

Toward Understanding Intracellular Signaling Events in Embryonic Stem Cells

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Introduction

Mouse embryonic stem cells (mESCs) are pluripotent cells that are derived from the inner cell mass of blastocyst-stage embryos¹. These undifferentiated cells retain the capacity to generate all cell types in the body, which was vividly demonstrated by their ability to contribute to all tissues of adult mice, including the germ cells, following injection into host blastocysts². Establishment of mouse ES cell lines, in combination with homologous DNA recombination, has allowed creation of mutant mouse strains with targeted gene elimination, which was credited by the 2007 Nobel Prize in Physiology and Medicine to Drs. Mario Capecchi, Martin Evans and Oliver Smithies.

In 1998, Thomson and colleagues successfully generated pluripotent human embryonic stem cell (hESC) lines, which has elicited a great deal of excitement in the fields of stem cell biology and regenerative medicine³. It has been widely recognized that hESCs and/or somatic stem cells might become potential sources of cells to regenerate damaged tissues. However, the molecular mechanisms underlying the control of hESC pluripotency and differentiation are not fully understood. In the past few years, much progress has been made in determining functional requirement of transcription factors Oct4, Sox2 and Nanog in maintenance of human and mouse ES cell identity. It is of interests to note that these factors are found to co-occupy a substantial portion of their target genes^{4,5}.

The essential role of these transcription factors in maintaining ES cell pluripotency was clearly demonstrated by Takahashi and Yamanaka⁶. These two researchers introduced retrovirus-carried transcription factors, *Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*, into mouse embryonic or adult fibroblast cells, and created so-called iPS (induced pluripotent stem) cells. The iPS cells exhibit ES cell-like morphology and growth properties, express ES cell markers and retain the potential to differentiate into three germ layer cells in vivo. Upon modification of in vitro selection conditions, several groups have shown that the iPS cells established using this approach retain germ-line competency^{7,8}. Immediately following the success of establishing mouse iPS cells, similar attempts by several groups have proven successful in establishing human iPS cells⁹⁻¹¹. The Yamanaka group again used the same group of four transcription factors (*Oct3/4*, *Sox2*, *Klf4* and *c-Myc*), while Yu and colleagues used another combination: *Oct4*, *Sox2*, *Nanog* and *Lin28*. Notably, these human iPS cells have normal karyotypes and telomerase activity, express unique ES cell markers and retain developmental potential to differentiate into all germ layer cells. Evidently, the iPS cell technology can now allow us to establish customized human ES cells using somatic cells derived from patients. This type of reprogrammed iPS cells will be useful for molecular

analysis of cellular events involved in pathogenesis of diseases and also for drug selection and toxicology tests.

Comparative dissection of signaling events in mouse and human ES cells

The intracellular signaling mechanism for control of human ES cell pluripotency remains to be elucidated. Since mESCs have received intensive attentions in the past twenty years and a great wealth of information is available, characterization of hESC properties has been naturally conducted by reference with mESCs. Comparative analyses between hESCs and mESCs by several groups have defined common and distinct marker gene expression patterns, cellular properties and signaling mechanisms¹²⁻¹⁵. A number of growth factors and cytokines, such as leukemia inhibitory factor (LIF), bone morphogenic protein 4 (BMP4), basic fibroblast growth factor (bFGF) and Wnt are known to play critical roles in regulation of mESC and/or hESC pluripotency in culture¹⁶⁻¹⁸.

In mouse ES cells, LIF is a most critical cytokine for maintenance of pluripotency and self-renewal. Binding of LIF with a complex consisting of the LIF receptor and gp130 induced recruitment of the JAK family kinases that in turn activate signal transducer and activator of transcription 3 (Stat3) and extracellular signal activated kinase 1/2 (Erk1/2). It appears that gp130-mediated activation of Stat3 is sufficient to maintain pluripotent mouse ES cells in serum-containing medium. However, LIF is insufficient to block neural differentiation and sustain self-renewal of mouse ES cells in serum-free medium. Ying et al. found that LIF works in concert with bone morphogenetic proteins (BMPs) to maintain pluripotency, and mouse ES cells can be cultured and propagated in serum-free medium supplemented with BMP and LIF without feeders¹⁹. Furthermore, forced expression of Id genes, downstream factors induced by BMP via the Smad pathway, liberated mouse ES cells from dependence on BMP or serum. Thus, BMP-induced expression of Id proteins support LIF/Stat3 mediated self-renewal of mouse ES cells by suppressing lineage-specific gene expression and cell differentiation.

Shp2, a cytoplasmic tyrosine phosphatase, acts in the molecular switch governing ES cell self-renewal versus differentiation²⁰. A targeted deletion of Shp2 gene in mouse ES cells results in more efficient self-renewal. LIF-stimulated phospho-Stat3 signals are higher in Shp2-deficient mES cells compared to wild-type cells, and expression of wild-type Shp2 in Shp2-deficient

mES cells downregulates LIF-stimulated phospho-Stat3 levels²¹. Consistent with this observation, mES cells engineered to express a G-CSF-gp130 chimeric receptor avoiding a Shp2 binding tyrosyl residue (Y757) of gp130 required lower levels of gp130 stimulation to maintain pluripotency²². Thus, Shp2 facilitates mouse ES cell differentiation by downregulating the Stat3 pathway, while promoting signaling through the Erk pathway. The balance between Stat3 activation, essential for mESC self-renewal, and Erk activation, which favors differentiation, may determine stem cell fate. Whether Shp2 has a similar function in control of human ES cell differentiation and self-renewal remains to be determined.

Interestingly, LIF is unnecessary for maintenance of human ES cell self-renewal²³. Human ES cells undergo rapid differentiation in feeder-free culture medium supplemented with LIF. A similar phenotype of LIF-independency was observed for cynomolgus monkey ES cell lines²⁴. Human ES cells are routinely maintained on fibroblast feeder layers or in fibroblast-conditioned medium (CM). Xu and colleagues showed that human ES cells cultured in unconditioned medium (UM) are subjected to high levels of BMP signaling activity, which is reduced in CM. Indeed, a BMP antagonist Noggin synergizes with basic fibroblast growth factor (bFGF) to suppress BMP signal and stimulate self-renewing proliferation of human ES cells in the absence of feeder cells. These findings suggest a basic difference in the self-renewal mechanism between mouse and human ES cells^{18,25}. Thus, the functional requirement of BMP signaling appears to be opposite between human and mouse ES cells. However, activation of the Erk pathway leads to differentiation in both human and mouse ES cells, as expression of dominant negative mutants or use of pharmaceutical inhibitors of Mek suppressed hESC and mESC differentiation²⁶.

Small molecules that suppress ES cell differentiation and sustain self-renewal

With the recognition that too many variable factors exist in culturing human and mouse ES cells with feeder cells and serum, attempts have been made to find small chemical molecules that are potent to sustain ES cell pluripotency in culture. Meanwhile, work using these molecules has also provided fundamental insights into the critical signaling pathways involved in ES cell differentiation versus self-renewal. Brivanlou's group isolated 6-bromoindirubin-3'-oxime (BIO) as a specific pharmacological inhibitor of glycogen synthase kinase 3 (GSK3) from mollusk Tyrian purple. Activation of

the Wnt pathway by use of BIO can sustain the undifferentiated status in both human and mouse ES cells, based on evaluation of expression of *Oct-3/4*, *Rex-1* and *Nanog*, marker genes for pluripotency²⁷. Removal of the compound from culture medium leads to restoration of multi-lineage differentiation program of human and mouse ES cells, suggesting inactivation of GSK3 by BIO is reversible.

Cheng's group compared a panel of human and mouse fibroblasts with capacities for supporting the prolonged growth of human ES cells, to search for growth factors required for hESC survival, proliferation, and self-renewal¹⁷. Their experiments suggest that supportive feeder cells secrete factors required for human ES cell survival/proliferation and also capable of blocking spontaneous differentiation thereby achieving self-renewal. By examining the effects of blocking or adding recombinant Wnt proteins into hESC culture, they found that recombinant Wnt3a induced human ES cell proliferation and also differentiation in the absence of feeder cell-derived factors. After treatment of Wnt3a for 4-5 days, human ES cells, while exhibiting an undifferentiated phenotype, fail to form undifferentiated hESC colonies. Results of a reporter assay indicate a low level of the beta-catenin-mediated transcriptional activation in the canonical Wnt pathway in undifferentiated hESCs, although upregulated during differentiation. Based on these observations, Cheng and colleagues proposed a model to explain the seemingly paradoxical roles of Wnt signaling in human ES cells¹⁷. Wnt, in combination with supportive feeder cells or conditioned medium, CM (containing anti-differentiation factors), supports self-renewing proliferation of undifferentiated human ES cells. However, in the absence of the "anti-differentiation factors", Wnt promotes ES cell proliferation and differentiation.

Ding's group launched a cell-based screen of chemical libraries to search for small molecules that promote self-renewal of ES cells²⁶. This search led to identification of a previously uncharacterized heterocycle, SC1, that supports propagation of mouse ES cells in an undifferentiated status in the absence of feeder cells, serum, and LIF. Long-term SC1-expanded murine ES cells maintain the differentiation capacity into all three primary germ layer cells in vitro. Biochemical analysis suggests that SC1 stimulates self-renewal of mouse ES cells through suppression of RasGAP and Erk1 activities²⁶. Ding's group also developed a simple chemically defined medium (CDM) that supports efficient self-renewal of human ES cells cultured on a Matrigel-coated surface over multiple passages²⁸. hESCs maintained under

such conditions express multiple hESC-specific markers, display the characteristic hESC morphology, maintain a normal karyotype in vitro, and form teratomas in vivo. This study also identified growth factors that direct monolayer differentiation of human ES cells toward neural, definitive endoderm/pancreatic and early cardiac muscle cell lineages in the CDM conditions²⁸.

In conclusion, elucidation of intracellular signaling pathways involved in decisions on self-renewal and differentiation of ES cells is a most critical issue in the field of stem cell biology. Toward this goal, combined use of genetic and chemical biology techniques is a most elegant approach.

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Stem Cell Research in China

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About the author: Guo-Tong Xu, MD, PhD, graduated from Harbin Medical University with a bachelor degree in 1982. He later received a MD degree from Peking Union Medical College, Chinese Academy of Medical Sciences, and a PhD in pharmacology from University of North Texas Health Science Center (UNTHSC), USA. After trained in Alcon Lab. and NEI/NIH, he was appointed as a Research Assistant Professor at UNTHSC. He returned to China in 1997, and served as the Director of Sino-American Rainbow EyeCare Center in Nanjing (1997-1999), the China Country Director of ORBIS International (1999-2001), and Special Professor in Shanghai Key Laboratory of Developmental Biology, Shanghai Second Medical University (2001-2003). In 2003, he joined the Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (CAS), and Shanghai Jiaotong University School of Medicine, and became a Principal Investigator, Professor and vice Director in 2005. In 2006, his lab joined the Key Laboratory of Stem Cell Biology of CAS. Recently, he was appointed as the Executive Vice President of Tongji University Medical School. Dr. Xu's research focuses on aging and metabolic eye diseases, including the development of new drugs and new therapies for diabetic retinopathy (DR) and age-related macular degeneration (AMD). He is now in charge of the establishment and development of a network of Stem Cell Banks in China. Dr. Xu served as the Secretary General and Chair of the Organizing Committee of the 2007 Shanghai International Symposium of Stem Cell Research. (Email: gtxu@sibs.ac.cn)

Background

Stem cells, in simple terms, are the cells capable of self-renewing and differentiating. By such definition, stem cells provide virtually unlimited sources for biomedical research and treatment of diseases through regenerative medicine. Stem cell research aspires the cure of many devastating illnesses such as Alzheimer's disease, Parkinson's disease and diabetes. It has the potential to revolutionize regenerative medicine which is currently limited by donor tissues and organs. Stem cells have provided experimental systems for studying early human development and mechanisms of genetic diseases. Stem cells are also becoming useful tools for drug discovery and development. It is therefore not surprising that stem cell research has been one of the hottest areas in life sciences. Progresses in stem cell research have been ranked among the major scientific breakthroughs in the past few years by top scientific journals.

The public interest in stem cell research goes beyond the scope of the science itself. China is a country where the general public is fascinated by stem cells and therapeutic cloning, and overwhelmingly supports stem cell research. In our history, one of the earliest concepts of cloning can be seen in the "Monkey King" published more than 400 hundred years ago. About 45 years ago, Professor Dizhou Tong, a Chinese embryologist at Institute of Oceanography, Chinese Academy of Science (CAS), cloned a fish by injecting a somatic cell of the Asian carp into an enucleated oocyte of the same species^[1]. Ten years later, Tong generated an interspecies hybrid fish by inserting a European carp nucleus into an Asia carp egg^[2]. In the 1980s, Dr. Daopei Lu at People's Hospital of Peking University performed the first syngeneic bone marrow transplantation in China^[3]. The hematopoietic stem cell from bone marrow was the first stem cell type being used for treating human diseases. These early examples signify the curiosity and fascination of Chinese people and their scientists in cloning and stem cell research.

In the past two decades, with the tremendous economic growth in China, the government significantly increased its supports to scientific research and technology development. Stimulated by the breakthroughs in mammalian cloning and human embryonic stem cells, Chinese government has provided generous support and ethical guidelines to regulate stem cell research. This encouraging environment has attracted many Chinese scientists abroad to return and establish research capabilities in stem cell research. Since several reviews on stem cell research in China have been published recently^[4-6], this article will primarily introduce the key stem cell players in China and their current works. We've learned and followed the progress of these scientists through organizing major research projects in China, academic exchanges and international stem cell research symposiums since 2001, and we hope that such information is valuable to the readers.

Stem cell researchers in various cities

The two 973 (basic research) projects for stem cell research initiated in 2001 represent a milestone in the development of stem cell