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Maintaining Embryonic Stem Cells and Induced Pluripotent Stem Cells

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1. Introduction

Early in 1981, the pluripotential cells were first established from mouse embryos by Evans et al (Evans and Kaufman, 1981). In the same year, Martin et al (Martin, 1981) named these pluripotential cells embryonic stem (ES) cells. In 1990s, human ES cells were first established by Thomson et al (Thomson et al., 1998). Such ES cells isolated from inner cell masses of blastocysts present the unique property of self-renewal and the ability to generate differentiated progeny in all embryonic lineages both in vitro and in vivo. The pluripotency of these ES cells was demonstrated conclusively by the observation that subclonal cultures, derived from isolated single cells, can differentiate into a wide variety of cell types including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). Due to these unique properties, ES cells may become an exceptional source of tissues for transplantation and have great potential for the therapy of incurable diseases.

2. The derivation of embryonic stem (ES) cells and induced pluripotent stem (iPS) cells

Recently, Laurent et al. (Laurent et al., 2010) determined the ethnic origins of the 47 commonly used human ES cell lines by genome-wide SNP genotyping and Bayesian analysis of population structure and found that the large majority of human ES cell lines (43 of 47) were of European and East Asian ethnicity. There was a notable lack of cell lines representing African ethnicity. Mosher and colleagues (Mosher et al., 2010) also described the lack of population diversity in widely distributed human ES cell lines and suggested deriving and disseminating new ES cell lines based on underrepresented populations or diverse donors to increase the ethnic diversity in human ES cell lines. Stem cell lines with a greater ethnic genetic diversity must be developed to optimize the use of such cells as research tools and in future therapies.
To increase the diversity of human ES cell lines, however, more emphasis should be put on the protocols involved in technique system, such as those for derivation, propagation, and long-term potency maintenance of human ES cells, which have still to be improved. Blastocyst-stage embryos donated for research after assisted reproductive techniques were used for new ES cell isolation. However, ethical or technical limitations restrict the research projects in derivation of new human ES cell lines. Establishment of human ES cells from

[1] The first 2 authors contributed equally to this work.

discarded poor-quality embryos (Lerou et al., 2008) (Chen et al., 2009) minimizes the ethical problem but requires further technical improvement and financial support. Defining protocols to derive and propagate high quality human ES cells from embryos should be well developed and promoted, especially in underdeveloped African countries.

Because of technical complications and ethical controversies of establishing human ES cell lines from embryos, the somatic cell in vitro reprogramming approach has become the most efficient and practical way to produce large banks of pluripotent cells. Recent breakthrough studies using a combination of four factors to reprogram somatic cells into induced pluripotent stem (iPS) cells without using embryos or eggs have led to an important revolution in stem cell research (Fig.1). Comparative analysis of human iPS cells and human ES cells using assays for morphology, cell surface marker expression, gene expression profiling, epigenetic status, and differentiation potential has revealed a remarkable degree of similarity between these two pluripotent stem cell types. These advances in reprogramming will enable the creation of patient-specific stem cell lines to study various disease mechanisms, offer valuable tools for drug discovery, and provide great potential to design customized patient-specific stem cell therapies with economic feasibility.

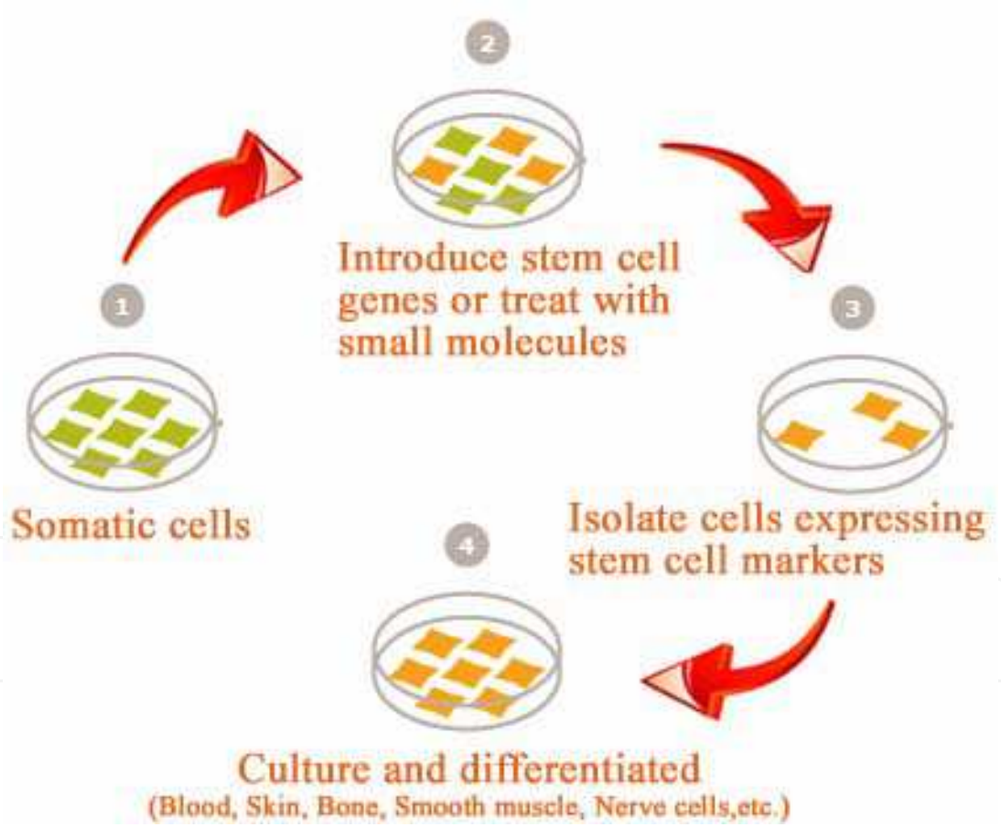

Fig. 1. Reprogramming somatic cells into induced pluripotent stem (iPS) cells.

Patient-specific pluripotent cell lines may provide a limitless source for human cell therapeutic application. However, although human ES cells and human iPS cells have been shown to share a number of similarities, there are still differences electrophysiology properties between human ES cells and human iPS cells (Jiang et al., 2010). It has been showed that foreign genes were silenced or removed after reprogramming, but those

approaches have low reprogramming efficiency, and either leave residual vector sequences, or require tedious steps. Whether reprogramming methods can be improved will depend on a better understanding on the molecular cell biology of pluripotent stem cells.

3. The cultivation of ES cells and iPS cells

ES cells were initially established and maintained by coculture with murine embryonic fibroblast (MEF) feeder cells (Evans and Kaufman, 1981). Subsequent studies identified that fibroblasts secrete multiple factors, including Leukemia inhibitory factor (LIF), fibroblast growth factors (FGFs), transforming growth factor b (TGFb), Activin, Wnts, insulin-like growth factor (IGF), and antagonists of BMP signaling. ES cells are normally derived and maintained in media containing these factors in combination. In stem cell cultures LIF is the essential media supplement for the maintenance of pluripotency of ES and iPS cells.
Practically, ES cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10~15% refined fetal calf serum, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 100U/mL leukemia inhibitory factor (LIF). Generally, pluripotent embryonic cells require a co-culture environment for their self-renewal in monolayer expansion, achieved by culturing on a layer of feeder cells. Mouse embryonic fibroblast cells obtained from 13.5-day embryos of mice were treated for 3 hours with mitomycin C (10μg/mL) as feeders for mouse or human ES cells and iPs cells (Fig.2). In our study, we have developed and validated a feeder-free culture model for ES cells propagation maintaining their pluripotency (Fig.3).

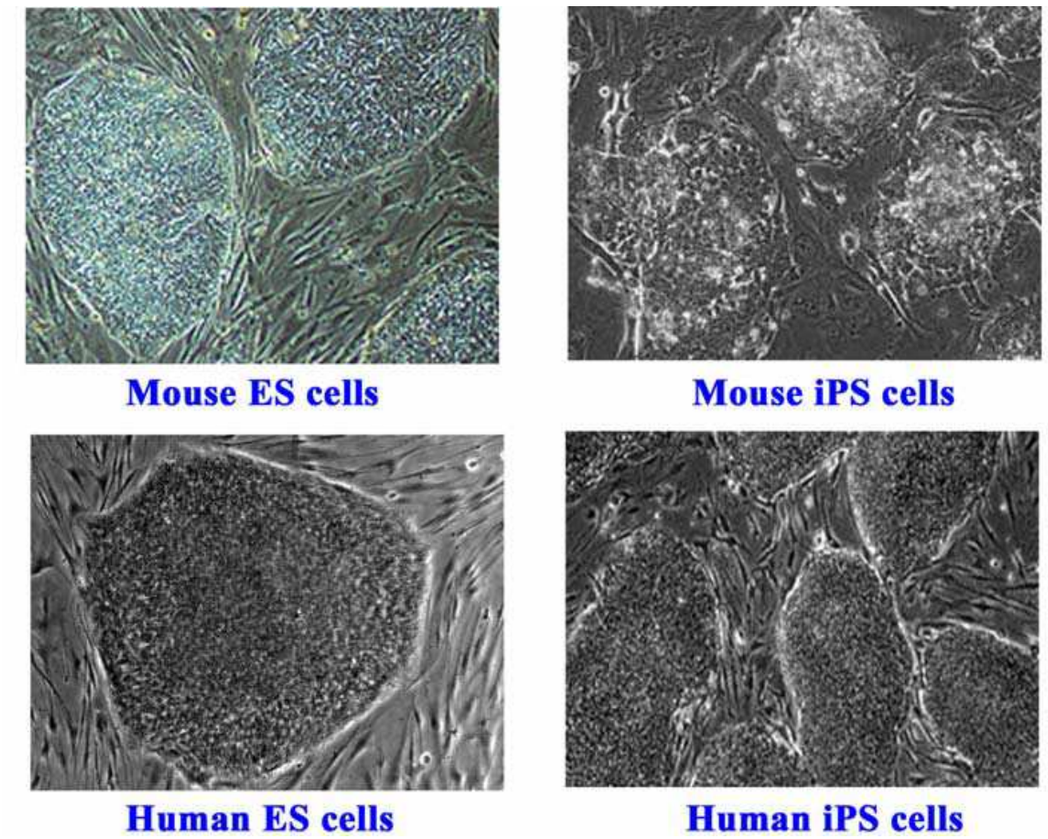

Fig. 2. The culture of ES cells and iPS cell with feeder cells.

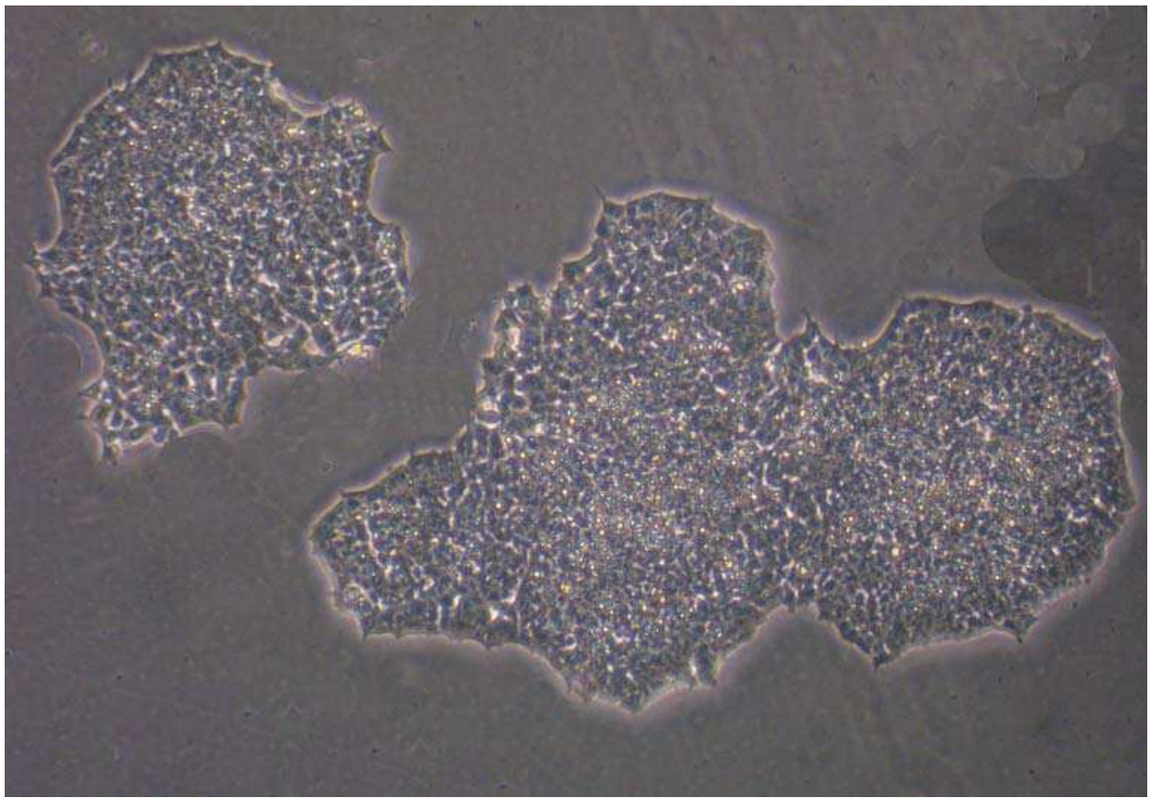

Fig. 3. The culture of mouse ES cells without feeder cells.

The ES cells grew in culture flask precoated with 0.1% gelatin and were maintained at less than 60% confluency to keep an undifferentiated phenotype. Once the cells were to reach 60% confluency, passage of cell was conducted at a 1:8 subculture ratio.

4. The molecular mechanisms and signaling pathways leading to maintain ES cells, the differences between mouse ES cells and human ES cells

The undifferentiated state of ES cells is maintained by the action of transcription factors, some of which are mouse specific and some are common to human and mouse. Oct4, Sox2, and Nanog are master transcription factors for maintenance of the undifferentiated state and self-renewal of ES cells. Regulatory mechanisms pertaining to the self-renewal of stem cells remain incompletely understood.

Signaling in stem cells maintenance includes LIF/Stat3 signaling, Wnt/b-catenin signaling, BMP signaling, and FGF signaling. The maintenance of mouse ES cells is synergistic signaling process. We have investigated the synergistic effect of retinol and leukemia inhibitory factor (LIF) on maintaining pluripotency of mouse ES cells and found that retinol showed a synergistic effect in maintaining pluripotency of mouse ES cells when combined with LIF in moderate concentration and the effect may be attributable to the over-expression of Nanog under retinol stimulation (Fig.4).

The key components that regulate the self-renewal of mouse ES cells have been deciphered and they are largely dependent on two key signaling pathways involving LIF and BMP signaling (Niwa et al., 1998) (Ying et al., 2003). However, human ES cells have significant

differences from mouse ES cells such as variations in the stage-specific antigens and in the ability of leukemia inhibitory factor (LIF) to maintain the undifferentiated state. In human ES cells, LIF receptors are expressed, and LIF can stimulate activation of Stat3 under experiment condition, but this pathway is not activated in the undifferentiated state, suggesting that the maintenance of human ES cells is Stat3 independent (Humphrey et al., 2004). The factors involved in human ES cells self-renewal still have not been elucidated, although significant progress has been made in recent years.

In contrast to mouse ES cells, human ES cells can induce trophoblast differentiation by BMP4 (Xu et al., 2005). The effect may at least partly owing to Smad 1/5/8 activation moderately represses Sox2 (Greber et al., 2008). Activin A, another TGFβ family member, is necessary and sufficient for the maintenance of self-renewal and pluripotency of human ES cells. It can induce the expression of Oct4, Nanog, Nodal, Wnt3, bFGF, and FGF8, and suppresses the BMP signal, support long-term growth of human ES cells on Matrigel coated flasks without either feeder cells or conditioned medium (Xiao et al., 2006).

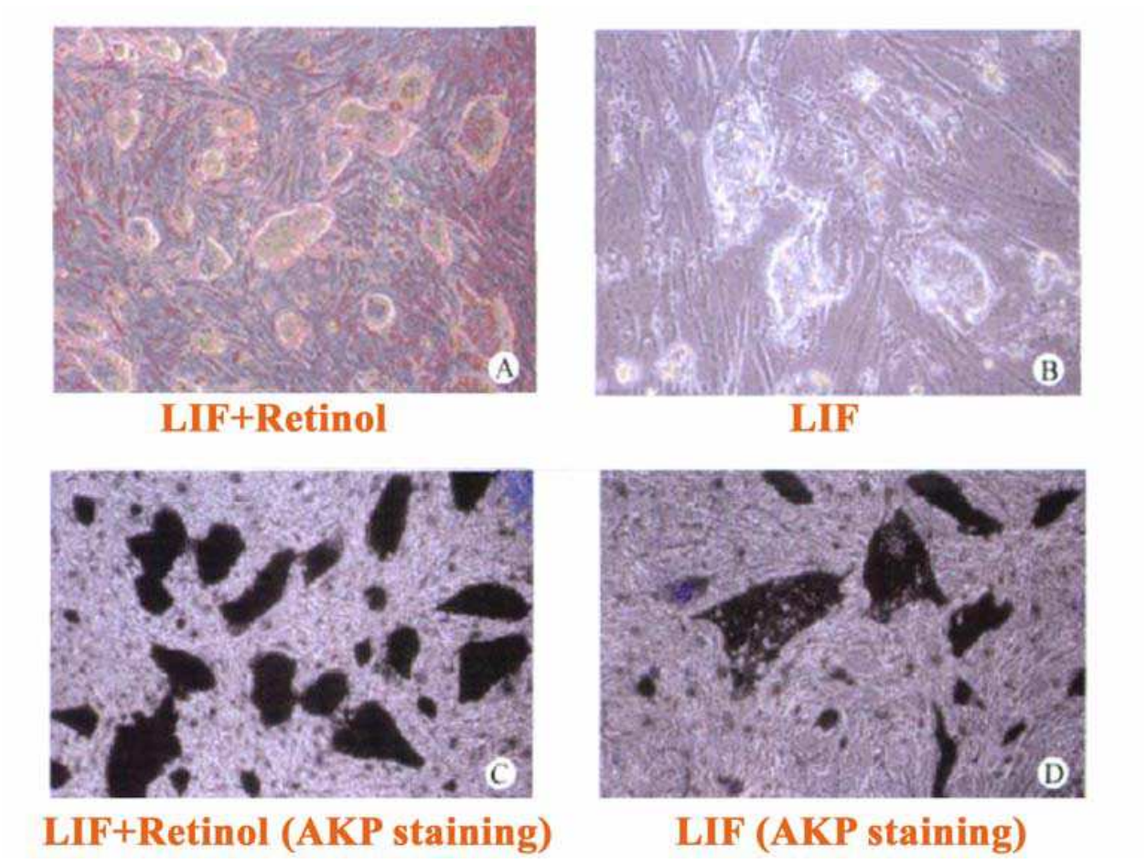

Fig. 4. Morphological analysis and alkaline phosphatase (AKP) assay of ES cell-S19 cultured for 14 days.

5. Support self-renewal and maintain potency of ES cells and iPS cells

Conventionally, pluripotent embryonic cells require a co-culture environment for their self-renewal in monolayer expansion, which is achieved by culturing on a layer of feeder cells. Mouse embryonic fibroblast (MEF) is used as typical feeders for ES and iPS cells. Many studies have explored several alternative cell sources as feeders to support human ES cells culture in monolayer and thereby limiting cross-species contaminations, which includes human embryo derived fibroblasts, foreskin fibroblasts, adult bone marrow cells, and visceral-endoderm (VE)–like cells (Amit et al., 2003) (Hovatta et al., 2003) (Cheng et al., 2003) (Richards et al., 2002) (Mummery et al., 2003).

A microporous poly (ethylene terephthalate) membrane-based indirect co-culture system for human pluripotent stem cells propagation in prolonged culture, which allows real-time conditioning of the culture medium with human fibroblasts while maintaining the complete separation of the two cell types, have developed and validated. This co-culture system is a significant advance in human pluripotent stem cells culture methods, providing a facile stem cell expansion system with continuous medium conditioning while preventing mixing of human pluripotent stem cells and feeder cells (Abraham et al., 2010).

Optimization and development of better defined culture methods for human ES and iPS cells will provide an invaluable contribution to the field of regenerative medicine. Recently, extracellular matrix supporting more undifferentiated growth of feeder-free human ES and iPS cells upon passaging was investigated (Pakzad et al., 2010). Extracellular proteome is found to maintain ES cells. It is reported that the pigment epithelium-derived factor receptor-Erk1/2 signaling pathway activated by the pigment epithelium-derived factor is sufficient to maintain the self-renewal of pluripotent human ES cells (Gonzalez et al., 2010). Synthetic substrate for culturing human ES cells and maintaining pluripotency was also developed (Mahlstedt et al., 2010).

6. Long-term culture and GMP standards

Prolonged culture of human ES cells may lead to adaptation and the acquisition of chromosomal abnormalities (Narva et al., 2010). In our study, the vulnerability of human ES cells and human iPS cells to apoptosis causes a low plating efficiency upon passaging was found. So far, no such small molecular events that promote self-regulation of human ES and iPS cells over a prolonged period of time have been reported in the literature. Maintaining the long-term potency of human ES and iPS cells in well state and produce more homogenous cell clones is still a grand challenge.

Therapeutic application of stem cell derivatives requires large quantities of cells produced in defined media that cannot be produced via conventional adherent culture. The use of feeder cells as well as animal-based products in ES or iPS cells culture may introduce batch-to-batch variations. The ideal culture method is developing feeder-free culture condition (Xu et al., 2001), even chemically defined culture conditions (Ludwig et al., 2006) for human ES cells or iPS cells expansion. But so far few human ES or iPS cell lines were produced without any exposure to animal-derived compounds or in accordance with good manufacturing practices (GMP) standards (Loser et al., 2010).

Therefore, culturing human ES or iPS cells in complete xeno-free conditions to reduce the risk of cross-transfer of pathogens without loss pluripotency would be a crucial prerequisite for clinical-grade applications. Nagaoka et al. (Nagaoka et al., 2010) cultured human

pluripotent stem cells using completely defined conditions on a recombinant E-cadherin substratum, which should facilitate growth of stem cells using GMP standards. Olmer et al. (Olmer et al., 2010) have applied a scalable suspension culture to expand single cell of undifferentiated human ES cells, which represents a critical step towards standardized production in stirred bioreactors. Rajala et al. (Rajala et al., 2010) demonstrated that human ES cells, iPS cells can be maintained in the same defined xeno-free medium formulation for a prolonged period of time while maintaining their characteristics, demonstrating the applicability of the simplified xeno-free medium formulation for the production of clinical-grade stem cells.

For cryopreservation, an effective serum- and xeno-free chemically defined freezing procedure for human embryonic and induced pluripotent stem cells is also needed (Holm et al., 2010).

7. Challenges and prospects

Research on the ES and iPS cells to develop stem cell-based regenerative medicine is still in its early stages and there are still many challenges, including standardization of protocols for cell derivation and cultivation, identification of specific molecular markers, development of new approaches for directed differentiation etc. Among them, culture scale-up ensuring maintenance of cell pluripotency is a central issue, because cell therapy is far more complex and resource-consuming process as compared to drug-based medicine; pluripotent stem cell biology and technology is in need of further investigation and development before these cells can be used in clinics safely and successfully.

In addition, to minimize the ethical controversies, unify guidelines for reviewing ES cell research is also important. Mandatory registration is required for stem cell lines.

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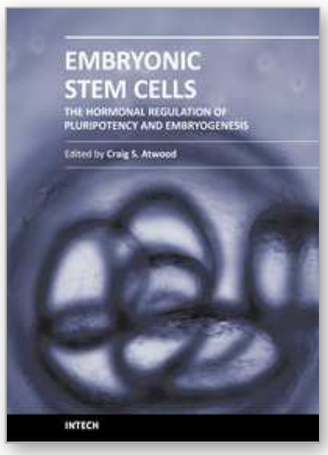

Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis

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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

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