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Efficient Derivation of Mesenchymal Stem Cells and Neural Precursor Cells From Human Embryonic Stem Cells Through Teratoma Formation*

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Abstract Many somatic cell types were obtained by *in vitro* differentiation or teratoma formation of human embryonic stem cells (hESCs). However, it is unclear whether specific cell types can be obtained from hESCs-derived teratoma. It was reported that many kinds of cells, including neural progenitor/precursor cells (NPCs) and mesenchymal stem cells (MSCs) were isolated efficiently from the teratoma of hESCs through *in vitro* selection. The teratoma-derived NPCs and MSCs showed specific characteristics of molecular markers similar to the primary NPCs and MSCs. Moreover, these teratoma-induced NPCs and MSCs can be further induced to differentiate into neurons, astrocytes, or adipose and bone cells, reflecting their inherent multi-potencies. Given that teratoma normally contains a mixture of ectoderm, mesoderm, and endoderm lineage cells at different differentiation stage, it was suggested that hESCs-derived teratoma could be an alternative source to generate a variety of uncommitted progenitor cells or terminally differentiated somatic cells, which may be otherwise difficult to obtain through direct *in vitro* differentiation.

Key words human embryonic stem cells, teratoma, mesenchymal stem cells, neural progenitor cells

Human embryonic stem cells(hESCs) are derived from human blastocysts[1], morulae[2] or single blastomeres[3]. The differentiation potential of hESCs provides valuable tools for understanding early human embryonic development and differentiation and holds great promises for regenerative medicine[4]. Numerous differentiation protocols have already been developed to produce various cell types from hESCs. *In vitro* differentiation methods can guide hESCs into multiple cell types, including neural progenitor/precursor cells (NPCs)[4, 5] and mesenchymal stem cells (MSCs)[6, 7]. Nevertheless, at present, not all cells or tissue types in the human body could be generated by hESC differentiation *in vitro*. It has been difficult to generate endoderm derivatives[8]. Moreover, different lines of hESCs can exhibit great variations in their efficiencies to differentiate into ectoderm and mesoderm lineage cells *in vitro* or even subtype of neuronal cells in the brain[9]. Therefore, alternative methods that can efficiently yield specific somatic cell types from undifferentiated hESCs remain to be developed.

Undifferentiated hESCs or mouse embryonic stem cells can generate teratoma when transplanted into immunocompromised rodents such as SCID and nude mice. The teratoma contains a mixture of amorphously differentiated or partially differentiated tissue progenitors or terminal differentiated cells of all three germ layers. Therefore, hESC-derived teratoma

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could be potentially an intermediate source for deriving uncommitted progenitor cells or terminally differentiated cell types. In this study, we document the derivation of NPCs and MSCs from hESCs as a proof of principle.

1 Materials and methods

1.1 hESC culture and teratoma formation

hESCs (HSF6 cell line with NIH code UC06) and hESCs-GFP[ubiquitously expressing Green-Fluorescent Protein (GFP) with lenti-GFP viral vector] was routinely cultured on mitomycin C-treated mouse embryonic fibroblasts (MEF) in KO-DMEM supplemented with 20% Knockout Serum Replacement (Invitrogen, San Diego, CA), 1 mmol/L L-glutamine (Invitrogen), 1% nonessential amino acid (Invitrogen), 0.1 mmol/L β-mercaptoethanol, 50 mg/L penicillin and 50 U/ml streptomycin (ICN biomedicals: Irvine, CA), 4 μg/L basic fibroblast growth factor (bFGF, Peprotech: UK). 5×10^6 hES cells were injected into hind-limb muscle or kidney capsule of 4- to 8-week-old male SCID-beige mouse. After 4 to 11 weeks, mice were sacrificed and teratoma tissues were dissected. A portion of teratoma tissues were examined histologically by staining sections with hematoxylin and eosin or immunohistochemistry. The rest of tissues were used to isolate NPCs or MSCs in culture.

1.2 Derivation and culture of NPCs from teratoma

The tissues were minced and digested with 0.05% trypsin and 0.01% EDTA (Invitrogen). Cells were collected and cultured in 15 mg/L polyornithine- (PO; Sigma) and 2 mg/L fibronectin (FN; Invitrogen)-coated dishes in serum-free medium DMEM/F12 supplemented with B27 supplement, 50 mg/L penicillin, 50 U/ml streptomycin and 10 μg/L bFGF.

1.3 Derivation and culture of MSCs from teratoma

After being digested, cells were cultured in MesenCult® medium (human MSC basal medium supplemented with Mesenchymal Stem Cell stimulatory Supplements; StemCell Technologies, Canada) and 10 μg/L bFGF.

Osteogenic or adipogenic differentiations of the teratoma-derived MSC (tMSCs) were determined as previously described[10]. The MSCs (P3 and P7) were inductively cultured in adipogenic differentiation medium: high-glucose DMEM (Invitrogen) medium supplemented with 1% fetal bovine serum (FBS, Invitrogen), 10^{-7} mol/L dexamethasone (Sigma), 6 μg/L insulin(Sigma) and 100 mg/L 3-isobutyl-1-methylxanthine (IBMX, Sigma). Induction medium was refreshed every 3 days. After 4 weeks in culture, the adipocytes formed were identified morphologically with Oil Red O staining. The MSCs were inductively cultured in osteoblast differentiation medium: DMEM/F12 medium supplemented with 10^{-8} mol/L dexamethasone, 10 mmol/L β-glycerophosphate (Sigma) and 50 mg/L vitamin C (Sigma). Induction medium was refreshed every 3 days. The osteoblast differentiations were identified with alkaline phosphatase staining and von Kossa staining, respectively.

1.4 Alkaline phosphatase staining, von Kossa staining, Oil Red O staining and colony forming efficiency

Alkaline phosphatase staining was performed using Vector® Blue Alkaline Phosphatase Substrate KitⅢ(VECTOR LABORATORIES, Burlingame, CA). The mineral was stained with silver nitrate using the von Kossa method. Fat granules were stained by Oil Red O staining solution (Sigma). Colony forming efficiency was determined according to the reported procedures[11].

1.5 Flow cytometry analysis

The tMSCs were incubated for 30 min at 4℃ with fluorescein-conjugate mouse anti-human antibodies: PE-CD105 (SH2) (50 μl/10^6 cells, eBioscience: San Diego, USA), PE-CD90 (Thy-1) (AbD Serotec, Europe), PE-CD73 (SH4), PE-CD29, PE-CD49b, FITC-CD14, PE-CD34 and FITC-CD45(50 μl/10^6 cells, BD Pharmingen, USA) or the corresponding isotype controls (FITC-conjugated mouse IgG isotype control; PE-conjugated mouse IgG isotype control, eBioscience). Cells were then washed twice in PBS and resuspended in 1% paraformaldehyde/PBS followed by a fluorescence-activated cell sorter (FACS; Becton Dickinson: Franklin Lakes, NJ) (GFP positive MSCs were not tested).

1.6 Immunocytochemistry

The teratoma derived NPCs (tNPCs) or their differentiated cells (after 4～7 days culturing without further bFGF supplement) were fixed in 4% paraformaldehyde/PBS for 20 min. After three washes in PBS, cells were permeabilized with 0.1% Triton X-100/PBS for 20 min. Cells were blocked in 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Cells were incubated overnight at 4℃ in primary antibody diluted in 5% BSA/PBS. The antibodies were used including mouse monoclonal anti-nestin (1∶20, ABcam, UK), mouse monoclonal

anti-βⅢ tubulin (TuJ1; 1∶400, Sigma) and mouse monoclonal anti-glial fibrillary acidic protein (GFAP; 1∶400, Sigma). Cells were washed three times in PBS and incubated with cy5-conjugated anti-mouse IgG or IgM (1∶200 in 5% BSA/PBS, Chemicon, USA) for 2 h at room temperature with protection from light. Cells were then in three washes with PBS. DAPI was added to label cell nuclei.

1.7 Karyotype analysis of NPCs and MSCs

To detect whether the mouse cells were mixed in the tNPCs or tMSCs, the karyotype of tNPCs (P2) and tMSCs (P3) were determined by standard G-banding procedure as described before[12].

2 Results

2.1 Teratoma formation

Teratoma model was constructed by injecting hESCs (Figure 1a and a′) into hind limb muscle of adult SCID-beige mouse. Two types of hESCs were used in this research (wild type and GFP positive). As expected, teratomas were primarily formed adjacent to the injection site. Histological analysis revealed that the teratoma contained representative tissues of the three germ layers, including ectoderm tissues such as neural epithelia, pavement epithelium-like structure, mesoderm-derivatives including smooth muscle, cartilage tissue, fat tissue, as well as endoderm tissues such as gut-like structure and gland epithelial cells (Figure 1). The resutls were consistent with the previous observations that multiple types of cells and tissues mingled together during teratoma formation of hESCs, suggesting that coordinated interactions between different cell types existed in teratoma. The interaction may help producing a variety of cells at different differentiation stages.

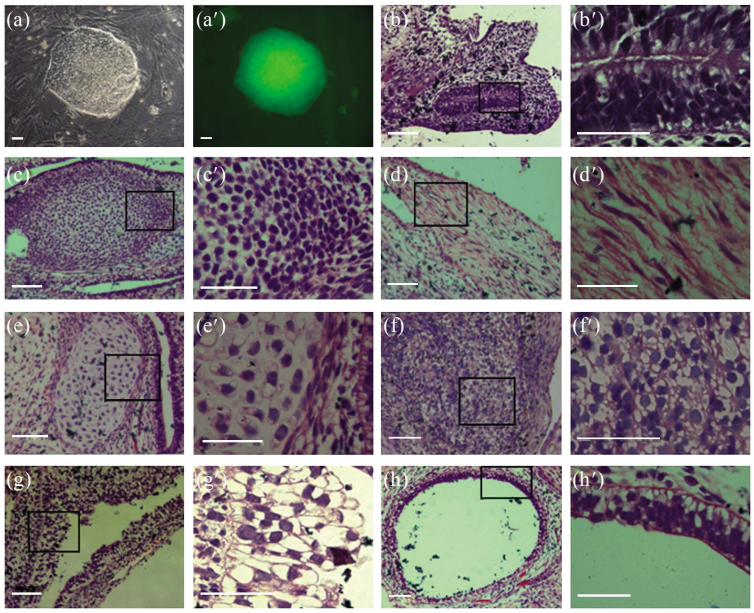

Fig. 1 Teratomas formed by the hESCs in SCID mice and examined histologically

(a) An undifferentiated hESCs colony on MEF and (a′) displayed green fluorescence. (b) and (b′) Neural epithilial. (c) and (c′) Pavement epithelium-like structure. (d) and (d′) Smooth muscle. (e) and (e′) Cartilage. (f) and (f′) Fat tissue. (g) and (g′) Gut-like structure (secretory epithelium rich in goblet cells). (h) and (h′) Gland epithilial. Scale bar, 50 μm.

2.2 Characterization of teratoma-derived NPCs

We next attempted to derive NPCs from the teratoma of hESCs through culturing dissociated teratoma cells in media suitable for the survival and expansion of human NPCs. One teratoma formed by 5×10^6 hES cells could derivate efficiently NPCs-P0 and the efficiency was (16.18 ± 2.42) cm^2/d corresponding to $(32.36\pm4.84)\times10^5$ cells/d (Table 1). After serial passaging in NPC media, we obtained a mono-layer cells exhibiting typical NPC morphology (Figure 2a). NPCs derived from GFP$^+$ hESCs displayed green fluorescence, indicating that lenti-viral vector was still active in NPCs (Figure 2b). The GFP$^+$ NPCs would be useful for tracing transplanted cells. Immunostaining indicated that 85% to 95% NPC-like cells were Nestin positive (Figure 2e), suggesting that teratoma-derived NPCs were highly homogenous. In late passage of teratoma-derived NPCs (≥P5) or upon withdrawal of

bFGF treatment, terminally-differentiated cells including neuronal marker TuJ1-positive neurons and GFAP-positive astrocytes were detected (Figure 2c, d, f, g) or GFAP-positive.

Table 1 Efficiency of derivation of NPCs-P0 from hESCs through teratoma formation

Teratoma	hESCs injected into one mouse	Age of teratoma in mice/d	Area of flask or dish for NPCs-P0 /cm^2	Days for NPCs-P0 to 100% confluence /d	Efficiency of derivation of NPCs- P0[1] /(cm^2•d^{-1})[2]
1	5×10^6	47	300	18	16.67
2	5×10^6	67	240	17	14.12
3	5×10^6	74	240	12	20.00
4	5×10^6	56	240	15	16.00
5	5×10^6	56	240	17	14.12

[1] Efficiency of derivation of NPCs-P0 from 5×10^6 hESCs through teratoma formation, [2] $\bar{x} \pm s$=16.18±2.42, the density of NPC-P0 was 2×10^5/cm^2.

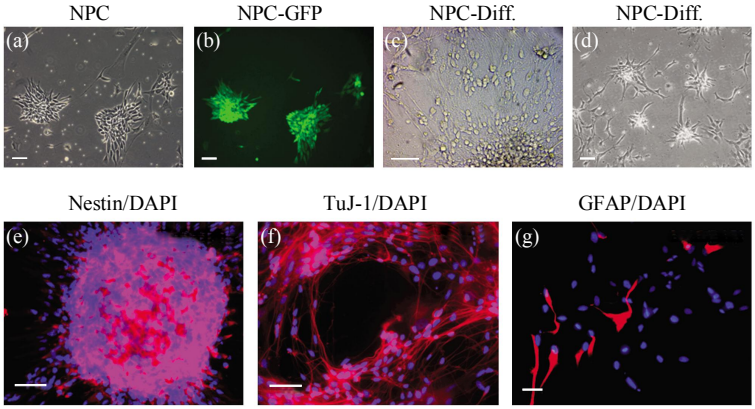

Fig. 2 Characterization of teratoma-derived NPCs

(a) NPCs in phase-contrast microscopic graph and (b) displayed green fluorescence. (c) and (d) Differentiated NPCs in phase-contrast microscopic graph. (e) Immunostaining of undifferentiated NPC with antibody against Nestin (in red). (f) Immunostaining of differentiated-NPCs with antibodies against TuJ1 (in red) and (g) GFAP (in red). DAPI counter-staining (in blue) shows nuclear morphology. Scale bar, 50 μm.

2.3 Characterization of teratoma-derived MSCs

We also derived MSCs from dissociated cultures of hESC teratoma through selection in Mesencult® medium. After selection, green fluorescent cells exhibited a fibroblast-like spindle shape in phase contrast microscope as described[13] (Figure 3a, a′,b and b′). Followed 2 weeks in cultures, primary MSCs formed distinct colonies and fused into a multicellular layer. One teratoma formed by 5×10^6 hES cells could derivate efficiently MSCs-P0 up to (20.97 ± 4.18) cm^2/d corresponding to (9.65 ±1.92) ×10^5 cells/d (Table 2). The colony forming efficiency of the teratoma-derived MSCs was 1/500 to 1/1 000 (Figure 3f). The teratoma-derived MSCs have been cultured in MesenCult® medium for 15 to 22 passages in high purity.

Functional differentiations of teratoma-derived MSCs were analyzed using osteogenic and adipogenic differentiation. Alkaline phosphatase (AP) staining was strongly positive after 10 to 14 days of osteogenic induction (Figure 3c). The average AP positive rate of

Table 2 Efficiency of derivation of MSCs-P0 from hESCs through teratoma formation

Teratoma	hESCs injected into one mouse	Age of teratoma in mice/d	Area of flask or dish for MSCs-P0 /cm^2	Days for MSCs-P0 to 100% confluence /d	Efficiency of derivation of MSCs- P0[1] /(cm^2•d^{-1})[2]
1	5×10^6	45	100	6	16.67
2	5×10^6	60	330	12	27.50
3	5×10^6	51	200	9	22.22
4	5×10^6	58	200	10	20.00
5	5×10^6	65	240	13	18.46

[1] Efficiency of derivation of MSCs-P0 from 5×10^6 hESCs through teratoma formation, [2] $\bar{x} \pm s$=20.97 ± 4.18, the density of MSC-P0 was 4.6×10^4/cm^2.

was more than 90%. After 4 weeks in culture, von Kossa staining showed mineralization deposits in osteogenic induction (Figure 3c′). Cells grown in adipogenic media formed lipid-filled adipocytes and lipid vacunoles after 1 week (Figure 3d). The lipid were stained by Oil Red O and showed salmon pink (Figure 3d′).

The results of flow cytometric analysis demonstrated that wild teratoma-derived MSCs (P3 to P7) were 85% to 97% homogeneous and positive for CD105, CD73, CD90, CD29 and CD49b, negative for CD14, CD34 and CD45 (Figure 3e). The results suggested that the teratoma-derived MSCs in the cultured conditions were highly enriched by the protocol based on their adherence to tissue culture surfaces as previously described[10, 13]. The negative staining cells might be differentiated-MSC cells or mixed other uncommitted stem cells that seem to be different in some surface molecules.

Karyotyping analysis of both teratoma-derived NPCs and MSCs show 46, XX normal female (Figure 3g). These data indicated that teratoma-derived progenitor cells maintain genome stability during *in vitro* expansion and selection.

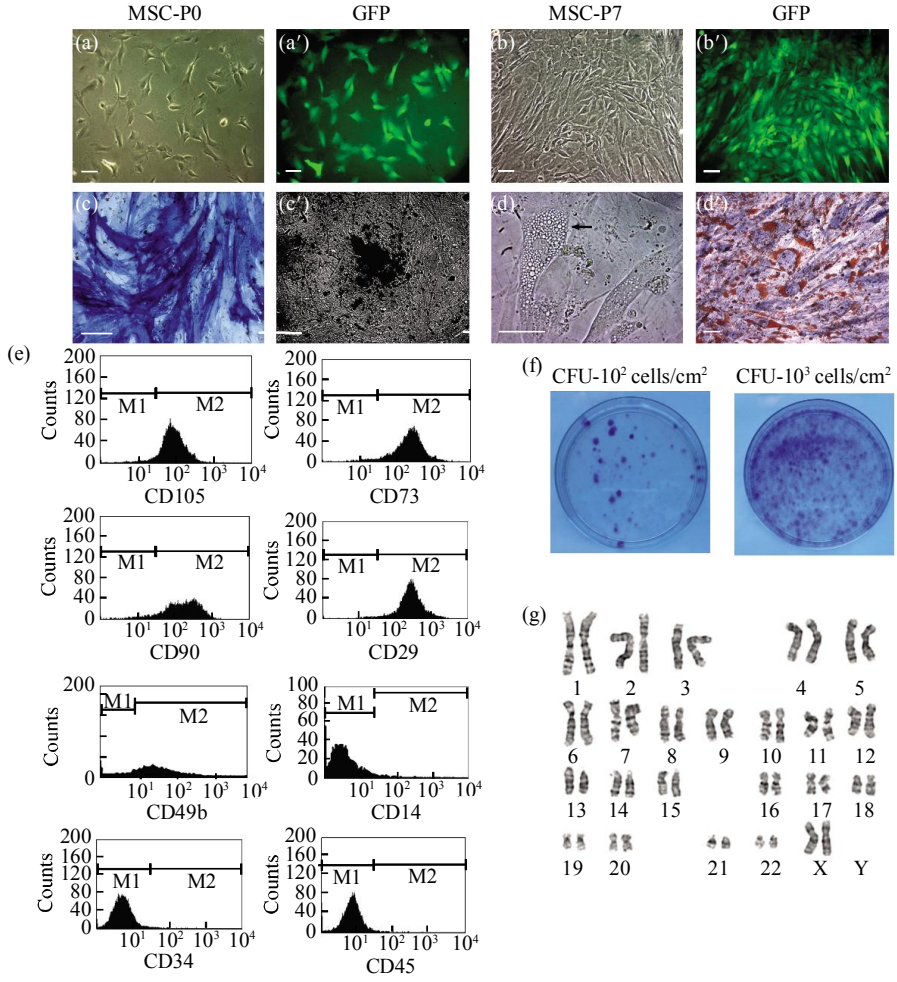

Fig. 3　Characterization of teratoma-derived MSCs

(a) Phase-contrast microscopic graph of primary teratoma-derived MSCs for 7 days cultured with MesenCult® medium, (a′) displayed green fluorescence. (b) Phase-contrast microscopic graph of confluence growth of teratoma-derived MSCs at passage 7 cultured with MesenCult® medium, (b′) displayed green fluorescence. (c) The cells layer displayed positive staining for alkaline phosphatase following 10 days of osteogenic induction (positive of AP staining was blue). (c′) Cells were harvested within 4 weeks of osteogenic-inducing medium and stained for mineralization deposits by von Kossa method. (d) Lipid vacuole (arrow) in MSCs after 7 d adipogenic induction. (d′) Adipogenic differentiation of MSCs was detected by Oil Red O staining and conterstaining by hematoxylin within 4 weeks of adipocyte induction. (e) Flow cytometry characterization of wild teratoma-derived MSCs. Cells were stained with surface markers CD105, CD73 and CD90 or CD29, CD49b, CD14, CD34 and CD45. From P3 to P7, the cells were 85% to 97% homogeneous and positive for CD105, CD73, CD90, CD29 and CD49b, negative for CD14, CD34 and CD45 (M1 indicates negative-stained with antibodies, but M2 positive-stained). (f) Colony forming unit assay of teratoma-derived MSCs with Giemsa staining. The colony forming effiency of the MSCs was 1/500 to 1/1000. (g) Karyotyping analysis of teratoma-derived cells using the G-banding method, and it was 46, XX normal female. Scale bar, 50 μm.

3 Discussion

hESCs have great potential in both basic research and clinical usages. Previous work using *in vitro*-methods have successfully differentiate hESCs into a variety of somatic cell types. However, alternative approach remains to be explored that would efficiently generate cell types that are otherwise difficult to obtain *in vitro*. Here, we described an efficient protocol to isolate NPCs and MSCs from dissociated cultures of hESC-derived teratoma. We found this method was highly efficient at least in producing NPCs and MSCs from dissociated cultures of teratoma.

3.1 The hESCs-teratoma is an alternative source to isolate many differentiating cell types including NPCs and MSCs

In this study, teratoma-derived NPCs and MSCs were obtained efficiently. The tNPCs, like primary NPCs, were bFGF dependent and nestin positive. These cells can further be differentiated into both neurons and glial cells. Likewise, the tMSCs have the potential of differentiation into osteoblast and adipocytes. The tNPCs or tMSCs in the cultured conditions were highly homogeneous and most likely absent of undifferentiated hESCs. Preliminary transplantation experiments showed that these differentiated tNPCs and tMSCs did not form any secondary teratoma when intramuscularly injected back to SCID mice (not shown).

In the hESC teratomas, we could presumably isolate many other kinds of specific cell types that are difficult to isolate from *in vitro* differentiation of hESCs. While many previous efforts have been focused on the *in vitro* culture conditions to differentiate specific progenitors and their progeny, our results suggest that teratoma can be an appropriate source for isolating gland cells, gut cells, follicle cells that so far are difficult to obtain during direct *in vitro* differentiation.

3.2 The hESCs teratomas were different to human embryonic carcinoma cells

Many human embryonic carcinoma (EC) cells lines were derived from human testicular germ cell tumors [14] that were usually malignant (invasive and disorganized in a large mass including mainly lowly differentiated cells or haphazardly arranged tissues). However, hESCs derived teratomas show a lot of arrayed tissues displaying organizational structures. Gland follicles and gut-like structures can be easily observed in teratoma sections. The entire hESCs teratoma was wrapped by an intact fibroid envelope and was regarded as benign ectopic heteromorphism of normal tissue. The reason behind the formation of teratoma was that the injected hESCs were grown in abnormal and unnatural conditions or places and become partially or terminally differentiated during slow growth *in vivo*. In contrast, a single injected EC cells could have the capability of regenerating a whole teratocarcinoma. At present, no evidence showed that a single hESCs had the capability of differentiation to a teratoma after grafting.

In this study, tNPCs and tMSCs have normal karyotypes. In contrast, human EC cells were highly aneuploid and included deletions or amplifications of various chromosomal regions[15]. Nevertheless, EC cell lines, *e.g.*, NTERA2 could be induced differentiated into neurons by retinoic acid and the NTERA2-derived neurons successfully engraft into the central nervous system (CNS) of rodent models after transplantation[16] or even shown to survive for more than 2 years in the human brain of a stroke patient[17]. It remains to be tested in cell transplantation experiments whether tNPCs and tMSCs can be efficiently integrated and differentiated into neural cells or mesoderm-derivatives *in vivo*.

3.3 hESCs-teratoma derived cells may be a source for cell transplantation

Currently, there are many factors that may limit the medical application of hESCs in cell therapy. One of those is animal-based ingredients with a risk of cross-transfer of pathogens or animal products, such as MEF associated pathogen transmission[18], a non-human sialic acid - Neu5Gc contamination[19]. Recent efforts have been made to use feeder-free culture, defined cultures, and certain types of primary human cells, such as fetal skin fibroblasts, foreskin fibroblasts, as feeder layers[18~20] to eliminating the risk of hESCs contamination adventitious agents. However, MEF cells seemed to support hESCs growth better than human feeders for some unknown reasons. hESCs under feeder-free conditions or with human serum[18, 20, 21] are prone to spontaneous differentiation or carry genetic and epigenetic abnormalities[22]. Moreover, to date, it has not been possible to fully eliminate all animal materials during both derivation and culturing of hESCs in order to create a completely xeno-free system, *e.g.*, many animal-based ingredients such as antibody, complement, extracellular matrix and serum

have still been used in derivation and culture of hESCs. However, it should be checked routinely whether rodent virus can be horizontally transferred into human cells. A recent study showed that there was no infection of hESCs by murine leukemia viruses (MuLV) in mouse feeders containing humantropic MuLV in hESCs cultures[23].

The MSCs and NPCs derived from teratoma were in the "surrogate matrix" mice-SICD mice. Unlike other normal mice, SCID mice do not harbor a variety of parasites, bacteria, and viruses that are potentially pathogenic for humans. In this study, hESCs were cultured with MEF feeders and tMSC and tNPCs were derived in the xeno-transplantion model. Nevertheless, the animal products or foreign cells could be diluted and removed after continuously passage of NPCs *in vitro* serum-free cultures. Thus, hESCs-teratoma derived cells may be a source for clinical-grade cells for cell transplantation.

3.4 The teratoma model system provides a probability to construct much more complex structure tissues or organs and mimic *in vivo* microenvironment or niches for cells differentiation

At present, *in vitro* hESCs differentiation take place in culture and cells grow in a 2-dimensional configuration. Some efforts have been tried with 3-dimensional scaffolds to direct morphogenesis during hESCs differentiation *in vitro* and to maintain the 3-dimensional structure and function of the construct as it is integrated with the host tissues after implantation [24]. However, it might shape the macro-configuration but not all micro-structures. This compromise in structure limits clinical viability. In hESCs teratomas, there were many small organizational structures that might be become large 3-dimensional cellular organization combining tissue engineering scaffolds and hESCs in "surrogate matrix".

In vitro differentiation NPCs of hESCs can lead to abnormalities in DNA methylation patterns in comparison to normal or primary cells *in vivo* [25]. Whole genome analysis of the NPCs and fetal tissue-derived NPCs showed that the major differences between them were mostly observed in genes related to the key neural differentiation pathways, such as Wnt, fibroblast growth factor, and leukemia inhibitory factor signaling pathways [26]. It will be of interests to determine whether teratoma-derived NPCs or MSCs are different or the same in these aspects when compared to primary NPCs.

Overall, we have demonstrated a new approach to isolate efficiengly MSCs and NPCs from teratoma of hESCs. Our study suggests that teratoma from hESCs could be a novel source to generate many other specialized human progenitor cell types for basic research or clinical applications.

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从人胚胎干细胞畸胎瘤中有效获取间充质干细胞和神经前体细胞 *

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摘要 通过人胚胎干细胞(human embryonic stem cells，hESC)体外分化方法和畸胎瘤形成可以分化获得多种成体细胞．但目前尚不清楚是否可以从 hESCs 畸胎瘤中分离某些特异性细胞．通过体外筛选方法，有效地从 hESCs 畸胎瘤中分离出神经前体细胞(neural progenitor cells，NPCs)和间充质干细胞(mesenchymal stem cells，MSCs)．这种 hESCs 畸胎瘤来源的 NPCs 和 MSCs 与体内神经前体细胞和间充质干细胞有着相似的分子标记和特性，并具有进一步的分化潜能——分别可以诱导成为神经元、神经胶质细胞、脂肪细胞和骨骼细胞等．根据人胚胎干细胞畸胎瘤中含有不同分化阶段的外胚层、中胚层和内胚层的组织或细胞，认为人胚胎干细胞畸胎瘤可以作为另一个细胞来源以获取多种(包括人胚胎干细胞体外分化难以得到的)各种前体 / 干细胞和终末分化细胞．

关键词 人胚胎干细胞，畸胎瘤，间充质干细胞，神经前体细胞
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