Supplementary materials

Methods

Characterization of hNCCs

FACS analysis and cell-sorting were performed using antibodies as shown in Supplementary Table 2. For hNCC identification, after 16 days of differentiation, cells were trypsinized and blocked with anti-human Fc-receptor (Miltenyi Biotec, Germany) in staining medium, and incubated with anti-human HNK-1 (Biolegend, SanDiego, CA, USA) and anti-human p75 (Biolegend) at 4 °C for 20 min with isotype antibodies as negative control. Percentage of positive cells was analyzed by LSRFortessa (BD San Jose, CA, USA). The identity of hNCCs was validated by various differentiation assays. For MSC induction, hNCCs were transferred from NC culture medium into α-MEM with 10% FBS and passaged every 4 days. Phenotype analysis of MSC surface markers was done at passage 2 using a human MSC Analysis kit (BD Biosciences, RUO) based on four positive markers CD44, CD73, CD90, CD105 and a combination of negative markers CD34/CD11b/CD19/CD45/HLA-DR by FACS. For osteogenic differentiation of MSC, cells were seeded into 6-well plates at a density of 4×10^5 cells per well and induced by addition of 5 nM dexamethasone (Sigma-Aldrich, D4902), 50 μg/mL ascorbic acid 2-phosphate (Sigma-Aldrich, A8960), and 10 mM glycerol 2-phosphate (Sigma-Aldrich, G9891) into the culture medium. Cells were fixed at day 16 and stained with Alizarin Red. For myofibroblast differentiation, DMEM with 10% FBS was added, and medium was changed every other day. Cells were stained with α-SMA at day 30. For peripheral neuron differentiation, hNCCs were seeded in 6 well plate and medium was changed into DMEM/F12 supplemented with 1× N2, Heregulin (20 ng/ml), FGF2 (10 ng/ml) and cAMP (5 μM). Cells were fixed and stained with peripherin (PRPH) at day 15.

Neuroblastoma cell lines

NB cell lines were kept in 5% CO_2 at 37 °C. Cells were passaged using 0.25% trypsin when researched 80% confluence. For CD55 characterization and isolation, cells were gently digested into single cells. APC-conjugated CD55 primary antibody was incubated with NB cells in 4 °C for half an hour. Cells were washed by PBS for 3 times before analysis or sorting by FACS. Mouse IgG-APC was used as negative control.

Lentiviral transduction

CD55 knockdown vectors (pPLK/GFP+Puro-*CD55* shRNA-1, 2) and control knockdown (pPLK/GFP+Puro-CTRL shRNA) vector were purchased from Public Protein/Plasmid Library (PPL, China) and packaged in 293FT cells according to the protocol as described in ViraPower ™ Lentiviral Expression Systems (Thermo Fisher). The sequences are shRNA1: TGGTCCACAGCAGTCGAATTTCAAGAGAATTCGACTGCTGTGGACCA; shRNA2 : ATGTGAAGAAAGCTTTGTGTTTCAAGAGAACACAAAGCTTTCTTCACAT. To knockdown CD55 expression in NBL, cells were plated at a cell density of 4×10^4 cells per well on 6-well-plates and transduced with control or CD55 shRNA virus at about a MOI of 5. 24 h after transduction, puromycin (2 μg/ml) was added to the culture medium to select transduced cells for 14 days.

Overexpression and knockdown

pCDH-CMYC plasmid and its corresponding control vector were purchased from Public Protein/Plasmid Library (PPL, China). pLKO.1-U6- cMYC-shRNAs-GFP-Puro (CMYC-shRNAs)

were purchased from Kidan Bio Co. Ltd., China. (sequence: shRNA1# ： CCCAAGGTAGTTATCCTTAAA; shRNA2#： TACGGAACTCTTGTGCGTAAC; Scrambled shRNA: GGTAAATCTCCAGTATACACT). For transfection, NBL-S cells were transfected with pCDH-cMYC or control vector (0.5 μg/ml) using lipofectamine 3000 (Invitrogen). 48 h after transfection, the transfected cells were harvested for real-time PCR. SKN-AS cells were transfected with cMYC-shRNAs or scrambled shRNA (0.5 μg/ml) using lipofectamine 3000. 72 h after transfection, the transfected cells were harvested for real-time PCR.

Cell proliferation assay

Cells were seeded at a cell density of 3000 cells per well onto 96-well-plates. At the end point of experiment, cells were incubated with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) for 4 h at 37 °C followed by absorbance read at 490 nm using a microplate reader.

Migration and invasion assay

To assess migration and invasion, Corning transwell (Cat#3422) with 8 μm pores were used. DMEM/F12 was used in the upper chamber and hNCC culture medium was used in the lower chamber. hNCCs (10000 cells for migration assay, 20000 cells for invasion assay) were added into the upper chamber for 24 to 48 h. Cells crossed the chamber were wash by PBS and fixed with 100% methanol for 15 min. The cells were stained with crystal violet (1 mg/ml) for 30 min and washed with PBS before taking photos. For invasion assay, Matrigel was diluted at 1:100 and added into the transwell and dried in the biosafety cabinet.

Colony formation assay

Cells were digested into single cells and 500 cells were seeded onto 6-well plates. Medium was changed every 2 days and colonies were stained using crystal violet (0.5%) 14 days after seeding. Colonies were counted, and photographs were taken.

Sphere formation assay

2000 hNCCs or 5000 NB cells were seeded into the ultralow-attachment 6-well plates and kept in sphere formation medium (RPMI-1640 medium with 1 × B27, FGF (20 ng/mL), EGF (20 ng/mL)). The number of spheres was counted after 2 weeks. For self-renewal experiments, spheres were collected and digested by trypsin, and re-suspended into single cells. 2000 hNCCs or 5000 NB cells were seeded for second passage using the same method. For single cell-derived sphere formation and self-renewal analysis, single cells were seeded and cultured in ultralow-attachment 96-well plates for 14 days and then single colonies were picked and digested by Accutase. The single cells were then split into the ultralow-attachment 96-well plates at the same density as in the primary passage. After 14 days, each well was calculated for sphere numbers.

Soft agar assay

The lower layer of agar was made by equal volume of 2 × culture medium containing 1.2% agar solution. This mixture was added into each well of a 6-well plate immediately. The upper layer of agar was carefully made by adding equal volume of 2 × culture medium and 0.6% agar solution. 2000 cells/well were added in the upper layer. Medium was changed every three days for 1 month.

At the end of experiment, cells were fixed by methanol at room temperature and stained with 0.5% crystal violet.

RT-qPCR analysis

The RT was done by using QuantiTect Reverse Transcription Kit (Qiagen, MD, USA). Primers used were summarized in Supplementary Table 1. qPCR was performed in triplicates on 96-well-plates and repeated at least three times using an Applied Biosystems Vii7 Real-Time PCR System. To calculate the relative gene expression, the Ct values were normalized to the Ct values of *GAPDH*. The relative transcriptional expression of interested genes was indicated with $2^{(-\Delta\Delta Ct)}$. Primers were listed in supplementary Table 1.

Western blotting

60 μg protein was separated on 8-10% SDS–PAGE gels which were subsequently transferred onto PVDF membranes (Sigma-Aldrich). After blocking with 5% milk in TBST at room temperature for 1 h, the membrane was incubated with primary antibody overnight at 4 °C on a horizontal rotor. Membrane was washed three times with TBST and incubated with secondary antibodies at room temperature for 1 h. The blots were subjected to chemiluminescent detection with ECL Detection Reagent (Amersham GE Care), and scanned for analysis. Antibodies used were summarized in Supplementary Table 2.

Immunohistochemistry (IHC) staining

IHC staining of tumors was done by using streptavidin− biotin− peroxidase complex method. Tumor tissues were fixed in 4% paraformaldehyde PBS solution at 4 °C for 24 h and then embedded in paraffin. Tissues were then cut into 6 μm sections and de-paraffined in xylene and rehydrated in gradient alcohols. Endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS for 30 min at room temperature, and sections were washed in PBS for three times. The sections were heated by microwave at 98 °C for 15 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval (PT Module, Thermo). Sections were blocked with 5% horse serum for 30 min at room temperature and incubated with primary antibodies at 4 °C overnight. Later, radish peroxidase-conjugated secondary antibodies (rabbit or mouse, Santa Cruz) were incubated at room temperature for 1 h. Sections were developed with diaminobenzidine and counterstained with hematoxylin using standard protocol. All experiments were conducted three times and each experiment was set up with triplicate samples. The IHC staining data were analyzed by two independent researchers. The antibodies used for staining were listed in Supplementary Table 2.

Plasmids and luciferase reporter assay

Plasmids used were the *CD55*-dependent pGL4.19 reporter construct (PPL, China) and pRenilla-TK vector (Promega, Madison, WI). pGL4.19/−805 and pGL4.19/+196 were generated by inserting the sequence fragment from −805 to −100 and +196 to +901 respectively into the control pGL4.19 vector. The mutant constructs were deletion mutants, which deleted the canonical E-box (pGL4.19/−805) or non-canonical E-box (pGL4.19/+196), respectively. pCDNA3-HA-human MYCN was a gift from Martine Roussel (Addgene plasmid # 74163; http://n2t.net/addgene:74163; RRID: Addgene74163). Cells were transfected with the CD55 promoter/luciferase reporter

constructs and the pRL-TK vector for normalization of the transfection efficiency using lipofectamine 3000. Stimulation experiments were done by transfecting the MYCN expressing vector or the control empty vector. 48 h after transfection, cells were lysed, and luciferase activity was determined using Dual-Luciferase Reporter Assay System (Promega).

ChIP-qPCR

Briefly, 1% formaldehyde cross-linked chromatin was immunoprecipitated by anti-MYCN (Abcam, #ab16898). The input (1%) DNA was used for normalization samples. The input DNA and precipitated DNA samples were quantified by qPCR with primers targeting on special sites with SYBR green fluorescent dye (Qiagen). Primers were listed in supplementary Table 1.

RNA-sequencing and cohort studies

For t-SNE analysis, normalized RNA-sequencing raw data of 2001 cancer samples were downloaded from TCGA, and t-SNE was run by R. Top 3000 genes comparing hNCC-tumors with different NBLs (fold change >2, FDR q-value<0.01) were analyzed in the Cancer Cell Line Encyclopedia (CCLE) database by the tool Enrichr. RNA-sequencing data comparing control hNCCs, *MYCN*-hNCCs and NBLs were normalized and PCA was performed using Partek Genomics Suit 7.0 software. GSEA was performed using DEGs (hNCC+MYCN vs. normal hNCCs, analyzed by Partek Genomics Suit 7.0 software) on MSigDB 6.2 from the GSEA website. For gene ontology analyses, list of genes were analyzed by Metascape (http://metascape.org/gp/index.html). Genomic data generated from this study are available in a public repository GEO (GSE137975).

Matched patient outcome and expression data were obtained from Leming Shi clinical cohort and Kocak cohort. Across samples, expression values were rank transformed and Kaplan scan of the gene set was used to optimize the most significant p-value, as the survival cut-off is modelled based on statistical testing instead of taking the average or median. The OS was calculated as the time between diagnosis and mortality, while the event-free survival EFS was calculated as the time between diagnosis and relapse, progression, secondary malignancy or mortality, or until the time of the last contact with the patient if none of these events occurred. The OS and EFS were calculated using the Kaplan and Meier method, and the curves were compared using the Mantel-Cox log-rank test.

Xenograft formation in vivo

NOD-SCID mice were provided by the Laboratory Animal Service Center of the Chinese University of Hong Kong. They were maintained in an air-conditioned room with controlled temperature of 24 ± 2 °C and humidity of 55 ± 15%, in a 12 h light/darkness cycle regulation and were fed laboratory chow and water *ad libitum*. For *in vivo* tumorigenic experiments of hNCCs, 2 × 10^6 control- or *MYCN*- hNCCs were re-suspended in PBS and Matrigel, and injected into renal capsule or the flanks of 4-6 week-old female NOD-SCID mice. Mice were fed doxycycline diluted in water daily (2 mg/mL) to induce *MYCN* gene expression to the end of experiment. For NB cell injection, female NOD/SCID mice between 4-6 week-old were used in the experiments. 100 µL PBS containing 5×10^6 SKN-BE(2)C or SKN-AS cells was injected into the flanks of the mice. Mice with tumor volume larger than 1.5 cm^3 were sacrificed immediately. For neutralizing antibody local treatment, when the tumors reached 100mm^3, 30µL IgG2B or CD55 NAb (100 µg/mL) were injected into tumors every other day. For intraperitoneal injection,

IgG2B or CD55 NAb (5 mg/kg) were injected intra-peritoneally every other day starting from day 15 after tumor inoculation. Increase in life span (ILS) was determined to compare the survival rates of the different treatment groups, and calculated based on the following equation: ILS = (DaysT – DaysC)/DaysC, where DaysC = days survived by control group mice and DaysT = days survived by treatment group mice. Mice were removed from the study if they were found dead in their cage, declining health necessitated killing or tumor diameter exceeded 15 mm. These parameters were used to construct a Kaplan–Meier survival curve.

Supplementary Figure legends

Figure S1. Induction and characterization of hESC-derived hNCCs. A) RT-qPCR analysis shows the dynamic mRNA change of neural plate border specifiers and neural crest specific markers during hNCC differentiation from two hESC lines (H7 and H9). Quantification data represent mean±SD, *p < 0.05, **p < 0.01, ***p < 0.001 by One Way ANOVA; **B)** Flow cytometry analysis of hNCCs using two neural crest markers, HNK1 and p75; **C)** Phenotypic characterization of hNCC-MSCs by MSC characterization kit using flow cytometry. hNCC-MSCs are positive for CD90, CD105, CD44 and CD73 and are negative for hematopoietic markers, CD34, CD11, CD19, CD45 and HLA-DR.

Figure S2. Deregulation of MYCN at the initiation stage of hESC differentiation leads to a dramatic and global reduction of neural plate border genes and NC specifiers. A) Map of DOX-inducible lentiviral vector containing the human *MYCN* gene; **B)** Representative photos and flow cytometry analysis showing the high transduction efficiency in hESC, scale bar = 100 μm; **C)** Induction of MYCN by 1 μg/mL DOX results in a more than 20-fold increase in *MYCN* mRNA expression as determined by qPCR. ***p < 0.0001 by Student's t test; **D)** RT-qPCR analysis reveals the reduction of neural plate border, NC specifiers and sympathetic neurogenesis genes along with NC differentiation after MYCN induction in day 0. *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test; **E)** Flow cytometry analysis shows that MYCN deregulation results in a significant reduction of $p75^+$ cells on day 16 after hESC induction. Quantification data is shown on the right. ***p < 0.001 by Student's t test.

Figure S3. Characterization of MYCN-hNCCs and tumors. A) Scheme illustrating the experimental design of the study. hNCCs were differentiated from hESC and MYCN was activated by DOX at different time points after hESC differentiation. For NB study, MYCN was activated at day 16, *in vitro* characterization of the MYCN-hNCCs was performed at day 45. Cells were propagated *in vitro* for two passages and implanted into the NOD-SCID mice at around day 60; **B)** The mRNA expression levels of neural markers are significantly reduced in MNA-NB compared to non-MNA-NB in Leming Shi cohort. Quantification data represent mean ± SD, ***p < 0.001 by Student's t test; **C)** Gene ontology analysis on the embryonic development genes; **D)** Heatmap of 1585 DEGs (fold change > 1.5, FDR q-value < 0.05) between control hNCCs and MYCN-hNCCs; **E)** Ontology analysis of the 1585 DEGs comparing control hNCCs and MYCN-hNCCs; **F)** Two-way Venn diagrams displaying the number of DEGs in MYCN-hNCCs Vs. control hNCCs compared with genes differentially expressed between MNA-NB and non-MNA-NB.

Figure S4. The expression levels of GNL3 and DCAF4 are associated with patient prognosis and disease progression of neuroblastoma. A) High *GNL3* expression level is correlated with poor survival rate, higher INSS stages, death from disease, high risk of disease and disease progression. Quantification data represent mean ± SD, *p < 0.05, ***p < 0.001 by Student's t test; **B)** High *DCAF4* expression is associated with poor survival rate, higher INSS stages, death from disease, high risk of disease and disease progression. Quantification data represent mean ± SD, *p < 0.05, ***p < 0.001 by Student's t test; **C)** 14 surface protein-encoding genes regulated by MYCN in MNA-NB.

Figure S5. Genes that correlate with MYCN and survival only in MNA-NB. A) *ABCC4, QSOX2, SCARB1, SLC7A5 and SLC19A2* positively correlate with *MYCN* expression level in MNA-NB but not non-MNA-NB in the Leming Shi cohort and Kocak cohort; **B)** Higher expression level of *ABCC4, QSOX2, SCARB1, SLC7A5 and SLC19A2* correlates with lower event-free survival rates in MNA-NB patients; **C)** Scheme indicating the design of ChIP-PCR primers and luciferase reporters of *CD55* promoter.

Figure S6. Knockdown of CD55 suppresses cell growth in MNA-NBL. A) Overexpression of CMYC in NBL-S cells does not affect CD55 mRNA expression; **B)** Knockdown of CMYC in SKN-AS cells by shRNAs does not affect CD55 mRNA expression; *p<0.05, ***p<0.001 by One Way ANOVA; **C)** Flow cytometry analysis shows different percentage of $CD55^+$ cells in 4 NBL; **D)** RT-qPCR analysis shows that CD55 is significantly knocked down in the two NBL. *p<0.05, **p<0.01, ***p<0.001 by Student's t test; **E)** MTT analysis shows that knockdown of CD55 suppresses cell growth in SKN-BE(2) cells but not SKN-AS cells. *p<0.05, ***p<0.001 by Student's t test; **F)** PCR-array (PAHS-020Z) analysis shows that knockdown of CD55 alters cell cycle- and cell growth-related genes; **G)** IHC staining of NANOG, CD133 and CD55 in either IgG2B- or CD55 NAb-treated tumors developed from SKN-AS cells, scale bar = 50 μm.

Figure S7. The expression of GPI and ROR2 are significantly upregulated in MNA-NB of Lemming Shi cohort and Kocak cohort.

Supplementary Tables

Supplementary Table 1:

Primers used in this study.

Supplementary Table 2:

Antibodies used in this study.

Supplementary Table 3:

Gene list of 1585 differentially expressed genes between control- and MYCN-hNCCs.