Expression of the G-CSF receptor, CD114, marks a novel, highly tumorigenic cell population in a neuroblastoma cell line

Danielle M. Hsu1†, Saurabh Agarwal2†, Ashley Benham3, Cristian Coarfa3, Denae N. Trahan2, Zaowen Chen2, Paris N. Stowers2, Amy N. Courtney2, Anna Lakoma1, Eveline Barbieri2, Leonid S. Metelitsa2, Preethi Gunaratne3, Eugene S. Kim1,* and Jason M. Shohet2,*

1 Division of Pediatric Surgery, Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, TX, 77030, USA

2 Department of Pediatrics, Section of Hematology-Oncology, Texas Children’s Cancer Center, and Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX 77030, USA

3 Department of Molecular & Human Genetics, Department of Pathology, Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX 77030 and Department of Biology & Biochemistry, University of Houston, Houston, TX 77204

†Denotes co-first authors

*Denotes co-senior authors

Co-senior authors:

Jason Shohet, MD, PhD
Department of Pediatrics
Section of Hematology-Oncology
Baylor College of Medicine
Houston, TX 77030
jmshohet@texaschildrenshospital.org

Eugene Kim, MD
Michael E. DeBakey Department of Surgery
Baylor College of Medicine
Houston, TX 77030
eskim@bcm.edu

The authors disclose no potential conflicts of interest.
Abstract
Neuroblastoma is a neural crest derived embryonal malignancy which accounts for 13% of all pediatric cancer mortality, primarily due to tumor recurrence. Therapy-resistant cancer stem cells are implicated in tumor relapse, but definitive phenotypic evidence of the existence of these cells has been lacking. In this study, we define a highly tumorigenic subpopulation in neuroblastoma with stem cell characteristics, based on the expression of CD114, which encodes the receptor for granulocyte colony-stimulating factor (G-CSF). CD114+ cells isolated from a primary tumor and the NGP cell line by flow cytometry were highly tumorigenic and capable of both self-renewal and differentiation to progeny cells. CD114+ cells closely resembled embryonic and induced pluripotent stem cells with respect to their profiles of cell cycle, microRNA and gene expression. In addition, they reflect a primitive undifferentiated neuroectodermal/neural crest phenotype revealing a developmental hierarchy within neuroblastoma tumors. We detected this de-differentiated neural crest subpopulation in all established neuroblastoma cell lines, xenograft tumors, and primary tumor specimens analyzed. Ligand activation of CD114 by the addition of exogenous G-CSF to CD114+ cells confirmed intact STAT3 upregulation, characteristic of G-CSF receptor signaling. Together our data describe a novel distinct subpopulation within neuroblastoma with enhanced tumorigenicity and a stem-cell like phenotype, further elucidating the complex heterogeneity of solid tumors such as neuroblastoma. We propose this subpopulation may represent an additional target for novel therapeutic approaches to this aggressive pediatric malignancy.
Introduction

High-risk neuroblastoma (NB) is an aggressive embryonal malignancy of young children arising from the embryonic neural crest. NB is the most common abdominal malignancy of childhood and accounts for 13% of all pediatric cancer deaths (1). Currently, overall survival is less than 50%, and treatment consists of dose-intensive chemotherapy, which confers major long-term side effects in many of these young survivors (2). As in adult cancers, the vast majority of deaths are due to relapsed, drug-resistant metastatic disease that may be driven by cancer stem cells.

Cancer stem cells (CSCs) have been defined as a multipotent subpopulation of cells within a tumor with the ability to self-renew, generate differentiated progeny, and recapitulate a heterogeneous cancer population (3, 4). In breast and colon cancer, multiple studies have demonstrated highly tumorigenic subpopulations within these tumors with enhanced tumorigenicity, resistance to chemotherapy, and the ability to establish metastatic foci in distant microenvironments (5, 6). Thus, most solid tumors likely represent heterogeneous populations of cells in various states of differentiation and with varying levels of tumorigenicity and chemosensitivity.

The translational potential of specifically targeting such chemoresistant and tumorigenic subpopulations has motivated a number of studies in neuroblastoma. These have included analyses of side population (SP) cells, a classic method used to isolate hematopoietic stem cells based on the efficient efflux of Hoechst 33342 (Hoechst) dye from stem cells (7), neurosphere assays (non-adherent, serum free conditions)(8), and purification of neuroblastoma subpopulations based on surface markers associated with stem cell populations in other types of malignancies (9, 10). These previous approaches have generated important data regarding the drug resistance and cytokine responsiveness of neuroblastoma, and in some cases have defined in vitro conditions that enrich for tumorigenic populations. However, these studies have all relied on extensive ex vivo cell culture of biopsy material or cell lines which is well known to extensively modify gene expression profiles(11) and have not consistently identified discrete subpopulations within primary neuroblastoma with enhanced tumorigenicity or self-renewal characteristics.

Neuroblastoma appears to arise within peripheral sympathetic ganglia throughout the body, including approximately 30% of cases that develop within the adrenal medulla (1, 12). The neural crest is a transient cell population originating from peripheral neuroectoderm of the dorsal tube, which further differentiates, delaminates and migrates to form multiple structures, including the peripheral sympathetic ganglia.

Neuroblastoma expresses GD2, Nestin, S100, and other markers of sympathetic neural crest lineage (13). Transgenic mice with MYCN expression targeted to neural crest (TH-MYCN mice) develop a high-grade malignancy, which closely models many aspects of human neuroblastoma including the presence of embryonic rests of crest-like precursors within peripheral ganglia (14). Thus both clinical and experimental models define neuroblastoma as a malignancy of the peripheral sympathetic nervous system linked to defective neural crest maturation.
A critical transcription factor controlling neural crest specification is STAT3; loss-of-function of STAT3 promotes apoptosis and loss of neural crest markers (e.g. SOX10 and snail2), while increased function promotes the maintenance of an undifferentiated early neural crest phenotype (15). STAT3 signaling has been found to be important for the maintenance of CSC-like subpopulations in several other malignancies, such as invasive bladder cancer (16), colon cancers (17), hepatocellular carcinoma (18), and malignant gliomas (19). Transcriptional activation of STAT3 is a downstream consequence of G-CSF (Granulocyte Colony-Stimulating Factor) mediated activation of the G-CSF receptor, typically found on granulocyte precursors. The receptor for G-CSF promotes neurogenesis and is found on a number of neuronal and neural crest derived cell types (20, 21). In addition, G-CSF signaling through the G-CSF receptor (aka CD114 encoded by CSF3R) promotes the survival and expansion of adult neural stem cells (21), has neurotrophic effects, and may promote regeneration of neural tissue after stroke (22). Expression of the receptor and this signaling pathway is implicated in the pathogenesis of multiple tumor types including ovarian (23), bladder (24) and squamous cell cancers (25). We thus hypothesized that surface expression of CD114 may distinguish a subpopulation of neuroblastoma with increased STAT3 responsiveness which could promote an undifferentiated phenotype similar to that found in early multipotent neural crest precursors.

To test this hypothesis we used fluorescence-activated cell sorting (FACS) to detect and isolate CD114+ cells in both clinical and experimental neuroblastoma tumor specimens. Of the human and mouse neuroblastoma cell lines, tumor xenografts, and primary clinical tumor samples tested, we detected a distinct subpopulation of CD114+ cells in all specimens, as well as in de novo tumors arising from TH-MYCN transgenic mice. Analysis of CD114+ subpopulations confirmed activation of STAT3 signaling in response to exogenous G-CSF. Subsequent characterization of this subpopulation, using in vivo limiting dilution and competitive lineage tracing studies, demonstrated that CD114+ cells are highly tumorigenic, self-renew, and give rise to more differentiated progeny. Within the normal developmental hierarchy of neural crest, genetic analysis demonstrates CD114+ cells are similar to (pre-migratory) neural crest cells, whereas the CD114- population represents a later stage of neural crest differentiation (migratory crest). Based on the differential expression of CD114, these studies define a novel de-differentiated subpopulation within neuroblastoma and provide further evidence that heterogeneous subpopulations within solid tumors differentially contribute to tumorigenicity.

**Materials and Methods**

**Cell culture and sources of lines**

Human neuroblastoma cell line, IMR-32, and HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA). The neuroblastoma cell lines SH-SY5Y/luc and NGP/luc were provided by Dr. J. Kandel and Dr. D. Yamashiro (Columbia University, New York, NY); CHLA-255/luc, SK-N-BE(2), and LA-N-5 were a gift of Dr. L. Metelitsa (Baylor College of Medicine, Houston, TX), SK-N-JF and SHEP provided by Dr. M. Brenner (Baylor Research Institute, Dallas, TX).
College of Medicine, Houston, TX), and NB-1691 a gift of Dr. J. Khan (National Cancer Institute, Gaithersburg, MD). Briefly, cell lines were maintained in Dulbecco’s modified Eagle’s medium (HEK293T), RPMI 1640 medium (IMR-32, LA-N-5, SH-SY5Y/luc, NGP/luc, SK-N-BE(2), NB 1691, SKN-JF, SHEP), or Iscove’s Modified Dulbecco’s Media (CHLA-255/luc). All media was supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, with the exception of CHLA-255 which was supplemented with 20% heat-inactivated fetal bovine serum. All cell lines have been validated by genotyping within the past 12 months. In addition, neuroblastoma origin of all lines has been confirmed by expression of CD56, Nestin, MYCN and tyrosine hydroxylase. All cell lines used in this manuscript were routinely tested for mycoplasma on a monthly basis.

Tissue culture of transgenic mouse tumors
Tumors resected from TH-MYCN +/+ mice were resected under sterile conditions. Tissue was minced, washed in cold PBS, treated with collagenase for 1 hour and filtered through a 70 micron cell filter. Cells where then placed in 500 ml of complete IMDM media (20% FBS, 2um beta-mercaptoethanol, 2% HEPES, 2% Na-Pyruvate and 1x non-essential amino acids). After 4 days, adherent cells were trypsinized and sorted for neuroblastoma specific markers (GD2 and CD24) and analyzed or replaced in culture. MYCN protein expression was confirmed by Western Blot in all mouse tumor materials.

Fluorescence-activated cell sorting
Flow cytometry was performed on an LSR II 5-laser flow cytometer (BD Biosciences). FACS was performed on a DAKO Cytomation MoFlo 9-color cell sorter. BD FACSDiva v6.1.2 (BD Biosciences) was used to analyze flow data and Summit v4.3 (Cytomation) software was used to analyze FACS data. All clinical specimens were obtained under protocol approved by the Institutional Review Board of Baylor College of Medicine (IRB protocol H-26515). A description of antibodies and techniques are found in Supplementary Materials and Methods.

In vivo tumorigenicity assay
Four to six week-old female non-obese diabetic (NOD)/SCID mice (NCI-Frederick, Frederick, MD) were used for all xenograft studies and cared for in accordance with the Institutional Animal Care and Use Committee of Baylor College of Medicine (IACUC protocol AN-4810, AN-3705). Utilizing our previously described orthotopic kidney capsule model (orthotopic model) of neuroblastoma, an inoculum of sorted CD114 positive or CD114 negative NGP cells in 0.1 ml of PBS was injected under the renal capsule (26). Three experiments were performed with decreasing numbers of implanted neuroblastoma cells: 1000 cells per mouse (CD114+ n=5; CD114- n=5), 100 cells per mouse (CD114+ n=10; CD114- n=10), 10 cells per mouse (CD114+ n=10; CD114- n=10). Starting three weeks
after tumor cell implantation, all mice were monitored weekly by bioluminescent imaging for development of tumor formation (Xenogen IVIS 100 System, Caliper Life Sciences, Hopkinton, MA).

**In vivo lineage tracing studies**

GFP+/CD114+ and GFP-/CD114- cells (NGP cell line) or tdTomato+/CD114+ and GFP+/CD114- cells (NB-1691 cell line) were sorted and mixed in a 1:1 ratio. An inoculum of 1,000 cells (500 CD114+ cells/500 CD114- cells) in 0.1ml of PBS was injected under the renal capsule of 4- to 6-week old female NOD/SCID mice (NGP line n=5, NB-1691 line n=6). All mice were sacrificed at 12 weeks and underwent complete necropsy examination, and xenografts underwent histologic confirmation. The end points evaluated were the percent tumor take (i.e., the percentage of animals that developed primary tumors) and tumor latency (i.e., the time from tumor cell injection to detection via bioluminescent imaging). For the lineage tracing experiments performed with the NGP cell line, anti-CD-56-APC was used to identify neuroblastoma cells.

**In vivo chemotherapy assay**

In vivo experiments were performed in 4 to 6 week-old female nude mice (athymic NCr-nu/nu, NCI-Frederick, Frederick, MD). Mice were housed in pathogen-free conditions and cared for in accordance with the Institutional Animal Care and Use Committee of Baylor College of Medicine (IACUC protocol AN-4810). Utilizing an orthotopic model of neuroblastoma, 15 mice were implanted with an inoculum of 1 million unsorted NGP neuroblastoma cells in 0.1 ml of PBS under the renal capsule (Patterson et al., 2011). Three weeks after tumor cell implantation and engraftment, xenograft tumors were confirmed by bioluminescent imaging and subsequently monitored weekly (Xenogen IVIS 100 System, Caliper Life Sciences, Hopkinton, MA).

The mice were then randomized into three treatment groups: control (n=3), VP-16 (n=6), and Cytoxan (n=6). Control mice were injected with vehicle (PBS) intraperitoneally (IP) on Monday, Tuesday, and Wednesday for three consecutive weeks. VP-16 group (Etoposide, 9 mg/kg/dose the first week and then 12 mg/kg/dose the next two weeks, Sigma, St. Louis, MO) was injected IP on Monday, Tuesday, and Wednesday for three consecutive weeks. The Cytoxan group (cyclophosphamide, 7.5 mg/kg/dose the first week and then 15 mg/kg/dose the next two weeks, Sigma, St. Louis, MO) was injected IP on Monday, Tuesday, and Wednesday for three consecutive weeks. At the end of six weeks, mice from each group were sacrificed over the course of six days to accommodate flow analysis of the 15 xenograft tumors. Xenograft tumors were resected, weighed, and processed for flow cytometry to assess CD114 population in each tumor of each treatment group. The % CD114+ population of each tumor was pooled into each treatment group and compared using Kruskal-Wallis method.

**Stat3 reporter assay**
The lentiviral Stat3 activation reporter used in the present study has an eGFP gene cloned downstream of a M67 responsive promoter, which contains four M67 binding motifs (TTCCCGTAA) and a TATA box (kindly provided by Dr. Michael T. Lewis, Baylor College of Medicine). Stat3.eGFP reporter-transduced neuroblastoma cells were harvested and 0.5 x 10^6 cells were either untreated or treated with 10 or 100 ng/ml of G-CSF (Neupogen, Amgen Inc., Thousand Oaks, CA) in combination with 0, 3, 10 μM Stattic (Calbiochem, EMD Millipore, Darmstadt, Germany) and then fixed at different time points (1% paraformaldehyde) and then stained and analyzed for CD114 and GFP expression by flow cytometry (full description of assay in Supplementary Materials and Methods).

**Statistical Analysis**

In vivo studies are expressed as mean ± SD. All in vitro assays, including flow cytometric analyses, cell cycle analyses, and Stat3 reporter assays, are expressed as mean ± SD, and all were performed in triplicate. Data was compared using Student’s t-test. Incidence of xenograft tumor formation was calculated with Fisher’s exact test. All animal and human data were compared using Kruskal-Wallis test.

Additional Supplementary Materials and Methods are included in Supplementary Data.

**Results**

**CD114+ cells are highly tumorigenic, can self-renew, and generate differentiated progeny tumor cells**

Initially, we used PE-conjugated monoclonal antibodies for flow cytometry to assess the expression of CD114 in neuroblastoma cell lines (Fig. 1A, 1B, and 1C). We consistently detect a small population of CD114+ cells (range 0.2-1.0%) in every neuroblastoma cell line examined regardless of phenotype (S or N type) or MYCN status (Fig. 1C). A distinct CD114+ subpopulation is also found in every primary human biopsy specimen obtained at initial diagnosis and in murine short-term culture cell lines derived from de novo neuroblastoma tumors arising in TH-MYC transgenic mice (Fig. 1B and Table 1). We confirmed the specificity of our analyses with competitive ligand-binding studies using PE-conjugated G-CSF. The positively stained subpopulation detected by flow cytometry binds specifically to G-CSF-PE-conjugate, which is competitively inhibited by non-conjugated G-CSF ligand or blocked by anti-receptor antibodies in both murine and human neuroblastoma cell lines (Supplementary Fig. S1A and S1B).

When placed in tissue culture, FACS-purified CD114+ cells rapidly differentiate, generating a mixed population of CD114- cells with a small percentage (0.2-1%) maintaining CD114+ expression. Despite this technical limitation, we used the following molecular, flow-cytometric, and in vivo xenograft studies to characterize the distinct phenotype of CD114+ subpopulations in neuroblastoma cell lines and tumors.

An orthotopic xenograft model was used to compare the in vivo tumorigenicity of FACS-sorted CD114+ and CD114- subpopulations (26). In vivo limiting dilution analysis was performed using binomial generalized linear
modeling with the complementary log-log link (Fig. 1D)(27, 28). After verifying goodness of fit to the Single Hit Poisson Model assumption (p > 0.15), CD114+ cells were greater than 10-fold tumorigenic than CD114- cells (p=0.008), with the calculated tumor initiating cell fraction of 1 in 583 for CD114+ cells and greater than 1 in 6050 for CD114- cells. We were able to generate tumors from as few as 10 CD114+ implanted cells (Fig. 1D).

Interestingly, FACS analysis of the resulting xenograft tumors, which were generated from highly purified CD114+ cells (>99% CD114+ cells), demonstrate a return to a baseline percentage of CD114+ cells as observed in the unsorted, parental cell line (0.2-1.0%)(Fig. 1E). This is similar to what is observed during attempts to culture CD114+ cells. While tumors primarily arose from CD114+ cells at limiting cell numbers (<100 cells), one xenograft tumor resulted from injection of 100 CD114- cells (Fig. 1D)(one tumor out of a total of 25 CD114- cell injections). However, analysis of this tumor again demonstrated a small CD114+ subpopulation (<1%) suggesting that either CD114- cells de-differentiated into CD114+ cells or contaminating CD114+ cell(s) was present in the negatively selected CD114- population injected.

We next performed competitive lineage tracing studies in mice by co-implanting mixtures of FACS-purified subsets of 50% CD114+ and 50% CD114- cells, differentially tagged with fluorescent markers. In two neuroblastoma cell lines (NGP and NB-1691), the resulting xenografts uniformly and exclusively contained the fluorescent tag derived from the CD114+ subset (Fig. 1F and 1G). Furthermore, flow cytometry of the xenograft tumors demonstrates that the percentage of CD114+ neuroblastoma cells again returned to levels found in the parental, unsorted cell line (0.2-1.0%)(Fig. 1F and 1G and Supplementary Table S1). Similar to the limiting dilution study, we discovered one exception of a single tumor (1 of 7) generated from the mixture of RFP+/CD114+ and GFP+/CD114- cells from the NB-1691 cell line. In this case, a small discrete focus of GFP+ tumor was found abutting a large, uniformly RFP+ tumor. Flow cytometry of the GFP+ population again demonstrated a small GFP+/CD114+ subpopulation (data not shown).

While the in vivo limiting dilution study and the in vivo lineage tracing study demonstrate a clear propensity and marked tumorigency of the CD114+ subpopulation, we identified tumors that were derived from the CD114- subpopulation yet recapitulated a small CD114+ subpopulation. Two possibilities may explain for this observation. One, the findings may represent contamination of the negatively selected CD114- population with CD114+ cells during the FACS isolation. Two, the observation may be explained by a phenomena of interconversion of CD114- cells to CD114+ cells. While this is something that has been previously described, the finding of a CD114- derived tumor was a rare occurrence.

**CD114+ cells do not segregate with CD133 populations or side-population cells**

Previously characterized tumor initiating populations in neuroblastoma have been defined by CD133 expression or using the “side population” (SP) cytometric assay (7, 8, 10). We therefore sought to determine if CD114 was found to be co-expressed with either of these populations of cells. We demonstrate that CD114+ cells do not co-
express CD133 (Supplementary Fig. S2A) and do not segregate with SP populations (based on Hoescht dye efflux)(Supplementary Fig. S2B and S2C). Our data suggests that these putative tumorigenic neuroblastoma populations which are primarily defined by in vitro assays, likely arise during tissue culture within the more differentiated CD114- population.

**Chemotherapy enriches for the CD114+ subpopulation in primary neuroblastoma**

We hypothesized that CD114+ cells may represent a chemoresistant subpopulation of neuroblastoma similar to cancer stem cell populations in other tumors (4, 6, 29). We therefore analyzed CD114 surface expression from primary human tumor samples (Table 1). All specimens were taken from the primary tumor site (except for one brain metastasis) during pre-chemotherapy biopsy, pre-chemotherapy resection, or post-chemotherapy surgical resection. Consistent with data from cell lines, the percentage of CD114+ cells were rare and ranged from 0.1 to 1.7% at diagnosis (0.75% ± 0.41%). In contrast, CD114+ cells were enriched 5 to 10 fold in tumors obtained from patients who had received multiple rounds of genotoxic chemotherapy (4.45% ± 2.36%, *p < 0.001) or at relapse (7.6%)(Fig. 2A and Table 1).

We experimentally replicated this treatment-dependent enrichment of CD114+ cells in vivo by treating tumor-bearing mice (NGP cell line) with three cycles of chemotherapy, using cyclophosphamide (Cytoxan) or VP-16 (Etoposide)(Fig. 2B and described in Supplementary Materials and Methods). Exposure to these genotoxic agents led to a statistically significant increase in the CD114+ subpopulation in xenograft tumors compared to control (Cytoxan **p < 0.05; Etoposide ***p < 0.04)(Fig. 2C).

**CD114+ cells demonstrate a cell cycle characteristic of pluripotent cells**

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) share characteristics associated with multi- or pluripotent ‘stemness’. An important feature is a cell cycle with a limited G1-S checkpoint, which yields a short G0/G1-phase and a prolonged S-phase (30, 31). In iPSCs this has been shown to be due to repression of p53 and p21 mediated checkpoint regulation (13). We therefore assessed the steady state cell cycles of CD114+ and CD114- populations within non-confluent neuroblastoma cell lines (representing a range of NB phenotypes). Utilizing BRDU pulse-labeling and CD114 FACS analysis, which permits us to concomitantly perform analysis of the total and CD114+ subpopulations, we uniformly demonstrate a distinctly altered cell cycle in the CD114+ subsets relative to the CD114- subsets (33% vs. 51% G0/G1-phase, respectively, *p < 0.0007; 54% vs. 36% S-phase, respectively, ** p < 0.0004)(Fig. 3B and 3C). These data are consistent with the cell cycles described for iPSCs and ESC lines (30)(Figure 3 and Supplementary Methods).

**Gene expression of the CD114+ subpopulation is consistent with an early neural crest derivation**
We next analyzed the gene expression differences between CD114+ and CD114- subpopulations in the NGP neuroblastoma cell line using low-density qPCR arrays (Supplementary Materials and Methods). These findings were validated for individual genes with TaqMan qPCR assays in CD114+ and CD114- subpopulations from NGP and SH-SY5Y cell lines (Fig. 5A, 5B, and 5C and Supplementary Table S2). We discovered that CD114+ cells overexpress markers of early pre-migratory crest (e.g. SOX10, NME1, TWIST, ISL), mixed epithelial and mesenchymal markers (e.g. CDH1, CDH2, VIM, MMPs), as well as positive cell cycle regulators associated with maintenance of pluripotency (e.g. CDC25, CDK1, Cyclin D1). Of note, SOX10 is known to promote the survival and inhibit the multi-lineage differentiation of neural crest stem cells (32). In contrast, CD114- cells overexpress mesenchymal genes involved in tissue invasion and angiogenesis (e.g. SNAIL-1, SNAIL-2, ZEB, MMPs, ANGPT1, TLK) and downregulated markers of neuroepithelium. These data clearly distinguish CD114+ and CD114- cells and support a model where CD114+ cells reflect an earlier state of neural crest development (Supplementary Fig. S3 and Discussion below).

**CD114+ cells overexpress microRNAs opposing neuronal differentiation and p53 activity**

MicroRNAs play an important role in reprogramming and stem cell maintenance and recent studies have identified a number of microRNAs which regulate neuronal lineage specification from embryonic precursors (33, 34). To evaluate differential microRNA expression, we performed small-RNA sequencing on RNA directly isolated from FACS-purified CD114+ and CD114- subpopulations from three neuroblastoma cell lines (NGP, IMR-32, LA-N-5). We found a highly consistent signature of 25 microRNAs differentially expressed in CD114+ versus CD114- subpopulations (Fig. 5A and Supplementary Table S3). This includes six upregulated microRNAs (miR-106b, 21, 25, 30e, 598, 93) that are also upregulated as mouse embryonic stem cells transition to neuronal precursors (Fig. 5B). Additional studies demonstrate that number of CD114+ upregulated microRNAs (miR-25, 106b, 17, 18, 19, 20a, 143, 27) are repressed upon neuronal lineage differentiation of somatic stem cells (33, 35, 36). In addition, microRNA pathway analysis with two independent prediction platforms (DIANA (DIANA Lab, Athens, Greece) and Ingenuity (Ingenuity Systems, Redwood City, CA) each demonstrated enrichment of p53, NGF, Notch and additional signaling pathways driving differentiation of neuronal and neural crest lineages (Supplementary Table S4 and Supplementary Table S5).

As shown in Supplementary Table S3, 15 of the CD114+ microRNAs are validated transcriptional targets of MYCN, an oncogenic transcription factor directly implicated in neural crest transformation and neuroblastoma tumorigenesis (37-39). Consistent with the emerging role of p53 repression in the maintenance of normal and malignant stem cell populations (13), most of these same microRNAs are known to target p53 and its downstream targets (Fig. 5C) and have been shown to correlate with poor prognosis in high-risk neuroblastoma microRNAs (miRs-17, -19, -20, -25, -92 and -93)(40). These data suggest a role of microRNAs in maintenance of a
dedifferentiated state in the CD114+ subpopulation of neuroblastoma and further distinguish CD114+ from CD114- neuroblastoma subpopulations.

**Exogenous G-CSF activates downstream STAT3 signaling in CD114+ cells**

While surface expression of the G-CSF receptor identifies a distinct, immature neuroblastoma subpopulation, additional analysis confirms intact downstream signaling upon ligation of G-CSF (Fig. 6). In the NGP and SH-SY5Y neuroblastoma cell lines, treatment with G-CSF leads to a transient increase in both CD114 expression and activation of a STAT3 responsive reporter construct containing four STAT3 response elements in a promoter upstream of eGFP (STAT3.eGFP-M67) in a dose-dependent manner (Fig. 6A and 6B). We confirmed that transcriptional activation correlated with increased phosphorylation of STAT3 within the CD114+ subpopulation as detected by flow cytometry (phospho-flow) 30 minutes after G-CSF exposure (Fig. 6G). The addition of a small molecule inhibitor, which prevents phosphorylation and dimerization of STAT3 (Stattic, EMD Biosciences), specifically abrogates the effect of exogenous G-CSF treatment (Fig. 6C, 6D, 6E, and 6F). As expected, this transient response of CD114+ cells was not detected in the CD114- population, suggesting that CD114 expression is both a marker of a stem cell subpopulation as well as a functional growth factor receptor for these cells.

**Discussion**

As with other aggressive malignancies which are composed of multiple heterogeneous cell types with differential tumorigenic and metastatic potential, we have identified a novel distinct subpopulation within neuroblastoma cell lines and tumors with similar properties. Our data demonstrates that expression of G-CSF receptor (CD114), consistently identifies a discrete de-differentiated subpopulation within neuroblastoma with markedly enhanced tumorigenicity. In addition, this population has many phenotypic similarities to iPSC and ESC cell types including similar microRNA expression, gene expression and cell cycle profiles. This subpopulation is distinct from previously characterized tumorigenic populations (tumor initiating cells) defined by CD133 expression, SP staining, or ‘neurosphere’ assays (7, 8, 10, 11). Moreover, for neuroblastoma, tumorigenicity of ‘neurosphere’ derived tumor initiating populations can be highly variable as well as cell line dependent, and these in vitro conditions dramatically alter gene expression and phenotype over time (11). In this study we utilized FACS to isolate CD114+ subpopulations directly from patient biopsies and animal tumors without additional ex vivo manipulation and validated these findings in multiple cell lines. The CD114+ subpopulation consistently represents approximately 0.5-1.5% of tumors at diagnosis, and the same percentage was found in all neuroblastoma cell lines analyzed. Lastly, CD114+ cells express a distinct set of genes consistent with an early neuroepithelial/neural crest phenotype, while CD114- gene expression correlates with the more differentiated CD114- mesenchymal neural crest populations. This places the CD114+ subpopulation within neuroblastoma in the appropriate developmental context for a putative neural crest derived tumor precursor population.
The distinct gene and microRNA expression profiles of CD114+ cells compared to CD114- cells support their role as putative neuroblastoma tumor initiating cells or cancer stem cells and also concur with current concepts of oncogene driven neuroblastoma pathogenesis. Neuroblastoma is uniformly p53 wild-type at diagnosis, and previous studies have shown that p53 repression is critical for MYCN driven tumor initiation (41, 42). Most recently, BMI-1 mediated repression of p53 has been shown to play a key role in this process in TH-MYCN transgenic murine neuroblastoma (43). There is increased expression of genes, which restrict neural crest differentiation and inhibit apoptosis (e.g. SOX10, STAT3, HIF-2α) and decreased expression of pro-apoptotic genes (e.g. CASP8, p53) in the CD114+ subpopulation. This is consistent with the resistance to apoptosis upon withdrawal of trophic factors observed in neuroblastoma precursors isolated from sympathetic ganglia of TH-MYCN transgenic mice (14). The CD114+ subpopulation displays increased expression of multiple microRNAs with validated targets in the p53 apoptotic response pathway, as well as microRNAs which promote reprogramming (44), stem cell cell-cycle regulation (30), and neuronal differentiation (33). Interestingly, repression of p53 through STAT3 activation appears to contribute to tumorigenesis (45), and small molecules which activate p53 and repress STAT3 can have potent antitumor effects (46).

As noted above, STAT3 is known to mediate chemoresistance, cancer stem cell maintenance (18, 19), and neural crest specification (15). We demonstrate that the CD114+ population is enriched by genotoxic chemotherapy in vivo (Fig. 2 and Table 1), and exogenous G-CSF promotes STAT3 transcriptional activity. These observations in a putative CSC population are further supported by recent observations in a murine stem cell reprogramming model (47) that G-CSF-dependent STAT3 activation is sufficient to drive the transition of epiblasts into pluripotent embryonic stem cells. Thus, the finding that the CD114+ subpopulation responds to G-CSF stimulation by upregulating STAT3 transcriptional activity is consistent with current models implicating STAT3 in normal neural crest and cancer stem cell biology.

Recently, genomic studies of neuronal differentiation pathways have better defined the earliest points at which neural crest specification and subsequent differentiation occur (48, 49). Neural crest is a transient embryologic tissue which arises from the neuroepithelium at the borders of the neural plate during neurulation (48). This pre-migratory epithelial neural crest represents the earliest specified neural crest and has been shown to be multipotent, capable of differentiating into many types of peripheral tissues (50). Integral to further neural crest differentiation is an epithelial-to-mesenchymal transition (EMT) of the pre-migratory neural crest into mesenchymal neural crest cells expressing MMPs, integrins and motility factors permitting their migration and differentiation into a host of tissues throughout the body (49). The molecular characterization of CD114+ and CD114- cells support a model where the transition from the CD114+ to the CD114- neuroblastoma phenotype recapitulates this early developmental pathway (illustrated in Supplementary Fig. S3). We speculate that oncogenic mutations at this critical juncture may block differentiation and generate a self-renewing, malignant,
CD114+ cancer stem cell population, which gives rise to migratory neural crest-like CD114- progeny (Supplementary Fig. S3).

Together these findings define a model of CD114+ cells as a novel highly tumorigenic subpopulation, arising from the early undifferentiated pre-migratory neural crest. These data are supported by the clinical data demonstrating CD114+ subpopulations in primary diagnostic and relapsed tumor biopsies. While CD114 receptor signaling is intact and responsive to exogenous ligand G-CSF, the role of G-CSF/G-CSF receptor binding in neuroblastoma tumorigenesis will require additional investigation. Endogenous cellular sources of G-CSF include mesenchymal stroma, endothelial cells, fibroblasts and monocytes/macrophages, and local signaling between these cells and CD114+ neuroblastoma precursors could possibly support the expansion of neuroblastoma in tumorigenic ‘niches’ in vivo. Of potential clinical significance, this hematopoietic cytokine is used extensively to limit neutropenia in children receiving chemotherapy for neuroblastoma and other solid tumors. However, it remains unclear whether in addition to endogenous sources, exogenous recombinant G-CSF could alter the biology of neuroblastoma in vivo. Both MYCN and ALK oncogenes drive neuroblastoma in children, and how these oncogenes promote the expansion and maintenance of a persistent CD114+ subpopulation within the neural crest with enhanced self-renewal potential and tumorigenicity also remain important questions.

Acknowledgments

This work was performed with support from the American Cancer Society (JS), Alex’s Lemonade Stand Foundation (JS), Gillson-Longenbaugh Foundation (JS), Children’s Neuroblastoma Research Foundation (JS), St. Baldrick’s Foundation (EK), and Texas Children’s Hospital Department of Surgery Seed Grant (EK).

The authors thank Dr. Malcolm Brenner, Dr. Jeffrey Rosen, and Dr. Oluyinka Olutoye (Baylor College of Medicine) for their comments and help with this manuscript and Dr. Susan Hilsenbeck (Baylor College of Medicine) for assistance with statistical analyses.
References:


<table>
<thead>
<tr>
<th>CD114 Positive (%)</th>
<th>Stage</th>
<th>Treatment</th>
<th>MYCN Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1*</td>
<td>IV</td>
<td>Pre-chemo biopsy</td>
<td>Amplified</td>
</tr>
<tr>
<td>0.4</td>
<td>IIA</td>
<td>Pre-chemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.5</td>
<td>IIA</td>
<td>Pre-chemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.6</td>
<td>IIA</td>
<td>Pre-chemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.6</td>
<td>IIB</td>
<td>Pre-chemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.8</td>
<td>IV</td>
<td>Pre-chemo biopsy</td>
<td>Amplified</td>
</tr>
<tr>
<td>0.8*</td>
<td>III</td>
<td>Pre-chemo biopsy</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.8</td>
<td>IIA</td>
<td>Pre-chemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.9</td>
<td>IV</td>
<td>Pre-chemo biopsy</td>
<td>Not amplified</td>
</tr>
<tr>
<td>1.0</td>
<td>IIA</td>
<td>Pre-chemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>1.7</td>
<td>III</td>
<td>Pre-chemo biopsy</td>
<td>Not amplified</td>
</tr>
<tr>
<td>2.8</td>
<td>IV</td>
<td>Post-chemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>3.4</td>
<td>IV</td>
<td>Post-chemo resection</td>
<td>Amplified</td>
</tr>
<tr>
<td>3.5*</td>
<td>III</td>
<td>Post-chemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>3.8</td>
<td>III</td>
<td>Post-chemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>4.0*</td>
<td>IV</td>
<td>Post-chemo resection</td>
<td>Amplified</td>
</tr>
<tr>
<td>7.6*</td>
<td>IV</td>
<td>Post-chemo resection of brain metastasis</td>
<td>Amplified</td>
</tr>
<tr>
<td>9.2</td>
<td>IV</td>
<td>Post-chemo resection</td>
<td>Not amplified</td>
</tr>
</tbody>
</table>
Figure / table legends:

Figure 1. CD114 expression marks a phenotypically distinct subset within primary neuroblastoma. A, using flow cytometry, the first panel demonstrates the bulk neuroblastoma cell population with gating around live cells. The second panel demonstrates isolation and detection of the CD114 cell population using a monoclonal anti-CD114-PE antibody. The third panel demonstrates a negative isotype control using an IgG-PE antibody. B, flow cytometry detects a small but discrete population of CD114+ cells in human biopsy specimens at diagnosis, neuroblastoma cell lines and short-term cultures derived from primary transgenic murine neuroblastoma tumors. C, analysis of CD114 expression in a panel of validated neuroblastoma cell lines. D, in vivo limiting dilution analysis comparing the tumorigenicity of CD114+ and CD114- subpopulations. The difference between the CD114+ and CD114- subpopulations is significant (p = 0.008) with a 10-fold increase in the frequency of tumor initiating cells in the CD114+ subpopulation compared to CD114- subpopulation. E, resulting neuroblastoma xenografts from FACS-purified subpopulations demonstrate homeostatic maintenance of a small (0.2-1.0%) population of CD114+ cells (FACS of parental cell line on left and resulting xenograft tumor on right). F and G, lineage specific tumorigenesis of CD114+ and CD114- subpopulations was assessed using competitive in vivo mixing of 500 CD114+ and 500 CD114- cells. Derived primarily from CD114+ cells, resulting xenograft tumors recapitulate a small population of CD114+ cells and are primarily composed of CD114- progeny cells (as indicated by fluorescent lineage marking). Error bars represent standard deviation (SD).

Figure 2. Human neuroblastoma tumors are enriched for CD114+ cells after treatment or at relapse. A, flow cytometric analysis of patient samples isolated at time of diagnosis or after multiple cycles of chemotherapy show a significant increase in the CD114+ population after treatment and in the case of one recurrent metastatic lesion. B, established orthotopic xenografts were treated with either Cytoxan (cyclophosphamide) or Etoposide (VP-16) as shown, and tumors were evaluated for CD114 expression using flow cytometry. C, a clear enrichment of the CD114+ subpopulation was demonstrated after chemotherapy exposure in vivo, as expected for a cancer stem cell population resistant to DNA damage. Kruskal-Wallis *p < 0.001 (pre-chemotherapy compared to post-chemotherapy clinical tumors); Kruskal-Wallis **p < 0.05 and ***p < 0.04 (compared to no treatment). Error bars represent standard deviation.

Figure 3. Cell cycle analysis of neuroblastoma subpopulations. A, representative analysis of neuroblastoma cell lines using BRDU/P.I. staining on CD114- and CD114+ subpopulations analyzed by multicolor flow cytometry. Total cell line gated for G01/S/G2-M and comparison of equivalent numbers of CD114+ and CD114- subsets are illustrated (1000 events). B, comparison of cell cycle distributions for CD114+ and CD114- subpopulations from four neuroblastoma cell lines. C, cumulative comparison of 4 cell lines shows highly significant and consistent differences (T-test *p < 0.0007 and **p < 0.0004. Error bars represent standard deviation). All experiments were
performed in triplicate and cells stained with PE-CD114+ cells prior to DAPI and anti-BRDU staining (Supplementary Methods).

Figure 4. Gene expression profiling of CD114+ and CD114- subpopulations with SABiosciences LDA pathway QPCR assays. A, the CD114+ cell population displays a pattern of increased self-renewal and proliferation markers (e.g. CDK1, CDH2, NME1). B, CD114- cells display increased migratory crest markers (e.g. FGFr, SOX2, NOTCH, BMPs). C, schema of neural crest differentiation, suggesting CD114+ cells arise earlier during differentiation. An EMT/MET barrier separates these distinct neural crest populations. (Red up in CD114+, Green up in CD114-. P < 0.05, 1.2 fold cut-off indicated on plots)

Figure 5. CD114+ cells have a microRNA expression signature consistent with stem cells. A, microRNA expression heat maps of CD114+ and CD114- subpopulations in 3 neuroblastoma cell lines (NGP, LA-N-5, IMR-32). B, the overexpressed 25 microRNAs in the CD114+ subpopulation and the involved pathways. Small-RNA sequencing reveals a consistent overexpression of microRNAs implicated in stem cell reprogramming and the maintenance of de-differentiated neuronal stem cells. C, CD114+ overexpressed microRNAs target p53 pathways and associated genes.

Figure 6. CD114 is activated by the ligand G-CSF. A, G-CSF increases the percentage of CD114+ cells in the NGP and SH-SY5Y (B) cell lines. Using flow cytometry, an increased CD114 population (PE) is observed with varying doses of G-CSF (0, 10, 100 ng/ml) and at varying time points (1/2 hour, 2 hours, 24 hours). C, the G-CSF-mediated increase in CD114+ cells is inhibited by the STAT3 inhibitor Stattic in the NGP cell line and the SH-SY5Y cell line (D). Using the 2-hour time point, flow cytometry demonstrates CD114 expression with varying doses of G-CSF (0, 10, 100 ng/ml) and varying doses of Stattic (0, 3, 10 uM). E, G-CSF induces STAT3 activation, which is inhibited by Stattic in the NGP cell line and the SH-SY5Y cell line (F). Using a STAT3-GFP reporter assay, flow cytometry at the 2-hour time point demonstrates CD114 and GFP co-expression with varying doses of G-CSF (0, 10, 100 ng/ml) and varying doses of Stattic (0, 3, 10 uM). G, G-CSF induces the phosphorylation and activation of STAT3. G-CSF increases the percentage of CD114+ cells (white bar) and the phosphorylation and activation of STAT3 in CD114+ cells (black bar) in the NGP cell line. Using flow cytometry, an increased CD114 population (PE) and corresponding levels of phosphorylated STAT3 (pY705) is observed with G-CSF (10 ng/ml) treatment. Black bars represent double positive population (CD114+ and pSTAT3+(Y705). T-test *p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent standard deviation.

Table 1. Clinical tumor samples analyzed for CD114 at pre-chemotherapy biopsy, pre-chemotherapy tumor resection, or at post-chemotherapy resection.
*= one patient with serial samples followed over the course of one year. + = another patient with matched pre- and post- treatment samples. We observed a clear trend of increased CD114% in tumors exposed to chemotherapy, which was recapitulated in orthotopic chemotherapy models (Figure 2).
Figure 1

A

NGP

CD114-PE

Isotype Control

0.5%

CD114+

0%

CD114+

FSC-A (x1000)

B

%CD114+ cells

Human cell lines
Mouse cell lines
Human primary tumors

C

% CD114+ Cells

CHLA-225
IMR-32
SK-NF
NGP
SF9
SH-SY5Y
SK-N-BE(2)C
LA-N-5
NB-1691

D

% positive tumors

10 cells
100 cells
1000 cells

No. of cells injected

E

NGP Cell Line

Xenograft Tumor

F

NGP

CD114+ GFP+ (500 cells)

CD114- (500 cells)

50% CD114+

6 weeks

fluorescent

G

NB-1691

CD114+ tdt+ (500 cells)

CD114- GFP+ (500 cells)

50% CD114+

6 weeks

fluorescent
Figure 2

A  Patient Samples

B  Treatment Schema

C  Treated Xenografts

CD114 Flow

Established xenograft

Xenograft

After treatment
Figure 3

A 100,000 events total  1000 events total  CD114+ Subset

B

% of total cell cycle

NGP  CHLA-255  LAN5  IMR32  NGP  CHLA-255  LAN5  IMR32

CD114- subset  CD114+ subset

C

% Cell Cycle

CD114+  CD114-

(4 cell lines: NGP, CHLA-255, IMR-32, LA-N-5)
Figure 4

A. Cancer Pathways

B. Stem Cell Pathways

C. EMT Pathways
Figure 5

A + CD114 - CD114 + CD114 -
hsa-let-7b
hsa-let-7c
hsa-let-7e
hsa-mir-106b
hsa-mir-1207
hsa-mir-133b
hsa-mir-132
hsa-mir-193a
hsa-mir-20a
hsa-mir-21
hsa-mir-25
hsa-mir-26b
hsa-mir-30d
hsa-mir-320a
hsa-mir-370
hsa-mir-590
hsa-mir-744
hsa-mir-199b
NGP
LA-N-5
IMR-32

B

Inhibition of Neuronal Differentiation
MiR-25, 106b, 17, 18, 19, 20a, 143, 27

ESC to Neuronal Precursors
MiR-106b, 21, 25, 30e, 598, 93

25 microRNAs overexpressed in CD114+ subpopulation

Neuroblastoma Oncogenesis
Mir-17-92 cluster, Mir-106-93 cluster

Reprogramming and de-differentiation
MiR-106b, 93, 17, 20a, 92

C

Functional Distribution of p53 Pathway Genes

APOTOPSIS
CELL CYCLE
METABOLISM
Other

# of genes targeted
# of targeting microRNA
Expression of the G-CSF receptor, CD114, marks a novel, highly tumorigenic cell population in a neuroblastoma cell line

Danielle M Hsu, Saurabh Agarwal, Ashley Benham, et al.

Cancer Res  Published OnlineFirst May 16, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-4056

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/05/16/0008-5472.CAN-12-4056.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/early/2013/05/16/0008-5472.CAN-12-4056. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.