum replacement and basic fibroblast growth factor, bFGF) that allowed hESCs to be cultured without direct contact with feeders.³³ Another approach to MEF removal from the culture system was to replace them with human feeders.³⁴ As the feeders are of human origin, the possibility of the transmission of foreign pathogens is limited, but unfortunately the secreted factors are still undefined and subject to large variation between batches.

True feeder-independent culture has been reported using an extracellular matrix and a combination of transforming growth factor β (TGF- β) and bFGF or high levels of bFGF alone^{35,36} together with an animal component-containing serum replacement. Recently, a number of publications have described defined xeno- or feeder-free medium formulations for the maintenance of hESCs.³⁷⁻⁴¹ mTeSR™1 is a serum-free, defined medium developed by Tenneille Ludwig and colleagues at the WiCell™ Research Institute (Madison, WI) that supports long-term, feeder-independent culture of hESCs and hiPSCs.⁴¹ The formulation of mTeSR™1 includes bFGF, transforming growth factor β (TGF- β), gamma-aminobutyric acid (GABA), pipercolic acid, and lithium chloride. Recently mTeSR™1 has been shown to be able to support the derivation and maintenance of human induced pluripotent stem cells.^{17,29} A completely animal protein-free system for hESC derivation and propagation was developed by the same group using a human serum albumin-containing formulation with a defined matrix consisting of human derived collagen, vitronectin, fibronectin and laminin.⁴⁰ A medium based on this formulation has been commercially released as TeSR™2.

A lot of effort has recently been focused on finding synthetic alternatives to Matrigel™. To this end, 3 publications have shown synthetic, fully defined surfaces that are able to support the long-term survival of undifferentiated hPSCs and show great promise for increased use in the culture of these cells.^{42,43,44}

Clinical Applications of hPSCs

Because of their differentiation potential, it is hoped that hPSCs may form the basis of cellular therapies in many diseases where tissue damage or malfunction is severe and irreversible. Cardiovascular diseases, type-1 diabetes, spinal cord injury, and Parkinson's disease are examples of diseases where it is hoped that hPSC-based therapies will provide a cure. Techniques have been developed to differentiate hESCs into a variety of adult cell types including hematopoietic,⁴⁵⁻⁴⁷ cardiac,^{48,49} neural, retinal pigmented epithelia⁵⁰⁻⁵² and osteogenic lineages.⁵³ However, a number of obstacles currently impede the clinical application of hPSC-based therapies. At present, only limited testing of hESC-derived cells has been performed to ensure full maturation and functionality of differentiated cells. All of these tests have been performed in laboratory animals and have had mixed results with respect to efficacy of treatment.⁵⁴ Furthermore, protocols for the differentiation of hPSCs to functionally relevant progeny are generally inefficient, resulting in low differentiated cell yields and contamination by other cell types as a result of aberrant differentiation. Of greater concern is the possibility of the persistence of undifferentiated hPSCs in transplanted populations which may result in teratomas.^{55,56}

Further concerns surround the possibility of immune rejection of transplanted cells either due to the expression of different major histocompatibility complex antigens on donor cells⁵⁷ or from the expression of foreign antigens as a result of culturing hPSCs in animal products.⁵⁸ Using patient-specific iPSCs as a starting population for cellular therapies would circumvent the need for histocompatibility matching.²⁻⁴ And while the potential of rejection due to expression of foreign animal antigens remains controversial,^{59,60} scientists and commercial entities are devoting much effort to developing xeno-free culture media and matrices for the expansion and subsequent differentiation of hPSCs.

While some differentiation protocols have been shown to be translatable to iPSCs, there is some concern that the originating cell type can influence the ultimate capacity of the reprogrammed cell to differentiate to specific lineages.⁶¹ A better understanding of iPSC biology and the mechanisms of reprogramming together with solutions to the above described obstacles for hESC-derived therapies must preclude any consideration of clinical based therapies of iPSC derived cells.

There are however examples of clinical application of hESC-based therapies that are moving closer towards becoming a reality. For example, transplant of ESC-derived oligodendrocyte progenitor cells into a rat model of spinal cord injury was able to significantly improve remyelination and locomotor recovery.⁶² Based on this result, Geron initiated the first clinical trial to test the safety and efficacy of this in humans. In November 2010, the FDA also approved the initiation of a clinical trial by Advanced Cell Technologies to utilize hESC-derived retinal pigmented epithelial cells to treat Stargart's macular dystrophy, a degenerative eye disease that causes blindness in children.

Use of hPSCs in Drug Screening and Toxicological Testing

The most immediate rewards are likely to be gained from the use of hPSCs in the fields of drug development or toxicity testing. It has been estimated that the cost of bringing a new drug to market through development, clinical trials and FDA approval can be upwards of 800 million to 1.3 billion USD.⁶³ Furthermore the number of drugs that are ultimately successful is very low. Given these costs and the high risk assumed by pharmaceutical companies, there are great advantages to having access to large numbers of biologically relevant human cells for early testing and screening. hPSCs in their undifferentiated state may be useful to identify teratogenic or toxic effects of potential compounds. Incorporating compounds into defined differentiation protocols may identify candidates that potentiate or skew differentiation towards a beneficial outcome. The potential to generate large numbers of end stage cells such as neurons and cardiomyocytes will ultimately provide directly relevant cell types for drugs being developed for cardiovascular or neurodegenerative disorders. In addition, the generation of cardiomyocytes and hepatocytes may be directly relevant to toxicity measurements. Finally, disease specific iPSCs made by reprogramming relevant cell types from patients has the potential of revealing not only fundamental biological defects but also providing potentially unlimited cells with which to investigate potential therapeutic approaches.

TABLE 1. Small Molecules Used to Reprogram Cells

MOLECULE	GENERAL FUNCTION	ROLE IN REPROGRAMMING	REFERENCE #
AZA	DNA methyltransferase inhibitor	Increases reprogramming efficiency of the 4 factors by overcoming partial reprogramming block	48
Valproic acid	Histone deacetylase inhibitor	Replaces KLF4 and C-MYC	49
BIX01924 BayK8644	Histone methyl transferase inhibitor L-type Ca ⁺⁺ channel activator	Replaces SOX2/C-MYC in NPC Both replace SOX2/C-MYC in MEF	50
E-616452, SB431542	TGF-b inhibitor	Replaces SOX2 in MEF	57
CHIR99021, Parnate	GSK-3 inhibitor, lysine specific demethylase 1	Replaces SOX2/C-MYC in MEF, both required for same in keratinocytes	52
SB431542, PD0325901, Thiazovivin	TGF-b inhibitor, MEK inhibitor Survival factor	Increases reprogramming efficiency of the 4 factors	53
Kenpaullone	Promiscuous kinase inhibitor	Replaces KLF4	54

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