Monoclonal antibody K312-based depletion of pluripotent cells from differentiated stem cell progeny prevents teratoma formation

Jongjin Park1, Dong Gwang Lee1, Na Geum Lee1, Min-Gi Kwon1,2, Yeon Sung Son1, Mi-Young Son3, Kwang-Hee Bae4, Jangwook Lee1, Jong-Gil Park1, Nam-Kyung Lee1,* & Jeong-Ki Min1,2,*

1Biotherapeutics Translational Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, 2Department of Biomolecular Science, KRIBB School of Bioscience, Korea University of Science and Technology (UST), Daejeon 34113, 3Stem Cell Convergence Research Center, KRIBB, Daejeon 34141, 4Metabolic Regulation Research Center, KRIBB, Daejeon 34141, Korea

INTRODUCTION

Human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells, have emerged as promising tools in the fields of regenerative medicine and tissue engineering (1, 2). As PSCs exhibit an unlimited self-renewal capacity and maintain pluripotency, leading to their differentiation into cells of all three embryonic germ layers, they are particularly attractive as well as suitable sources for cell replacement therapy (3, 4). However, a few obstacles hinder the clinical use of PSCs after differentiation induction. In particular, the teratoma risk raised by undifferentiated PSCs is a major limitation in applying PSCs for transplantation (5, 6). Hence, various strategies have been developed to remove undifferentiated PSCs, including treatment with cytotoxic reagents or sorting of undifferentiated cells through flow cytometry (7). Nevertheless, improving the efficiency of undifferentiated cell removal remains essential. Thus, developing an efficient tool to distinguish undifferentiated PSCs with tumorigenic potential from heterogeneous PSC populations is indispensable for the progression of PSC application in clinical settings.

Discovering surface markers specifically expressed on PSCs is a prerequisite to not only assess the pluripotency level of PSCs but also isolate the undifferentiated population using antibody-based separation methods. Several cell-surface markers specific to PSCs, such as stage-specific embryonic antigen (SSEA)-3, SSEA-4, Tra-1-60, and Tra-1-81, have been identified to date and commonly used for detecting PSCs (8). However, the expression of these markers is not completely restricted to PSCs, and hence, for isolating the undifferentiated cell population, an antibody targeting one of these markers to distinguish completely the teratogenic cells would be insufficient. For example, Tang et al. reported that an anti-SSEA-5 monoclonal antibody (mAb) alone reduces the tumorigenic potential, but complete removal of teratoma formation is achieved by targeting three pluripotent cell-surface markers, SSEA-5, CD9, and CD90 (9). Owing to this limitation, identifying a more reasonable cell surface marker to distinguish the teratogenic cell population...
from heterogeneous PSCs remains necessary. Previously, we developed a mAb that binds to PSCs and identified L1 cell adhesion molecule (L1CAM) as a target antigen maintaining the pluripotency and self-renewal activity of PSCs (10). In addition, another cell surface molecule, desmoglein-2 (DSG2), has been identified as a novel PSC-specific marker, and DSG2-negative cell population, obtained through anti-DSG2 mAb targeting, exhibits a remarkable suppression of teratoma formation (11).

In this study, we generated a novel mAb, K312, which bound to a glycan moiety specifically presented on undifferentiated PSCs. We evaluated the specificity of K312 in separating a cell population with high pluripotency and tumorigenic potential from differentiated PSCs. Our study might provide a new strategy for effectively managing the risk of teratoma formation with stem cell therapy in clinical settings.

RESULTS

Identification of a mAb specific to human PSCs

We generated a panel of hybridomas, which was raised against undifferentiated H9 human ESCs (hESCs). On screening mAbs that bind to H9 hESCs, we identified a novel mAb, K312, and evaluated its selectivity in determining the PSC population. As shown in Fig. 1A, K312 strongly binds to H9 hESCs, similar to mAbs against other cell-surface pluripotency markers, such as Tra-1-60, Tra-1-81, SSEA-3, and SSEA-4. The specific binding of K312 to pluripotent cells was further validated in two induced pluripotent stem cell lines (iPS-FAD and iPS-SPD), as well as two embryonic carcinoma cell lines (NTERA-2 and NCCIT), but K312 does not bind to J1 mouse ESCs (Fig. 1B). We also observed that K312 correlatively binds to H9 hESCs harboring each of the indicated pluripotency markers (Fig. 1C). Subsequently, immunofluorescence analysis of H9 hESCs showed that K312 is co-localized with E-cadherin, a well-known cell-surface pluripotency marker, thereby, verifying the selective binding of K312 to pluripotent cells (Fig. 1D). To identify a target antigen of K312, we performed mass spectrometry analysis of a protein fraction, corresponding to approximately 60 kDa, immunoprecipitated using K312 (Fig. 1E). However, no meaningful candidate protein target was identified as K312 antigen (Supplementary Fig. 1 and 2). As PSC surface markers are occasionally composed of complex glycan structures (12), we hypothesized that K312 might interact with polysaccharide structures of a specific target antigen expressed on PSCs. To assess the ability of K312 to bind glycan, H9 hESCs or NCCIT lysates were incubated with PNGase F for deglycosylation and pulled down by K312. Interestingly, the target protein band disappeared after PNGase F treatment, even with 30 min incubation (Fig. 1F). Thus, we could suggest that K312 specifi-
K312 binding is dependent on the pluripotency of hESCs

We next investigated whether the cell-binding activity of K312 was limited to pluripotent cells. To this end, H9 hESCs were differentiated through embryoid body (EB) formation or retinoic acid (RA) treatment, and then, K312 binding to the cells was evaluated by fluorescence-activated cell sorting (FACS). As shown in Fig. 2A, the binding capacity of K312, as well as of mAbs targeting SSEA-3 and Tra-1-60, gradually decreases as H9 hESCs are differentiated to form EBs until 9 days. In addition, the protein expression levels of various pluripotency markers were evaluated by western blotting. As expected, the expression levels of the K312 target, E-cadherin, EpCAM, Oct4, Nanog, and Sox2 were notably reduced in the differentiated hESCs (Fig. 2B). To validate the loss of binding of K312 on differentiated hESCs, H9 hESCs were treated with RA for 12 days. Consistent with the EB formation assay, RA treatment downregulated the expression of the target of K312 and other pluripotency markers in a time-dependent manner (Fig. 2C, D). Therefore, we demonstrate that K312 specifically binds to the undifferentiated hESCs, and can be used for identifying cells in the pluripotent state.

K312 distinguishes a highly pluripotent ESC population from heterogeneous hESCs

Given the selective binding of K312 to pluripotent hESCs, we sought to examine the ability of K312 in classifying hESCs into two cell populations with high or low pluripotency. Undifferentiated H9 hESCs were gated based on an isotype control antibody and forward scatter, and by probing the cells with K312, we obtained two different cell populations, K312-high and K312-low (Fig. 3A). Next, the expression of pluripotency markers was analyzed by western blotting in these cell subsets. As shown in Fig. 3B, a clear difference exists between K312-high and K312-low cells in the expression of Oct4, Nanog, and E-cadherin, with the expression being higher in K312-high cells than K312-low cells. Furthermore, the clonogenic activity of these cell populations was assessed by colony formation assays. As expected, K312-high cells developed into compact colonies, whereas K312-low cells rarely formed colonies (Fig. 3C, D). These results indicated that K312 successfully distinguishes a cell population with high pluripotency from heterogeneous hESC populations.

K312 effectively removes teratogenic cells from differentiated PSC progeny

We next investigated whether K312 was utilizable in separating undifferentiated cells from the differentiated progeny. To this end, H9 hESCs were differentiated under RA treatment for 12 days. Under the differentiation conditions, H9 hESCs were converted into a differentiated phenotype characterized by...
and ectoderm (BMB http://bmbreports.org). The markers specific to the endoderm, mesoderm, and ectoderm, respectively, were developed from K312-high cells, but not K312-low cells or control mouse embryonic fibroblasts (MEFs), indicating that the target antigen of K312 is a crucial marker for separating tumorigenic cells from the differentiated progeny (Fig. 4C). To evaluate the differentiation of K312-high cells into the three germ layers, the testicles excised from mice were subjected to histological analysis. As shown in Fig. 4F, the neural epithelium, respiratory endothelium, and myxoid tissue, representing the ectoderm, endoderm, and mesoderm, respectively, were developed from K312-high cells, but were not observed in the testicles treated with control MEFs or K312-low cells. In addition to histology, expression of the representative molecular markers of the three germ layers was analyzed by RT-PCR. The markers specific to the endoderm (GATA6, FN1, AMY2A), mesoderm (ACAN, HAND1, MSX1), and ectoderm (MAP2, NESTIN, TUBB3) were markedly expressed in the testicle tissues of mice belonging to the K312-high group but not the K312-low or control group (Fig. 4G). Thus, K312 effectively eliminates the risk of teratoma formation by sorting out teratogenic cells from the differentiated cell progeny after in vitro differentiation of hESCs.

**DISCUSSION**

Numerous studies have been conducted on the clinical application of PSCs, especially for stem cell transplantation. However, given the plasticity and heterogeneity of PSCs after *in vitro* differentiation, safety, especially that pertaining to tumorigenicity, is a major concern when differentiated PSCs are transplanted *in vivo* (14, 15). To address this issue, several antibodies targeting PSC-specific antigens have been developed to isolate fully differentiated PSCs by depleting undeveloped PSCs (8). In this study, we demonstrate that a novel mAb K312 specifically binding to human PSCs could separate the pluripotent cell population from differentiated PSCs. We found that pluripotent cells are considered to be depleted from the differentiated PSC progeny when K312 fails to bind to the differentiation-induced cells (Fig. 2A, C). This implies that the target antigen of K312 is specifically expressed on PSCs and can be a cell-surface pluripotency marker useful for antibody-based cell sorting approaches. Consistent with the decreased binding of K312, the pluripotency markers were barely detected in the cells negatively isolated using K312 (Fig. 2B, D), indicating that the surface expression of the K312 target is intimately correlated with the pluripotency of cells. Furthermore, K312 clearly distinguished the K312-low and K312-high cell populations from undeveloped hESCs (Fig. 3A). These results suggest that K312 is also utilizable for assessing whether PSC lines stably maintain their pluripotency.

Well-defined cell-surface pluripotency markers precisely indicate the pluripotent state of PSCs. As is generally known, glycan moieties are incorporated in common cell-surface pluripotency markers, such as glycolipids SSEA-3 and SSEA-4 or keratan sulfate proteoglycan Tra-1-60 and Tra-1-81, and various mAbs binding to glycan in these markers have been developed (8, 16). However, these markers tend to be expressed on tumor cells and are occasionally not suitable to describe the identity of PSCs (17, 18). Thus, it is necessary to discover a valuable surface antigen specific to PSCs. In this study, we determined that the expression of a PSC-specific surface antigen targeted by K312 is well representative of the pluripotent state of various PSCs, with its expression being downregulated in differentiation-induced hESCs, indicating that the antigen could be a novel marker specific for PSCs but not differentiated PSC progeny (Fig. 1B and Fig. 2). Moreover, H9 hESCs expressing SSEA-3, SSEA-4, Tra-1-60, or Tra-1-80 were counterstained with K312, implying that the antigen of K312 is expressed independently of the other markers (Fig. 1C). Although the target of K312 was not identified here, we demonstrated that K312 binds to the glycan moiety of a target specifically expressed on PSCs. Thus, future investigation is warranted to identify the target of K312.

Prior to the emergence of various efforts for the clinical application of PSCs, the tumorigenic potential of differentiated PSCs should be assessed *in vivo*. In this regard, the teratoma assay is the most reliable method because it directly monitors the differential development of PSCs into a wide range of tissues (13). In terms of assessing the risk of teratoma formation with PSCs differentiated *in vitro*, several studies have shown that an antibody targeting a PSC-specific marker, such as SSEA-5 or claudin-6, can sort teratogenic cells from differentiated progeny, and thereby manage the teratoma risk accompanied with differentiation-induced PSCs (9, 19). Although these studies demonstrate the substantial utilization of antibody-based depletion of tumorigenic PSCs, complete elimination of teratoma formation is observed when the tumorigenic cells are immunodepleted using antibodies against SSEA-5 and two additional pluripotency markers (CD9 and CD30) or are killed by claudin-6-targeting cytotoxic drugs. Interestingly, we observed that the K312-low cell population did not completely result in developing a teratoma, same as the control MEFs, which indicates that managing the risk of teratoma formation is possible just by K312-based depletion of teratogenic cells (Fig. 4D, E). These data...
also imply that the target of K312 is essential for maintaining the self-renewal capacity and tumorigenic activity of PSCs.

In conclusion, K312, which specifically targeted PSCs, not only identified the pluripotent state of PSCs, but also separated undifferentiated tumorigenic PSCs from differentiated PSCs, suggesting that it is valuable to further the clinical application of stem cell-based therapy by eliminating the risk of teratoma formation.

MATERIALS AND METHODS

Cell culture

H9 hESCs, iPSC-JAD and iPSC-SPD, were cultured in DMEM/F12 supplied with 20% serum replacement and 10 ng/ml basic fibroblast growth factor (bFGF). For culturing J1 mouse ES cells, leukemia inhibitory factor was added instead of bFGF in the medium as described above. These feeder-based cell lines were cultured on irradiated MEFs. Embryonic carcinoma cell lines, NTERA-2 and NCCIT, were cultured as previously described (11). For feeder-free culture, H9 hESC clumps were transferred onto Matrigel (BD Bioscience)-coated plates and cultured with mTeSR medium (STEMCELL Technologies). EB formation assay was performed using an AggreWell plate (STEMCELL Technologies) as described previously (11). For in vitro differentiation, H9 hESCs were cultured in the medium not containing bFGF and treated with 10 μM RA (Sigma-Aldrich).

Hybridoma generation and mAb purification

BALB/c mice were immunized with H9 hESCs as described previously (10). Hybridomas were generated and screened to select mAbs binding to H9 hESCs via flow cytometry. mAbs secreted from hybridoma cells were purified using Protein G-Sepharose column chromatography as described previously (10).

FACS analysis

Cells were dissociated using Accutase (STEMCELL Technologies) for 10 min and washed with 3% bovine serum albumin in PBS. Cells (1 × 10^7) were incubated with 1 μg of the indicated antibody for 1 h at room temperature. K312 was detected using a FITC-labeled secondary antibody, and phycoerythrin-labeled antibodies against SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 (BioLegend) were used for double staining. Cell sorting was performed using a FACSAria Cell sorter (BD Bioscience).

Immunofluorescence staining

H9 hESCs were incubated with K312 and anti-E-cadherin (Cell Signaling Technology) antibodies for 16 h at 4°C after fixing. Secondary antibodies labeled with FITC and phycoerythrin were used to detect K312 and anti-E-cadherin antibodies, respectively. Fluorescence images were captured using a Zeiss 510LSM META laser-scanning microscope (Carl Zeiss).

Immunoblotting

Cells were dissolved in RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) with protease and phosphatase inhibitors to isolate cell lysates. Cell lysates (20 μg) were mixed with SDS sample buffer, heated for 10 min, and analyzed by SDS-PAGE. Separated proteins were transferred onto a PVDF membrane and blocked with 3% skim milk in PBS for 1 h. The membrane was incubated with K312 or primary antibodies against pluripotency markers for 16 h at 4°C. After incubating the membrane with appropriate secondary antibodies conjugated with horseradish peroxidase, ECL solution (GE Healthcare) was added and the immunoreactive bands were visualized by FUSION SOLO S chemiluminescence system (Vilber).

Immunoprecipitation

Cell lysates, as prepared for immunoblotting, were precleared of protein G beads for 1 h. The bead-unbound fractions were incubated with K312 for 4 h at 4°C, and then, K312 was precipitated by protein G beads for 12 h. Target protein-antibody reactants were released by heating, and the samples were centrifuged to remove the beads. Collected supernatant was analyzed by SDS-PAGE, followed by immunoblotting.

Deglycosylation assay

H9 hESCs or NCCIT cells were lysed in RIPA buffer, and 10 μg of lysate was incubated with 500 units of PNGase F (NEB) at 37°C for 0.5, 1, 2, and 4 h. Samples reacting or not reacting with PNGase F were immunoblotted using K312 as described earlier (Immunoblotting section).

Alkaline phosphatase staining

Colonies-forming cells were fixed in 10% formalin solution and stained using an alkaline phosphatase staining kit (Sigma-Aldrich), as recommended by the manufacturer. Alkaline phosphatase-positive colonies were manually counted under light microscope and images were captured using HP Scanjet (Hewlett-Packard).

Teratoma assay

Six-week-old NSG mice (Jackson Laboratory) were cared for, following the guidelines of the Animal Care Committee of the Korea Research Institute of Bioscience and Biotechnology. The teratoma assay was performed as described previously (11).Briefly, control irradiated MEFs, K312-low cells, or K312-high cells (5 × 10^5) were injected into each side of the mouse testes using a 31-gauge Ultra-Fine™ syringe (BD Bioscience). Teratomas were excised 8 weeks from injection, and their weights and sizes were measured. For histology, tissue fixation, paraffin processing, embedding, sectioning, and hematoxylin and eosin staining were performed as described previously (20).

Reverse transcription PCR (RT-PCR)

Total RNA was extracted from mice testicle tissues using TRIzol reagent (iNtRON Biotechnology) and cDNA was synthesized as described previously (20). For RT-PCR, primers to detect GATA6, FN1, AMY2A, HAND1, MSX1, MAP2, NESTIN, TUBB3,
and GAPDH gene expression were used with primer sequences obtained from a previous study (19). Primer sequences used to analyze ACAN gene expression were 5'-TCTGTAACCAGGC TCCAAC-3' (sense) and 5'-CTGGCAAAATCCCCACTAAA-3' (anti-sense).

ACKNOWLEDGEMENTS

This research was supported by the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Research Initiative Program (KGM5272221) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1I1A2057698).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

REFERENCES

1. Trounson A and DeWitt ND (2016) Pluripotent stem cells progressing to the clinic. Nat Rev Mol Cell Biol 17, 194-200
2. Park J, Lee Y and Shin J et al (2019) Mitochondrial genome mutations in mesenchymal stem cell derived from human dental induced pluripotent stem cells. BMB Rep 52, 689-694
3. Graf T and Stadtfeld M (2008) Heterogeneity of embryonic and adult stem cells. Cell Stem Cell 3, 480-483
4. Yamanaka S (2020) Pluripotent stem cell-based cell therapy promise and challenges. Cell Stem Cell 27, 523-531
5. Blum B and Benvenisty N (2008) The tumorigenicity of human embryonic stem cells. Adv Cancer Res 100, 133-158
6. Ben-David U and Benvenisty N (2011) The tumorigenicity of human embryonic and induced pluripotent stem cells. Nat Rev Cancer 11, 268-277
7. Wuputra K, Ku CC, Wu DC, Lin YC, Saito S and Yokoyama KK (2020) Prevention of tumor risk associated with the reprogramming of human pluripotent stem cells. J Exp Clin Cancer Res 39, 100
8. Choi HS, Kim WT and Ryu CJ (2014) Antibody approaches to prepare clinically transplantable cells from human embryonic stem cells: identification of human embryonic stem cell surface markers by monoclonal antibodies. Biotechnol J 9, 915-920
9. Tang C, Lee AS, Volkmer JP et al (2011) An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. Nat Biotechnol 29, 829-834
10. Son YS, Seong RH, Ryu CJ et al (2011) Brief report: L1 cell adhesion molecule, a novel surface molecule of human embryonic stem cells, is essential for self-renewal and pluripotency. Stem Cells 29, 2094-2099
11. Park J, Son Y, Lee NG et al (2018) DSG2 is a functional cell surface marker for identification and isolation of human pluripotent stem cells. Stem Cell Rep 11, 115-127
12. Satomaa T, Heiikanen A, Mikkola M et al (2009) The N-glycome of human embryonic stem cells. BMC Cell Biol 10, 42
13. Wesselschmidt RL (2011) The teratoma assay: an in vivo assessment of pluripotency. Methods Mol Biol 767, 231-241
14. Narsinh KH, Sun N, Sanchez-Freire V et al (2011) Single cell transcriptional profiling reveals heterogeneity of human induced pluripotent stem cells. J Clin Invest 121, 1217-1221
15. Nussbaurn J, Minami E, Lallamme MA et al (2007) Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J 21, 1345-1357
16. Lanctot PM, Gage FH and Varki AP (2007) The glycans of stem cells. Curr Opin Chem Biol 11, 373-380
17. Schopperle WM and DeWolf WC (2007) The TRA-1-60 and TRA-1-81 human pluripotent stem cell markers are expressed on podocalyxin in embryonal carcinoma. Stem Cells 25, 723-730
18. Brimble SN, Sherrer ES, Uhl EW et al (2007) The cell surface glycosphingolipids SSEA-3 and SSEA-4 are not essential for human ESC pluripotency. Stem Cells 25, 54-62
19. Ben-David U, Nudel N and Benvenisty N (2013) Immuno logic and chemical targeting of the tight-junction protein Claudin-6 eliminates tumorigenic human pluripotent stem cells. Nat Commun 4, 1992
20. Park J, Lee NG, Oh M et al (2020) Selective elimination of human pluripotent stem cells by anti-Dsg2 antibody-doxorubicin conjugates. Biomaterials 259, 120265