Th-MYCN Mice with Caspase-8 Deficiency Develop Advanced Neuroblastoma with Bone Marrow Metastasis

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Abstract

Neuroblastoma, the most common extracranial pediatric solid tumor, is responsible for 15% of all childhood cancer deaths. Patients frequently present at diagnosis with metastatic disease, particularly to the bone marrow. Advances in therapy and understanding of the metastatic process have been limited due, in part, to the lack of animal models harboring bone marrow disease. The widely used transgenic model, the Th-MYCN mouse, exhibits limited metastasis to this site. Here, we establish the first genetic immunocompetent mouse model for metastatic neuroblastoma with enhanced secondary tumors in the bone marrow. This model recapitulates 2 frequent alterations in metastatic neuroblastoma, overexpression of MYCN and loss of caspase-8 expression. Mouse caspase-8 gene was deleted in neural crest lineage cells by crossing a Th-Cre transgenic mouse with a caspase-8 conditional knockout mouse. Although overexpression of MYCN by itself rarely caused bone marrow metastasis, combining MYCN overexpression and caspase-8 deletion significantly enhanced bone marrow metastasis (37% incidence). Microarray expression studies of the primary tumors mRNAs and microRNAs revealed extracellular matrix structural changes, increased expression of genes involved in epithelial to mesenchymal transition, inflammation, and downregulation of miR-7a and miR-29b. These molecular changes have been shown to be associated with tumor progression and activation of the cytokine TGF-β pathway in various tumor models. Cytokine TGF-β can preferentially promote single cell motility and blood-borne metastasis and therefore activation of this pathway may explain the enhanced bone marrow metastasis observed in this animal model. Cancer Res 73(13); 4086–97. © 2013 AACR.

Introduction

Neuroblastoma, a peripheral neural crest-derived childhood solid tumor, is a major medical challenge (1, 2). Half of all patients with neuroblastoma have metastatic disease at diagnosis, which carries a poor prognosis. Primary human neuroblastoma tumors arise in the paraspinal ganglia or the adrenal medulla, whereas disseminated disease appears in the bone marrow (71% of patients), bones (56%), lymph nodes (31%), lungs (3%), and other internal organs (15%–45%). The new International Risk Group classification system of the disease divides the patients to 16 risk groups, with the highest risk group being the one that presents with metastasis to the bone marrow and has only 40%–50% survival rate (2, 3). The most commonly used preclinical transgenic mouse neuroblastoma model, the Th-MYCN model (4), exhibits a limited capacity for metastasis to the bone marrow (<5% incidence). In an attempt to establish an immunocompetent genetic metastatic model for neuroblastoma, we crossed 2 genetically engineered mouse lines, each with a known molecular alteration, common to the aggressive disease, amplification of MYCN, and loss of expression of caspase-8.

MYCN oncogene amplification is frequently seen in aggressive neuroblastoma and occurs in 25%–35% of human patients (5). Caspase-8 is a cysteine endoproteinase that cleaves peptide bonds after aspartic acids (6). In addition to its proapoptotic function as an initiator caspase in the extrinsic receptor-mediated death pathway (6), caspase-8 plays important roles in mediating migration, adhesion, growth, immune response, differentiation, wound healing, fibrosis, and necroptosis in certain cell types (7–9). Suppression of caspase-8 expression by epigenetic silencing occurs in approximately 70% of human
neuroblastomas (10). Loss of caspase-8 has also been associated with enhancement of the tumorigenic potential of SV40 T-antigen transformed mouse embryonic fibroblasts (11) and with providing an advantage in survival and metastasis of engrafted neuroblastoma cell lines (12, 13). Nevertheless, the role of caspase-8 expression has not been tested thus far in vivo in an immunocompetent mouse model, which could have significance given the roles caspase-8 plays in the immune system.

To circumvent the lethality in CASP8−/− mice, Salmena and colleagues developed a conditional knockout mouse in which LoxP sites were introduced into the DNA flanking exons 3 and 4 of the mouse caspase-8 gene (14). We mated these mice with Th-Cre transgenic mice, which express Cre recombinase only in the peripheral neural crest cells and in the brain catecholaminergic neurons starting on day E9.5 (15), to selectively delete caspase-8 in the cells that give rise to neuroblastoma.

Our preliminary results suggested that conditional knockout of caspase-8 alone was insufficient to induce tumor formation. We studied 30 mice (129 × 1/SvJ background) harboring 2 floxed caspase-8 alleles and Th-Cre for 6 to 9 months. Tumors did not develop in any of these mice, suggesting that additional genetic alterations are needed. Thus, we tested the hypothesis that the loss of caspase-8 facilitates MYCN-induced tumor formation or metastasis by using the Th-MYCN transgenic mouse neuroblastoma model (4). Because mouse genetic background influences tumor penetrance in this model (4), we backcrossed all mouse lines at least 6 generations (6−12) to the 129 × 1/SvJ background to ensure that differences in tumor formation were not due to strain variability.

Materials and Methods

Mouse strains

Th-MYCN hemizygote mice were purchased from National Cancer Institute (NCI, Bethesda, Maryland) mouse repository (strain #01XD2) on genetic background 129 × 1/SvJ and kept on this background. Floxed caspase-8 mice were received from Razqallah Hakem (14) on 129 × 1/SvJ, C57BL6 background and backcrossed 6 to 12 generations to the 129 × 1/SvJ background. Th-Cre hemizygote mice were received from Dr. Marcello Rubinstein (15) on a B6.CBF2 background and backcrossed 6 to 12 generations to the 129 × 1/SvJ background. This study was carried out in strict accordance with the instructions in the Guide to Care and Use of Laboratory Animals of the NIH (Bethesda, MD). The protocol was approved by the Institutional Animal Care and Use Committee at St. Jude Children's Research Hospital (IACUC protocol #20). All efforts were made to minimize suffering.

Genotyping of mouse tissues and tumors

Caspase-8 primers:
Sense primer−5‘-CCAGGAAAAAGTTTGGTCTTAGC-3‘
Antisense primer−5‘-GCGCTTTCGTTAGTCTACCTGT-3‘

PCR amplification of the wild-type caspase-8 allele gives a 650-bp band; the caspase-8 floxed (exons 3 and 4) produces a band of 850 bp. Thus, the deleted cre recombinase digested DNA band is 200 bp.

Th-Cre primers:
Sense primer−5‘-ATGTCCCAATTATACCTGACCTACAC-3‘
Antisense primer−5‘-CTAATCGGACCTTCC-3‘

Th-MYCN primers:
Sense primer−5‘-CGACCACAGGCCCCCTCAGTA-3‘
Antisense primer−5‘-CAGGCTCAAGTCATCTTCCA-3‘

Quantitative RT-PCR for mouse caspase-8

Total RNA was extracted from mouse tumors with Trizol reagent (Life Technologies) and reverse transcribed with Superscript II (Life Technologies). Primers for mouse caspase-8 were used to PCR amplify a 120-bp fragment from exon 1 to exon 2 of the transcript, thus recognizing wild-type message and floxed caspase-8 message if stable. Primers used: sense primer−5‘-CCCTACAGGGCTAGCTCTTT-3‘ antisense primer−5‘-CAGGCTCAAGTCATCTTCCA-3‘.

Antibodies and immunohistochemistry

For Western blots, we used antibodies: mouse caspase-8, (Cell Signaling cat. no. 4927, Dil 1:1,000), MYCN (Cell Signaling cat. no. 9405, Dil 1:1,000), and Actin (Santa Cruz cat. no. 1616, Dil 1:2,000). For staining paraffin-embedded formalin-fixed tumors and tissues we used antibodies: caspase-8 IH11 (Abcam cat. no. ab119892, dilution 1:200) and anti-caspase-3, Ki67, PGP9.5, synapthophysin, chromogranin A, and NFP as described previously (16).

Ultrasound imaging

Ultrasound imaging of the mouse tumors was carried out as described (16) using the VisualSonics VEVO-770 high frequency ultrasound system (VisualSonics).

Microarray analysis of neuroblastoma tumor samples

Total RNA was extracted from primary mouse tumors using TRIzol reagent (Life Technologies). Samples were assayed with the Affymetrix Mouse 430v2 GeneChip array and the Agilent mouse microRNA v18 array microRNA. Data was summarized by the RMA protocol (17) using Partek Genomics suite 6.6. Outlier samples were detected and removed by principal component analysis (PCA) and a batch correction corresponding to hybridization date was applied. The data were defined by class and a series of unequal variance t tests were applied to compare classes. Data was visualized and filtered by P value (0.05) and log ratio and submitted to Gene Set Enrichment Analysis (GSEA, www.broadinstitute.org/gsea) to assess Gene Ontology (GO) enrichment. The mRNAs targeted by down-regulated microRNAs in the primary tumors samples with bone marrow metastasis were predicted using MirTarget2 (18), and followed by an enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) at the National Institute of Allergy and Infectious Diseases at the National Institutes of Health (19). All array data have been deposited in Gene Expression Omnibus at National Center for Biotechnology Information, and are accessible through GSE42548 (mRNA) and GSE42254 (microRNA). Select
array data were validated by quantitative real-time PCR (qRT-PCR) using TaqMan gene expression assays as described in Supplementary Fig. S1.

Results
Establishing a neuroblastoma mouse model with MYCN amplification and caspase-8 deficiency

Neural crest-specific deletion of caspase-8 in mice was achieved by mating floxed and/or knockout caspase-8 alleles mice with hemizygous Th-Cre mice and then with hemizygous Th-MYCN mice (Fig. 1A). Four genotypes of mice were established: caspase-8<sup>fl/fl</sup> (floxed/floxed), caspase-8<sup>fl/ko</sup> (floxed/knockout), caspase-8<sup>wt/ko</sup> (wild-type/knockout), and the control caspase-8<sup>wt/wt</sup> (wild-type/wild-type). Cre-mediated caspase-8 deletion in the caspase-8<sup>fl/fl</sup> primary tumors was assayed by genomic PCR (Fig. 1B, TU lanes). Caspase-8 was deleted in the primary tumors at varying efficiencies ranging from a low percentage of cells to complete deletion (Fig. 1B).

Figure 1. Characterization of the tumors formed in the Th-MYCN, Th-Cre, caspase-8 deleted mice (fl/fl, fl/ko, or wt/ko caspase-8). A, deficient caspase-8, Th-MYCN mice were obtained by mating floxed or heterozygous caspase-8 alleles mice with hemizygous Th-Cre mice and then with hemizygous Th-MYCN mice. B, validation of caspase-8 deletion status in the primary Th-MYCN, Th-Cre, caspase-8<sup>fl/fl</sup> neuroblastoma tumors using genomic PCR. Tail DNA (TA) from mice with tumors and primary tumor DNA (TU) was amplified using primers flanking exons 3 and 4 of mouse caspase-8 gene. Only the Casp8 bands were detected in the tail samples, whereas the Casp8<sup>fl</sup> and the deleted caspase-8 bands (Casp8<sup>del</sup>) were detected in varying levels in the primary tumors. PCR amplification of the casp8<sup>3-4</sup> allele (Casp8<sup>fl</sup>) generates a band of 850 bp and the deleted band (Casp8<sup>del</sup>) is 200 bp. C, Western blot analyses of primary neuroblastoma tumors with an anti-mouse caspase-8 antibody. /<sup>-/-</sup> mice are the Casp8<sup>fl/fl</sup> and caspase8<sup>fl/ko</sup> mice. D, qRT-PCR analysis for mouse caspase-8 was conducted on total RNA extracted from the primary tumors of the following Th-MYCN, Th-Cre mouse genetic groups: wt/wt caspase-8, <i>n</i> = 8; wt/ko caspase-8, <i>n</i> = 5; *, <i>P</i> = 0.007 compared with wt/wt, fl/ko caspase-8, <i>n</i> = 6; †, <i>P</i> = 0.003 compared with wt/wt; <i>P</i> values determined by t test. E, Kaplan–Meier survival curves of the Th-MYCN, Th-Cre mice with wt/wt caspase-8 (<i>n</i> = 85) or fl/fl caspase-8 (<i>n</i> = 90, <i>P</i> = 0.12 compared with wt/wt), or fl/ko caspase-8 (<i>n</i> = 90, <i>P</i> = 0.30 compared with wt/wt), or caspase-8 heterozygous wt/ko (<i>n</i> = 43, <i>P</i> = 0.21 compared with wt/wt). <i>P</i> values were determined by Gehan–Breslow–Wilcoxon log-rank test.
Caspase-8 expression was also assessed in the primary tumors at the mRNA and protein levels. Most Th-MYCN, Th-Cre, wt/wt caspase-8, or Th-MYCN, Th-Cre, caspase-8 deficient mice (fl/fl and fl/ko caspase-8) in the paravertebral ganglia, either surrounding or near the aorta (purple; 81 ± 7% of the cases) or near the kidney (green) and adrenal gland (19 ± 7% of the cases); n = 28 for the wt caspase-8 group and n = 41 for the caspase-8-deficient group. B, typical examples of preneoplastic islets observed in mice with caspase-8 wt/wt, Th-MYCN (n = 10) or deficient caspase-8, Th-MYCN (n = 9) at ages 10, 21, and 49 days are shown (H&E). No preneoplastic islets were observed in mice without MYCN. Arrows point to LN, lymph node; PN, preneoplastic lesions; BM, Bone marrow; A, aorta; VE, vertebra; GN, ganglia. Scale bar, 50 μm.

Figure 2. Caspase-8 deficiency does not change the incidence, latency, location, or growth rate of the mouse primary neuroblastoma tumors. A, primary tumors could be identified by ultrasound imaging starting at mouse age 6 to 7 weeks. Tumors (red) arose in Th-MYCN, Th-Cre, wt/wt caspase-8, or Th-MYCN, Th-Cre, caspase-8 deficient mice (fl/fl and fl/ko caspase-8) in the paravertebral ganglia, either surrounding or near the aorta (purple; 81 ± 7% of the cases) or near the kidney (green) and adrenal gland (19 ± 7% of the cases); n = 28 for the wt caspase-8 group and n = 41 for the caspase-8-deficient group. B, typical examples of preneoplastic islets observed in mice with caspase-8 wt/wt, Th-MYCN (n = 10) or deficient caspase-8, Th-MYCN (n = 9) at ages 10, 21, and 49 days are shown (H&E). No preneoplastic islets were observed in mice without MYCN. Arrows point to LN, lymph node; PN, preneoplastic lesions; BM, Bone marrow; A, aorta; VE, vertebra; GN, ganglia. Scale bar, 50 μm.

Caspase-8 expression was also assessed in the primary tumors at the mRNA and protein levels. Most Th-MYCN, Th-Cre, caspase-8 wt/wt (labeled +/+ ) primary abdominal tumors (19/20) expressed endogenous caspase-8, as determined by RNA protection (data not shown) and immunoblot assays (Fig. 1C). Caspase-8 expression in the wt/ko (+/−) tumors was reduced to approximately 50% of wild type, whereas the Th-MYCN, fl/fl, and fl/ko caspase-8 alleles tumors (labeled −/− ) had lower caspase-8 expression, around 20% average, suggesting that the floxed allele is hypomorphic (Fig. 1C and D). Levels of caspase-8 protein in the primary tumors was also determined by immunohistochemistry with anti-caspase-8 antibody and found to be low or undetectable (Supplementary Fig. S2). The primary deficient caspase-8 tumors stained for neuronal markers typical to neuroblastoma as synapthophysin and PGP9.5 (Supplementary Fig. S3) and were indistinguishable from the wild-type tumors in their incidence, latency, mass (Figs. 1E, 2, 3A and Supplementary Fig. S4). In addition, no statistical significant differences were found in the number of proliferating cells in the primary tumors as determined by Ki-67 staining (both groups had >95% positive cells, data not shown), or in the number of primary tumor cells undergoing apoptosis as determined by immunostaining with cleaved caspase-3 (Supplementary Fig. S5). MYCN expression comparing primary tumors with and without caspase-8 was also not significantly changed (Supplementary Fig. S6).
Figure 3. Deficiency of caspase-8 in the Th-MYCN mouse model enhances metastasis preferentially to the BM. A, no significant difference in the mean mass of the primary neuroblastoma tumors of the Casp8wt/wt, Th-MYCN, mouse group, and the caspase-8 deficient (Casp8fl/fl and Casp8fl/mice, labeled /−/−), Th-MYCN mice at the time interval metastasis was assayed (14 ± 6-week-old mice). Data are expressed as mean ± SEM (n = 12 for both mouse groups). B, a significantly higher incidence of metastatic neuroblastoma was found in the BM of the caspase-8–deficient mouse group compared with the wt caspase-8 group (n = 21, wt caspase-8 mice and n = 28, caspase-8–deleted; *P = 0.014; P determined by Fisher exact test). C, metastatic incidence to the ovaries did not differ statistically between the wt caspase-8 group and the deficient caspase-8 group (n = 7, wt caspase-8; n = 9, caspase-8–deleted; P = 0.10 by Fisher exact test). D, typical examples of H&E staining of the secondary tumors in the BM of caspase-8–deficient mice in long bones, such as the femur, tibia, and humerus. The BM metastatic tumor cells stained positive for the neuronal markers synaptophysin and tyrosine hydroxylase. Areas of tumor cells in the BM are marked with arrows and asterisks indicate bones. Th-MYCN mouse BM with no metastasis served as control. Scale bar is 50 μm except in images 1, 3, 4, and 6 from top left it is 200 μm. BM, bone marrow.
Growth characterization of the primary neuroblastoma tumors in the genetically engineered caspase-8-deficient Th-MYCΝ mouse model

Comparison of overall survival and primary tumor onset between the different mouse genetic groups, all on the 129/SvJ background, and harboring wild-type or deficient caspase-8 yielded no significant statistical difference (Fig. 1E and Supplementary Fig. S4) with the limitation that all mice had to be sacrificed after 16 to 17 weeks due to high primary tumor burden. To determine whether there were any changes in the growth or location of the primary tumors before this point, we monitored weekly the tumors in both caspase-8-expressing and caspase-8-deleted Th-MYCΝ mice by ultrasound imaging (Fig. 2A). No significant differences were found in the frequency or initial location of the tumors. Primary tumors in all mice groups were located in areas surrounding the aorta (81 ± 7% in both groups) or near the adrenal gland or kidney (19 ± 7% in both groups; Fig. 2A). In addition, we examined mice at earlier time points (ages 10, 21, and 49 days) to determine whether there were variations in the number of initiating preneoplastic hyperplasia cells arising during development in the paraspinal ganglia (Fig. 2B). These studies were based on previous work that showed higher frequencies of hyperplastic cells in the paraspinal ganglia of heterozygous Th-MYCΝ mice compared with 129/SvJ wild-type mice littersmates 1 to 5 weeks after birth (20, 21). These experiments as well did not reveal any differences in the appearance or incidence of hyperplastic neuroblasts between Th-MYCΝ mice with and without caspase-8 expression (Fig. 2B). Thus, we conclude that caspase-8 deficiency does not significantly contribute to initial primary tumor formation.

Caspase-8-deficient Th-MYCΝ mice have preferentially enhanced neuroblastoma metastasis to the bone marrow

We then screened Th-MYCΝ mice harboring advanced-stage primary tumors (ages 9–17 weeks, Fig. 3A) from the wild-type and caspase-8-deficient groups for secondary metastatic tumors to determine whether caspase-8 plays a role in metastasis in vivo. Detailed necropsy of all major organs was carried out (Fig. 3B–D). In agreement with the data described above, no statistical significant difference was found in the average size of the initial primary tumors (Fig. 3A). We did detect, however, a significant difference in the frequency of secondary tumors in the bone marrow (Fig. 3B and Table 1). Eighty sections were cut from various bones of the mice, including the sternum, the long bones, and the vertebra. Tumor cells were identified and the number of bone marrow tumor foci was determined by hematoxylin and eosin (H&E) staining and immunohistochemistry with the neuroblastoma markers synapthophysin, tyrosine hydroxylase, and/or PGP9.5 (Table 1). From 27 mice with deficiency in caspase-8 (16 fl/fl and 11 wt/wt, labeled −/−), 10 mice (37%) had metastatic foci in their bone marrow within 10 to 17 weeks. The size of the foci ranged from clusters of 5 to 10 tumor cells to large sheets compromising up to a quarter of the bone marrow cells’ population. Twenty one mice with Caspase-8+/+ were screened for bone marrow metastasis and only one mouse (4.5% incidence) had bone marrow metastasis at weeks 10 to 17 (P = 0.014, Fig. 3B and D and Table 1). Bone marrow tumor cells were negative for caspase-8 expression as determined by immunostaining (Supplementary Fig. S2) and had very low apoptosis levels (about 1%) measured by immunostaining for cleaved caspase-3 protein (Supplementary Fig. S5).

Table 1. Metastasis to the bone marrow in the Th-MYCΝ-deleted caspase-8 mice

<table>
<thead>
<tr>
<th>Casp8 genotype</th>
<th>Mouse number(s)</th>
<th>Age (weeks)</th>
<th>Sternum</th>
<th>Long bones</th>
<th>Vertebra</th>
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<tr>
<td>wt/wt</td>
<td>1–20</td>
<td>10–17</td>
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<tr>
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<td>10</td>
<td>N</td>
<td>2a</td>
<td>1</td>
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<tr>
<td>fl/ko</td>
<td>1–10</td>
<td>10–17</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>fl/ko</td>
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<td>9</td>
<td>N</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
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<td>N</td>
<td>1</td>
<td>1</td>
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<tr>
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<td>17</td>
<td>N</td>
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<td>1</td>
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</table>

NOTE: Mice were generated from at least 3 to 4 mating combinations during a 2-year period.

n = 21 for wt/wt casp-8 mice; n = 16 for fl/ko casp-8 mice; and n = 11 for fl/fl casp-8 mice

Abbreviation: N, No tumor cells detected.

aNeuroblastoma cells were confirmed by immunohistochemistry with synapthophysin, tyrosine hydroxylase, and/or PGP9.5 antibodies and by morphology after H&E staining. Numbers in table indicate independent foci ranging in size from a cluster of 5 to 10 tumor cells to large sheets of cells.
The incidence of metastatic dissemination to other organs of the mice, other than the bone marrow (Supplementary Fig. S7) was not significantly enhanced in the caspase-8–deficient group (shown for ovaries, Fig. 3C). The secondary metastatic lesions stained positive for neuronal markers typical for neuroblastoma (Supplementary Fig. S7 shown for PGP9.5). Metastatic ovaries were stained for cleaved caspase-3 and had 1% to 2% of cells undergoing apoptosis, equal to the percentage of cells undergoing apoptosis in the matching primary tumors (Supplementary Fig. S5).

Array expression analysis of the caspase-8–deficient primary tumors reveals changes in extracellular matrix proteins and tumor cell–ECM interacting proteins

We compared the mRNA and microRNA expression profiles of a group of 6 primary tumors with wild-type caspase-8 levels in animals with no bone marrow metastasis to a group of 7 primary tumors with deficient caspase-8 levels in animals that had bone marrow metastasis. Heat map of the top 20 statistically significant genes that differ in expression between the 2 groups are shown in Fig. 4A. The list included genes known to have a role in metastasis, epithelial-to-mesenchymal transition (EMT), cell detachment from the extracellular matrix (ECM), fibrosis, wound healing, and inflammation as Timp2 (tissue factor pathway inhibitor-2, a serine protease inhibitor (22), Snai2 (snail homolog 2 known also as SLUG, a neural crest transcription factor (23), Myct1, myc target 1, a direct c-myc target gene (24), Serpinh1, a serine or cysteine peptidase inhibitor known also as Hsp47, a collagen-specific molecular chaperone (25), Emcn, a mucin-like sialoglycoprotein that interferes with the assembly of focal adhesion complexes and inhibits interaction between cells and the ECM (26) and Fos (27, 28). The complete list of genes that are different between the groups and had statistical significance above \( P = 0.05 \) is in Supplementary Table S1. The list included MMP-15, matrix metalloproteinase 15 (29), 1.3-fold increase in the primary tumors with bone marrow metastasis. The complete list of mRNAs was submitted to GSEA and the top enriched gene set was the ECM structural constituents with a nominal \( P \) value of 0.008 (Fig. 5). This gene set was included upregulation in expression of Timp2, LAMA4 (laminin alpha 4; refs. 30, 31), FBLN2 (fibulin 2; ref. 32), PRELP (ECM protein that functions to anchor basement membranes to the underlying connective tissues; ref. 33), COL4A2 (collagen type 4 alpha 2, (refs. 34, 35; Fig. 5), mRNA analysis was done also on a group of 10 deficient caspase-8 mice that did not show metastasis to bone marrow by histology (Fig. 4B). The top genes that came up are in Fig. 4B and all the gene changes with \( P < 0.05 \) are included in Supplementary Table S2. This tumor group had 4 genes that overlapped with the metastatic group: Fos (downregulation), Lnc11 (downregulation), Emcn (upregulation), and Myct1 (upregulation), suggesting these gene expression changes occur before metastasis is observed in the bone marrow.

Analysis of microRNA expression was done on primary tumors with wild-type caspase-8 and no bone marrow metastasis compared with caspase-8–deficient primary tumors with detected bone marrow metastasis (Supplementary Table S3). The top microRNAs changes (fold changes >30%) are shown in Fig. 6, and included downregulation of miR-29b (1.86 fold) and miR-7a (1.43-fold) expression in the deficient caspase-8 group. Suppression of miR-29 by TGF-β1/Smad3 signaling has been shown to promote collagen and other ECM components expression and to promote renal fibrosis (36, 37). MiR-7 was shown to be suppressed in human neuroblastoma (38), breast cancer, and glioblastoma and its downregulation was associated with tumor metastasis (38, 39). Its forced expression in tumor cells inhibited EMT transition and metastasis of breast cancer cells via targeting focal adhesion kinase expression (39). The microRNAs targeted by the downregulated microRNAs in the primary tumors with bone marrow metastasis in this study were predicted by MirTarget2 and analyzed by GO enrichment. Interestingly, this analysis showed highly enriched expression (\( P < 10^{-6} \)) for the identical gene set that came up in the mRNA analysis, the gene set of the ECM structural components as collagens and laminins.

Discussion

Tumor suppressor genes are defined as genes whose loss-of-function in tumor cells contribute to the formation and/or maintenance of the tumor phenotype. The findings presented in this report provide proof that caspase-8 function as a metastatic bone marrow tumor suppressor gene in neuroblastoma. Loss of caspase-8 expression does not affect primary tumor formation in the Th-MYCN mouse but it does promote selective metastasis formation and maintenance in the bone marrow. These results are in accordance with previous studies in mice that showed that a deficiency of TRAIL-R, a protein at the apex of the caspase-8–signaling pathway, enhances metastasis of squamous cell carcinoma to lymph nodes without affecting primary tumor development (40). It also supports the recent clinical findings that a lack of caspase-8 correlates with relapse in human neuroblastoma patients evident by bone marrow metastatic disease (10).

Caspase-8 suppression by epigenetic silencing has also been reported in other human tumors including small cell lung carcinoma, primitive neuroectodermal tumors, alveolar rhabdomyosarcoma, medulloblastoma, and retinoblastoma (41). It will be of great interest to investigate whether caspase-8 loss can also enhance bone marrow metastasis in these tumors.

Deletion of caspase-8 in the mouse primary tumor in this animal model was a driver genetic event that led to approximately 7-fold increase in Th-MYCN-induced preferential metastasis incidence to the bone marrow (from 5% average incidence to 37%). Interestingly, metastasis to other organs including the abdominal and pulmonary lymph nodes or lungs was not significantly different. This is in contrast to what we previously found by engrafting human cells in the chick embryo or injecting human neuroblastoma tumor cells deficient in caspase-8 expression directly to the blood stream of immunodeficient mice (12). In these experiments, metastasis incidence of cells with decreased caspase-8 expression was increased to both the bone marrow and to
the lungs at the same frequency. In the animal model studied in this work, we concentrated on the effect of the developing primary tumors on metastasis and determined the transcriptional changes occurring at the primary tumor before and after metastasis are detected in the bone marrow. Although we did not find statistical significant changes in the percentage of cells undergoing apoptosis in the primary tumors, we found changes in the ECM structure of the caspase-8-deficient primary tumors as upregulated expression of collagen 4A2 and laminin α4 once metastasis is detected in the bone marrow.

Figure 4. Heat map of the top 20 genes that are differentially expressed in the primary tumors (P < 0.05).
marrow. These ECM changes are likely to cause increased stiffness of the primary tumor and changes in mechanotransduction properties, which have been shown in different tumor types to correlate with advanced stage of disease (42–44). In addition, we see transcriptional changes that would suggest increased motility and migration of the caspase-8-deficient tumor cells by upregulated expression of genes involved in EMT (as Snai2, Twist1, and TfpI2), enhanced detachment of the tumor cells from the ECM (effected by Emcn, PRELP, miR-7), and increased fibrosis (accumulation of ECM proteins and downregulation of miR-29b). Interestingly, EMT changes have been observed recently in vivo in a breast cancer animal model specifically when the oncogene myc was amplified (45). ECM constituents change as accumulation of collagens and laminins has been described in fibrosis of tissues and tumors (9, 34, 35, 37) and in wound response processes (8). Caspase-8 downregulation has been linked to wound processes in vivo in which accumulation of collagen and other ECM structural proteins occur (8) and fibrosis is seen in mice that have deficient caspase-8 in their epidermal tissues (9). In addition, caspase-8 has direct interaction with the ECM proteins by being in complexes with integrins (12, 46) and as a part of the focal adhesion complex (7). Deficiency of caspase-8 in the primary tumors thus could cause changes in the ECM structure and/or possible activation of a wound-like process that triggers deposition of ECM proteins by fibroblasts in the stroma and can activate production of various cytokines. The cytokine TGF-β is one of the major cytokines to be activated in response to wound/injury processes (47), fibrosis (36, 37), or as direct changes in the stiffness and mechanotransduction properties of the ECM (48). Importantly for the bone marrow preferential metastasis, TGF-β has been shown by intravital imaging experiments to be transiently and locally activated in breast cancer motile cells and switch the cells from cohesive to single cell motility (49). Cells restricted to collective invasion were capable of lymphatic invasion but not blood borne metastasis (49). Thus transient activation of TGF-β preferentially in the caspase-8 primary tumors as result of ECM remodeling and/or fibrosis can potentially promote single cell motility, increase invasion to the blood vessels, and enhance bone marrow metastasis. Interestingly, we see upregulated expression in the caspase-8-deficient primary tumors of genes known to be induced by TGF-β as Tgif2 and Tgfβ1i1 (Supplementary Table S1) and downregulation of miR-29b associated with TGF-β activation (36, 37). Intravital imaging experiments in our caspase-8-deficient mouse model could shed light whether indeed increased blood borne metastasis of single cells contributes to the preferred metastasis to the bone marrow.
In this work, we observed downregulation of microRNA-7 expression in the caspase-8–deficient mouse primary tumors, which has been described recently in human neuroblastoma and was associated with metastatic advanced stage (38).

Finally, our finding that the loss of caspase-8 in the mouse primary tumor cells significantly promotes metastasis to the bone marrow, the most common site for metastasis in human neuroblastoma (71% of patients at diagnosis; refs. 1–3), indicates that this Th-MYCN/caspase-8–deleted animal model should be useful for testing therapies for metastatic neuroblastoma. Ongoing experiments in our laboratory are aimed at purifying the metastatic bone marrow cells for gene expression analysis and surgically resecting or debulking the primary tumors to allow even further progression of the metastatic process in the bone marrow. Our preliminary experiments also indicate the feasibility of establishing orthotopic allograft models by passaging the primary tumor cells from mice to mice and thus establishing uniform animal cohorts suitable for drug screenings (16). Labeling the primary and secondary metastatic tumor cells in vivo in this animal system by breeding to a fluorescence and/or a luciferase mouse reporter line in which expression of the reporter gene is cre-recombinase mediated (50) is currently in progress and should facilitate the monitoring of tumor cell growth and responses to therapeutic modalities.

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

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Acknowledgments
The authors thank all members of Dr. Jill Lahti’s laboratory for discussion, Joshua Stokes from St. Jude BMC and Judith Hyle for help with preparing the figures, the staff at the St. Jude Veterinary Pathology Core including Sean Savage, Patricia J. Varner, Dorothy Bush, and Pamela Johnson, the staff at the St. Jude Hartwell Center for Bioinformatics and Biotechnology including Dr. Geoffrey Neale, Ridout Granger, Deanna Narve, Melanie Loyd, and John Morris, and the staff at St. Jude Animal Imaging Shared Resource for assistance.

Grant Support
The work was funded by NIH grant RO1 CA067938 (J.M. Lahti), Comprehensive Cancer Center Support Grant P30 CA021765, the American Lebanese Associate Charities (ALSAC), and NCI grants PO1 CA18403 and RO1 CA102321 (W.A. Weiss.)

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Received July 6, 2012; revised March 13, 2013; accepted March 14, 2013; published OnlineFirst March 27, 2013.

www.aacrjournals.org
Cancer Res; 73(13) July 1, 2013
4095

Figure 6. MicroRNAs that are differentially expressed in the caspase-8–deficient primary tumors.
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Cancer Res 2013;73:4086-4097. Published OnlineFirst March 27, 2013.

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