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Safe Transplantation of Pluripotent Stem Cell by Preventing Teratoma Formation

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Abstract

As the renewable source of all cell types in the human body, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) hold great promise for regenerative medicine and cell therapy. However, one major obstacle to the clinical application of these pluripotent stem cells (PSCs) is that these kinds of stem cells remaining with their differentiated derivatives pose cancer risk by forming teratomas after transplantation. The microenvironment niches of PSCs are crucial for teratoma formation and its progression. The high expression of some oncogenes like *cMyc* and *Klf4* are involved in the teratoma formation process. The kinetics of the teratoma and tumor formation after transplantation is depends on the number of remaining PSCs and it could take a long time for a small number of PSCs to form teratomas. Therefore, the batch-to-batch deviation in the lineage specific differentiation will make it a tediously long and not decisive attempt to evaluate the teratoma risk of the PSC-derived cells prepared for therapeutic approaches. The removal of undifferentiated PSCs could be achieved by some ways such as destroying the remained undifferentiated PSCs from tissue and the differentiated cell population, removing PSCs during the differentiation procedure, inducing complete differentiation of leftover undifferentiated PSCs and inhibition from the dedifferentiating process for committed cells. Therefore for this purpose we could use many techniques including monoclonal antibodies, small molecules, anti-angiogenic agents, suicide genes and pharmacological agents to eliminate undifferentiated PSCs and inhibit teratomas. Overalls, an efficient approach beyond the mentioned points in this article to eliminate the teratoma risk associated with PSCs would greatly facilitate the development of the ESC/iPSC-based cell therapy.

Keywords: Cell therapy; iPS; Regenerative medicine; Pluripotent; Stem cell; Teratoma; Transplantation

Pluripotent Stem Cells and Teratoma Formation

Stem cells continuously turn toward the choices of self-renewing, differentiation, migration, quiescence and finally death [1-3]. The embryonic stem cells (ESCs) and their counterparts induced pluripotent stem cells (iPSCs) go through unlimited self-renewal and pluripotency to differentiate into all cell types from all lineages in the body. Therefore, as a renewable source of cells, ESCs and iPSCs possess great promise for the regenerative medicine and cell therapy of human diseases that have no cure in the current state. Human iPSCs are the potential source of patient-specific pluripotent stem cells (PSCs) and have tremendous value for therapeutic purposes [4,5]. However, some serious obstacles must be overcome before the PSC therapies could enter the clinics. These main obstacles include immunogenicity and the teratoma risk that even a few contaminating undifferentiated PSCs within their differentiated derivatives may form teratomas (A non-cancerous tumor) after transplantation [6,7]. As PSCs can form teratomas *in vivo*, this may cause cancer when differentiated cells contaminated with ESCs are transplanted into human organs (Figure 1). Teratoma formation and spreading requires some molecular processes such as self-renewal, lack of contact inhibition and telomerase activity [6]. Also the degree of difference in histocompatibility between donor cells and recipients, the immunosuppressive regiments of the recipients, differentiation protocols and selection strategies has significant importances. The site of transplantation (immune-privilege organs) is a main factor in the teratoma formation. In 2006, Cooke et al., described that the injection under the kidney capsule having the highest success rate and producing the largest teratoma sizes due to its rich vascular bed [8]. Teratoma formation in the lung and thymus had the highest while the pancreas was partially site privileged [9]. Based on some researches the rate of teratoma formation is as follows: subcutaneous 25-100%; intratesticular 60%; intramuscular 12.5% and under the kidney capsule 100% [10]. Also the high expression of some oncogenes like *cMyc* and *Klf4* are involved in the teratoma formation process [11]. In fact formation of three somatic germ layers within the teratoma is taken as the best indicator of the pluripotency of cell lines [12]. But a case report on the teratoma development in a child receiving fetal neural stem cell for treatment of ataxia telangiectasia emphasizes this risk [13], although it has been reported previously that sorting of cells expressing the neural precursor marker Sox1 before transplantation has been reduced the risk of teratoma formation [14,15]. In 2009, Geron Company received a temporary FDA hold on the first ever FDA-approved clinical trial using ESC for spinal cord injury after observing a few incidents of animals developing microscopic, nonproliferative cysts at the lesion sites [16]. The situation for iPSCs is more complex as they integrated by viruses carrying *Oct4*, *Sox2*, *Myc* and krupple-like factor 4 (*Klf4*) transcription factors [4]. Although the viral integration has no direct effect to the reprogramming process, the genomic alterations and the risk of reactivation of the transgenes during the propagation of the undifferentiated cells, is an important threat that prevents any clinical use of such genetically altered cells. Also, all these four transcription factors are highly expressed in cancer tissues [17-20]. Reactivation

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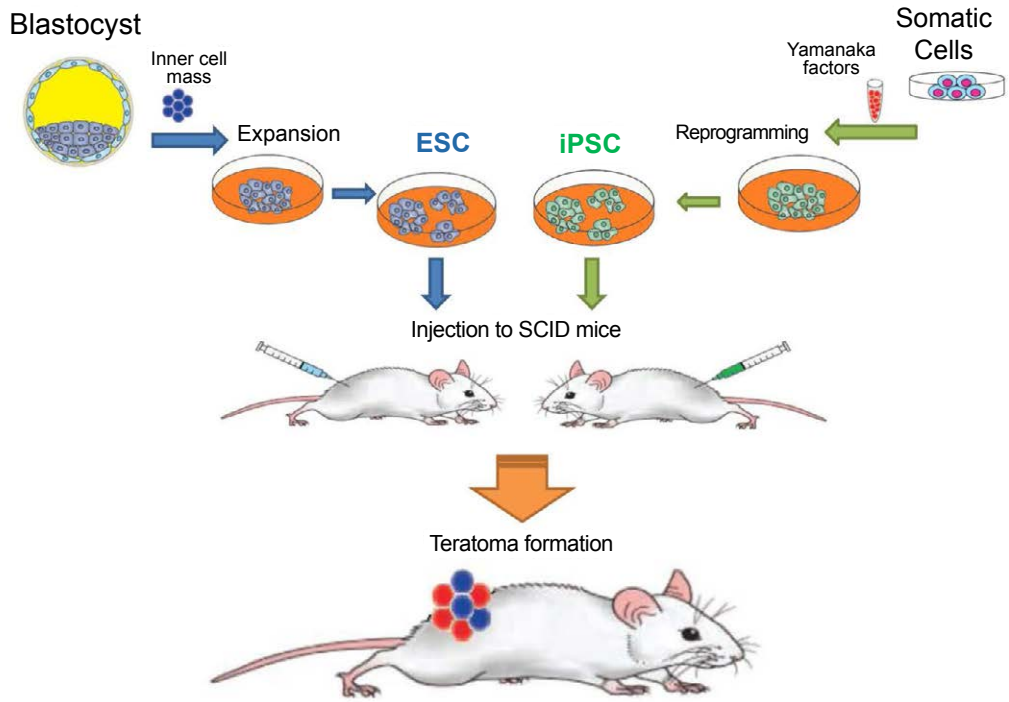

Figure 1: Schematic of teratoma formation. An overview of teratoma formation: culture of PSCs until the desired amount is achieved. The cells are harvested and with equal volume of liquid Matrigel injected to the SCID mice models for teratoma formation.

of the reprogramming transcripts can dispose iPSCs to genomic instability [21]. In 2011, Gore et al. have been shown that the mutations occur in the somatic codons during reprogramming [22] and a study by Hussein et al demonstrated the copy number variation of iPSCs during the reprogramming process [23]. The comparison of teratoma formation between ESCs and iPSCs is indicated by some details in the Table 1.

In most reports, teratoma formation was not reported after transplantation of differentiated grafts derived from ESCs [24,25]. These studies, however, were xenograft transplantations (i.e., human cells into a rat host), suggesting that the lack of tumor formation might be caused by severe immune-rejection due to transplantation from different species. Indeed, allograft experiments resulted in tumor formation even though the cells had been differentiated for more than 14 days *in vitro*, possibly due to a small number of undifferentiated pluripotent cells in the graft [26]. Some observations have been shown that not only undifferentiated human ESCs but also ESCs proliferating neural progenitors can produce tumors [27,28]. The study by Lee and colleagues shows that a critical threshold of undifferentiated human ESCs is required for teratoma formation to occur. Theoretically, a single undifferentiated ESC should be sufficient to populate a tumor; however, various factors may prevent teratoma formation [29]. Depends on the delivery site of pluripotent stem cell and also method of administration, it is possible that even fewer numbers of pluripotent cells lead to tumor formation. Several studies on allogeneic models of ESC transplantation was revealed that as few as 500 ESCs can lead to teratoma formation in immunodeficient state and between 50,000 and 100,000 cells are required for tumor formation in immunocompetency [30]. However there are differences between mouse and human pluripotent stem cells also this phenomenon could be related to the heterogeneity of phenotypically defined ESC population. For instance the *E-Ras* gene promotes the formation of teratomas by mouse ESCs but its human orthologue is not expressed in human ESCs [31]. So the behavior of transplanted mouse ESCs would not accurately reflect the behavior of transplanted human ESCs. Therefore the benefit of nonhuman primate models is more appropriate to demonstrate the safety of human pluripotent stem cell-based therapies. Interestingly, it has been reported that the teratoma growth is accelerated in male immunocompetent mice and xenogeneic rats. It means the teratoma growth might be supported by male hormones. The risk of teratoma formation appears to be influenced by a complex interaction between sex and immune status that needs further exploration [32].

Inflammation is the main driving force for the commencement of tumor development. Macrophage migration inhibitory factor (MIF) is an important regulator of host immune responses and also is recognized as a pro-tumorigenic factor [33] and is over-expressed in many tumors. Monocyte chemo-attractant protein 1 (MCP-1) is an essential chemokine, which acts through its receptor CCR2 to induce the migration and activation of macrophages and thus tumor progression [34]. In 2012, Wang et al demonstrated that PSCs transplantation stimulates an inflammatory response that involves the rapid recruitment and activation of macrophages, which may be a very important driving force in the formation of teratomas [35].

Antibody-based Strategy for Preventing Teratoma

The removal of rogue un-differentiated PSCs could be achieved by 1) destroying the remained undifferentiated PSCs in the differentiated tissue with specific antibodies, 2) removing the undifferentiated PSCs from the differentiated cell population, 3) removing pluripotent cells during the differentiation procedure, 4) inducing complete differentiation of leftover undifferentiated PSCs and 5) Inhibition from the dedifferentiating process for committed cells. To overcome the problem of teratoma formation, antibody based strategies has

Factors influencing tumorigenicity	HESCs	HiPSCs
Origin	Inner Cell M ass cells that have undergone very few divisions	Mature somatic cells that have undergone many cell divisions
Derivation	A relatively minor selection pressure*	A major selection pressure owing to forced drastic change of epigenetic landscape May result in mutations and/or aberrations owing to reprogramming stress
Viral integration	Not applicable	Most of the methods use viral vectors for reprogramming
Oncogenes activation	Not applicable	Current methods induce and up-regulate oncogenes in the reprogramming process
Cellular adaptation	Prolonged growth in culture often results in gains of chromosomes 12, 17, 20 and X	Prolonged growth in culture often results in gains of chromosome 12

Table 1: Comparison of the tumorigenicity between ESCs and iPSCs.

been used to remove the undifferentiated ESCs before transplantation. Among these a cytotoxic antibody against podocalyxin-like protein-1(PODXL) can induce death to ESCs [36] but PODXL is expressed in many of human tissues and making it improper for elimination of the ESCs within the differentiating culture [37]. The immune-depletion of ESCs with the antibody mixture against multiple specific surface markers such as stage specific embryonic antigen 5 (SSEA5), CD9, CD30, CD50 and CD200 that referred to as the pluripotency surface markers could remove ESCs from differentiating cultures and reduce the teratoma risk [38]. The expression of SSEA5 is relatively specific for human ESC but the other surface marker is expressed in differentiated tissues [39], so the antibody-selection of ESCs could be limited by the specificity of the expression of their specific surface markers.

There is another limitation for the antibody based strategies and the problem is that some progenitor cells could dedifferentiate into pluripotent cells after transplantation and leading to teratoma formation [40]. It has been reported that natural killer cells are not important for the rejection of human PSCs *in vivo* [41]; however, in addition to antibody based strategies there are reports on the complement dependent control of teratoma formation that involves the innate immunity. In 2006 Koch et al. demonstrated that the complement system contributes to the control of teratoma growth after transplantation of ESCs [42]. In 2011, Kim et al presented a protocol that could inhibit teratoma formation near 99%. They claimed that two consecutive MACS (magnetic activated cell sorter) separations upon simultaneous staining of two different ESC markers, SSEA-3 and TRA-1-60, completely depleted undifferentiated ESCs [43]. Recently it was demonstrated that an antibody against Claudin-6 (a cytotoxin-conjugated antibody that targets undifferentiated cells; and *Clostridium perfringens* enterotoxin, a toxin that binds several Claudins) efficiently kills undifferentiated cells, thus eliminating the tumorigenic potential and teratoma formation of human pluripotent stem cell-containing cultures [44].

Small molecules for Preventing Teratoma

Small molecule targeting of pluripotent stem cell specific anti-apoptotic factors is an effective strategy to eliminate the risk of teratomas occurrence in pluripotent stem cell-based therapy. In 2004, Bieberich and colleagues reported the selective apoptosis of pluripotent stem cells by novel ceramide analogue that prevents teratoma formation. They were reported that undifferentiated ESCs could be eliminated from cultures of ESC-derived neuronal cells by treatment with ceramide analogue N-oleoyl serinol [45]. Increased level of ceramide is a part of a mechanism that promotes programmed cell death during the development of the mouse brain. The addition of ceramide analogues to mouse ESCs eliminates the undifferentiated cells, but leaves those that have differentiated without any cytotoxic effect [46]. In a study concerning teratoma formation, the levels of a growth factor from the EGF-CFC family (Cripto) were down regulated by generating a Cripto knock-out mouse. Cripto is over-expressed in a wide range of epithelial cancers [47] and its repression enhances neuronal differentiation. When Cripto knock-out ESCs were grown in the presence of FGF-8, and after grafting to immunosuppressed hemiparkinsonian rats, the Cripto negative ESC-derived grafts contained large numbers of dopaminergic-positive neurons without any teratomas [48]. In 2013, researchers in Korea found that quercetin and YM155-induced selective cell death is enough to inhibit teratoma formation after transplantation of human pluripotent stem cell-derived cells [49]. YM155 is an antagonist for surviving effect in pluripotent cells and quercetin (QC), a dietary flavonoid, is widely found in fruits (like apple), vegetables and green tea. QC has the ability to suppress various types of cancer cells and tumor growth, which are related, to expression of surviving [50]. PSCs are likely critically dependent, for their survival and self-renewal, on relatively few anti-apoptotic genes like *Bcl10* and survivin that are highly expressed in ESCs. ABT737 and QC, inhibiting Bcl-2 family proteins and survivin, respectively, triggered apoptosis of ESCs and iPSCs but not that of their differentiated derivatives. QC treatment does not seem to influence the differentiation of PSCs into three germ layer lineages [49]. FTY720 (the pro-drug analogue for sphingosine-1-phosphate) is already used in clinical trials to treat multiple sclerosis, mainly because of its immunosuppressing activity (Davis et al., 2005). The experiments showed that FTY720 eliminates teratoma-forming cells and protects neural progenitor cells from N-oleoyl serinol-inducible apoptosis [51].

It has been shown that encapsulation of ESC with membranes (2.2% barium alginate) prevented the teratomas formation up to three months. Also it has been reported the mouse ESCs but not the human ESCs formed aggregates within the alginate capsules, which remained free of fibrosis [52].

Using suicide Gene into the Pluripotency Locus

The marker genes for ESCs and those required for iPSCs production including *Oct4*, *Sox2*, *Klf4*, *cMyc*, *Lin28*, and *Nanog*. These genes that related to the pluripotency are linked to stem cell tumorigenesis [6]. It was reported that gancyclovir treatment could prevent the teratoma formation of ESCs with a transgenic thymidine kinase gene driven by a mouse *Nanog* promoter [53], however the random integration of the transgene in the genome could change the promoter activity and increase the risk of cancer. In 2012, Rong et al., have been designed a strategy to introduce a suicide gene into a genetic locus that is specifically expressed in pluripotent cells but not in their differentiated derivatives [54]. They chose the pluripotency gene *Nanog* locus that is specifically expressed in pluripotent cells. The *Nanog* is rapidly down-regulated post-epiblast stage during embryonic development [55]. *Nanog*, is phosphorylated at multiple Ser/Thr-Pro motifs and the phosphorylation stimulates the interaction between Nanog and the prolyl isomerase Pin1, that cause *Nanog* stabilization by suppressing

its ubiquitination. Inhibition of Pin1 activity and destroying the Pin1–Nanog interaction in ESCs suppresses their capability to self-renew and to form teratomas [56].

Cell Signaling and Teratoma

Previously it was thought that the pluripotent marker Oct-4 is enough to predict the formation of teratomas and the removal of Oct-4-positive cells could prevent teratomas from developing after transplantation [57]. But we know that the Oct-4 negative stem cells could contribute to the teratoma formation. Various signaling pathways and cytokines, including fibroblast growth factor (FGF), bone morphogenic protein (BMP4) [58,59], transforming growth factor beta (TGFβ), p38 MAPK [60], Janus kinase (JNK) pathway and ERK pathway [61] regulates ESC self-renewal and survival. Some growth factors also influence apoptosis via PKC, PI3K (Phosphatidyl-Inositol kinase), and Akt pathways [62] and the decrease in the levels of these factors could initiate teratoma formation and enhance its size and incidence.

The cyclin dependent kinase (CDK) inhibitor, p18INK4c, is a known tumor suppressor that can inhibit self-renewal of tumor cells or adult stem cells [63]. Also experiments have been shown that the teratoma volume from p18-overexpressing ESCs was found to be significantly reduced compared to the control group [64]. So, p18 inhibits teratoma growth, which is consistent with a role for p18 as a tumor suppressor in somatic tissues [65].

The B-cell lymphoma 2 (*Bcl-2*) families, consisting more than 25 pro- and anti-apoptotic members, regulates the caspase cascade and apoptosis [66]. These proteins maintain a balance between new cells and old one. ESCs over-expressing *Bcl-2* proliferate in feeder-free cultures when supplemented with leukemia inhibitory factor (LIF) [67], indicating that attenuation of apoptosis is critical for ESCs survival and self-renewal. The teratomas generated from *Bcl-2* over-expressing cells are significantly larger than control cells, suggesting that *Bcl* enhances ESCs survival and teratoma formation *in vivo* [68]. Some studies demonstrated that a distinct mitochondrial p53 function regulates apoptotic signals in ESCs [69,70]. The mitochondrial localization of p53 was suggested to be the result of a post-translational modification of undifferentiated ESCs [69]. The p53 dependent transcription under certain stress condition could induce differentiation by blocking *Nanog* expression. Interestingly, down-regulation of tumor suppressors in the p53 pathway increases the efficiency of the reprogramming and enables it with only two factors including *OCT4* and *SOX2* [71,72]. Also in 2008, Schieke et al. have been demonstrated that the degree of overall mammalian target of rapamycin (mTOR) activation and the mTOR inhibitor rapamycin reduces metabolic rate, augments differentiation, and inhibits teratoma formation of the embryonic stem cells with a high metabolic rate. They concluded that mitochondrial metabolism modulates differentiation and teratoma formation capacity in mouse embryonic stem cells [73]. In 2012, Hannesdottir et al. have been found a role for signal transducer and activator of transcription 1 (STAT1) in protecting from teratoma formation by inducing apoptosis and eliminating premature or aberrantly formed follicles which have the potential to transform into teratomas [74]. STAT1 encodes an inducible transcription factor and mediates the intracellular response to type I/II interferons [75], and serves as an important effector in the innate immune response [76] and a regulator of cell death [77].

Teratoma Restriction by preventing Angiogenesis

Angiogenesis is the proliferation of a network of blood vessels that penetrates into tumors, supplying nutrients and oxygen and removing leftover products. We now show that β1 integrin plays an essential role during angiogenesis. In 1997, researchers have been shown that β1 integrin is essential for teratoma growth and angiogenesis [78]. Anti-angiogenic therapies to limit the growth of tumors are under investigation [79]. Usually the host vasculatures expanded into teratoma and support the nutriention supply of teratoma [80]. The discovery and characterization of teratoma-derived angiogenesis processes will contribute to our understanding of how teratoma regulates angiogenesis and provide a therapeutic strategy for teratoma formation after ESCs therapy. Based upon the investigations, the agents targeting endothelial cells differentiated from ESCs or preventing host vasculatures from expanding into teratomas may be a choice to prevent and treat teratomas formed by undifferentiated ES cells.

The ESCs that passaged by collagenase could not to generate teratoma when single cell suspension is prepared using trypsin digestion, dissimilar to efficient teratoma formation by trypsin adapted ESCs [81]. Given that teratoma formation must be prevented in clinical cases, this result in the single cell suspension containing ESCs provides promise for a cell therapy strategy.

Diagnosis of Teratoma Formation

As clinical translation of pluripotent stem cells progresses, the use of "gold standard" teratoma formation for examination of pluripotency and/or other PSCs manners in acting *in vivo* will continue to be essential for researchers (Figure 2). In all the conventional teratoma assays using severe combined immunodeficient (SCID) mice and teratomas are formed after injection of clusters of PSCs. The teratoma usually expanded and shows cystic elements that are histologically characterized as nervous tissue, keratinizing stratified squamous epithelium (ectoderm), smooth muscle, striated muscle, cartilage, bone (mesoderm), and glandular tissue in the form of gut- and respiratory-like epithelia (endoderm) (Figure 2).

Teratoma tissues are composed of undifferentiated cells, which expressing SSEA-3, SSEA-4, Tra-1–60 and Tra-1–81. These cells are more differentiated but have less proliferation capacity in compare to ESCs and they would be difficult to form teratoma like masses *in vivo*. Also fluorescence activated cell sorter (FACS analysis) revealed that ESCs derived teratomas express a high level of CD56, and CD56 would be a good indicator for teratoma formation of ESCs *in vivo*. CD56 is a surface glycoprotein that frequently expressed on the surface of neurons, glia, skeletal muscle and natural killer cells [82]. It is intensely expressed in the mesenchyme and is a marker of early neuro-ectodermal differentiation [83], which has been used to identify neural differentiation of human ESCs [84].

Flow-cytometry assay is limited by the sensitivity and its reliable limit is at 10^4 in the background of 10^6 cells with two color assay. Also it requires large sample sizes for testing. Polymerase chain reaction (PCR) similarly needs large sample sizes for statistically reliable testing and is not truly quantitative.

As we know the reporter gene imaging is an ideal technology for monitoring long-term stem cell viability, death, and proliferation. In 2011, Su et al. were developed a bioluminescence assay for detection of teratoma formation. They transduced ESCs with a double fusion reporter gene that consists of firefly luciferase and enhanced green fluorescent protein (Fluc-eGFP) [80]. There were no apparent side effects of the reporter gene on maintaining stem cell state, cell viability and cell proliferation. Therefore this technique enables us to track cells by noninvasive imaging.

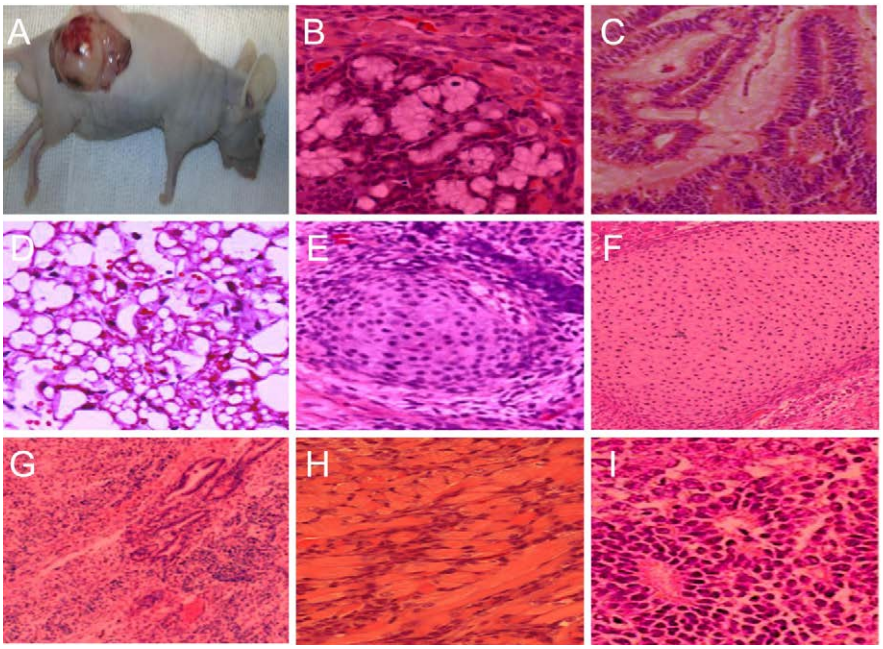

Figure 2: Teratoma growth in subcutaneous tissue of nude mice and histological characterization of a teratoma formed from undifferentiated mouse ESCs. (A) The teratoma mass formed in the subcutaneous region of a nude mouse, (B) Glandular epithelium (endoderm), (C) guts epithelium (endoderm), (D) adipocyte (mesoderm) tissue, (E) Cartilage (mesoderm) tissue, (F) an osteoid like (mesoderm) tissue, (G) smooth muscle cell (mesoderm), (H) striated muscle tissue (mesoderm) and (I) neural epithelium (ectoderm).

Another method to survey the teratoma production could be the transplantation of PSCs onto the chorio-allantoic membrane (CAM) of chicken embryos. This method possess the advantages of the *in vivo* experiment with the simplicity of an *in vitro* conditions such as quick and cheap without ethical concerns. The CAM is located at the periphery of the embryo as a vascularized extraembryonic natural immunodeficient tissue. Tumor growth in CAM could be observed within days (~ 5–10 mm in Size) and much similarity to teratomas [85].

Conclusion

Now there are some methods available to eliminate undifferentiated cells remained from pluripotent stem cells. Some of these methods are efficient in some extent but we still need an efficient and cost effective method for this purpose. The kinetics of the teratoma formation is depend on the number of remaining PSCs and it could take a long time for a small number of PSCs to form teratomas. Therefore, the batch-to-batch deviation in the lineage specific differentiation will make it a tediously long and not decisive attempt to evaluate the teratoma risk of the PSC-derived cells prepared for therapeutic approaches. Therefore, an efficient approach beyond the mentioned points in this article to eliminate the teratoma risk associated with PSCs would greatly facilitate the development of the ESC/iPSC-based cell therapy.

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