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Temporal Expression of Transcription Factor *ID2* Improves Natural Killer Cell Differentiation from Human Pluripotent Stem Cells

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Abstract

Natural killer (NK) cells are one type of innate lymphoid cells, and NK cell-based immunotherapy serves as a potentially curative therapy for cancers. However, the lack of reliable resources for a large amount of NK cells required for clinical infusion has limited the broader application of

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[#]**Author Contributions** J.J. and Y.C. are co-first authors. J.J., Y.C., and X.B. conceived and designed the experiments. Y.C., G.J., and X.L. designed and conducted genetic-engineered experiments. J.J. and Y.C. conducted NK cell differentiation and function analysis. J.J., Y.C., and X.B. wrote the manuscript with support from all authors.

Supporting Information

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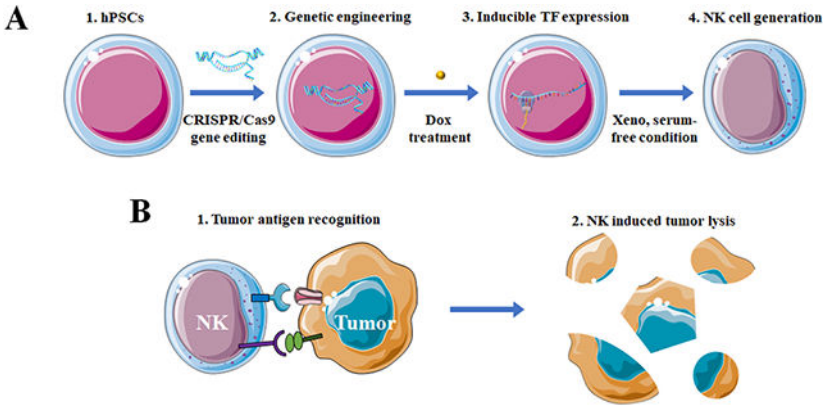
Table of antibodies used in this study; characterization of an all-in-one, Tet-on 3G inducible eGFP system; representative karyotyping analysis of ID2-H9 cells; time-dependent ID2 expression on ID2-hPSCs with doxycycline (dox) treatment; representative flow cytometry analysis of CD45 and CD56 expression on hPSC-derived NK cells; biocompatible analysis of hPSC-NK cells for normal somatic cells (PDF)

The authors declare no competing financial interest.

NK cells in targeted immunotherapy. Substantial effort has thus been made to generate NK-like cells from human pluripotent stem cells (hPSCs), but detailed molecular mechanisms regulating NK cell differentiation remain elusive, preventing us from developing robust strategies for NK cell production. Here, we genetically engineered hPSCs with inducible overexpression of transcription factors *NFIL3, ID2*, or *SPI1* via CRISPR/Cas9-mediated gene knock-in and investigated their temporal roles during NK cell differentiation. Our results demonstrated *ID2* overexpression significantly promoted NK cell generation compared with *NFIL3* and *SPI1* overexpression under a chemically defined, feeder-free culture condition. The resulting ID2 hPSC-derived NK cells exhibited various mature NK-specific markers and displayed effective tumor-killing activities, comparable to NK cells derived from wildtype hPSCs. Our study provides a new platform for efficient NK cell production, serving as a realistic off-the-shelf cell source for targeted cancer immunotherapy.

Graphical Abstract



Keywords

human pluripotent stem cell (hPSC) differentiation; natural killer (NK) cells; immunotherapy; genetic engineering

INTRODUCTION

Natural killer (NK) cells are one kind of lymphocytes that are differentiated from hematopoietic stem cells (HSCs) in the bone marrow and become mature in lymph nodes.[1,2] NK cells present characteristics of both innate and adaptive lymphoid cells,[2,3] demonstrating superior ability to attack tumor cells and suppress their growth *in vivo*.[4,5] Unlike T cells, prior sensitization and antigen exposure are not required for NK cells.[6,7] Activating receptors, such as CD16 (FcγRIII) and NK group 2D (NKG2D), and inhibitory receptors expressed on NK cells work synergistically to distinguish normal from tumor cells, triggering cytolytic programs and cytokine release against abnormal cells.[8] Importantly, allogeneic NK cells are free of graft-versus-host diseases (GvHD) that are commonly associated with allogeneic T cell-based cancer therapies.[9] This unique characteristic opens the possibility to develop universal NK cells, which could be used to treat any patient without human leukocyte antigen matching.

Given their unique ability to self-renew and differentiate into all kinds of somatic cells, human pluripotent stem cells (hPSCs) are emerging as a promising cell source for scalable production of NK cells. As compared to primary NK cells or NK-92 cell line, hPSCs are more accessible to genetic modifications, such as chimeric antigen receptor (CAR) engineering, to potentially produce off-the-shelf genetically enhanced NK cells for cancer immunotherapy.[10,11] However, there are several obstacles to overcome before realizing the full potential of hPSC-NK cells.[12] First, many established differentiation protocols require embryoid body formation, feeder cells, and/or stromal cells,[13-16] reducing the reproducibility and standardization of massive NK cell production from hPSCs.[17,18] Second, more cost-effective protocols are needed, as the employment of expensive growth factors and animal-derived components in current protocols may limit NK cell manufacturing to achieve a clinically relevant dosage (*i.e.*, 10^7 NK cells per kilogram of a patient).[19] Notably, the long NK cell derivation period of seven or more weeks from hPSCs also makes it difficult for cell preparation and increases contamination risk. These studies suggest that our knowledge of human NK cell development is limited, and we need a better understanding of and control over NK cell differentiation from hPSCs.

To address these challenges, we propose here a transcription factor (TF)-mediated cell fate engineering approach, aiming to develop a robust and rapid platform for scalable production of NK cells from hPSCs. *NFIL3, SPI1*, and *ID2* were previously identified as key factors during NK cell generation and maturation.[20-23] Thus, we genetically modified hPSCs with CRISPR/Cas9-mediated gene knock-in technique to introduce inducible *NFIL3, SPI1*, and *ID2* constructs into the *AAVS1* safe harbor locus,[24] and demonstrated that *ID2* overexpression significantly promoted NK cell generation compared with *NFIL3* and *SPI1* overexpression under a chemically defined, feeder-free culture condition. The resulting hPSC-derived NK cells exhibited various mature NK-specific markers and displayed effective tumor-killing ability across various cancer cells *in vitro*. Collectively, our study provided a new platform for efficient NK cell production, serving as a realistic off-the-shelf cell source for targeted cancer immunotherapy.

RESULTS

Targeted Gene Knock-in in hPSCs for Inducible Expression of *NFIL3*, *SPI1*, and *ID2*.

In order to temporally activate key TFs in a manner representative of native NK cell development, an all-in-one, Tet-On 3G doxycycline-inducible expression system was employed, which contains two promoters, Tet-on 3G transactivator protein driven by the constitutive EF1α promoter, and a transgene of interest driven by the TRE3G promoter (Figure 1A and Supporting Information, Figure S1A). This all-in-one inducible system effectively expressed eGFP in H9 hPSCs under doxycycline (dox) treatment (Figure S1B,C). We next replaced eGFP with *NFIL3*, *SPI1*, and *ID2*, and knocked each of them into the endogenous *AAVS1* safe harbor locus in H9 hPSCs via CRISPR/Cas9-mediated homology-directed repair (HDR) (Figure 1B). After nucleofection, puromycin-resistant single cell-derived hPSC clones were isolated and subjected for PCR genotyping with a successful targeted knock-in efficiency of 87.5% (7 out of 8 clones), 87.5% (7 out of 8 clones), and 83.3% (10 out of 12 clones) (Figure 1C) for *NFIL3, SPI1*, and *ID2*, respectively.

Successfully targeted clones were then subjected to homozygosity assay, and 28.5% (2 out of 7 clones), 14.3% (1 out of 7 clones), and 40% (4 out of 10 clones) of *NFIL3*, *SPI1*, and *ID2* knock-in clones were homozygous (Figure 1C). We picked heterozygous C7, C8, and C6 of *NFIL3, SPI1*, and *ID2* knock-in hPSCs for NK cell differentiation. Genetically modified hPSCs displayed strong expression of pluripotency markers, stage-specific embryonic antigen-4 (SSEA-4) and octamer-binding transcription factor-4 (OCT4) (Figure 1D). Importantly, these hPSCs retained a normal karyotype after CRISPR/Cas9-mediated genome editing (Figure S2). Similar to inducible eGFP expression, the resulting knock-in hPSCs expressed high levels of *NFIL3*, *SPI1*, and *ID2* in response to dox treatment (Figure 1E, Figure S3A-C).

Overexpression of *ID2* Promotes NK Cell Differentiation from hPSCs.

To investigate the function of *NFIL3, SPI1*, and *ID2* during *in vitro* NK cell development, we adapted and modified a previous chemically defined NK cell differentiation protocol[10] (Figure 2A). Under dox treatment during the whole differentiation, about 0.6%, 14.6%, 9.0%, and 65.1% CD45+CD56+ cells were generated from wildtype hPSCs, NFIL3-hPSCs, SPI1-hPSCs, and ID2-hPSCs, respectively (Figure 2B), suggesting that overexpression of NK-specific TFs improves *in vitro* NK cell differentiation from hPSCs. Notably, forced expression of *ID2* yielded the highest percentage of CD45+CD56+ cells under the chemically defined, feeder-free monolayer culture condition, consistent with enhanced *ID2* expression during NK cell differentiation from hPSCs.[25,26] Collectively, our preliminary results suggest the potential of using forced TF expression in enhancing NK cell differentiation from hPSCs.

ID2 plays stage-specific functions during NK cell development and maturation *in vivo*.[27-29] Thus, we investigated the stage-specific effects of forced *ID2* expression in NK cell generation from hPSCs to develop an optimized differentiation protocol (Figure 3A). Temporal treatment of dox significantly affects the generation of CD45+CD56+ cells (Figure 3B,C), confirming the stage-specific roles of *ID2* during NK cell development. Among all tested conditions, dox treatment from day 12 to day 22 (Group #2) yielded highest percentage (~73.7%) of CD45+CD56+ cells (Figure 3B,C). The resulting cells derived under the optimized condition were further characterized, and they displayed high expression levels of typical NK cell surface markers, including CD16, KIR3DL1, NKp46, NKG2D, and NKp44 (Figure 3D), consistent with previously reported hPSC-derived NK cells (Figure S4).[10,11,16,18] Taken together, our results demonstrated the stage-specific roles of *ID2* overexpression in enhancing NK cell differentiation from hPSCs.

hPSC-Derived NK Cells Display Cytotoxicity against Cancer Cells.

We next investigated the expansion and transmigration ability of hPSC-derived NK cells. Similar expansion fold was observed in hPSC-derived and primary NK cells (Figure 4A) in the presence of *IL-2*, *IL-15*, and *OKT3*.[30] Furthermore, *ID2* overexpression-induced hPSC-NK cells exhibited similar transmigration ability as wildtype hPSC-derived and primary NK cells (Figure 4B,C). To further explore their potential in cancer immunotherapy, *ID2* overexpression-induced NK cells were cocultured with different cancer cells for tumor-killing analysis. Two hours following coculture with U87MG glioblastoma cells,

immunological synapses were formed between NK and tumor cells (Figure 4D), facilitating cytotoxicity activities of NK cells against tumor cells. As expected, hPSC-derived NK cells via *ID2* overexpression or feeder layer coculture expressed IFN-γ and CD107a in response to tumor cells (Figure 4E,F), indicating cytotoxic granule release. We next assessed tumor-killing ability of hPSC-derived NK cells against different tumor cells, including LNCaP, A549, U87MG, and MDA-MB-231, and demonstrated that similar to their counterparts in peripheral blood, hPSC-derived NK cells displayed a broad antitumor cytotoxicity at various effector-to-target ratios (Figure 4G-I). Notably, hPSC-derived NK cells did not kill normal H9-derived somatic cells (Figure S5), suggesting their safety in future clinical application.

DISCUSSION

Adoptive NK cell-based immunotherapies hold great promise for clinical cancer treatment, given their unique innate tumor-killing ability and safety in allogeneic transplantation. In order to meet clinical needs (10^7 cells/kg for a patient[19]), several human sources of NK cells, including peripheral blood (PB) and umbilical cord blood (UCB), were investigated in cancer immunotherapy. However, primary NK cells isolated from PB and UCB sources were heterogeneous,[31] and these cells were not sufficient to treat many patients.[19,28] In contrast, hPSCs could be expanded unlimitedly and differentiated into NK cells to meet the clinical needs,[14,25] providing a realistic cell source for off-the-shelf cancer immunotherapy. In order to realize the therapeutic application of hPSC-derived NK cells, however, we need a better understanding of the molecular mechanisms and control over the specification of NK cells from hPSCs.

TF-mediated forward programming approach has been recently used to efficiently differentiate hPSCs into neural, glial, liver, skeletal and cardiac muscle cells.[32] However, such an approach has not yet been applied to NK cell induction. In this study, we genetically engineered hPSCs with doxycycline-inducible expression of *NFIL3, SPI1*, and *ID2*, and demonstrated TF-mediated forward programming enhanced NK cell differentiation, in which inducible *ID2* expression yielded the highest percentage of CD45+CD56+NK cells. This result is consistent with enhanced *ID2* expression during NK cell differentiation from hPSCs.[25,26] The resulting hPSC-derived NK cells also displayed NK-specific surface markers and cytotoxic activities against various tumor cells *in vitro*.

In summary, our all-in-one inducible expression system described in this study can serve as a modular strategy to screen more transcription factors for robust NK or T cell induction from hPSCs. The engineered *ID2*-expressing hPSCs may also provide a scalable strategy to generate off-the-shelf NK cells as potentially standardized cellular products for clinical applications in cancer treatment. Given the relative ease of genome editing in hPSCs, other genetic modifications, such as chimeric antigen receptor (CAR) expression and/or inhibitory receptor deletions, can also be performed to achieve optimal therapeutic effects in hPSC-derived NK cells.

METHODS

Donor Plasmid Construction.

To construct AAVS1-Puro XLone-NFIL3, SPI1, and ID2 plasmids, fragments of human *NFIL3, SPI1*, and *ID2* genes were amplified from Addgene plasmids (NOs. 82985, 97039, and 98394) and used to replace eGFP in the AAVS1-Puro XLone-eGFP donor plasmid (Addgene NO. 136936).

Maintenance and Differentiation of hPSCs.

H9 hPSCs were obtained from WiCell and maintenance on Matrigel- or iMatrix-511-coated plates in mTeSR plus or E8 medium. For NK cell differentiation, hPSCs were dissociated with 0.5 mM EDTA and seeded onto iMatrix-511-coated 24-well plate at a cell density between 10 000 and 80 000 cell/cm^2 in mTeSR plus medium with 5 μM Y27632 for 24 h (day – 1). Afterward, NK cell differentiation was performed according to a previous report[10] with modifications shown in Figure 2A and 3A. Briefly, 6 μM CHIR99021 was used to induce mesoderm differentiation from day 0 to day 2 in LaSR basal medium,[33] followed by 10 μM SB431542, 50 ng/mL SCF, and VEGF treatment from day 2 to day 4. To induce hematopoiesis, 50 ng/mL SCF and FLT3L were used in Stemline-II medium from day 4 to day 12.[34] Floating day 12 hematopoietic stem and progenitor cells (HSPCs) were collected and treated with 50 ng/mL *SCF, FLT3L, IL-3, IL-7*, and *IL-15* from day 15 to day 23. From day 23 to day 30, differentiated cultures were treated with 50 ng/mL *SCF, FLT3L, IL-7*, and *IL-15* as well as 5 μg/mL heparin. For feeder layer-based NK cell differentiation,[17] day 12 HSPCs were collected and transferred on OP9 stromal feeder cells, which were cultured in the α-MEM medium containing 20% FBS, 10 ng/mL *SCF*, 10 ng/mL *FLT3L*, 5 ng/mL *IL-7,* and 10 ng/mL *IL-15*. After coculturing for 7 days, differentiated cells were collected and transferred on fresh OP9 feeder cells, and NK cell differentiation was continued for 4 weeks under the same conditions.

hPSC-NK Cell Purification.

hPSC-derived NK cells were purified using EasySep FITC Positive Selection Kit (StemCell Technologies) according to the manufacturer's instruction. Briefly, differentiated NK cells were centrifuged at 200*g* for 5 min, washed twice with 10 mL of PBS −/− solution containing 1% bovine serum albumin (BSA) (FlowBuffer-1), and then pelleted by centrifugation. After aspirating the supernatant, cell pellet was resuspended in 100 μL of FlowBuffer-1 at a cell concentration of 1 × 10^8 cells/mL with 1:50 CD56-FITC antibody, and incubated in the dark at room temperature for 30 min. Afterward, cell and antibody mixtures were washed once with 2 mL of FlowBuffer-1 and incubated with 10 μL of EasySep FITC Selection Cocktail in 100 μL of FlowBuffer-1 at room temperature for 15 min. A total of 5 μL of well-mixed magnetic nanoparticles was then added to the 100 μL cell mixture and incubated at room temperature for another 10 min. The resulting cell suspension was then brought to a total volume of 2.5 mL FlowBuffer-1 in a flow tube, and then placed into the magnet without a cap for 5 min. The magnet was then inverted in one continuous motion to pour off the supernatant and then returned to an upright position. The flow tube was moved from the magnet and washed with 2.5 mL FlowBuffer-1 to resuspend the cells on the flow tube wall by gently pipetting up and down for 2–3 times. The magnet treatment

was repeated for two to three times, and enriched NK cells were then resuspended in an appropriate amount of desired medium for further application.

Nucleofection and Genotyping of hPSCs.

To increase cell viability, hPSCs were treated with 10 μM Y27632 3–4 h before nucleofection or overnight. Following, 1–2.5 × 10^6 singularized hPSCs were nucleofected with 6 μg of AAVS1 XLone donor plasmids along with 6 μg of SpCas9 AAVS1 gRNA T2 (Addgene; #79888) in 100 μL of human stem cell nucleofection solution (Lonza; #VAPH-5012) using program B-016 in a Nucleofector 2b. Nucleofected hPSCs were then plated into one well of a Matrigel-coated 6-well plate in 3 mL of prewarmed mTeSR plus with 10 μM Y27632. Twenty-four hours later, the medium was changed with mTeSR plus containing 5 μM Y27632, followed by a daily medium change. When cells reach about 80% confluency, 1 μg/mL puromycin (Puro) was applied for drug selection for about 1 week. Individual clones were then picked and expanded for 2–5 days in each well of a Matrigel-coated 96-well plate, followed by PCR genotyping using QuickExtract DNA Extraction Solution (Epicenter; #QE09050) and 2 × GoTaq Green Master Mix (Promega; #7123). For positive genotyping, the following primer pair was used: CTGTTTCCCCTTCCCAGGCAGGTCC and TCGTCGCGGGTGGCGAGGCGCACCG (T_m = 65 °C). For homozygous genotyping, the following set of primer sequences was used: CGGTTAATGTGGCTCTGGTT and GAGAGAGATGGCTCCAGGAA (T_m = 60 °C).

Tumor Cell Line Culture.

U87MG, A549, LNCaP, and MDA-MB-231 tumor cells were kindly provided and cultured by the laboratories of Drs. Sandro Matosevic, Chang-Deng Hu, and Philip Low at Purdue University. U87MG, A549, LNCaP, and MDA-MB-231 cells were cultured in Eagle's Minimum Essential Medium (EMEM) (containing 10% fetal bovine serum, 100 units mL^{-1} penicillin and 100 mg mL^{-1} streptomycin), Kaighn's Modification of Ham's F-12 Medium (F-12K) (containing 10% fetal bovine serum, 100 units mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin), RPMI-1640 Medium (containing 10% fetal bovine serum, 100 units mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin), and Leibovitz's L-15 Medium (containing 10% fetal bovine serum, 100 units mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin), respectively. All tumor cell lines were incubated at 37 °C in a humidified 5% CO_2 atmosphere. The medium was changed every 2 days and cells passaged at 70%–80% confluency.

Expansion of NK Cells.

Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoperp (StemCell Technology, 07851) gradient centrifugation in SepMate (StemCell Technology, 85450) tubes. Primary human cells were isolated from PBMCs by magnetic bead CD3 depletion (Miltenyi Biotec, 130-050-101), followed by CD56 (Miltenyi Biotec, 130-111-553) isolation. Purified NK cells were cultured in AIM-V (Invitrogen) medium with 500 U/mL *IL-2* (Peprotech, 200–02), 2 ng/mL *IL-15* (Peprotech, 200–15), and 100 ng/mL *OKT3* (Ortho Pharmaceuticals, Raritan, NJ, USA) at the concentration of 1 × 10^6 cells/mL for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. The cells were cultured in AIM-V medium supplemented with *IL-2* and *IL-15* at 37 °C in a humidified 5% CO_2 atmosphere for further analysis.

Flow Cytometry Analysis.

Differentiated cells were gently pipetted and filtered through a 70 or 100 μm strainer sitting on a 50 mL tube. The cells were then pelleted by centrifugation and washed twice with PBS −/− solution containing 1% bovine serum albumin (BSA). The cells were stained with appropriate conjugated antibodies (Table S1) for 25 min at room temperature in the dark, and analyzed in an Accuri C6 plus cytometer (Beckton Dickinson) after washing with BSA-containing PBS −/− solution. FlowJo software was used to process the flow data.

Transwell Migration Analysis.

For transwell assays, 600 μL of serum-free medium was placed in the lower chamber of a 24-well transwell plate (Corning). NK cells (2.5×10^5) were added in 100 μL of serum-free medium to the upper chamber (5 μm pore size), and the plate was incubated at 37 °C with 5% CO_2 for 5 h. The number of NK cells that migrated to the lower chamber was determined by flow analysis (Accuri C6 plus cytometer, Beckton Dickinson). Data are presented as percentage of migration based on total cell input.

NK Cell-Mediated *In Vitro* Cytotoxicity Assay.

The cell viability was analyzed by flow cytometry according to a previous protocol.[35] Briefly, tumor cells were stained with 2 μM Calcein-AM in MEM medium at 37 °C for 10 min in the dark, followed by 10% FBS treatment for 10 min in the dark at room temperature. Labeled tumor cells were pelleted at 300x*g* for 7 min and resuspended in culture medium with 10% FBS at a density of 50, 000 cells/mL. Tumor cells (100 μL) were then mixed with 100 μL of 150, 000, 250, 000, and 500, 000 cells/mL NK cells in 96 well plates, and incubated at 37 °C, 5% CO_2 for 12 h. To harvest all the cells, cell-containing media were first transferred into a new round-bottom 96-well plate, and 50 μL of trypsin-EDTA was added to the empty wells to dissociate attached cells. After 5 min incubation at 37 °C, dissociated cells were transferred into the same wells of round-bottom 96-well plate with floating cells. All cells were pelleted by centrifuging (300x*g*, 4 °C, 5 min), and washed with 200 μL of PBS −/− solution containing 0.5% BSA. Pelleted cells were stained with propidium iodide (PI) for 15 min at room temperature, and analyzed in the Accuri C6 plus cytometer (Beckton Dickinson).

Conjugate Formation Assay.

To visualize immunological synapses, 100 μL of tumor cells (50, 000 cells/mL) were seeded onto wells of 96-well plate and incubated at 37 °C for 12 h, allowing them to attach. NK cells (100 μL, 500, 000 cells/mL) were then added onto the target tumor cells and incubated for 6 h before fixing with 4% paraformaldehyde (in PBS). Cytoskeleton staining was then performed using an F-actin Visualization Biochem Kit (Cytoskeleton Inc.).

Statistical Analysis.

Three to five samples were analyzed for each group, and data are presented as mean ± standard deviation (SD). Statistical significance was determined by Student's *t*-test (two-tail) between two groups, and three or more groups were analyzed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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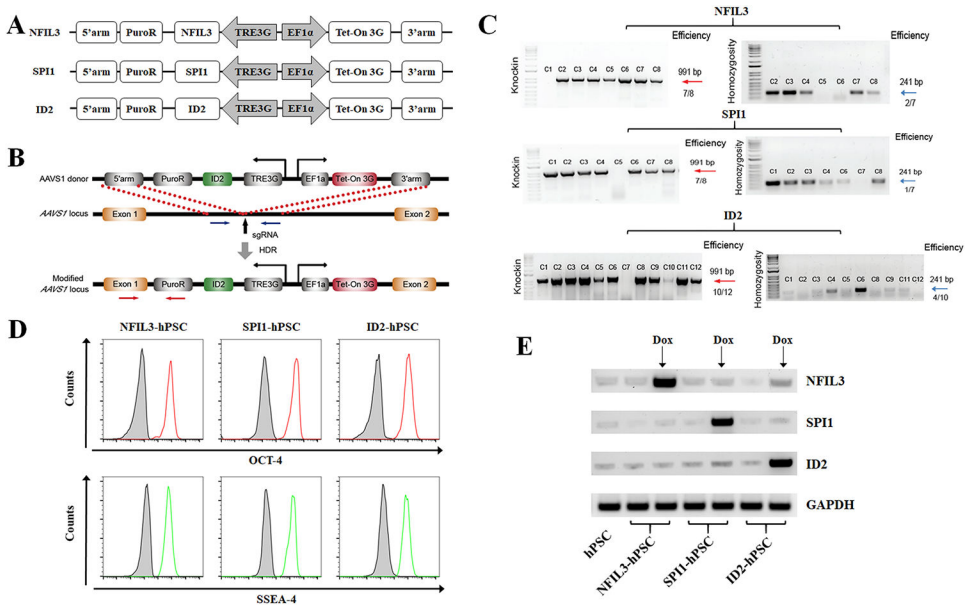

Figure 1.

Targeted gene knock-in in H9 hPSCs for inducible expression of *NFIL3, SPI1*, and *ID2*. (A,B) Schematic of all-in-one, Tet-on 3G inducible system construct and targeted knocked-in strategy at the endogenous *AAVS1* safe harbor locus via CRISPR/Cas9-mediated homologous recombination shown in panels A and B. (C) PCR genotyping of hPSC clones after puromycin selection is shown, and the expected PCR product for correctly targeted *AAVS1* site is 991 bp (red arrow). A homozygosity assay was performed on the knock-in clones, and those without ~204 bp PCR products were homozygous (blue arrow). (D) Flow cytometry analysis of OCT4 and SSEA4 expression in the indicated hPSC lines. (E) RT-PCR analysis of *NFIL3, SPI1*, and *ID2* expression in the indicated hPSC lines with or without doxycycline (dox) treatment.

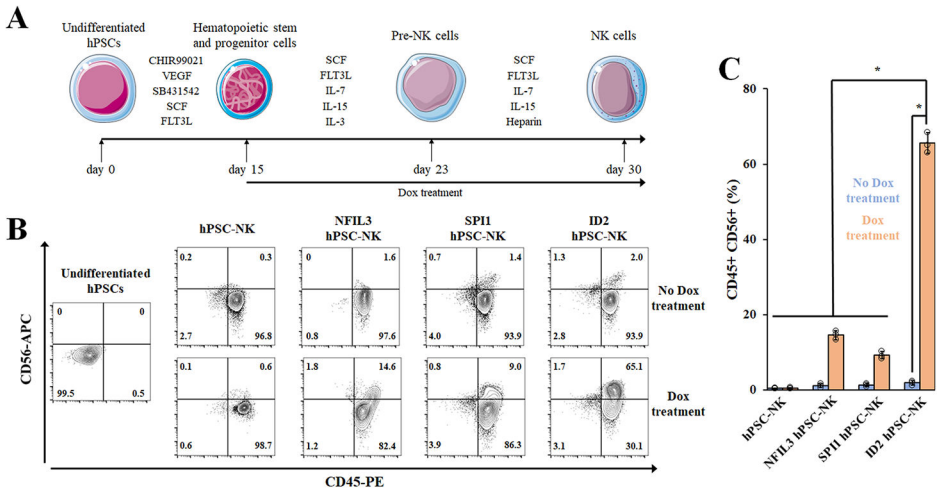

Figure 2.

Inducible overexpression of transcription factors during NK cell differentiation. (A) Schematic of NK cell differentiation from human pluripotent stem cells (hPSCs) with or without doxycycline (dox) treatment is shown. (B,C) Representative flow cytometry analysis of CD45 and CD56 expression in day 30 NK cell differentiation cultures from indicated hPSC lines is shown in (B) and quantified in (C). Three wells for each condition, and data are presented as mean ± s.d. of three independent replicates, $*p < 0.05$.

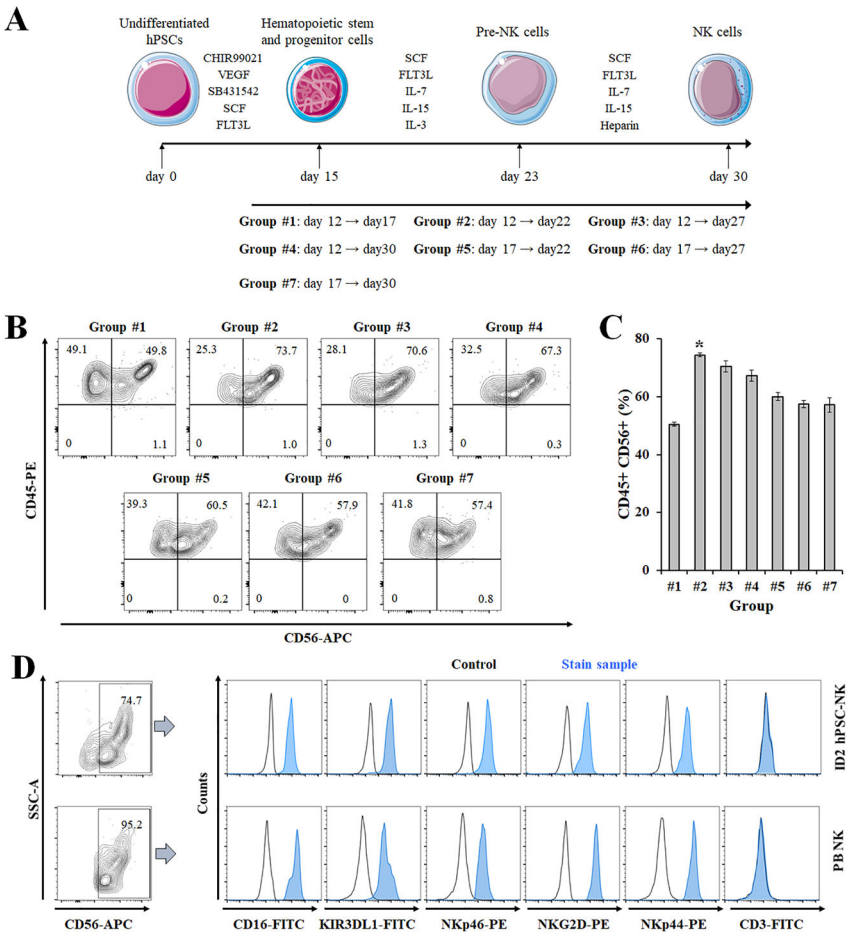

Figure 3.

Stage-specific effects of *ID2* overexpression during NK cell differentiation. (A) Schematic of NK cell differentiation from human pluripotent stem cells (hPSCs) with stage-specific overexpression of *ID2* via doxycycline (dox) treatment. (B,C) Representative flow cytometry analysis of CD45 and CD56 expression on day 30 NK cell differentiation cultures with indicated dox treatment is shown in (B) and quantified in (C). Three wells for each condition, and data are presented as mean ± s.d. of three independent replicates, $*p < 0.05$. (D) Representative histogram plots of indicated NK cell markers and corresponding isotype controls are shown.

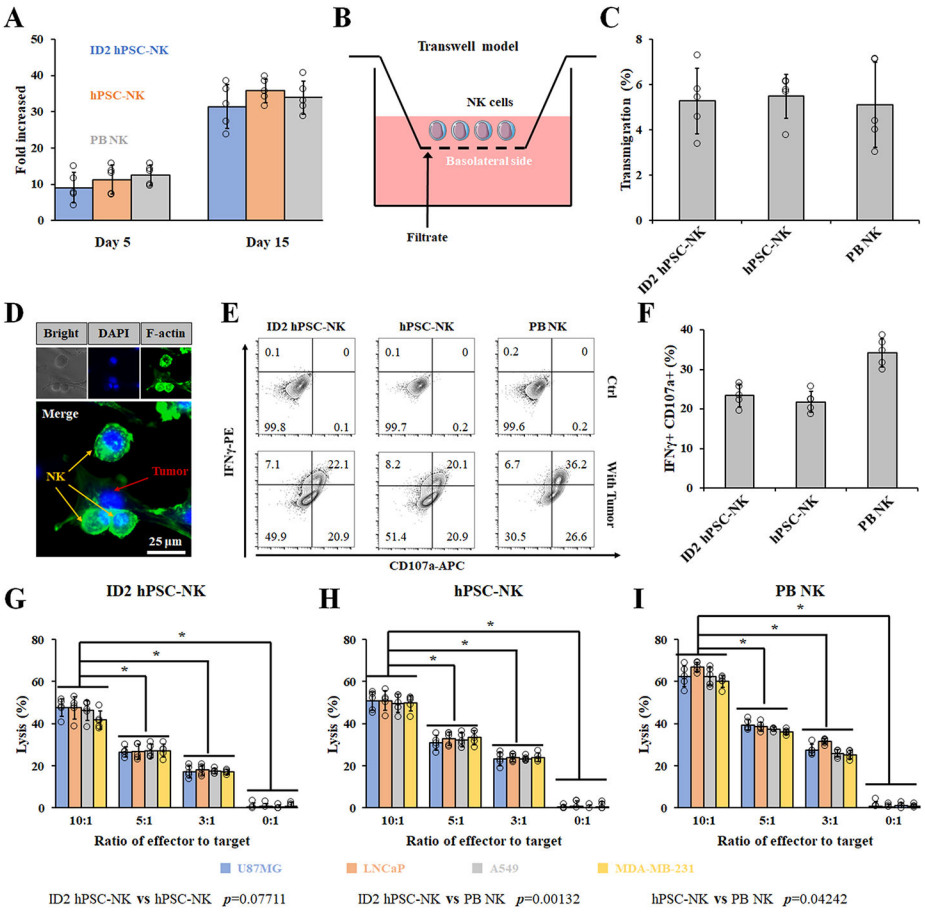

Figure 4.

hPSC-derived NK cells effectively kill various tumor cells. (A) Expansion of indicated NK cells at day 5 and day 15 were shown. (B,C) Schematic of *in vitro* transwell model for transmigration study (B) and transmigrated NK cells were quantified (C). (D) Representative images of polarized F-actin accumulation at the interface between indicated NK cell and targeted U87MG glioblastoma cell are shown. Scale bar, 25 μm. Representative flow cytometry analysis of IFNγ/CD107a in ID2-hPSC-derived, wildtype hPSC-derived NK cells and PB NK cells with or without glioblastoma cell stimulation is shown in (E) and quantified in (F). Five replicates for each condition, and data are presented as mean ± s.d. of five independent replicates. Cytotoxicity of *ID2*-hPSC-derived (G), wildtype hPSC-derived NK cells (H), and PB NK cells (I) against indicated tumor cells at ratios of 0:1, 3:1, 5:1, and 10:1 was quantified. Data are presented as mean ± s.d. of five independent replicates, *$p <$ 0.05.