Regenerative Therapy 5 (2016) 31–39

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original article

In vivo maturation of human embryonic stem cell-derived teratoma over time

Hidenori Akutsu [a], Michiyo Nasu [a], Shojiroh Morinaga [d], Teiichi Motoyama [e], Natsumi Homma [a, f], Masakazu Machida [a], Mayu Yamazaki-Inoue [a], Kohji Okamura [b], Kazuhiko Nakabayashi [c], Shuji Takada [b], Naoko Nakamura [a], Seiichi Kanzaki [a], Kenichiro Hata [c], Akihiro Umezawa [a, *]

[a] Department of Reproductive Biology, National Research Institute for Child Health and Development, Tokyo, Japan
[b] Department of Systems BioMedicine, National Research Institute for Child Health and Development, Tokyo, Japan
[c] Department of Maternal-Fetal Biology, National Research Institute for Child Health and Development, Tokyo, Japan
[d] Department of Pathology, Kitasato Institute Hospital, Tokyo, Japan
[e] Department of Pathology, Yamagata University School of Medicine, Yamagata, Japan
[f] School of BioMedical Science, Tokyo Medical and Dental University, Tokyo, Japan

ARTICLE INFO

Article history:
Received 5 May 2016
Accepted 19 June 2016

Keywords:
Pluripotent stem cell
Next generation sequencing
Comparative genomic hybridization
Single nucleotide mutation
Nucleotide substitution rate
Morphometry

ABSTRACT

Transformation of human embryonic stem cells (hESC) is of interest to scientists who use them as a raw material for cell-processed therapeutic products. However, the WHO and ICH guidelines provide only study design advice and general principles for tumorigenicity tests. In this study, we performed in vivo tumorigenicity tests (teratoma formation) and genome-wide sequencing analysis of undifferentiated hESCs i.e. SEES-1, -2 and -3 cells. We followed up with teratoma formation histopathologically after subcutaneous injection of SEES cells into immunodeficient mice in a qualitative manner and investigated the transforming potential of the teratomas. Maturity of SEES-teratomas perceptibly increased after long-term implantation, while areas of each tissue component remained unchanged. We found neither atypical cells/structures nor cancer in the teratomas even after long-term implantation. The teratomas generated by SEES cells matured histologically over time and did not increase in size. We also analyzed genomic structures and sequences of SEES cells during cultivation by SNP bead arrays and next-generation sequencing, respectively. The nucleotide substitution rate was 3.1×10^{-9}, 4.0×10^{-9}, and 4.6×10^{-9} per each division in SEES-1, SEES-2, and SEES-3 cells, respectively. Heterozygous single-nucleotide variations were detected, but no significant homologous mutations were found. Taken together, these results imply that SEES-1, -2, and -3 cells do not exhibit in vivo transformation and in vitro genomic instability.

1. Introduction

Human embryonic stem cells (hESCs) have unique properties such as their pluripotent differentiation potential and immortality [1,2]. This capability to become a series of somatic cell types within the human body garnered significant attention and interest in the fields of regenerative medicine. The first clinical trial of hESC-based therapy started with injection of a glial cell product into patients with subacute spinal cord injury [3]. hESC-derived retinal pigmented epithelium was then developed for patients with Stargardt's macular dystrophy and dry age-related macular degeneration [4,5]. Because hESCs are used as a raw material of these cell-based products, the residual undifferentiated hESCs in the final products need to be examined for in vivo tumorigenicity [6]. Using conventional assays, malignant transformation

* Corresponding author. Department of Reproductive Biology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo, 157-8535, Japan. Tel.: +81 3 5494 7047; fax: +81 3 5494 7048.
E-mail address: umezawa@1985.jukuin.keio.ac.jp (A. Umezawa).
Peer review under responsibility of the Japanese Society for Regenerative Medicine.

http://dx.doi.org/10.1016/j.reth.2016.06.003

(generation of carcinomas, sarcomas or germ cell tumors) from hESC-teratomas has not yet been reported. When designing tumorigenicity assays, important parameters to examine include characterization of the transplanted stem cells, quantification of the number of engrafted cells, the mode and site of transplantation, monitoring of tumor formation and growth, and pathological analysis [7]. Monitoring teratomas as such is crucial, since theoretically, hESC-derived cells survive over time.

Genomic mutations may arise in hESC-derived cells during cultivation, posing an additional risk for their clinical use. Karyotypic analysis and comparative genomic hybridization (CGH) are commonly used to detect chromosomal and sub-chromosomal aberrations. Genome-wide sequencing analysis has also been applied to monitor cells in this context [8,9]. Mutation rates have been determined by various methods in human cells [10–12], and genomic sequence of hESCs has been monitored as an index for transformation and for determining nucleotide substitution rates of hESCs as compared with other cell types.

In this study, we monitored histo–pathological parameters of hESC-teratomas in vivo over time, and examined their genomic alteratiosn. We found that hESC-teratomas histologically matured over time, and that the nucleotide substitution rate in hESCs was comparable with that of somatic cells.

2. Results

Tests to detect malignant transformations include tumorigenicity test by subcutaneous implantation to immunodeficient mice. In addition to these tests, “genetic abnormality that induces persistent cell proliferation” and “genomic instability” are additional concerns. From the viewpoints of tumorigenicity of ESCs used as a starting material for manufacturing of regenerative medicine products, we herewith investigated teratoma analysis by subcutaneous implantation of SEES cells and genome-wide analysis, i.e. CGH and whole exome analysis to detect the transforming activity of the SEES cells.

2.1. Teratoma formation of SEES cells

To address whether the SEES cells (SEES-1, -2 and -3) have the competence to differentiate into specific tissues, teratomas were formed by subcutaneous implantation of SEES cells (1.0×10^7 cells/site) into immunodeficient mice (Fig. 1). Three independent clones of SEES cells induced teratomas within 6–10 weeks after implantation. Histological analysis of paraffin-embedded sections demonstrated that the three primary germ layers were generated as shown by the presence of ectodermal epidermis, glomerulus-like structure, retina, retinal pigmented epithelium, ganglion and neuroepithelium, mesodermal muscle and cartilage, and endodermal ciliated epithelium, proper gastric glands and hepatocytes in the teratomas. Thus, all SEES cell clones examined had potential for multi-lineage differentiation in vivo. We did not detect germ cell tumors, sarcomas or carcinomas in SEES-derived teratomas by histopathological analysis [13].

2.2. Morphometric analysis of teratomas

To investigate the predisposition of SEES cells to differentiate in vivo, we measured the areas of histological components such as cartilage, epidermis and intestine (Fig. 2A–E). The ratios of each component did not differ among teratomas generated by SEES-1, -2, and -3 cells (Fig. 2F–H). We also performed the same experiments using SEES cells at different PDs, i.e. short- and long-term cultivation, to investigate whether long-term cultivation affects in vivo differentiation (Fig. 3). The in vivo differentiation of SEES cells was independent of the cultivation period or passage number.

2.3. Long-term observation of teratomas generated by SEES cells

We followed up the teratomas generated by SEES cells for up to 32 weeks (Fig. 4). The maturity of hESC-teratomas clearly increased after long-term implantation, while the total area made up of each component remained unchanged. Epidermal cysts and intestines included keratinous and secretory material, respectively, and the cyst and intestinal lumen became enlarged due to accumulation of the keratinous and secretory material (Fig. 4G–J). We found neither atypical cell/structure nor cancer in the teratomas after long-term implantation.

2.4. Structural alteration analysis and exome analysis in hESCs

We performed CGH with the dye swap method on the SEES cell lines to investigate whether they had structural alterations (Fig. 5A, B). Compared to each hESC line at an early passage, we detected no chromosomal aberrations, including gain and loss, in all stocks of hESC lines (SEES-1, -2, -3). We then performed whole exome sequence analysis on the SEES cell lines to clarify the number of genetic alterations that occurred during cultivation (Fig. 5C–F). The exome analysis targeted 93.91 Mb in the genome; 21.78 Mb function as protein-coding sequences and start or stop codons (Fig. 5C). This exome study also covered a large part of the non-coding DNA sequences (non-CDS). Mapping to the reference genomic sequence hs37d5, single-nucleotide variations (SNVs) and small indels were detected at two different passages (Fig. 5D). Subsequently, the two identified genotypes at earlier and later passages were compared to find the de novo mutations occurred during the cell culture period. Because platform-specific or method-specific errors are offset in this process, reliable mutation data can be obtained. In the initial analysis of the SEES-1 line, 325 single-nucleotide and 69 indel mutations had been identified. When each genotype was examined, we noticed that a chromosome-wide mutational event causing loss of heterozygosity (LOH) occurred on the X chromosome. Hence, we also performed a structural alteration analysis by using a SNP genotyping array for all the samples. In contrast to CGH, it can also detect copy-neutral LOH with higher resolution. In SEES-1, the sole detected mutation was a deletion of one of the two X chromosomes after passage 10. Most of hetero-to-homo genotype calls on the chromosome turned out to be caused by the chromosomal deletion rather than single-nucleotide mutations. We excluded such calls and instead calculated the mutation rate of single-nucleotide substitutions. Under our cultivation conditions, one passage corresponds to approximately three population doublings (PDs). In total, 99 single bases mutated during 168 PDs in the diploid sequences of the exome targets, i.e. 3.1×10^{-9} mutations per site per PD. The numbers of mutations of SEES-2 during passages 4 and 45 were 93 single-nucleotide and 18 indel mutations (Fig. 5E). Those found in SEES-3 during passages 4 and 24 were 52 and 12 mutations, respectively. No structural mutations were detected by SNP array in the SEES-2 and SEES-3 lines. Similarly, the mutation rates of SEES-2 and SEES-3 were calculated as 4.0×10^{-9} and 4.6×10^{-9}, respectively. The regional annotation of single-nucleotide mutations shows that the number of non-synonymous bases is larger than that of synonymous bases (Fig. 5F).

We also investigated whether SEES-2 cells have mutations in cancer-related genes that are listed in the literature [14] (Supplemental Table 1). We did not find any significant homozygous nonsynonymous genetic alterations of the above-mentioned cancer-related genes in the SEES-2 cells used for teratoma formation.