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Tumorigenic risk of human induced pluripotent stem cell explants cultured on mouse SNL76/7 feeder cells

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ABSTRACT

The potential for tumor formation from transplanted human induced pluripotent stem cell (hiPSC) derivatives represents a high risk in their application to regenerative medicine. We examined the genetic origin and characteristics of tumors, that were formed when 13 hiPSC lines, established by ourselves, and 201B7 hiPSC from Kyoto University were transplanted into severe combined immune-deficient (SCID) mice. Though teratomas formed in 58% of mice, five angiosarcomas, one malignant solitary fibrous tumor and one undifferentiated pleomorphic sarcoma formed in the remaining mice. Three malignant cell lines were established from the tumors, which were derived from mitomycin C (MMC)-treated SNL76/7 (MMC-SNL) feeder cells, as tumor development from fusion cells between MMC-SNL and hiPSCs was negative by genetic analysis. While parent SNL76/7 cells produced malignant tumors, neither MMC-SNL nor MMC-treated mouse embryo fibroblast (MEF) produced malignant tumors. When MMC-SNL feeder cells were co-cultured with hiPSCs, growing cell lines were generated, that expressed genes similar to the parent SNL76/7 cells. Thus, hiPSCs grown on MMC-SNL feeder cells have a high risk of generating feeder-derived malignant tumors. The possible mechanism(s) of growth restoration and the formation of multiple tumor types are discussed with respect of the interactions between MMC-SNL and hiPSC.

1. Introduction

The safety of hiPSC lines produced by genetic introduction remains a great concern for their use in regenerative medicine, since there is the risk of tumorigenesis through insertion mutations and re-activation of transgenes [1,2] or from contamination with undifferentiated cells [3]. While one of the criteria of pluripotency is that hiPSCs can form teratomas in immune-deficient mice [1,2], Yamashita et al. used an *in vitro* cartilage differentiation system in place of the teratoma formation and identified abnormal hiPSC lines with tumorigenic potential [4]. Examination of transplants of secondary neurospheres that were generated from tail tip fibroblast-derived iPSC, showed that 84% of mice died or became weaker because of the development of tumors [3]. Such a high incidence of tumorigenicity in mouse iPSC-derivatives was not well explained, as they attributed this property as being due to the persistence of undifferentiated cells.

hiPSCs in culture generally require the presence of feeder cells and embryonic stem cell (ESC) medium containing leukemia inhibitory factor (LIF) and fibroblast growth factor 2 to maintain pluripotency and self-renewal, though feeder cell-free culture methods are under extensive development [5,6]. Once hiPSC colonies are developed on the mouse feeder cell layer, hiPSCs or their derivatives are intertwined with the feeder cells during harvesting, and mixtures of these cells are naturally injected for transplantation *in vivo*. Mitomycin C (MMC) treatment is used to arrest the proliferation of feeder cells since it causes growth arrest by damaging DNA after crosslinking [7]. MMC-treated feeder cells have been believed to grow no further *in vitro* and *in vivo*. Therefore, MMC-treated feeder cells are considered to be safe for hiPSCs transplantation *in vivo*. SNL76/7 cells, which are derived from a mouse fibroblast STO cell line transfected with neomycin-resistance (*neo*^{r}) and *Lif* genes [8], are used as feeder cells for hiPSCs [2]. These feeder cells release LIF and unidentified substances that allow stem cells to propagate and maintain their pluripotency. However, it is unclear whether hiPSCs exert any influence on the feeder cells. The significance of interactions between hiPSCs and feeder cells in tumor development has not been reported. Furthermore, the

Abbreviations: DMEM, Dulbecco's modified eagle medium; iPS, induced pluripotent stem; MEF, mouse embryonic fibroblast; SCID, severe combined immune-deficient.

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genetic origins of tumors derived from these cells have not been thoroughly elucidated.

We established the normal human lung fibroblast, TIG-1 cell line, for the study of cellular aging [9], immortalized these cells by introducing the *hTERT* gene for the study of malignant transformation [10] and also generated 22 hiPSC lines from TIG-1 following the original demonstration by Yamanaka et al. [2] to reveal the molecular differences between differentiation and cellular aging [11]. We confirmed their pluripotency by teratoma formation and isolated differentiated human cells from the teratoma tissues. We observed re-emergence of hiPSC-like cells from the differentiated cells *in vitro*, thus demonstrating their tumorigenic risk [12]. During the course of the investigation to isolate malignant human cells from explanted hiPSC derivatives, we observed that some of the formed tumors were not teratomas but rather malignant tumors that originated genetically from mouse cells. These unexpected findings urged us to re-examine the tumorigenic risk of hiPSC derivatives grown on mouse feeder cells. Thus, we thoroughly examined the formation of tumors from the 17 TIG-1-derived hiPSC lines generated by ourselves [11] and from the 201B7 hiPSC line established by the Yamanaka group [2], focusing on their malignancy and genetic origins. The possible mechanism(s) of diverse malignant tumor formation from hiPSC explants are discussed in conjunction with the interactions between hiPSCs and feeder cells.

2. Materials and methods

2.1. Cell lines and cultures

TIG-1, normal human fetal lung fibroblasts, were cultured as described previously [9]. We used 17 hiPSC lines we generated from TIG-1 and 201B7 cells, which were established by Yamanaka at Kyoto University, who kindly provided us with the cell line through the RIKEN Cell Bank [2,11]. The cells were cultured as described previously with the feeder cell line, SNL76/7, which was provided (clone: CL-AB2.2) from the Welcome Trust Sanger Institute (Cambridge, UK) [11]. They were cultured with DMEM containing 7% fetal bovine serum (FBS). The second and third passage cells that were obtained after receiving the cells were stored in liquid nitrogen until use. In preparation of the feeder cell layer, the thawed cells were grown and treated with MMC (Wako Pure Chemical) for 2 h at a concentration of 12–20 μg/ml to achieve mitotic inactivation. They were stored at −150 °C until use. Feeder dishes were prepared by seeding the thawed feeder cells into gelatin-coated culture dishes. Growth arrest was confirmed for one week before replacing the medium with human ESC medium for co-culture with the hiPSC line, as described previously [11]. Tumor-derived cells were cultured with MCDB 131 medium (Sigma) containing 10% FBS.

2.2. Tumor formation, histopathology and cell isolation

To test for tumor formation, approximately 1×10^6–2×10^7 cells were transplanted into both flanks of SCID mice (CREA), as described previously [11]. The tumors were dissected and fixed in 4% paraformaldehyde (Sigma) for histo-pathological analyses with hematoxylin and eosin (HE) staining and immunohistochemical staining, which were performed at Sapporo General Pathology Laboratory Co. Ltd. (Sapporo, Japan) using primary and second antibodies with detection markers (listed in Table S1).

Cell lines were isolated from fresh tumor tissues and cultured, as described previously [12]. All surgical procedures were performed under ether anesthesia, and all efforts were made to minimize animal suffering. This study, including all the animal experiments, was specifically approved by the Animal Care and Use Committee of Tokushima Bunri University.

2.3. Long-term co-cultivation of feeder cells with hiPSC

Four independent cultures were tested for the emergence of colony growth. Half of feeder cell layer in the 10 cm dish was inoculated with K4 hiPSCs, with a glass bar barrier placed in the middle of the dish at the time of inoculation so that the remaining half of the feeder cells were subsequently exposed to the hiPSC culture medium. One of the growing colonies was scraped off for expansion and further culture.

2.4. Analysis of anchorage-independent growth

The soft agar assay was performed, as described previously [10]. Some of the colonies were picked and expanded in culture for further study.

2.5. Genomic PCR and RT-PCR

Genomic DNA was isolated from the tumor tissues and cultured cells and total RNA and cDNA were prepared as described previously [12]. PCR amplification was performed using Primestar DNA polymerase and appropriate primer pairs (listed in Table S2). The mouse *Gapdh* primers crossed over with human *GAPDH*.

3. Results and discussion

3.1. Identification of various malignant tumors formed by hiPSC transplantation

Suspensions of 201B7 hiPSCs were transplanted into SCID mice to confirm their ability to form teratomas. However, histopathological analyses by HE staining and immunostaining identified the tumor type as a malignant solitary fibrous tumor, which was composed of spindle shape cells with high frequent mitosis and irregular sized nucleus (Fig. 1A), expressing mild levels of CD34 (Fig. 1B) and αSMA (Fig. 1C). These findings were unexpected, since the 201B7 cell line was the hiPSC line that Yamanaka group had proven to exhibit pluripotency. At the outset of the study, we suspected that this was so called cancerization of hiPSC. However, we did not observe any human chromosomes (data not shown) in the B7t-1 cell line that was established from this tumor (Fig. 2A). This finding urged us to thoroughly examine the tumor formation of 13 hiPSC lines that we had established previously. Histopathological analyses of the 15 tumors showed eight teratomas, five angiosarcomas (malignant hemangio-endothelioma), one malignant solitary fibrous tumor and one undifferentiated pleomorphic sarcoma, as summarized in Table 1. The characteristic features of teratomas with three or two germ layers formed in seven tumors were described previously [11]. The remaining tumors, other than the teratoma, were malignant tumors (Table 1 and Fig. 1A–K). All the tissues were negative for Factor VIII antigen (expressed in mature endothelial cells) (data not shown). Features characteristic of angiosarcoma, as in the case of K4 (1st), were strong expression of CD34, hematopoietic stem cell marker (Fig. 1E) and negative expression for αSMA, smooth muscle actin (Fig. 1F), in many growing endothelial-like cells. Thus, K6, K17 (2nd), K18 and K22 were identified as angiosarcomas. Furthermore, the undifferentiated pleomorphic sarcoma in K11 was characterized by various types of cells (Fig. 1G) expressing CD34, αSMA, S-100 (expressed typically in smooth muscle cells) and CD31 (Fig. 1H–K, respectively).

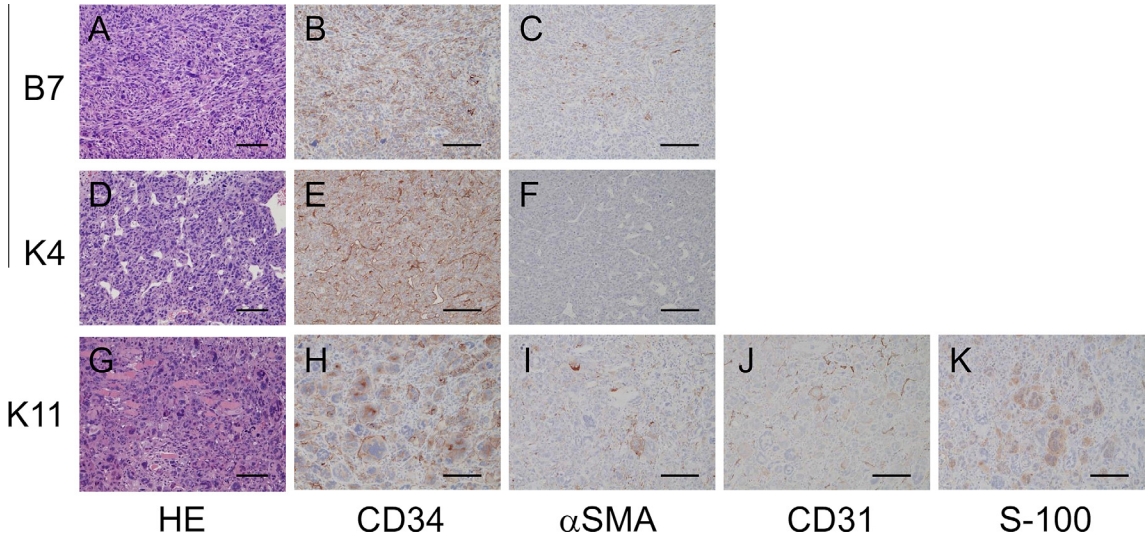

Fig. 1. Histopathological and immunohistochemical analyses of tumors formed by hiPSC transplantation. HE staining of 201B7 (B7), K4 and K11 iPSC tumors are shown in A, D and G, respectively. CD34, and αSMA immunostaining of B7, K4 and K11 iPSC tumors are shown in B, E and H, and C, F and I, respectively. CD31 and S-100 immunostaining of K11 iPSC tumors are shown in J and K, respectively. B7, K4 and K11 tumors were identified as malignant solitary fibrous tumor, angiosarcoma and undifferentiated pleomorphic sarcoma, respectively. The bars indicate 100 μm.

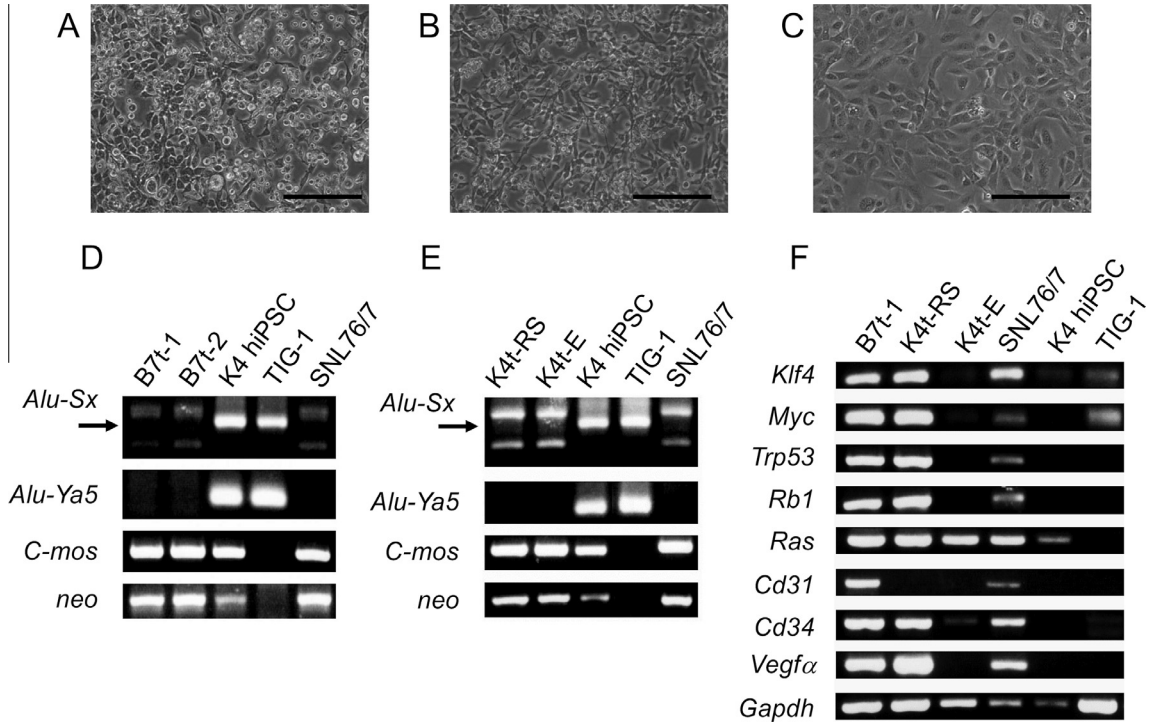

Fig. 2. Tumor-derived cell lines and their genomic PCR and RT-PCR findings. Phase contrast photographs of cell line (B7t-1) from 201B7 tumor, K4t-RS and K4t-E from K4 tumor are shown in A–C, respectively. Scale bars indicate 200 μm. (D) Genomic PCR analysis of B7 tumor-derived cell lines (B7t-1 and B7t-2) was performed using human-specific *Alu* and mouse-specific *C-mos* and feeder-specific *neo-resistant* gene primers. They were compared with those of control cell lines (SNL76/7, TIG-1 and K4 hiPSC). (E) Genomic PCR of K4t-RS and K4t-E derived from the K4 tumor was performed. Note that genomic DNA from K4 iPSC culture contains some SNL76/7 DNA, since SNL76/7 feeder cells were used. (F) RT-PCR of B7t-1, K4t-RS and K4t-E was performed using mouse gene primers.

CD31, which is known as PECAM1, an endothelial cell marker, was also expressed in hematopoietic progenitor cells.

We then considered that abnormal hiPSC lines with tumorigenic properties have been generated previously, as reported by Yamashita et al. [4]. Therefore, we tested the reproducibility of teratoma or malignant tumor formation using the same hiPSC lines with a difference of only a few passages. As shown in Table 1, K4 hiPSC produced angiosarcoma in the first transplantation, but formed teratomas in the second transplantation. Similarly, K17 hiPSC generated teratomas in the first transplantation, but angiosarcomas in the second, revealing that the formation of malignant tumors from hiPSC is not due to specific properties of the hiPSC lines, but likely occurs by chance.

3.2. Genetic origins of malignant tumor cells

Since we could not identify human chromosomes in the cultured cells isolated from the 201B7 tumor, we suspected the

Table 1
Types of tumor examined by HE staining and immune-histopathological analysis as described in text.

iPSC line	Malignant	iPSC line	Teratoma
201B7	Malignant solitary fibrous tumor	K1	Gland epithelium, melanocyte vascular tube (3 germ)
K4 (1st)	Angiosarcoma	K4 (2nd)	Gland epithelium, vascular tube (2 germ)
K6	Angiosarcoma	K5	Gland epithelium, cartilage like, retina like (3 germ)
K11	Undifferentiated pleomorphic sarcoma	K9	Gland epithelium, connective tissue, striated muscle (2 germ)
K17 (2nd)	Angiosarcoma	K12	Gland epithelium, connective tissue, vascular tube (2 germ)
K18	Angiosarcoma	K13	Melanocyte, gland epithelium, vascular tube (3 germ)
K22	Angiosarcoma	K17 (1st)	Gland epithelium, cartilage like, vascular tube (2 germ)
		K19	Hepatocyte like, gland epithelium, vascular tube (2 germ)
		K21	Gland epithelium (1 germ)

possibility that the tumor cells were derived from mouse cells and decided to examine their genetic origin by using mouse- or human-specific repetitive genes and marker genes inserted in the SNL76/7 feeder cells. Therefore, we established two cell lines (B7t-1 and B7t-2) from a 201B7 hiPSC tumor. From B7t-1, we cloned a round cell line (Fig. 2A) and sub-cultivated them up to 120 population doubling levels (PDLs). They formed colonies in soft agar, indicating transformation with anchorage-independent growth ability.

We then checked the genomes of B7t-1 and B7t-2 in comparison with those of human TIG-1, mouse SNL76/7 and K4 hiPSCs grown on feeder cell layer by *examining short interspersed nuclear elements* (*SINE*). Human-specific sequences of *Alu-Sx* and *Alu-Ya5* were detected in K4 hiPSC and TIG-1. A mouse-specific *C-mos* sequence was detected in B7t-1, B7t-2, and K4 hiPSC, as well as in the SNL76/7 cells. In addition, a selection marker of the *neo*^{r} gene from the SNL76/7 cells was detected in these cell lines (Fig. 2D). Thus, B7t-1 and B7t-2 were derived from SNL76/7 cells, but not from the host SCID mouse, suggesting that the 201B7 tumor was formed from MMC-treated feeder cells. Moreover, it was noticeable that K4 hiPSC cultured on feeder cells also contained a marker gene from the SNL76/7 cells. We acknowledge that the hiPSCs prepared for transplantation are mixed with some SNL76/7 feeder cells, which occurs naturally when feeder cell layers are used for hiPSC cultures. To confirm these results, we cloned two cell lines (K4t-RS and K4t-E) from another type of tumor, angiosarcoma, formed by an explant of the K4 hiPSC line (Fig. 1D).

The K4t-RS cell line exhibited low adherent round cells with interchangeable spindle shape cells (Fig. 2B) and the K4t-E cells were morphologically endothelial-like cells (Fig. 2C). They were sub-cultivated up to 140 PDLs with no decline of growth rate, suggesting their immortalization. To examine their malignancy, we transplanted them again into SCID mice. The K4t-RS formed a tumor after 3 weeks with the injection of up to 1×10^5 cells. The tumor was identified as angiosarcoma with many mitotic and heteromorphic figures compared with the first angiosarcoma (Fig. 2D). The K4t-E, however, did not develop into a malignant tumor, but rather soft tissue with irregular vascular tubes after 7 months (Supplementary Fig. S1). Analyses of the genetic origins of these malignant cell lines indicated that they contained the *neo*^{r} gene (Fig. 2E), suggesting that the K4 hiPSC-derived tumor was formed from MMC-SNL.

These findings urged us to examine the genetic origins of all tumors formed from explants of the hiPSC lines. By performing genomic PCR, 11 tumors were proved to be teratomas with human origins. The remaining eight tumors were malignant and originated from the mouse SNL76/7 cells (Table 2). It was noticeable that none of the malignant tumors contained human *Alu* sequences. Thus, tumors from the hiPSC lines developed by chance into either teratomas with the human genome or malignant tumors with the mouse feeder cell genome. In other words, there were no cases of mixture between the human and mouse feeder genomes, implying that hybrid cells between human cells and mouse cells were not generated during the tumor development. We also suspected the possibility that the concentration of MMC was not sufficient to inactive the growth ability of the SNL76/7 cells. However, the formation of malignant tumors or teratomas was not affected by increasing the MMC concentration from 12 to 20 μg/ml (data not shown), suggesting the existence of some unknown factors in the formation of malignant tumors.

Table 2
Genetic origin of tumor.

Identified origin	Transplanted hiPSC lines
Human	K1, K3, K4 (2nd), K5, K9, K12, K13, K16, K17 (1st), K19, K21
Mouse (SNL76/7)	201B7, K4 (1st), K6, K8, K11, 17 (2nd), K18, K22
Mixture of human and mouse	None

3.3. Diverse types of tumors and cell lines with different expression profiles from MMC-SNL

Since malignant tumors were formed from the same SNL76/7 cell line, it would be natural to form an identical type of tumor, such as fibrosarcoma, in every case. However, we observed three distinct types of malignant tumors among the eight cases; malignant solitary fibrous tumor (Fig. 1A–C), angiosarcoma (Fig. 1D–F) and undifferentiated pleomorphic sarcoma (Fig. 1G–K). In addition, we have established three morphologically different malignant cell lines from the tumors, which included a round cell line (Fig. 2A), a round/spindle cell line (Fig. 2B) and an endothelial-like cell line (Fig. 2C). In order to confirm the diversity of these cell lines, B7t-1, K4t-RS and K4t-E, we compared their gene expression profiles with those of the SNL76/7, K4 hiPSC and TIG-1 cell lines. RT-PCR analyses revealed that B7t-1 expressed all genes examined including proto-oncogenes of *Klf4*, *c-Myc* and *Ras*, tumor suppressor genes of *Rb1* and *Trp53*, and differentiation-related genes, *Cd31*, *Cd34* and *Vegfα* (*vascular endothelial growth factor alpha* gene), consistent with the SNL76/7; thus, confirming their similarity (Fig. 2F). K4t-RS expressed most of these genes, but a significant difference from B7t-1 was the lack of expression of *Pecam1* (*Cd31*). In contrast with these cell lines, K4t-E did not express most of these genes, except for *Ras*, while a constitutively expressed marker, *Gapdh*, was positive. K4t-E appears to exhibit suppression of the expression of most genes except for housekeeping genes during its differentiation. Thus, we were able to establish cell lines with diverse gene expression from MMC-SNL. The diversity of tumors, however, was not particularly broad, but was mostly limited to the mesenchymal lineage.

3.4. Generation of malignant tumors from the parent SNL76/7 cells

Since the malignant tumors originated from MMC-SNL, we tested the anchorage-independent growth in soft agar and the

tumor forming ability of MMC-SNL, MMC-MEF and MMC non-treated parent SNL76/7 cells. There were no signs of colonies in soft agar at three weeks and no tumors were formed in the SCID mice after 8 months from either the MMC-SNL or MMC-MEF cells. However, the parent SNL76/7 cells produced a large number of colonies in soft agar (Fig. 3A) and tumors in mice (Fig. 3B). Histopathological examination of the tumor tissue by HE staining (Fig. 3C) and CD34- (Fig. 3D) and αSMA- (Fig. 3E) immunostaining revealed that SNL76/7 cells produced a malignant solitary fibrous tumor. Since tumor formation was confirmed at the 2nd passage after their supply, we suspected that the SNL76/7 cells were provided with transformants at the time of delivery, although MMC-SNL did not form tumors. Thus, the above demonstration that explants of hiPSC with some contaminated MMC-SNL produced tumors suggest that there exists an intimate interaction between MMC-SNL and hiPSCs during malignant tumor formation.

3.5. Growth restoration of MMC-SNL co-cultivated with hiPSCs

Therefore, we examined the effects of the interaction of MMC-SNL with hiPSC on its recovery from growth arrest. After MMC-SNL were co-cultured with K4 iPSCs with ES medium for one week, the growth medium was changed to DMEM containing 7% FBS and the cells cultured for up to one month. Three colonies grew in the dish and one of them (K4c-3) was isolated for further expansion in a larger dish. Examination of the genetic origin of K4c-3 confirmed that it was derived from the SNL76/7 feeder cells (data not shown). The K4c-3 cells (Fig. 3F) looked morphologically like the parental SNL76/7 cells, but not like K4t-RS (Fig. 2B) or K4t-E (Fig. 2C), suggesting that K4c-3 simply regained their growth ability and did not convert into other types of malignant cell lines *in vitro*. To confirm this finding, we compared the gene expression profiles of these cell lines (Fig. 3G). Expression profiles including *Oct4*, *Klf4*, *c-Myc* and *Nanog* were virtually the same between K4c-3 and the SNL76/7 cells. However, K4t-RS was unique, with strong positive expression of *Lgr5* (intestinal stem cell marker) and negative expression for *Oct4* and *Nanog*. No colony growth was observed from the cultures of MMC-SNL without contact of hiPSCs even after three months. These results are consistent with the hypothesis that MMC-SNL restored their growth ability by interactions with hiPSC *in vitro*.

3.6. Possible mechanisms of diverse tumor formation from MMC-SNL

There are at least two steps involved in the generation of diverse tumors; growth recovery of MMC-SNL and their diverse tumor formation. It is now apparent that MMC-SNL can restore their growth ability through their interactions with hiPSCs. The MMC treatment was confirmed to be sufficient for arresting cell growth through the testing of increased concentration of MMC and longer duration of cultivation. Since hiPSCs are interwoven with MMC-SNL at the time of harvesting and transplantation, it is expected that MMC-SNL have interacted with hiPSCs during their cultivation *in vitro* and growth expansion of the implanted cells in a SCID mouse.

The most natural explanation for their interaction is cell fusion between the MMC-SNL and hiPSCs, and proliferation of the resultant hybrids. A fusion of MEF or adult somatic cells with ESC has been reported to generate pluripotent hybrid cell lines [13]. However, we did not detect any sign of human-specific *Alu* sequences in the malignant tumors and tumor-derived cell lines, showing that the possibility of cell fusion must be very low, although this possibility could not be completely excluded.

Another possible mechanism of interaction is a metabolic cooperation between hiPSCs and MMC-SNL through membrane transport. Double strand breaks of DNA (DSB) caused by mild γ-ray or MMC treatment can be rescued by repair-related enzymes. Furthermore, it has been reported that proteins can be transported to neighboring cells through cytoplasmic membrane in the absence of cell fusion [14]. Thus, it is our present hypothesis that the severe DSB in MMC-SNL might have been rescued by the provision of repair enzymes from the interacting hiPSCs via membrane transport; although this remains to be proven in future.

Next, it is necessary to explain how diverse types of malignant tumors were generated. We obtained at least three types of malignant tumors and three types of tumor-derived cell lines (Table 1, Fig. 2A–C), although the parent cell line, SNL76/7 cells, produced a tumor that was composed of homogenous fibrocyte like cells (Fig. 3). One explanation is that this cell line with malignant properties was converted into cancer-like stem cells by the transfer of reprogramming factor(s) from hiPSCs gaining multi-potency, which generated diverse cancer progenitor like cells with expression of some differentiation marker genes. A report that showed

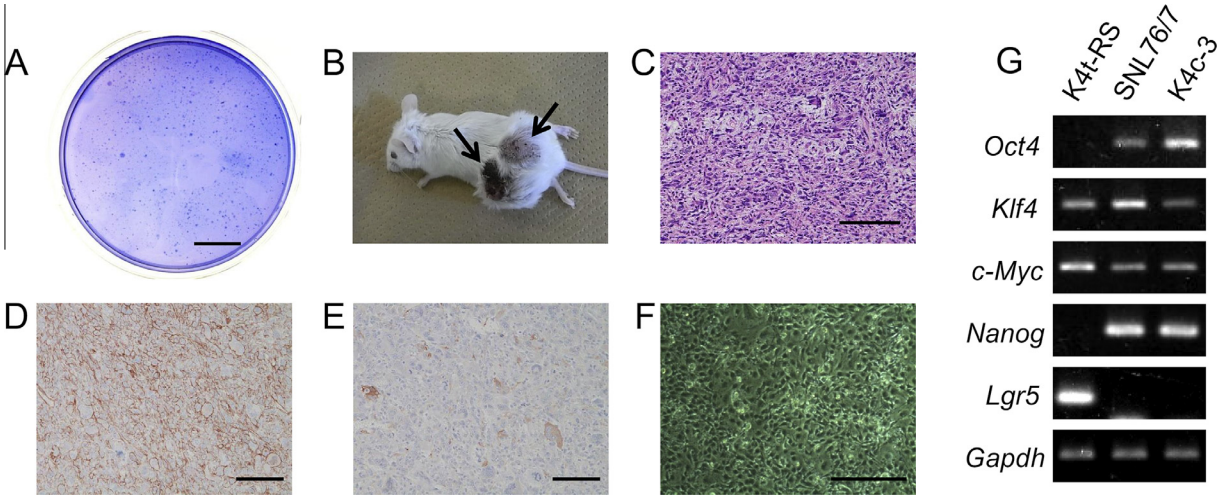

Fig. 3. Tumor formation of the parent SNL76/7 feeder cells and cell lines generated by co-culturing of MMC-treated SNL76/7 with hiPSCs. Untreated SNL76/7 feeder cells formed colonies in soft agar (A) and produced tumors (B). Scale bar in (A) indicates 2 cm. Histopathological examination by HE staining (C) and immunohistochemical staining for CD34 (D) and αSMA (E). Bars (in C – F) indicate 200 μm. A cell line, K4c-3 (F) was established by expanding the growing colony after co-culturing MMC-SNL with K4 iPSC. RT-PCR was performed to compare between SNL76/7 and K4t-RS. Similar expression profiles were observed between K4c-3 and SNL76/7, but not K4t-RS.

that direct delivery of transcription proteins could induce pluripotent cells also supports this possibility [15]. However, the limited features of these tumor cells might reflect properties of mesenchyme stem like cells in MEF, from which the SNL76/7 cells were originated.

Taken together, we conclude that transmission of repair enzymes and initializing factors from hiPSCs through the membrane to MMC-SNL produced diverse malignant tumor cells from MMC-SNL. The exact mechanisms, however, remained to be determined. Thus, previous work on tumor formation from explants should be re-evaluated by examining the genetic origin of the cells. The present study also indicates that we should not utilize these feeder cell lines for culture of hiPSCs, even for research purposes, since mouse cell lines tend to transform into malignant cells without artificial treatment in culture.

Author contributions

M. Kamada, T. Kumazaki and Y. Mitsui conducted most of the experiments. Y. Kawahara performed the iPSC transplantation into the SCID mice for tumor formation. T. Takahashi contributed to the pathological analysis. Y. Mitsui, M. Kamada and T. Matsuo wrote most of the manuscript. T. Takahashi and Y. Mitsui conceived and supervised the entire project.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.009.

References

[1] J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, I.I. Slukvin, J.A. Thomson, Induced pluripotent stem cell lines derived from human somatic cells, Science 318 (2007) 1917–1920.
[2] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872.
[3] K. Miura, Y. Okada, T. Aoi, A. Okada, K. Takahashi, K. Okita, M. Nakagawa, M. Koyanagi, K. Tanabe, M. Ohnuki, D. Ogawa, E. Ikeda, H. Okano, S. Yamanaka, Variation in the safety of induced pluripotent stem cell lines, Nat. Biotechnol. 27 (2009) 743–745.
[4] A. Yamashita, S. Liu, K. Woltjen, B. Thomas, G. Meng, A. Hotta, K. Takahashi, J. Ellis, S. Yamanaka, D.E. Rancourt, Cartilage tissue engineering identifies abnormal human induced pluripotent stem cells, Sci. Rep. 3 (2013) 1978.
[5] V. Akopian, P.W. Andrews, S. Beil, N. Benvenisty, J. Brehm, M. Christie, A. Ford, V. Fox, P.J. Gokhale, L. Healy, F. Holm, O. Hovatta, B.B. Knowles, T.E. Ludwig, R.D. McKay, T. Miyazaki, N. Nakatsuji, S.K. Oh, M.F. Pera, J. Rossant, G.N. Stacey, H. Suemori, Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells, Vitro Cell Dev. Biol. Anim. 46 (2010) 247–258.
[6] S. Yamasaki, Y. Taguchi, A. Shimamoto, H. Mukasa, H. Tahara, T. Okamoto, Generation of human induced pluripotent stem (Ips) cells in serum- and feeder-free defined culture and TGF-Beta1 regulation of pluripotency, PLoS One 9 (2014) e87151.
[7] M. Tomasz, Mitomycin C: small, fast and deadly (but very selective), Chem. Biol. 2 (1995) 575–579.
[8] A.P. McMahon, A. Bradley, The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain, Cell 62 (1990) 1073–1085.
[9] M. Ohashi, S. Aizawa, H. Ooka, T. Ohsawa, K. Kaji, H. Kondo, T. Kobayashi, T. Noumura, M. Matsuo, Y. Mitsui, S. Murota, K. Yamamoto, H. Ito, H. Shimada, T. Utakoji, A new human diploid cell strain, TIG-1, for the research on cellular aging, Exp. Gerontol. 15 (1980) 121–133.
[10] M. Kamada, T. Kumazaki, T. Matsuo, Y. Mitsui, T. Takahashi, Establishment of ultra long-lived cell lines by transfection of TERT into normal human fibroblast TIG-1 and their characterization, Cell Biol. Int. 36 (2012) 519–527.
[11] T. Kumazaki, S. Kurata, T. Matsuo, Y. Mitsui, T. Takahashi, Establishment of human induced pluripotent stem cell lines from normal fibroblast TIG-1, Hum. Cell 24 (2011) 96–103.
[12] T. Kumazaki, T. Takahashi, T. Matsuo, M. Kamada, Y. Mitsui, Re-emergence of undifferentiated cells from transplants of human induced pluripotent stem cells as a possible risk factor of tumourigenesis, Cell Biol. Int. Rep. 21 (2014) 17–24.
[13] C.A. Cowan, J. Atienza, D.A. Melton, K. Eggan, Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells, Science 309 (2005) 1369–1373.
[14] Y. Bi, D. Mukhopadhyay, M. Drinane, B. Ji, X. Li, S. Cao, V.H. Shah, Endocytosis of collagen by hepatic stellate cells regulates extracellular matrix dynamics, Am. J. Physiol. Cell Physiol. (2014).
[15] D. Kim, C.H. Kim, J.I. Moon, Y.G. Chung, M.Y. Chang, B.S. Han, S. Ko, E. Yang, K.Y. Cha, R. Lanza, K.S. Kim, Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins, Cell Stem Cell 4 (2009) 472–476.