Supplementary Information

G-CSF promotes neuroblastoma tumorigenicity and metastasis via STAT3-dependent cancer stem cell activation

Running Title: G-CSF is a cancer Stem cell (CSC) specific growth factor

Keywords: Neuroblastoma; Cancer stem cells; G-CSF; G-CSF receptor; STAT3 inhibitors

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Supplementary Methods:

Phospho-flow assay

Phospho-flow assay was performed as described previously (11). Briefly, untreated or treated NGP cells were fixed using 1% paraformaldehyde followed by surface staining for CD114 and cell permeabilization using cold methanol. Intracellular pSTAT3 (Y705) was then stained using Pacific Blue-conjugated anti-STAT3 (Y705) antibody (BD Biosciences, 560312).

G-CSF ELISA

Estimation of G-CSF in human and mouse NB cell lines supernatant and serum samples were performed by Quantikine Mouse G-CSF immunoassay kit (MCS00, R&D Systems) and Human

G-CSF ELISA kit (ab100524, Abcam, Cambridge, MA) according to manufacturer recommendations. Briefly, cell supernatant and serum samples were diluted two times in recommended buffers and ELISA was developed and read at 450 nm using microplate reader, as described above.

Immunohistochemistry

Cohorts of mice used in the present study were sacrificed on day 29 post-implantation (72 hours post-treatment with last dose of drug). Complete necropsy was performed and tumor tissues were collected and preserved in 4% paraformaldehyde for immunohistochemical analysis. Immunostaining was performed on 5 μm sections of paraffin embedded xenograft tumor tissues using anti-Cleaved Caspase-3 antibody (9579, Cell Signaling, Danvers, MA) and anti-phospho STAT3 (Y705) (9145, Cell Signaling). Scoring of slides was performed by randomly selecting ten high power fields (HPF) on each slide and counting for positive stained cells.

Gene expression analysis

JAK/STAT signaling pathway array (PAHS-039ZC, SABiosciences) was used to analyze JAK/STAT pathway genes in NB subpopulations in response to G-CSF and Stattic treatments. NB cells were untreated or treated with G-CSF (20 ng/ml) and Stattic (1μM) for 2 h followed by fixation with 1% paraformaldehyde. Fixed cells were stained with anti-CD114 antibody and CD114+ and CD114- cells were flow sorted. Extraction of RNA from FACS-sorted cells, cDNA synthesis and real time RT-PCR was performed as described previously (11). Array results were initially analyzed using RT^2 profiler PCR array data analysis v3.5 (Qiagen) then heat maps were generated using GeneSpring GX software (Agilent Technologies). Pathway analyses were generated through the use of IPA (Ingenuity Systems,

http://www.ingenuity.com). PCR array results were further validated on NB subpopulations by performing qPCR for individual genes in triplicates for each sample using Power SYBR Green PCR Master Mix (Applied Biosystems) and normalized to β-actin and GAPDH. The primers used are listed in Supplementary Table S3. Dissociation curves were analyzed for each primer pair as a means to ensure the quality of amplicon and to monitor primer dimers.

Supplementary Figure legends:

Figure S1. Effect of G-CSF on neuroblastoma subpopulations

Schematic representation of experimental plan to determine effects of G-CSF on NB colony formation. As represented, single cells of stably transduced GFP+ NB subpopulations were sorted in wells of 96-well plate followed by treatment with varying doses of G-CSF and weekly monitoring. **(B)** Effect of G-CSF on increasing the cell number count of CD114+ cells. Each condition is replicated in at least 20 individual wells and data is represented as number of cells/well (mean ± SEM). NGP CD114+ cells showed significant increase in cell counts at day 21 with 10 and 20 ng/ml of G-CSF. SH-SY5Y CD114+ cells showed vigorous growth in response to increasing doses of G-CSF. CD114- cells from both cell lines under similar culture conditions did not show any major effect for G-CSF. (t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). **(C)** Representative photographs of CD114+ cells at different time points in response to G-CSF treatment. (Day 7, day 14, scale bar= 100 μm, day 21, scale bar= 200 μm).

Figure S2. Phospho-flow assay in response to G-CSF, Stattic and anti-G-CSF treatment

(A) Effect of different amounts of G-CSF on the percentage of CD114+ and pSTAT3 (Y705)+ cells. NGP cells were treated with different amounts of G-CSF (ng/ml) for 2 h and percentage of CD114+ cells and pSTAT3 (Y705)+ cells were determined by phospho-flow cytometry assay. **(B)** Effect of Stattic (+=1 μM, ++=3 μM for 2 h) alone or combined with G-CSF (10 ng/ml

for 2 h) on the percentage of CD114+ and pSTAT3 (Y705)+ cells. **(C)** Effect of anti-G-CSF antibody (10 ng/ml for 2 h) alone or combined with G-CSF (10 ng/ml for 2 h) or Stattic (1 μM for 2 h). Data are mean ± SEM and repeated twice with three replicates for each condition. (t-test, *p<0.05, **p<0.01, ***p<0.001).

Figure S3. Estimation of G-CSF levels

(A) G-CSF ELISA assay of different human NB cell lines. Supernatant from confluent grown human NB cell lines was collected and used to determine G-CSF (pg/ml). Data represented as mean ± SD of assay performed in triplicates and repeated three times. Human G-CSF ELISA kit was used. **(B)** G-CSF ELISA assay on serum samples drawn from $CSF^{-/-}$ mice expressing no endogenous G-CSF. Control-/- is unimplanted homozygous ($CSF^{-/-}$) mice, Het-/+ is heterozygous ($CSF^{-/+}$) mice, Tumor-/- is NB975 implanted homozygous ($CSF^{-/-}$) mice untreated. Data represented as mean ± SD of ELISA performed in triplicates on samples from four respective mice. Quantikine Mouse G-CSF immunoassay kit was used. (t-test, ***p<0.001)

Figure S4. Cleaved Caspase-3 staining in tumor xenografts

(A) Immunohistochemistry analysis of Cleaved Caspase-3 in representative tumor sections of xenografts treated with vehicle control, G-CSF, Stattic, Etoposide and combination of Stattic and Etoposide in the present study. Scale bar, 100μm. **(B)** Pathological scoring of Immunohistochemistry slides of Cleaved Caspase-3 as shown in (A). Data represented as mean ± SEM and calculated with scoring of ten randomly selecting high power fields (HPF) on each slide. Each slide was scored twice independently by pathologists and each group represents scoring of randomly selected three tumors. (t-test, *p<0.05)

Figure S5. Phospho-STAT3 (Y705) staining in tumor xenografts

(A) Immunohistochemistry analysis of phospho-STAT3 (Y705) in representative tumor sections of xenografts treated with vehicle control, G-CSF, Stattic, Etoposide and combination of Stattic and Etoposide in the present study. Scale bar, 100μm. **(B)** Pathological scoring of Immunohistochemistry slides of phospho-STAT3 (Y705) as shown in (A). Data represented as mean ± SEM and calculated with scoring of ten randomly selecting high power fields (HPF) on each slide. Each slide was scored twice independently by pathologists and each group represents scoring of randomly selected three tumors. (t-test, *p<0.05, **p<0.01, ***p<0.001).

Figure S6. Gene-expression analysis in CD114+ subpopulation

(A) (B) (C) Scatter plot analysis using low-density qPCR array for JAK/STAT pathway genes in the CD114+ subpopulation from the NGP cell line treated with G-CSF (20 ng/ml for 2 hrs) and Stattic (1 μM for 2 hrs). Results shows that G-CSF upregulate while Stattic downregulate the expression of JAK/STAT pathway genes. Some key genes are highlighted in plots. The red star shows upregulation and green start shows down regulation of gene. **(D)** Ingenuity pathway analysis of the CD114+ subpopulation with G-CSF treatment showing interaction of different key genes. Analysis indicates that upregulation of STAT3 directly downregulate p53 and related genes.

Figure S7. STAT3 knockdown analysis in human neuroblastoma cell lines

(A) STAT3 stable knockdown (KD) in different NB cell lines and analysis of percentage of CD114+ cells. Results showing significant decrease in percentage of CD114+ cells with STAT3-KD in comparison to untreated controls. Data are mean ± SEM and repeated twice with three replicates for each condition. (t-test, *p<0.05, **p<0.01). **(B)** Western immunoblots of stable STAT3 KD neuroblastoma cell lines confirming significant decrease in STAT3 expression with KD. CyPB was used as a loading control. C=control, KD= STAT3 knockdown.