Differentiation of human pluripotent stem cells into functional cells

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Abstract
Introduction
Generation of functional cells from human pluripotent stem cells is the key for their application. Here, we review representative differentiation protocols of human pluripotent stem cells into neural and cardiac lineages. These protocols can be divided into three groups: embryoid body formation, monolayer culture and coculture with stromal cells. The former two are the mainstream methods, while coculture is seldom used now. We also discuss further improvements needed to be achieved.

Conclusion
We summarised the main differentiation protocols of human pluripotent stem cells into neural and cardiac lineages. However, some general problems exist for the current differentiation system and need to be solved in the future.

Introduction
Embryonic stem cells (ESCs) are derived from inner cell mass of preimplantation embryos and can generate more than 200 cell types of the three embryonic germ layers while maintain the ability of self-renew. In 1998, Thomson et al. successfully established ESC lines from human embryos, lighting the enthusiasm of regenerative medicine. The invention of induced pluripotent stem cell (iPSC) technology by Yamanaka et al. brings further the application of human pluripotent stem cells (PSCs), including in vitro model of human development and pathogenesis, drug screening and cell therapy for degenerative diseases.

To accomplish the full promise of human PSCs, we need to differentiate them towards functionally specialised cell and tissue types in a consistent and efficient manner. Since derivation of human ESC lines, hundreds and thousands of differentiation protocols have been published, which can be divided into three groups: embryoid body (EB) method, coculture with stromal cells and monolayer culture method. A general guideline for differentiation protocol design is to mimic embryonic development processes, which was elaborated in this review. Here, we focused on the evolution of human PSC differentiation conditions, summarised the progresses of this filed and remaining shortcomings and discussed future tendency.

Discussion
Neural differentiation of human PSCs
Neural differentiation system based on EB formation
As early as 2001, Zhang et al. reported a neural differentiation protocol based on EB formation. After optimising the original method for more than 10 years, his lab has established protocols for human PSC differentiation into various neuronal and glial subtypes (glutamatergic, GABAergic, dopaminergic and spinal cord motor neurons; astrocyte, oligodendrocyte, etc.)7–11. These protocols mainly consist of five stages: (1) ESC clones are detached from mouse embryonic fibroblasts (MEFs) and suspended as aggregates in ESC medium without bFGF for 4 days; (2) ESC aggregates are first suspended for 2 days and then attached to laminin-coated plates for 3 days in neural induction medium. At this period, cells in the colony become elongated and arrange radically like rosettes, called primitive neuroepithelia, which can be patterned by morphogens (sonic hedgehog (SHH), retinoic acid (RA), etc.) to acquire ventral and posterior characteristics; (3) primitive neuroepithelia are further cultured in the same medium for another 4–5 days, and the area and thickness of rosettes increase to form neural tube-like rosettes with lumens, called definitive neuroepithelia; (4) at day 15, rosettes are lifted by force, and non-neural cells at the outer edge of colonies remain attached to plates. The rosettes can be passaged as suspended aggregates in neural induction medium supplemented with bFGF and EGF, and their developmental potential changes during culture (projection neurons first, followed by interneurons and glia) and (5) neural spheres are digested into single cells, seeded onto laminin-coated plates or polyornithine-treated coverslips and culture with neural differentiation medium.

The neural differentiation protocol of Zhang’s lab has the following advantages: (1) no growth factor is used to induce neural fate, reducing cost and (2) cell–cell interaction in EB mimics in vivo embryonic developmental processes to some extent. On the other side, it also has some drawbacks: (1) the heterogeneous nature of EB differentiation limits neural...
induction efficiency, and non-neural cells account for a substantial proportion; (2) human PSC state is the key to success of this protocol, which may be too tricky to handle for a beginner and (3) the differentiation process is very long, especially for glia. Although it is reasonable, considering human embryonic development takes 9 months, the long process affects its application under acute conditions.

Neural differentiation system based on monolayer culture

The ‘default model’ of neural induction proposes that neural induction occurs as a result of the inhibition of BMP signalling in the embryonic ectoderm, and that in the absence of cell–cell signalling, ectodermal cells will adopt a neural fate. In 2009, Studer’s lab found that dual inhibition of both BMP and activin signalling with Noggin and SB431542 can induce rapid and efficient neural differentiation of human PSCs. This protocol consists of three stages: (1) human PSCs are dissociated into single cells and cultured as adherent monolayer with MEF conditioned media supplemented with bFGF and ROCK inhibitor, Y27632; (2) when human PSCs reach certain confluence, the culture is switched into KSR media supplemented with Noggin and SB431542 to induce neural fate. N2 media is mixed with KSR media at increasing ratios from day 4 after adding Noggin and SB431542 and reaches 100% at day 10. The initial cell density determines the ratio of central nervous system and neural crest progeny. When PSC confluence is 50%–70%, both neuroectoderm and neural crest are induced, while above 90% confluence primarily lead to neuroectoderm and (3) the neuroectoderm is passaged as clumps or single cells and differentiated into neurons in N2 media supplemented with BDNF and ascorbic acid. Based on this protocol, his lab further demonstrated that functional floor plate tissues can be derived from human ESCs, when patterning with SHH from day 1. They also employed a floor plate-based strategy to derive human dopaminergic neurons that efficiently engraft in vivo and GABAergic neurons. Melanocytes of neural crest origin can also be generated based on original protocol.

High efficiency and rapidness are the obvious advantages of dual inhibition protocol. However, it also has some limitations: (1) dissociation human PSCs into single cells may cause cell death and sometimes genomic instability, and culture with conditioned media brings variability; (2) neural induction by Noggin increases cost, while chemical BMP inhibitors (like LDN-193189) may have off target effects and (3) compared with the EB method, the dual inhibition protocol has a short history, and more work needs to be done to find the right condition to generate each neuronal and glial subtype.

Neural differentiation system based on coculture

Coculture with stromal cell lines is an efficient PSC differentiation strategy, especially for certain cell types. For neural differentiation, a classic example is PA6 cells’ inductive role in dopaminergic neurons. As early as 2004, Zeng et al. reported a dopaminergic differentiation protocol of human ESCs, based on coculture with PA6 cells. They just seeded human ESCs onto PA6 cells and cultured with GEMEM supplemented with 10% SR. After 3 weeks, most colonies contained a high percentage of tyrosine hydroxylase (TH, a marker of dopaminergic neurons) positive cells. In the same year, Perrier et al. reported another coculture-based dopaminergic differentiation protocol of human ESCs, based on coculture with PA6 cells. They seeded human ESCs onto M5 cells and cultured with DMEM supplemented with 15% SR for 16 days and then in N2 media for 12 days. SHH and FGF8 were added between days 12 and 20 to pattern neuroectoderm. Rosettes were harvested and passaged twice in N2 media containing SHH, FGF8, BDNF and ascorbic acid. Then the cells were cultured with neuronal maturation media without SHH and FGF8.

The advantage of the coculture method is simple and efficient for certain cell types. The drawback is unclear inductive activity, hindering the mechanism study. The use of stromal cell lines also makes generated cells unsuitable for transplantation. With the rapid advancement of the chemical defined differentiation system, the coculture method becomes unpopular. However, it can act as a backup choice for certain human PSC lines, for which existing protocols do not work.

Differentiation of human PSCs into cardiomyocytes

Like neural lineage, cardiomyocyte differentiation protocols of human PSCs can also be divided into three categories, and EB or monolayer-based systems are equally popular. Coculture with a visceral endoderm-like cell line (END-2) was first reported by Christine Mummery et al. in 2003. Its efficiency is lower compared with the other two methods and seldom used. Here we only discussed some classical examples of EB or monolayer methods.

Generate cardiomyocyte through EB formation

As early as 2001, Kehat et al. established a cardiomyocyte differentiation protocol of human ESCs, with EB formation first in foetal bovine serum (FBS) containing medium for 7–10 days and then plated on gelatin-coated dishes. Since then, many publications reported improvement of the original protocol. Gordon Keller’s group established a serum-free, chemical-defined protocol, which is highly effective for analysing growth factor variables and timing during cardiac differentiation and will be elaborated below.
In 2008, Lei Yang et al. reported a three-step protocol to generate cardiovascular progenitors from human ESCs\(^\text{21}\): (1) EBs are formed by plating small aggregates of human ESCs for 1 day and treated with optimised levels of BMP4, activin A and bFGF from days 1 to 4 to induce a primitive-streak-like population; (2) VEGF and DKK1 are added between days 4 and 8 for induction and specification of cardiac mesoderm; (3) cardiovascular progenitors are expanded by culturing with VEGF, DKK1 and bFGF, and can be isolated through sorting out the KDR\(^{\text{neg}}/\text{C-KIT}^{\text{neg}}\) population. EBs are maintained under hypoxic conditions for the first 10–12 days, and cardiomyocytes are generated by culturing the KDR\(^{\text{neg}}/\text{C-KIT}^{\text{neg}}\) population either as aggregates in suspension cultures or as adherent monolayers. Three years later, the same group found that the emergence of cardiac mesoderm could be monitored by coexpression of KDR and PDGFRa and each human PSC line required optimisation of activin and BMP signalling for efficient cardiac differentiation\(^\text{24}\).

One major limitation of the EB-based differentiation system is heterogeneous EB size. To overcome this, several groups developed a 'forced aggregation' protocol. Elefanty's group developed a chemically defined medium (APEL, albumin polyvinylalcohol essential lipids) and used it in spin EB differentiation of human ESCs\(^\text{25,26}\). In their protocol, 3000 cells are aggregated at the bottom of the 96-well plates by centrifugation at 480g for 2 min at 4°C to form spin EBs, and BMP4, activin A, WNT3A, SCF and VEGF are added for the first 3 days to induce cardiac differentiation. Burridge et al. reported a 96-well V-bottom uncoated plate aggregation system in 2007 and modified it in 2011\(^\text{27,28}\). In their improved protocol, 5000 cells in a chemically defined medium (RPMI containing polyvinyl alcohol and other components) are aggregated by centrifugation at 950g for 5 min at room temperature, and mesoderm cells are induced by BMP4 and bFGF for 2 days under hypoxia\(^\text{29}\).

The EB method has a long history and is well studied. It can be applied to most human PSC lines after optimisation. The disadvantages include such aspects: (1) heterogeneity exists even using a 'forced aggregation' protocol to form EB, since the exterior and interior of EBs have different access to growth factors in medium; (2) growth factors (activin A, BMP4, bFGF, VEGF, etc.) are used to induce cardiac fate, increasing the cost of differentiation protocol.

**Generate cardiomyocyte under monolayer culture**

In 2007, Laflamme et al. established the first monolayer culture-based cardiomyocyte differentiation protocol of human ESCs\(^\text{29}\). To induce cardiac differentiation, they cultured the cells in the RPMI-B27 medium, and added a high dose of activin A for 1 day, followed by BMP4 for 4 days. Beating cells emerged by day 12 after addition of activin A. Compared with EB-based protocols, this method is convenient, without time-consuming steps of forming EBs first and then replating-treated EBs. However, the efficiency is about 30%, lower than the EB method.

Recently, two significant improvements were achieved for the monolayer culture method. Zhang et al. reported a 'matrix sandwich' protocol, generating cardiomyocytes with high purity (upto 98%) and yield (up to 11 cardiomyocytes/input PSC) from multiple PSC lines\(^\text{30}\). Human PSCs are first cultured on matrigel in the mTeSR1 medium. When cells reach 80%–90% confluence, a thin layer of matrigel is overlaid by replacing the original medium with mTeSR1 medium containing matrigel to promote an epithelial-to-mesenchymal transition as in gastrulation. However, the use of matrigel increases cost and also brings uncertainty to the differentiation process, due to the undefined component of matrigel, even for growth factor-reduced matrigel.

Another highly efficient cardiac differentiation protocol is by modulating Wnt signalling at different stages. Minami et al. found a compound (KY02111) through screening, which could induce efficient cardiac differentiation when combined with other Wnt agonists and antagonists\(^\text{31}\). Although they did not know the direct target of KY02111, their data suggested that KY02111 promotes differentiation by inhibiting Wnt signalling. In another study, Lian et al. demonstrated that temporal modulation of Wnt signalling is both essential and sufficient for efficient cardiac induction in human PSCs under-defined, growth factor-free conditions\(^\text{32}\). These two protocols are the most efficient and also cost-effective cardiac differentiation protocols up-to-date and need to be tested on more human PSC lines.

**Conclusion**

Here we summarised the main differentiation protocols of human PSCs into neural and cardiac lineages. However, some general problems exist for the current differentiation system and need to be solved in the future. First, current differentiation protocols need to be adapted to human PSCs cultured under chemically-defined conditions; second, variation of differentiation efficiency among different human PSC lines should be minimised; and third, functional tissues should be generated from human PSCs for transplantation.

**References**