Tumor and Stem Cell Biology

G-CSF Receptor Positive Neuroblastoma Subpopulations Are Enriched in Chemotherapy-Resistant or Relapsed Tumors and Are Highly Tumorigenic

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Introduction

High-risk neuroblastoma (NB) is an aggressive embryonal malignancy of young children arising from the embryonic neural crest. Neuroblastoma 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 (CSC).

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 shown 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 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 (nonadherent, serum-free conditions; ref. 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 about 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 seems 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), whereas 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 granulocyte colony-stimulating factor (G-CSF)–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-positive (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, showed 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 shows CD114+ cells are similar to (premigratory) neural crest cells, whereas the CD114-negative (CD114−) population represents a later stage of neural crest differentiation (migratory crest). On the basis of the differential expression of CD114, these studies define a novel dedifferentiated 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. The neuroblastoma cell lines SH-SY5Y/luc and NGP/luc were provided by Drs. J. Kandel and 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 College of Medicine), and NB-1691 a gift of Dr. J. Khan [National Cancer Institute (NCI), Gaithersburg, MD]. Briefly, cell lines were maintained in Dulbecco’s Modified Eagle Medium (HEK293T), RPMI-1640 medium [IMR-32, LA-N-5, SH-SY5Y/luc, NGP/luc, SK-N-BE(2), NB-1691, SKN-JF, and SHEP], or Iscove’s Modified Dulbecco’s Medium (IMDM; CHLA-255/luc). All media was supplemented with 10% heat-inactivated FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, with the exception of CHLA-255, which was supplemented with 20% heat-inactivated FBS.

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 article 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-μm cell filter. Cells where then placed in 500 mL of complete IMDM media (20% FBS, 2-μm β-mercaptoethanol, 2% HEPES, 2% Na-pyruvate, and 1x nonessential amino acids). After 4 days, adherent cells were trypsinized and sorted for neuroblastoma-specific markers (CD2 and CD24) and analyzed or

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replaced in culture. MYCN protein expression was confirmed by Western blot analysis in all mouse tumor materials.

**Fluorescence-activated cell sorting**

Flow cytometry was conducted on an LSR II 5-laser flow cytometer (BD Biosciences). FACS was conducted 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 (IRB) of Baylor College of Medicine (IRB protocol H-26515). A description of antibodies and techniques are found in the Supplementary Materials and Methods.

**In vivo tumorigenicity assay**

Four- to 6-week-old female nonobese diabetic/severe combined immunodeficient (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 (IACUC) of Baylor College of Medicine (IACUC protocol AN-4810, AN-3705). Using our previously described orthotopic kidney capsule model (orthotopic model) of neuroblastoma, an inoculum of sorted CD114+ or CD114− NGP cells in 0.1 mL of PBS was injected under the renal capsule (26). Three experiments were carried out with decreasing numbers of implanted neuroblastoma cells: 1,000 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 3 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).

**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 0.1 mL 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 endpoints evaluated were the percentage 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 carried out with the NGP cell line, anti-CD-56-APC was used to identify neuroblastoma cells.

**In vivo chemotherapy assay**

In vivo experiments were carried out in 4- to 6-week-old female nude mice (athymic NCr-nu/nu; NCI-Frederick). Mice were housed in pathogen-free conditions and cared for in accordance with the IACUC of Baylor College of Medicine (IACUC protocol AN-4810). Using 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 (26). 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).

The mice were then randomized into 3 treatment groups: control (n = 3), VP-16 (n = 6), and Cytoxan (n = 6). Control mice were injected with vehicle (PBS) intraperitoneally on Monday, Tuesday, and Wednesday for 3 consecutive weeks. VP-16 group (etoposide, 9 mg/kg/dose the first week and then 12 mg/kg/dose the next 2 weeks; Sigma) was injected intraperitoneally on Monday, Tuesday, and Wednesday for 3 consecutive weeks. The Cytoxan group (cyclophosphamide, 7.5 mg/kg/dose the first week and then 15 mg/kg/dose the next 2 weeks; Sigma) was injected intraperitoneally on Monday, Tuesday, and Wednesday for 3 consecutive weeks. At the end of 6 weeks, mice from each group were sacrificed over the course of 6 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 percentage 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 4 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 × 10^6 cells were either untreated or treated with 10 or 100 ng/mL of G-CSF (Neupogen; Amgen Inc.) in combination with 0, 3, and 10 μmol/L Static (Calbiochem, EMD Millipore) 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 the 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 conducted in triplicate. Data were compared using Student t test. Incidence of xenograft tumor formation was calculated with Fisher exact test. All animal and human data were compared using Kruskal–Wallis test.

Additional Supplementary Materials and Methods are included in the Supplementary Data.

**Results**

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

Initially, we used phycoerythrin (PE)-conjugated monoclonal antibodies for flow cytometry to assess the expression of CD114 in neuroblastoma cell lines (Fig. 1A–C). 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-MYCN 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 nonconjugated G-CSF ligand or blocked by antireceptor 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 conducted using binomial generalized linear modeling with the complementary log-log link (Fig. 1D; refs. 27, 28). After verifying goodness of fit to the Single Hit Poisson Model assumption (P > 0.15), CD114⁺ cells were more 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 more than 1 in 6,050 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), show 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. Although 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 showed a small CD114⁻ subpopulation (<1%) suggesting that either CD114⁻ cells dedifferentiated into CD114⁺ cells or contaminating CD114⁻ cell(s) was present in the negatively selected CD114⁻ population injected.

We next conducted competitive lineage-tracing studies in mice by coimplanting mixtures of FACS-purified subsets of 50% CD114⁻ and 50% CD114⁺ cells, differentially tagged with fluorescent markers. In 2 neuroblastoma cell lines (NPS and NB-1691), the resulting xenografts uniformly and exclusively contained the fluorescent tag derived from the CD114⁻ subset (Fig. 1F and G). Furthermore, flow cytometry of the xenograft tumors shows 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 G 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 showed a small GFP⁺/CD114⁻ subpopulation (data not shown).

Although the in vivo limiting dilution study and the in vivo lineage-tracing study show a clear propensity and marked tumorigenicity 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. First, the findings may represent contamination of the negatively selected CD114⁻ population with CD114⁺ cells during the FACS isolation. Second, the observation may be explained by a phenomenon of interconversion of CD114⁻ cells to CD114⁺ cells. Although 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” cytometric assay (7, 8, 10). We therefore sought to determine if CD114 was found to be coexpressed with either of these populations of cells. We show that CD114⁺ cells do not coexpress CD133 (Supplementary Fig. S2A) and do not segregate with side populations (based on Hoechst dye efflux; Supplementary Fig. S2B and S2C). Our data suggest 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 CSC 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 prechemotherapy biopsy, prechemotherapy resection, or postchemotherapy surgical resection. Consistent with data from cell lines, the percentage of CD114⁻ cells was 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 (NPS cell line) with 3 cycles of chemotherapy, using cyclophosphamide (Cytoxan) or VP-16 (etoposide; Fig. 2B and described in the Supplementary Materials and Methods). Exposure to these genotoxic agents led to a statistically significant increase in the CD114⁻ subpopulation in xenograft tumors compared with control (Cytoxan ***, P < 0.05; etoposide *** P < 0.04; Fig. 2C).

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Table 1. Clinical tumor samples analyzed for CD114 at prechemotherapy biopsy, at prechemotherapy tumor resection, or at postchemotherapy resection

<table>
<thead>
<tr>
<th>CD114⁺, %</th>
<th>Stage</th>
<th>Treatment</th>
<th>MYCN status</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1⁺</td>
<td>IV</td>
<td>Prechemo biopsy</td>
<td>Amplified</td>
</tr>
<tr>
<td>0.4</td>
<td>IIA</td>
<td>Prechemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.5</td>
<td>IIA</td>
<td>Prechemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.6</td>
<td>IIA</td>
<td>Prechemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.6</td>
<td>IIB</td>
<td>Prechemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.8</td>
<td>IV</td>
<td>Prechemo biopsy</td>
<td>Amplified</td>
</tr>
<tr>
<td>0.8⁺</td>
<td>III</td>
<td>Prechemo biopsy</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.8</td>
<td>IIA</td>
<td>Prechemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>0.9</td>
<td>IV</td>
<td>Prechemo biopsy</td>
<td>Not amplified</td>
</tr>
<tr>
<td>1.0</td>
<td>IIA</td>
<td>Prechemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>1.7</td>
<td>III</td>
<td>Prechemo biopsy</td>
<td>Not amplified</td>
</tr>
<tr>
<td>2.8</td>
<td>IV</td>
<td>Postchemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>3.4</td>
<td>IV</td>
<td>Postchemo resection</td>
<td>Amplified</td>
</tr>
<tr>
<td>3.5⁺</td>
<td>III</td>
<td>Postchemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>3.8</td>
<td>III</td>
<td>Postchemo resection</td>
<td>Not amplified</td>
</tr>
<tr>
<td>4.0⁺</td>
<td>IV</td>
<td>Postchemo resection</td>
<td>Amplified</td>
</tr>
<tr>
<td>7.6⁺</td>
<td>IV</td>
<td>Postchemo resection of brain metastasis</td>
<td>Amplified</td>
</tr>
<tr>
<td>9.2</td>
<td>IV</td>
<td>Postchemo resection</td>
<td>Not amplified</td>
</tr>
</tbody>
</table>

*One patient with serial samples followed over the course of 1 year.
*Another patient with matched pre- and posttreatment samples. We observed a clear trend of increased CD114% in tumors exposed to chemotherapy, which was recapitulated in orthotopic chemotherapy models (Fig. 2).

CD114⁺ cells show a cell-cycle characteristic of pluripotent cells

Embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) 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 nonconfluent neuroblastoma cell lines (representing a range of neuroblastoma phenotypes). Using bromodeoxyuridine (BrdUrd) pulse-labeling and CD114 FACS analysis, which permits us to concomitantly conduct analysis of the total and CD114⁻ subpopulations, we uniformly show 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 C).

These data are consistent with the cell cycles described for iPSCs and ESC lines (ref. 30; Fig. 3 and Supplementary Materials and 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 neuroblasto-ma cell line using low-density quantitative PCR (qPCR) arrays (Fig. 4 and 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–C and Supplementary Table S2). We discovered that CD114⁺ cells overexpress markers of early premigratory crest (e.g., SOX10, NME1, TWIST, and ISL), mixed epithelial and mesenchymal markers (e.g., CDH1, CDH2, VIM, and MMPs), as well as positive cell-cycle regulators associated with maintenance of pluripotency (e.g., OCT4, UTF1, and LIN28). These findings are consistent with the cell cycles described for iPSCs and ESC lines (ref. 30; Fig. 3 and Supplementary Materials and Methods).
pluripotency (e.g., CDC25, CDK1, and cyclin D1). Of note, SOX10 is known to promote the survival and inhibit the multilineage 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, ans 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 later).

CD114^+ cells overexpress miRNAs opposing neuronal differentiation and p53 activity

miRNAs play an important role in reprogramming and stem cell maintenance and recent studies have identified a number of miRNAs, which regulate neuronal lineage specification from embryonic precursors (33, 34). To evaluate differential miRNA expression, we conducted small RNA sequencing on RNA directly isolated from FACS-purified CD114^+ and CD114^- subpopulations from 3 neuroblastoma cell lines (NGP, IMR-32, LA-N-5). We found a highly consistent signature of 25 miRNAs differentially expressed in CD114^+ versus CD114^- subpopulations (Fig. 5A and Supplementary Table S3). This includes 6 upregulated miRNAs (miR-25, 106b, 17, 18, 20a, 143, and 27) that are repressed upon neuronal lineage differentiation of somatic stem cells (33, 35, 36). In addition, miRNA pathway analysis with 2 independent prediction platforms DIANA (DIANA Lab) and Ingenuity (Ingenuity Systems) each showed enrichment of p53, NGF, and Notch and additional signaling pathways driving differentiation of neuronal and neural crest lineages (Supplementary Tables S4 and S5). As shown in Supplementary Table S3, 15 of the CD114^+ miRNAs 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 miRNAs are known to target p53 and its downstream targets (Fig. 5C) and have been shown to correlate with poor prognosis in high-risk neuroblastoma miRNAs (miRs-17, -19, -20, -25, -92, and -93; ref. 40). These data suggest a role of miRNAs 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

Although 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 4 STAT3 response elements in a promoter upstream of eGFP (STAT3.eGFP-M67) in a dose-dependent manner (Fig. 6A and B). 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–F). 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 that 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 show that expression of G-CSF receptor (CD114), consistently identifies a discrete differentiated subpopulation within neuroblastoma with markedly enhanced tumorigenicity. In addition, this population has many phenotypic similarities to iPSC and ESC cell types including similar miRNA expression, gene expression, and cell-cycle profiles. This subpopulation is distinct from previously characterized tumorigenic populations (tumor-initiating cells) defined by CD133 expression, side population 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 used FACS to isolate CD114\(^+\) subpopulations directly from patient biopsies and animal tumors without additional \textit{ex vivo} manipulation and validated these findings in multiple cell lines. The CD114\(^+\) subpopulation consistently represents approximately 0.5\% to 1.5\% of tumors at diagnosis, and the same percentage was found in all neuroblastoma cell lines analyzed. Finally, CD114\(^+\) cells express a distinct set of genes consistent with an early neuroepithelial/neural crest phenotype, whereas 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 miRNA expression profiles of CD114\(^+\) cells compared with CD114\(^-\) cells support their role as putative neuroblastoma tumor-initiating cells or CSCs 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, and HIF-2\(\alpha\)) and decreased expression of proapoptotic genes (e.g., CASP8 and p53) in the CD114\(^+\) subpopulation. This is consistent with the resistance to

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\caption{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, and NME1). B, CD114\(^+\) cells display increased migratory crest markers (e.g., FGFs, SOX2, NOTCH, and BMPs). C, CD114\(^+\) cells express neuroectodermal markers and CD114\(^-\) cells express increased mesenchymal markers, reflecting the inherent EMT transition occurring during neural crest development (details in Supplementary Fig. S3). Red, up in CD114\(^+\); green, up in CD114\(^-\); \(P < 0.05, 1.2\)-fold cutoff indicated on plots.}
\end{figure}
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 miRNAs with validated targets in the p53 apoptotic response pathway, as well as miRNAs that promote reprogramming (44), stem cell cycle regulation (30), and neuronal differentiation (33). Interestingly, repression of p53 through STAT3 activation seems to contribute to tumorigenesis (45), and small molecules, which activate p53 and repress STAT3 can have potent antitumor effects (46).

As noted earlier, STAT3 is known to mediate chemoresistance, CSC maintenance (18, 19), and neural crest specification (15). We show 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 ESCs. 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 CSC 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 premigratory 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 premigratory 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.
CD114 is activated by the ligand G-CSF. A and B, G-CSF increases the percentage of CD114+ cells in the NGP (A) and SH-SY5Y (B) cell lines. Using flow cytometry, an increased CD114 population (PE) is observed with varying doses of G-CSF (0, 10, and 100 ng/mL) and at varying time points (30 minutes, 2, and 24 hours). C and D, the G-CSF-mediated increase in CD114+ cells is inhibited by the STAT3 inhibitor Stattic in the NGP cell line (C) and the SH-SY5Y cell line (D). Using the 2-hour time point, flow cytometry shows CD114 expression with varying doses of G-CSF (0, 10, and 100 ng/mL) and varying doses of Stattic (0, 3, and 10 μmol/L). E and F, G-CSF induces STAT3 activation, which is inhibited by Stattic in the NGP cell line (E) and the SH-SY5Y cell line (F). Using a STAT3-GFP reporter assay, flow cytometry at the 2-hour time point shows CD114 and GFP coexpression with varying doses of G-CSF (0, 10, and 100 ng/mL) and varying doses of Stattic (0, 3, and 10 μmol/L). 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]. The t test; *, P < 0.05; ***, P < 0.01; ****, P < 0.001. Error bars represent SD.

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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+ CSC 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 premigratory neural crest. These data are supported by the clinical data showing CD114+ subpopulations in primary diagnostic and relapsed tumor biopsies. Although 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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G-CSF Receptor Positive Neuroblastoma Subpopulations Are Enriched in Chemotherapy-Resistant or Relapsed Tumors and Are Highly Tumorigenic

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