Activation of CXCL12-CXCR4 signalling induces conversion of immortalised embryonic kidney cells into cancer stem-like cells

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ABSTRACT

Cancer stem cells (CSCs) have been implicated in the growth and progression of several types of human cancer. The technology to derive and establish CSCs in vitro could be a critical tool for understanding cancer and developing new therapeutic targets. In this study, we developed expandable CD15+ induced CSCs (iCSCs) from immortalised 293FT human epithelial cells by co-culture with human bone marrow-derived mesenchymal stem cells (BM-MSCs) as feeder cells in vitro. The iCSCs converted through an epithelial-mesenchymal transition program acquired mesenchymal traits, the expression of stem cell markers, and epigenetic changes. Moreover, the iCSCs not only efficiently formed tumourspheres in vitro but also induced tumours in immunocompromised mice injected with only 10 of the iCSCs. Furthermore, we showed that the expression of the chemokine CXCL12 and its receptor CXCR4 by the iCSCs resulted in the activation of the Fut4 gene through CXCR4/ERK/ELK-1-signalling pathways and the maintenance of the iCSCs in the undifferentiated state through CXCR4/AKT/STAT3-signalling. These findings suggest that immortalised 293FT cells may acquire potential oncogenicity through molecular and cellular alteration processes in microenvironments using BM-MSCs, and could represent a valuable in vitro model as a cancer stem cell surrogate for studying the pathophysiological properties of CSCs.

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ARTICLE HISTORY

Received 1 November 2019 Revised 28 September 2020 Accepted 20 October 2020

KEYWORDS

iCSCs; BM-MSC; 293FT; CXCR4/CXCL12; ERK/ELK1; AKT/STAT3

ARTIFICIAL CELLS, NANOMEDICINE, AND BIOTECHNOLOGY

2020, VOL. 48, NO. 1, 1303-1313

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Introduction

There is increasing evidence for the existence of cancer stem cells (CSCs) within a population of cancer cells, which are defined by specific cell surface antigen expression and are believed to initiate and sustain new tumour growth and mediate chemoresistance [1,2]. The development of a CSC-targeting strategy for the elimination of the CSC population is critical for improving the treatment of cancer patients. However, it is difficult to isolate pure populations of CSCs to expand into large numbers for the characterisation of their biological properties and for high throughput screening of CSC-targeting compounds.

Currently, there are several techniques [3–5] for isolating CSCs from tumour cell lines or tissues including cell sorting technologies by magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) using antibodies against CSC-specific surface antigens [6]. These methods have provided much information about cancer stem cell isolation. However, it is often not feasible to obtain tumour tissues from cancer patients due to legal or administrative limitations, and it has not yet been possible to isolate sufficient numbers of CSCs. Bone marrow-derived mesenchymal stem cells (BM-MSCs), as part of the cancer cell niche, expressing SDF-1α (CXCL12) are multipotent cells, which are also able to enhance the stemness of cancer cells through multiple signalling pathways in vivo [7–9].

In this study, we focussed on generating CSC-like cells, such as CSC surrogates, from non-CSC like cells under a certain cell culture condition. To do this, we used a co-culture method with BM-MSCs to convert an immortalised epithelial 293FT cell line into CSCs. Our newly developed method for the induction and maintenance of CSCs would be very useful to overcome the current limitations of the study with CSCs and expedite the investigation of significance of CSCs in cancer development and treatment.

Methods

Animal studies

All animal protocols were approved by the Korea University Institutional Animal Care and Use Committee (KUIACUC-2015-67) and were performed in accordance with government and
institutional guidelines and regulation. Six-week-old male BALB/c nude mice (n = 70) were purchased from Central Lab Animal Inc (Seoul, Korea) were housed under a 12-h light/dark cycle (08:00–20:00) with free access to water and food in pathogen-free conditions. The various ranges of unsorted-iCSCs or CD15⁺ or CD15⁻ or 293FT cells with Matrigel (Corning) were injected subcutaneously at abdomen in Balb/c nude mice, respectively. Number of the injected cells was ranged from 1 × 10⁴ to 5 × 10⁶. The tumours were detected from about 4 weeks after cell implantation, but the exact time of tumour formation was dependent on the cell type or number. Tumours size was measured every 2–3 days and expressed as volume (length × width × height). Mice were sacrificed when tumour size reached 1.5 cm in the largest diameter.

**Cell line and culture conditions**

Human bone marrow-derived MSC cell lines were purchased from LONZA (Walkersville, MD, USA), human immortalised cell lines (HEK293 and 293T) were obtained from American Tissue Type Culture Collection (Manassas, VA, USA), 293FT cell lines were obtained from Invitrogen, and DP (dermal papilla) cells were from Dr. Sung (Kyungpook National University, Daegu, Korea). NSC (neural stem cells) were cultured in DMEM media containing 1% GlutaMAX™-I, 10% FBS, and 1% penicillin/streptomycin (all from Gibco) at 37 °C in 5% CO₂/95% air and the medium was replaced every third day. Approximately 70–80% confluence of the BM-MSC lines were cultured in LONZA MSCBM (mesenchymal stem cell basal medium) supplemented with 10% MCGS supplement, 2% L-glutamine and 0.1% GA-1000. For iCSC generations, less than 50 cells of each human immortalised cell line were co-cultured with iCSC media (DMEM media containing 1% GlutaMAX™-I, 10% FBS, and 1% penicillin/streptomycin plus 2 mM Glutamine (Gibco) in the presence of BM-MSC feeder cells (passage number 3) for more than 20 days to form colonies, which were sub-cultured by mechanical passaging. Then, established iCSCs and their control cells were expanded up to more than 23–25 passages for further analyses.

**Statistics**

Data were analysed by two-tailed Student’s t-test. *p* values < .05 (*) and < .01 (***) were considered statistically significant. Data are presented as the mean ± standard deviation of mean. Survival curves of the mice were analysed by the Kaplan–Meier method and compared with the Log-rank test. Statistical analysis were conducted using SPSS software (IBM).

**Results**

**Conversion of the immortalised and cancer cell lines into CSC-like cells in vitro**

To determine whether CSC-like cells can be generated from immortalised and cancer cell lines, we selected five human immortalised cell lines (HEK293, 293T, 293FT, DP, and NSC) and five human cancer cell lines (U87-MG, MCF7, HeLa, SW480, and A549) and co-cultured a small number of cells (less than 50, approximately) of each cell line on BM-MSCs in our culture system using DMEM-GlutaMAX™-I as the basal media supplemented with 10% FBS, 25 mM glucose, and 6 mM glutamate (we call this the CSC media for induction and maintenance) for 20 days (Figure 1(a,b); Supplementary Figure S1(a)). BM-MSCs were used as feeder cells to constitute an *in vitro* microenvironment to induce and maintain CSCs. Seven days after co-culture with BM-MSCs, some colonies with mesenchymal cell morphology appeared on feeder cells from nine cell lines (HEK293, 293T, 293FT, DP, U87-MG, MCF7, HeLa, SW480, and A549), which were visible under a phase-contrast microscope (Figure 1(b)). However, no colonies were apparent from the NSC line (Figure S1(a)).

Subsequently, the colonies derived from nine cell lines were maintained for 20 days, and then a fraction of the cells from growing colonies was picked up (P0, passage number 0) and expanded up to P4 without feeder cells as described in the supplementary methods (Figure S1(a)). Next, these subculture cells at P4 originated from different cell lines were analysed by FACS analysis with the CD133 antibody, a broad-spectrum marker of CSCs. Interestingly, the number of CD133 + cells was 6 times increased in the 293FT cell line-derived cells compared to the parental 293FT cell line (Figure S1(b)). Similarly, HEK293 cell-derived cells showed a 2.86-fold increase in the number of CD133 + cells compared to the HEK293 cell line; the other cell line-derived colonies did not show any significant increase (Figure S1(b)). We further analysed the cells that were derived from five immortalised cell lines with the CD15 CSC marker. In our FACS analysis, the percentage of CD15 + cells was strikingly increased in the 293FT-derived colonies, which showed a 16.37-fold increase compared to the 293FT cell line cell, but was not significantly increased in the other immortalised cell line-derived cells (Figure S1(c)). Taken together, these results demonstrate that most of the human immortalised and cancer cell lines were morphologically converted to CSC-like cells in our co-culture system with BM-MSCs, but only 293FT immortalised cell line can develop to the induced CSC (iCSC)-like cell lines (named iCSC1 and iCSC2) expressing CSC marker CD133 and CD15 (Figure S1(d)).

To further characterise the established iCSC lines, the gene expression levels of *Fut4* and *Prom1* (CD15 and CD133 marker genes, respectively) from the same passage number (P7) of the iCSC lines were analysed by real-time RT-PCR (Figure 1(c)). *Fut4* and *Prom1* were highly up-regulated in iCSC lines compared to 293FT cells, and the expression levels of the two genes were significantly higher in iCSC2 than iCSC1 (Figure 1(c)). In addition, our immunostaining and FACS results using CD133 and CD15 antibodies were consistent with those obtained by real-time RT-PCR analysis (Figure 1(c), Figure S1(b,c)), showing that the established iCSC lines acquired the properties of CSCs. Interestingly, we found that while the 293FT cell line showed the typical epithelial-like morphology with regular dimensions [10], the iCSC lines showed mesenchymal cell morphology under the culture
condition with the CSC media for 36 days (Figure 1(b) and Figure S1(d)).

To further examine whether the observed mesenchymal cell morphology of iCSC was originated via the process of an epithelial-to-mesenchymal transition (EMT), we analysed the expression levels of EMT-related genes. The iCSC lines showed high expression levels of the mesenchymal-associated genes such as Twist1, Snail, Slug, Zeb1, vimentin, and fibronectin compared to the 293FT cell line (2.77 ± 2–4.54 ± 2-fold), while the expression level of E-cadherin was dramatically decreased in the 293FT cell line (Figure 1(e)). These data were consistent with those from our immunostaining analysis (Figure 1(f) and Figure S1(g)) although there was no significant difference in N-cadherin expression between the iCSCs and the 293FT cell line (Figure 1(e)). In addition, we confirmed that the iCSC lines were derived from 293FT cells via short tandem repeat (STR) analysis with the 16 polymorphic DNA markers (Table S1). Taken together, we concluded that the iCSC lines were generated from 293FT cells through an EMT process under coculture with BM-MSCs.

Next, to explore whether upregulation of Fut4 in iCSC1 and iCSC2 lines is caused by epigenetic change in 293FT cells, we examined the methylation patterns on Fut4 promoter of the 293FT cell line and the iCSC1 and iCSC2 lines. The results showed that the levels of methylation of Fut4 of the iCSC lines was much reduced compared to that of the 293FT cell line (17.1% for iCSC1 and 10.0% for iCSC2 versus 95.0% for 293FT cells, respectively) (Figure 1(h)). Interestingly, our Bisulphite Sequencing PCR (BSP) analysis data showed that the ELK-1 binding consensus sequence containing one CpG dinucleotide was unmethylated in both iCSC1 and iCSC2 (Figure 1(h) arrows), but methylated in the 293FT cell line...
Furthermore, we found that mRNA levels of Tet1-3 and Idh1-2, known as the key regulators of DNA demethylation [11], were dramatically increased in the iCSC lines compared to the 293FT cell line (Figure 1(i)). Taken together, our results demonstrate that the established iCSCs were epigenetically converted from the 293FT cell line in vitro, and that the iCSC lines may be surrogates for authentic CSCs.

Conversion of the 293FT cell line to CSC-like cells by the SDF-1α/CXCR4 signalling pathway

To understand the underlying mechanism by which BM-MSC converted the 293FT cell line to CSC-like cells, we examined the BM-MSC secreted factors such as chemokines that are involved in the signalling pathways promoting cancer initiation and progression. It is well known that the CXCL12/SDF-1α/CXCR4 signalling pathway plays a critical role in regulating various cellular activities [12].

Thus, we first examined the expression levels of CXCR4 and SDF-1α in the 293FT cell line, BM-MSCs, and iCSC lines by FACS analysis. The result showed that CXCR4 was highly expressed in 293FT cells (81.2%) compared to BM-MSCs (0.19%) (Figure 2(a-c)), and the population of CXCR4+/CD15− cells was enriched approximately 31-fold higher in the iCSC lines (91–92.23%) compared to the 293FT cell line (2.9%) (Figure 2(d)). Western blot and ELISA analyses revealed that both BM-MSCs and the iCSC lines secreted higher levels of SDF-1α compared to the 293FT cell line (Figure 2(e-f)). Thus, we hypothesised that the conversion of the 293FT cell line to a CSC phenotype could be associated with the activated CXCR4 signalling pathway induced by secreted SDF-1α from BM-MSCs feeder cells.

To test this hypothesis, we first excluded potential cancer stem cells in 293FT cells by isolating 293FT CD15− cells (293FTCD15− cells) using MACS and confirmed that the isolated 293FT CD15− cells (P0) were negative for CD15 antibody (Figure 3(a)). Then, we cultured these cells without BM-MSC feeder cells in our iCSC media up to 10 passages (approximately 30 days) to avoid autonomously converted 293FT CD15− cells emerged during long-term culture. We did not observe the significant increase of 293FT CD15− cell populations under this culture condition by FACS analysis (Figure 3(b)). Next, a small fraction of 293FT CD15− cells was co-cultured with BM-MSC feeder cells for more than 20 days in the presence or absence of AMD3100 (10 μM), a specific inhibitor of CXCR4 (Figure 3(c)). FACS analysis showed that the CD15+ cells were 9.33% in the culture condition without AMD, while CD15+ cells were only 0.33% in the culture condition with AMD at 20 days after co-culture with BM-MSCs (Figure 3(d)). Consistent with this result, immunostaining analysis also revealed that the CD15+ cells could be detected at about 15 days after co-culture of 293FT CD15− cells (P0) with BM-MSC in a condition without AMD, while the CD15− cells were not detected in the culture condition with AMD (Figure S2). After small clumps were mechanically passage (P1) onto a petri-dish without BM-MSC feeder cells at day 21, the CD15+ cells were cultured for more than 7 days and then were clearly detected in a condition without AMD as shown in Figure 3(e). Our FACS analysis with the CSC markers CD15 and CD133 showed that the majority (89.58%) of these cells were CD15+/CD133− at 58 days (P3), and some (9.85%) of these cells were CD15+/CD133− (Figure 3(f)). The morphology of this established iCSC cell line (named the iCSC3 cell line) was similar to the iCSC1 and iCSC2 lines (Figure 3(g)). These results demonstrate that the secreted factor SDF-1α from co-cultured BM-MSCs feeder cells contributes to the conversion of 293FT CD15− to iCSCs, which may be mediated by activating CXCR4 signalling in 293FT CD15− cells.

Next, we examined the molecular mechanism underlying SDF-1α-mediated iCSC transition of 293FT cells under our experimental condition. Interestingly, we observed that pELK-1 (S383) protein was increased in the nucleus of the iCSC lines treated with recombinant SDF-1α (Figure 4(a) and b) whereas the protein was completely prevented in the presence of AMD3100 or PD98059, which are specific inhibitors of CXCR4 and ERK1/2, respectively [13] (Figure 4(b)). In addition, we found that ELK-1 protein directly bound to a region of the Fut4 promoter, using a chromatin immunoprecipitation (ChIP) assay (Figure S3(a–c)). These results indicate that the Fut4 gene is transcriptionally upregulated by activated ELK-1, and in turn increases the expression of CD15 proteins on the cell surface of iCSC.

Next, we investigated whether the SDF-1α/CXCR4 signalling pathway is directly linked to ERK/ELK-1-activation [14,15] or AKT/STAT3-activation [16–19] in the iCSC lines. To this end, we treated iCSC1 line with SDF-1α in the presence and absence of AMD3100 and examined phosphorylation status of these signalling proteins by immunoblotting analysis with phospho-specific antibodies. We observed that activation of CXCR4 signalling pathway by SDF-1α markedly increased the phosphorylation levels of both the ERK1/2 (T202-204) and ELK-1 (S383) proteins (Figure 4(c)). Similarly, the phosphorylation levels of both AKT (S473) and STAT3 (Y705) proteins were also significantly increased under the same condition (Figure 4(d)). Taken together, these results demonstrate that SDF-1α activated the ERK/ELK-1 and/or AKT/STAT3 proteins as an autocrine signalling effect.

Self-renewal and differentiation potentials of the iCSC lines in vitro

To evaluate the self-renewal ability of the established iCSC lines, we performed tumorsphere formation assays with the iCSC lines and the 293FT cell line in the NSC culture medium (DMEM/F12, 1% N2, 1% ITSA, 1% B27, 1% penicillin/streptomycin, 20 ng/ml EGF, 20 ng/ml FGF2). A U87-MG human glioblastoma cell line [20] was used as a positive control (Additional file 4: Figure S4(a)). After 10-day suspension culture, all cell lines including the iCSC lines were grown as floating spheres, but the majority of the 293FT and U87-MG cell lines were attached to both mammalian bacterial culture dishes after a 15-day induction (Figure 5(a) and Figure S4(a)). The primary spheres from the iCSC lines showed a fast growth rate compared to the others (Figure 5(b)).
Next, we analysed the cells that were dissociated from the primary spheres of the iCSC lines by FACS with CD15 and CD133 antibodies. Interestingly, most cells dissociated from the iCSC line-derived spheres were CD15\(^+\) and CD133\(^+\), but the cells from the 293FT cell- or U87-MG cell-derived primary spheres were CD15\(^-\) and CD133\(^-\) (Figure 5(c) and Figure S4(c)). The primary spheres from iCSC lines were also CD15\(^+\) and CD133\(^+\) in our immunostaining assays, but the spheres from 293FT and U87-MG cell lines were CD15\(^-\) and CD133\(^-\) (Figure 5(d) and Figure S4(d)). These results demonstrate that the iCSC lines have self-renewal ability and are clonogenic in vitro.

To examine the level of ABC-binding cassette transporters, known to be highly expressed in CSCs [21], we performed real-time RT-PCR experiments with the total RNA isolated from suspension-cultured iCSC lines. The expression levels of Abcc1, Abcc2, Abcc3, Abcc5, and Abcb1 genes were significantly increased, but not Abcc4, Abcc6, Abcg2, and Abca2 (Figure 5(e)). Taken together, these results demonstrate that the cells constituting the spheres were highly chemoresistant, indicating that our iCSC lines possess the typical properties of CSCs. Interestingly, we found that the cells in the primary spheres were strongly positive against NSC markers (CD15, CD133, NESTIN, SOX2 or NCAM) [22] and stem cell marker (OCT4) antibodies (Figure 6(a)) when the iCSC lines were grown as spheres, suggesting that a population of cells consisting of iCSC may be CSCs with neural lineage properties.

Next, to examine the differentiation potentials of the iCSC lines, the iCSC-derived spheres were cultured in DMEM/F-12 medium supplemented with B27, bFGF, and EGF for 7 days, and then subsequently cultured in the medium supplemented with 10% FBS for 7 days (Figure 6(b)). Differentiated cells from the iCSC-spheres were positive with neuronal (Tuj1 and MAP2), oligodendrocyte (GFAP), and astrocyte marker (OCT4) antibodies, but were negative with Oct4, CD15, and CD133 marker antibodies (Figure 6(c)). Interestingly, some differentiated cells were positive for both neuronal and glial markers (Tuj1/GFAP or MAP2/GFAP) (Figure 6(c) and 6(d)), indicating that some cells from iCSC-spheres can possess two lineages, such as neuronal and glial. These results demonstrate that the iCSC lines were directly differentiated into the mature cancer cells with neural lineage properties.
Figure 3. iCSC generation from 293FT<sup>CD15</sup>-cells. (a) Flow cytometry analysis of CD15/SSEA-1 protein expression in 293FT<sup>CD15</sup>-icsc cells after purification of 293FT<sup>CD15</sup>-cells from 293FT<sup>CD15</sup>-cells. (b) Flow cytometry analyses of sorted 293FT<sup>CD15</sup>-icsc cells over time in culture in a representative time course experiment: passage number 0, 5, and 10 (P0, P5, and P10). (c) Phase-contrast images of iCSCs generated from 293FT<sup>CD15</sup>-icsc cells co-cultured with BM-MSC in the presence or absence of AMD3100. Insets in bottom left corner, original magnification ×400. (d) Flow cytometry analyses of the CD105 and CD15 protein expression in 293FT<sup>CD15</sup>-icsc cells co-cultured with BM-MSC in the presence or absence of AMD3100 at P0. (e) Immunofluorescence images of CD15 and CXCR4 protein expression in iCSC3 at P1. (f) Flow cytometry analysis of CD15 and CD133 protein expression in iCSC3 cells at P3. (g) Images show the colonies formed by 293FT<sup>CD15</sup>-cells with BM-MSC in the presence or absence of AMD3100 at P0.

Figure 4. CXCL12/CXCR4 activates ERK/ELK-1 and AKT/STAT3-signalling pathways in iCSCs. (a) Experimental procedure for the activation of CXCL12/CXCR4 in iCSCs. (b) Immunofluorescence images of pELK-1 subcellular distribution. (c) Western blot images of ERK1/2 (Thr202-204) and ELK-1 (Ser383) phosphorylation in response to SDF-1α/CXCL12 in iCSCs. A graph shows quantification of the western blotting results. (d) Western blot images of AKT (Ser473) and STAT3 (Tyr705) phosphorylation in response to SDF-1α/CXCL12 in iCSCs. A graph shows quantification of the western blotting results.
To demonstrate the tumorigenic potentials of the iCSC lines, we first isolated the CD15$^+$ and CD15$^-$ cells from the iCSC1 line using FACS. The unsorted-iCSC1, CD15$^+$, CD15$^-$, and 293FT cell lines were subcutaneously injected into BALB/c nude mice. The unsorted-iCSC1 line and the CD15$^+$ cells induced tumours at a very low dose (1:133 for iCSC1 cells versus 1:134 for CD15$^+$ cells, $p = .995$), while the CD15$^-$ cells induced tumours only at a high dose (1:28,044 for CD15$^-$ cells versus 1:134 for CD15$^+$ cells, $p = 2.08e-7$), and a high dose of 293FT cells (1 × 10$^6$) did not induce tumours (Figure 7(a)). In addition, as few as ten CD15$^+$ cells or ten cells from the unsorted iCSC1 line were sufficient to initiate tumours in immunocompromised mice, suggesting that the expression of CD15 proteins may be directly involved in maintaining stem cell properties.

We also evaluated the relationship between the tumorigenic potentials of CD15$^+$ or CD15$^-$ cells and their subsequent effects on mortality at the same cell dose (Figure 7(b)). Three weeks after injection, the tumours were palpable. Some mice injected with the iCSC1 line were euthanized to compare their gross tumour size at 71 days post-injection (Figure 7(c)). We checked tumour size once every three days; the tumours from the iCSC1 line and CD15$^+$ cells grew at an extremely rapid rate and reached a tumour volume of 0.5 cm$^3$ by 46 days post-injection. However, the tumours from the CD15$^-$ cells were slowly developed compared to those from the iCSC1 line and CD15$^+$ cells and reached a volume of 0.5 cm$^3$ volume on day 61 after the initial injection of CD15$^-$

Figure 5. Sphere formation ability is markedly elevated in iCSCs. (a) Sphere comparison of 293FT-icsc cells and iCSC lines in suspension culture. Representative photos (magnification ×100; black scale bars, 10 μm). (b) Numbers of primary spheres (>10–40 μm or >40 μm) generated from 293FT-icsc cells and iCSCs. (c) Flow cytometry analyses of brain cancer stem cell marker protein levels, CD15 and CD133, in 293FT-icsc cells and iCSCs. (d) Immunofluorescence images of CD15 and CD133 protein expression in 293FT-icsc cells and iCSCs. (e) Real-time PCR analyses of multi-drug resistant gene expression. Results are means ± SD calculated from triplicates.

**Tumorigenicity and self-renewal of the iCSC lines in vivo**

To demonstrate the tumorigenic potentials of the iCSC lines, we first isolated the CD15$^+$ and CD15$^-$ cells from the iCSC1 line using FACS. The unsorted-iCSC1, CD15$^+$, CD15$^-$, and 293FT cell lines were subcutaneously injected into BALB/c nude mice. The unsorted-iCSC1 line and the CD15$^+$ cells induced tumours at a very low dose (1:133 for iCSC1 cells versus 1:134 for CD15$^+$ cells, $p = .995$), while the CD15$^-$ cells induced tumours only at a high dose (1:28,044 for CD15$^-$ cells versus 1:134 for CD15$^+$ cells, $p = 2.08e-7$), and a high dose of 293FT cells (1 × 10$^6$) did not induce tumours (Figure 7(a)). In addition, as few as ten CD15$^+$ cells or ten cells from the unsorted iCSC1 line were sufficient to initiate tumours in immunocompromised mice, suggesting that the expression of CD15 proteins may be directly involved in maintaining stem cell properties.

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cells (Figure 7(b)). We further examined cumulative survival rates comparing CD15\(^+\) cell tumours and CD15\(^-\) cell tumours along with control cells. The results showed that the median overall survival (T50) among mice injected with cells from the iCSC1 line and the CD15\(^+\) cells was less than 16 weeks (16 weeks for iCSC1 cells vs 15 weeks for CD15\(^+\) cells; 95% confidence interval [CI] of ratio, 0.86 - 1.27; \(p = 0.79\); Figure 7(d)) compared to 53 weeks among mice injected with the CD15\(^-\) cells at the same cell dose. In contrast, the median overall survival among mice injected with a high dose of CD15\(^-\) cells was 20 weeks, suggesting that CD15\(^-\) cells can also initiate tumorigenesis in immunocompromised mice.

Because a key property of CSCs is the ability to self-renew in tumours, we examined whether CD15\(^+\) cells from primary tumours formed by the iSCs could reconstitute secondary tumours. In addition, we also examined whether CD15\(^+\) cells from secondary tumours could form tertiary tumours. The CD15\(^+\) iSCs from primary and secondary tumours were subsequently transplanted into new recipient mice and formed secondary and tertiary tumours, respectively. The percentage of CD15\(^+\) cells in the tumours was increased (\(~59\%\), 84\%, and 86\%) (Figure 7(e)). These results indicate that the iSCs can self-renew in vivo, and be passaged several times. In aggregate, these results demonstrate that the iCSC lines and

Figure 6. iSCs are differentiated into both glial and neuronal lineages in vitro. (a) Immunofluorescence images of neural stem cell marker protein expression in iSCS-spheres. Representative photos (magnification \(\times 100, \times 200\); scale bars, 10 \(\mu\)m). (b) Experimental procedure for differentiation. (c) Immunofluorescence images of neural lineage marker protein expression in iSCS-spheres. Representative photos (magnification \(\times 200\); white scale bars, 10 \(\mu\)m). (d) Quantification of the results of c.
the FACS-sorted CD15^+ cells have strong tumorigenicity and self-renewal capacity in vivo.

**Discussion**

In this study, we developed a method for converting immortalised epithelial 293FT^CD15^- cells to mesenchymal CSC-like cells through epithelial to mesenchymal transition (EMT). During the conversion process, BM-MSCs and their secreted components such as SDF-1^a were required, and simple CSC medium consisting of DMEM/F12-Glutamax, glutamine, and high glucose supported the conversion and maintenance of the CSC-like cells. The iCSCs were characterised to the neural lineages by various defined CSC markers, and also showed self-renewal capacity in vitro and in vivo. In addition, they were differentiated into glial and neuronal cells in vitro and formed hierarchically organised tumours in vivo that may act as cells of origin of cancer.

A previous report by Woodward et al. showed that 293 T human embryonic kidney cells have cancer stem cell-like features characterised by spheres formed in serum-free stem cell promoting three-dimensional culture conditions containing EGF and FGF2 [23]. The spheres contained a population of higher ALDH1 and CD44^+/CD24^- cells compared to monolayer, which has been used to define cancer stem cells population in solid tumours including breast, colon, liver, and pancreatic cells [24–26], but not for brain tumours. CSCs may be developed from stem cells [27], may be generated from differentiated somatic cells with oncogenic mutations [27, 28], or may be enriched by microenvironmental influences [7, 8, 29, 30]. In our study, the CSC-like cells were directly derived from immortalised epithelial cells in a microenvironment using BM-MSCs without additional genetic manipulation for any artificial mutations, supporting the idea that the microenvironment can regulate the CSC phenotype [31, 32]. Importantly, we demonstrated that CXCR4/SDF-1^a signalling activation by a ligand, SDF-1^a, is an important mechanism by which the iCSCs were generated from the immortalised epithelial 293FT cells.

Our EMT results (Figure 1(e)) showed that several transcription factors that regulate EMT, such as Twist1, Snail1, and Zeb1, were dramatically expressed in iCSCs; these may endow iCSCs with stem cell properties [33] and subsequently, make them more chemoresistant (Figure 5(e)).
In our study, BM-MSCs actively secreted SDF-1α ligand proteins and the 293FT cells highly expressed CXCR4 receptor proteins, which suggests that the process of converting the epithelial 293FT cells into iCSCs is likely to involve a CXCR4/SDF-1α signalling pathway that is linked to PI3K/AKT/NF-κB, ERK/Elk-1, and JAK/STAT3. Interestingly, we observed that treating 293FT<sup>CΔ15</sup>-cells with the SDF-1α inhibitor AMD3100 during co-culture dramatically suppressed their conversion into iCSCs, and the SDF-1α in iCSC lines rapidly promoted nuclear ELK-1 activation in our western blot. In our ChIP assays, the binding of ELK-1 to the Fut4 enhancer was identified in iCSCs, and it was decreased by AMD3100 or PD98059 treatment. Additionally, we observed the methylation dynamics of the ELK-1 binding site in the Fut4 promoter by bisul- 

Disclosure statement

The authors indicated no potential conflicts of interest.

Funding

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare (HI14C3477), and the Ministry of Science and ICT (2019M3ESD0565399) of the Government of the Republic of Korea.

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