c-MYC Drives Breast Cancer Metastasis to the Brain, but Promotes Synthetic Lethality with TRAIL

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Abstract

Brain metastasis in breast cancer is particularly deadly, but effective treatments remain out of reach due to insufficient information about the mechanisms underlying brain metastasis and the potential vulnerabilities of brain-metastatic breast cancer cells. Here, human breast cancer cells and their brain-metastatic derivatives (BrMs) were used to investigate synthetic lethal interactions in BrMs. First, it was demonstrated that c-MYC activity is increased in BrMs and is required for their brain-metastatic ability in a mouse xenograft model. Specifically, c-MYC enhanced brain metastasis by facilitating the following processes within the brain microenvironment: (i) invasive growth of BrMs, (ii) macrophage infiltration, and (iii) GAP junction formation between BrMs and astrocytes by upregulating connexin 43 (GJA1/Cx43). Furthermore, RNA-sequencing (RNA-seq) analysis uncovered a set of c-MYC-regulated genes whose expression is associated with higher risk for brain metastasis in breast cancer patients. Paradoxically, however, increased c-MYC activity in BrMs rendered them more susceptible to TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis. In summary, these data not only reveal the brain metastasis-promoting role of c-MYC and a subsequent synthetic lethality with TRAIL, but also delineate the underlying mechanism. This suggests TRAIL-based approaches as potential therapeutic options for brain-metastatic breast cancer.

Implications: This study discovers a paradoxical role of c-MYC in promoting metastasis to the brain and in rendering brain-metastatic breast cancer cells more susceptible to TRAIL, which suggests the existence of an Achilles’ heel, thus providing a new therapeutic opportunity for breast cancer patients.

Introduction

To successfully form distant metastases, migrating cancer cells must adapt to new microenvironments within secondary organs. This process of adaptation typically involves changes in gene expression and in the signal transduction profiles of cancer cells that will eventually give rise to the distinct features of organ-tropic metastatic cells (1). Although several genes that contribute to organ-specific metastasis have been identified, little is known about whether these genes can cause synthetic lethality in organ-specific metastatic cancer cells when combined with anticancer agents.

Synthetic lethality in cancer drug discovery refers to the selective killing of cancer cells harboring a specific mutation by compromising the function of a secondary gene (2). This approach is widely used as an alternative to directly targeting the oncogene, which is often challenging. For example, inhibition of PLK1, STK33, or TBK1 can induce death in several types of cancer cells containing KRAS mutations (3–5). Synthetic lethal approaches are also being used to discover therapeutic agents that cause lethality in cancer cells containing molecular lesions in tumor suppressor genes. The best-known example is the synthetic lethality of cancers lacking breast cancer 1 or 2 (BRCA1 or 2) induced by treatment with an inhibitor of poly (ADP-ribose) polymerase 1 (PARP1; refs. 6, 7).

Although the studies mentioned above provide insight into the synthetic lethality of cancers with specific genetic lesions, synthetic lethal interactions in organ-tropic metastatic cancer cells are largely unexplored.

Breast cancer metastasizes to several organs including lungs, brain, bones, and liver, and a majority of breast cancer mortality is attributed to metastasis (8, 9). Especially, brain metastasis is generally associated with shorter survival (10) and there are no effective treatments for this disease. Thus, in the present study, we investigated synthetic lethal interactions in brain-metastatic breast cancer. We found that c-MYC function is essential for breast cancer metastasis to the brain, but increased c-MYC activity makes brain-metastatic breast cancer cells (BrM-BCC) highly sensitive to
TRAIL-induced apoptosis. Thus, our study provides evidence of a synthetic lethal interaction between c-MYC–induced brain-metastatic potential and TRAIL.

**Materials and Methods**

**Expression constructs**

**Cell viability assays**
MDA-MB-231, MCF7, and their metastatic derivatives were treated with TRAIL of for 24 and 72 hours, respectively. Cell viability was measured using the CellTiter-Glo system according to the manufacturer's protocol (Promega).

**Animal studies**
All animal works were done in accordance with a protocol approved by the KAIST Institutional Animal Care and Usage Committee. For subcutaneous injections, 4 × 10^5 of 231-BrM cells were mixed with growth factor-reduced matrigel (3:1), followed by injection into the lower flanks of 6- to 8-week-old BALB/c Nude mice (Orient Bio). Tumor volumes were calculated using (width)^2 × (length)/2. For brain metastasis assays, 1 × 10^3 of 231-BrM or 2 × 10^5 of 231-Par cells were intracranially injected and extracted brains were imaged with IVIS system. MCF7-HER2 cells (1 × 10^6) were intracranially injected after pretreating mice with 10 μg of β-Estradiol (Sigma-Aldrich) for 3 days. β-Estradiol was administered everyday of imaging until the completion of the experiments. Brain metastases were assessed based on the weight loss as well as the presence of behavioral abnormalities. For TRAIL treatment assays, mice were treated with 500 μg of APO2/TRAIL by intraperitoneal bolus injection every day for 14 days, starting 8 days after intracardiac injection of 231-BrM.

**Cell culture and cell line generation**
MDA-MB-231 and MCF7-HER2 cell lines were cultured in Dulbecco's modified Eagles medium (DMEM) and RPMI1640 with 10% FBS, respectively. Human astrocytes were purchased from ScientCell and cultured in complete Astrocyte Media (ScienCell). c-MYC knockdown and rescued 231-BrM cell lines were generated by using retro- and lentiviral system, respectively. c-MYC–overexpressing 231-Par and MCF7-Par and Cx43-rescued cell lines were generated by a lentiviral system. Cell lines were recently authenticated by DNA fingerprinting analysis, and Mycoplasma was periodically tested by e-myco plus detection kit (Intron).

**Glutamine consumption assay**
Conditioned media (CM) were collected 24 hours after cell plating and glutamine concentration were measured with Glutamine/Glutamate-Glo Assay kit (Promega) according to the manufacturer's protocol. Glutamine consumption rate was calculated by [glutamine]_control media − [glutamine]_CM and normalized by cell numbers.

**Bax oligomerization assays**
Cells were homogenized by using a 23-gauge syringe and centrifuged at 2,500 RPM for 10 minutes at 4°C. Supernatant was collected andcentrifuged at 8,000 RPM for 10 minutes at 4°C. Pellet was resuspended with DPBS and treated with BMH (final 1 mmol/L) for 30 minutes in ice. Samples were quenched with 25 mmol/L DTT for 10 minutes at room temperature and subjected to Western blotting analysis.

**Immunostaining and microscopic analysis**
Brains were isolated and embedded in OCT (Leica). Ten-μm-thick sections were subjected to immune-fluorescent staining with CD45 and F4/80 antibodies and microscopic analysis was performed using Zeiss Imager M1 microscope at ×20 magnification.

**Macrowell-based three-dimensional (3D) tumor sphere formation assay**
The agarose was dissolved by boiling in DMEM and stained to fabricate microwell to generate nonadherent surfaces. Single cells were seeded onto microwell and cultured for 7 days. Microscopic analysis was performed with phase-contrast microscope (Leica). The area and perimeter of each sphere was measured by using ImageJ software. The circularity of spheres was calculated by 4π × (area)/(perimeter)^2.

**3D invasion assays**
Tumor spheres were embedded in hyaluronic acid (HA)-collagen1 hydrogels. After 72 hours, spheres were fixed with 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with Triton X-100 (Sigma-Aldrich). Spheres were then stained with DAPI and Phalloidin (Sigma-Aldrich). Microscopic analysis was performed with phase-contrast microscope (Leica) and a confocal microscope (Nikon). The invading area for initial and final time point was measured using ImageJ software. The invasion was quantified by calculating the ratio of invading area changes from initial to final time point, comparing with the initial invading area (A = (Afinal − Ainitial) × 100/Ainitial).

**Macrophage (RAW 264.7) recruitment assay**
Tumor spheres and RAW 264.7 cells were labeled with CellTracker Green CMFDA and Red CMTPX (Invitrogen), respectively. Tumor spheres were embedded with HA-collagen type I hydrogels. RAW 264.7 cells mixed with the same matrix were added onto tumor sphere–containing HA-collagen matrix. RAW cell infiltration was observed for 24 hours using confocal microscope (Nikon). The infiltrated RAW 264.7 cells were quantified by counting the infiltrated cells area inside the boundary of microwell.

**Dye transfer assay**
Suspended cancer cells were labeled with 3 μmol/L of Calcein Red-Orange, AM dye (Invitrogen) for 30 minutes at 37°C. Labeled cancer cells were mixed with unlabeled human astrocytes at a ratio of 4:1 and incubated for 5 hours at 37°C. Dye transferred astrocytes were analyzed using FACS LSFortessa.

**qRT-PCR**
Four nanograms of cDNA was subjected to qRT-PCR by using an CFX-96 Real-Time PCR System (Bio-Rad). HPRT was used as an endogenous control. Primer sequences are provided in Supplementary Materials and Methods.

**Genome-wide RNA-seq analysis and MYC-BrMGS derivation**
RNAs isolated from 231 cell lines were subjected to strand-specific whole transcriptome analysis by Illumina Next-seq 500 using 75 nt single-end methodology (SE75). The RNA sequence
reads were aligned to the human GRCh38 using the STAR software (ver. 2.5). For quantification of mRNA, the counts per million fragments mapped of each sample were calculated. EdgeR package that uses a negative binomial model was used to detect differentially expressed genes from RNA-seq count data. Genes with the fold difference of >1.5 and P value <0.001 between shcntr and shMYC as well as shMYC-Vec and rescue groups were selected. A gene set composed of these genes (total 125) and c-MYC was defined as MYC-BrMGS. The data are available in the NCBI Gene-Expression Omnibus public database under accession number GSE106312.

Clinical sample analysis
EMIC 192 (GSE12276) and EMC 286 (GSE2034) data sets were used to analyze association between c-MYC-BrMGS and brain metastasis-free survival (BrMFS). To divide breast cancer patients into subgroups based on expression levels of c-MYC-BrMGS, a hierarchical clustering algorithm was applied that used the centered correlation coefficient as the measure of similarity and centroid linkage clustering. For cluster analysis, gene-expression data were normalized by the quantile method, log₂-transformed, and median-centered across genes and samples. The Kaplan–Meier method was used to calculate the time to metastasis, and differences between the times were assessed using log-rank statistics.

FACS analysis for apoptosis
Cells were treated with 10 ng/mL of TRAIL for 2 hours and stained with Annexin V-Alexa488 (Invitrogen) and 7-AAD solution (BD), followed by flow cytometry analysis.

Statistical analysis
Statistical analysis was performed by an unpaired Student t test, two-way ANOVA, Mann–Whitney tests, or log-rank test as indicated in each figure legend. P values of <0.05 were considered as statistically significant. Results are reported as mean ± SEM. Detailed information is available in Supplementary Materials and Methods.

Results
Elevated c-MYC activity in highly BrM-BCCs is required for their brain-metastatic ability
The oncogenic function of c-MYC is well established, and its role in cancer metastasis has been documented in some cancer types (14–17). In the case of breast cancer metastasis, both lung metastasis-promoting and -suppressive functions of c-MYC have been reported (18–20). However, whether or not c-MYC activity is altered in BrM-BCCs and this is required for breast cancer metastasis to the brain remains mostly unexplored.

To investigate this, we first examined whether highly brain-metastatic BCCs have differential expression levels of c-MYC compared with poorly brain-metastatic BCCs. We used MDA-MB-231 and MCF7-HER2 (Erb-B2 Receptor Tyrosine Kinase 2) human breast cancer cell lines as model systems because of the availability of the low metastatic parental population (231-Par and MCF7-Par) and highly brain-metastatic sublines (231-BrM and MCF7-BrM3; refs. 21, 22). c-MYC protein levels were increased in 231-BrM cells compared with 231-Par cells with no increase in its transcript levels (Fig. 1A).

Figure 1. Elevated c-MYC activity in BrM-BCCs is required for their brain-metastatic ability. A, Relative c-MYC protein (left) and mRNA levels (right) in 231-Par and -BrM cells by immunoblotting analysis and qRT-PCR, respectively. B, Subcutaneous tumor growth rates of control (shcntr) and c-MYC knockdown (shMYC) 231-BrM cells. Tumor volumes were measured at the indicated days. C and D, Relative brain-metastatic abilities of c-MYC knockdown 231-BrM (C) and c-MYC-overexpressing 231-Par (D), compared with their corresponding control cell lines. Top, c-MYC protein levels in the indicated cells prior to the injection. Bottom, cells were injected into the left ventricle of immunodeficient mice. At the indicated days, luminescent signals from extracted brains were measured by using bioluminescence imaging (BLI) system. Normalized BLI signals in extracted brains and representative BLI images of brains are shown. P values, one-tailed Mann–Whitney test. All data represent mean ± SEM. *, P < 0.05.
231-BrM #2 cells, another independent brain-metastatic variant of MDA-231, was also observed but from the transcript level (Supplementary Fig. S1A). In the MCF7-HER2 system, MCF7-Par and BrM3 showed similar total c-MYC protein levels, but MCF7-BrM3 exhibited an increased pS62/pT58-MYC ratio compared with MCF7-Par (Supplementary Fig. S1B). It has been shown that activity of c-MYC can be regulated by relative levels between phospho-S62-MYC (pS62-MYC) and phospho-T58-MYC (pT58), in which a high pS62/pT58-MYC ratio indicates the activated c-MYC pathway (23). Thus, our data suggest that the c-MYC pathway is more active in MCF7-BrM3 compared with the Par cells. Further supporting this, MCF7-BrM3 showed increased glutamine consumption rates, which is consistent with previous data that overexpression of c-MYC promotes glutamine consumption in MCF7 (refs. 24, 25; Supplementary Fig. S1C).

On the basis of our data indicating the activated c-MYC pathway in BrM-BCCs, we tested whether c-MYC is required for cancer metastasis to the brain. c-MYC knockdown in 231-BrM had no effect in their general growth rate as subcutaneous tumors (Fig. 1B). However, c-MYC knockdown led to a significant decrease in the formation of brain metastases (Fig. 1C; Supplementary Fig. S1D). Consistent with this, overexpression of c-MYC in 231-Par cells increased their ability to form brain metastases (Fig. 1D). Similar results were observed with c-MYC–overexpressing MCF7-Par (Supplementary Fig. S1E). Collectively, our data suggest that c-MYC is a key player in breast cancer metastasis to the brain.

Increased c-MYC function in BrM-BCCs is essential for invasive outgrowth in the brain microenvironment

Next, we investigated the mechanism by which increased c-MYC activity enhances the ability of BrM-BCCs to form brain metastases. Brain-metastatic cancer cells exhibit expansive growth when growing as spheres, and this phenotype is correlated well with their invasiveness in a 3D matrix (26, 27). To determine whether c-MYC contributes to this process, we used a nonadherent microwell platform, each well of which is designed to contain a single tumor sphere (Fig. 2A, schematic). In this assay, whereas control 231-BrM cells formed spheres with disorganized morphologies (i.e., expansive or grape-like structures; ref. 27), c-MYC–depleted 231-BrM cells produced much more compact and circular spheres, which was reversed upon c-MYC rescue (Fig. 2A and B; Supplementary Fig. S2A). Consistent with this, overexpression of c-MYC in MCF7-Par cells led to formation of disorganized spheres as demonstrated by decreased circularity (Supplementary Fig. S2B).

On the basis of the aforementioned association between expansive growth and invasiveness, we next investigated the role of c-MYC in the invasive growth of BrM-BCCs. We used a published spheroid-based invasion assay in which tumor spheres are embedded in a 3D matrix composed of collagen (type 1) and HA, one of the major components in brain extracellular matrix (28, 29). 231-BrM cells exhibited more invasive behavior in this HA-collagen matrix compared with the 231-Par (Supplementary Fig. S2C), but invasive growth of

Figure 2.
Increased c-MYC function in BrM-BCCs is essential for invasive outgrowth in the brain microenvironment. A (top), A simplified schematic of the microwell system used in this study. Single cells were seeded onto nonadherent microwell, and bright field images were taken at day 7. A (bottom), Representative images of spheres formed by the indicated 231-BrM cells. B, The quantification of sphere circularity from A. Lower circularity indicates less compact and disorganized spheres. The circularity of spheres was calculated by $4\pi \times (\text{area})/ (\text{perimeter})^2$, $n = 3$. C and D, Comparison of invasive growth of the indicated 231-BrM cells. Tumor spheres were embedded within the matrix composed of collagen (type 1) and HA and stained with Phalloidin. Images were taken by using a confocal microscope (C). The invasion was quantified by calculating the changes of invaded areas from 0 hours ($A_0$) to 72 hours ($A_{72}$). D, Invaded area ($A = (A_{72} - A_0) \times 10^6/A_0$). $n = 3$. $P$ values, two-tailed unpaired Student $t$ test. All data represent mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.
231-BrM was significantly reduced upon knockdown of c-MYC and restored upon c-MYC rescue (Fig. 2C and D). Together, these data suggest c-MYC promotes the invasive growth of BrM-BCCs in the brain microenvironment.

**c-MYC creates a favorable brain microenvironment by promoting macrophage infiltration**

In addition to changes in their intrinsic properties such as invasive growth, BrM-BCCs can also modify their own microenvironment by modulating the infiltration of inflammatory cells (e.g., macrophages) to improve their survival and growth in the brain (10, 30). To determine whether c-MYC also plays a role in modulation of the brain microenvironment, we looked for changes in the stromal composition of brain lesions arising from c-MYC–depleted 231-BrM cells. Similar to our observation in vitro (refer to Fig. 2A), lesions formed by control 231-BrM cells exhibited more disorganized and expanded shape whereas those formed by c-MYC–depleted BrM cells showed more compact structure (Fig. 3A, green). More importantly, immuno-stained brain sections revealed that lesions formed by control 231-BrM cells contained large numbers of leukocytes (CD45⁺), including macrophages (F4/80⁺; Fig. 3A, red). In contrast, brain lesions formed by c-MYC–depleted 231-BrM cells only contained very few CD45⁺ and F4/80⁺ cells (Fig. 3A, red).

To further support c-MYC's role in macrophage recruitment in the brain, we used a 3D macrophage recruitment assay (Fig. 3B, experimental scheme). Briefly, macrophages (RAW 264.7) were added to a HA–collagen matrix in which tumor spheres were embedded, and their migration toward tumor spheres was monitored alongside the migration of control cells (Fig. 3B, left). Consistent with our experimental data, 231-BrM cells caused rapid infiltration of RAW 264.7 toward tumor spheres in the HA–collagen matrix. Importantly, c-MYC knockdown dramatically reduced this macrophage infiltration, but it was restored upon c-MYC rescue (Fig. 3B). Similarly, overexpression of c-MYC in MCF7-Par increased RAW cell recruitment (Supplementary Fig. S3). Our data suggest that elevated c-MYC in BrM-BCCs induce the infiltration of macrophages within brain metastases.

**c-MYC promotes GAP junction formation between BrM-BCCs and astrocytes via upregulation of Cx43**

To better understand how c-MYC promotes brain metastasis in breast cancer, we compared the transcriptomes of 231-BrM cells expressing shCtrl, shMYC, shMYC-Vec, and c-MYC rescue constructs by using genome-wide RNA-sequencing. Through this analysis, we identified 20 and 105 genes whose expression were upregulated and downregulated by c-MYC, respectively (P<0.001 and fold change > 1.5; Supplementary Table S1).

To identify the genes that mediate c-MYC's role in promoting brain metastasis, we compared our gene set with a previously reported list of genes that are differentially expressed in 231-BrM and CN34-BrM (BrM subtype derived from CN34 breast cancer cell line) compared with their corresponding Par lines (21). This analysis identified 4 genes (1 up and 3 down) that were differentially expressed in 231-BrM cells compared with 231-Par and regulated by c-MYC (Fig. 4A). c-MYC–dependent expression of these four genes was verified by qRT-PCR (Fig. 4B; Supplementary Fig. S4A and S4B, Supplementary Table S1). From this analysis, we found that major gap junction protein GJA1 (Cx43, connexin43) was upregulated by c-MYC in BrM-BCCs. Cx43-mediated gap junction formation between BCCs and astrocytes has recently been suggested to support BCC growth in the brain (13). However, the upstream regulator of Cx43 in BrM-BCCs has not been identified. Thus, we hypothesized that c-MYC may promote brain metastasis by stimulating gap junction formation between BrM-BCCs and astrocytes and that Cx43 functions as a downstream mediator of c-MYC in this process.

To test this hypothesis, we performed dye (calcein) transfer assays, which allowed us to monitor gap junction formation (13). Although c-MYC knockdown significantly reduced dye transfer from 231-BrM to astrocytes, c-MYC rescue restored the dye transfer activity (Fig. 4C and D). This indicates that c-MYC function is required for the gap junction formation between BrM-BCCs and astrocytes. Furthermore, the lost dye transfer ability of c-MYC–depleted 231-BrM cells was fully recovered upon the ectopic expression of Cx43 (Fig. 4E and F, Supplementary Fig. S4C). Collectively, these data suggest that c-MYC promotes gap junction formation through upregulating Cx43 expression.

**Activated c-MYC pathway predicts brain metastasis in breast cancer patients**

On the basis of our discovery of a role for c-MYC in promoting brain metastasis, we next asked whether c-MYC is clinically associated with the risk of brain metastasis in breast cancer patients. To this, we used publicly available breast cancer microarray data sets (EMC192 and EMC286) that included information about BrMFS (21, 31). In both EMC192 and EMC286, c-MYC transcript levels showed no association with BrMFS (Fig. S5A).

We suspected that c-MYC transcript levels may not represent a sufficient reflection of active c-MYC signaling because our experimental data suggest that the c-MYC pathway in BrM-BCCs can also be activated by an increased c-MYC protein level and by changes in phosphorylation status of the protein (refer to Fig 1A; Supplementary Fig. S1B).

To address this, we performed a similar analysis as above but using a gene set that is composed of 125 c-MYC–regulated genes, identified in our RNA-seq analysis (Supplementary Table S1), and c-MYC. We named this gene set the "c-MYC brain metastasis gene set (MYC-BrMGS)." Compared with c-MYC expression alone, MYC-BrMGS showed a significant association with the risk of brain metastasis in EMC192 and EMC286 data sets (Fig. S5B). In contrast to increased risks for brain metastasis in MYC-BrMGS–positive patients, the association between MYC-BrMGS and lung metastasis-free was observed only in EMC 192 (Supplementary Fig. S5A). In case of bone metastasis-free survival (BoMFS), MYC-BrMGS–positive group showed lower risks for the bone metastasis in EMC 286, which is opposite of what we found with brain metastasis (Supplementary Fig. S5B).

Thus, clinical data analyses in combination with our experimental data strongly suggest that increased c-MYC activity is required for the brain metastasis in breast cancer.

**Increased c-MYC activity in BrM-BCCs sensitizes them to TRAIL-induced apoptosis**

Changes in molecular features of cancer cells can alter their drug responsiveness. Because we found that the c-MYC pathway is highly activated in BrM-BCCs, we wondered whether this causes changes in their sensitivity to anticancer drugs. To test this, we first compared the responsiveness of 231-Par and BrM to the most commonly used chemotherapeutic agents for breast cancer (docetaxel, doxorubicin, and methotrexate) and observed no differences between 231-Par and BrM (Supplementary Fig. S6A).

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However, 231-BrM cells, but not 231-LM cells (a highly lung-metastatic MDA-MB-231 derivative), exhibited dramatically increased susceptibility to TRAIL-induced cell death compared with 231-Par cells (Fig. 6A). FACS analysis confirmed a greater increase in apoptotic cells in 231-BrM upon TRAIL treatment (Supplementary Fig. S6B), which is in accordance with previous reports that c-MYC can activate TRAIL-induced apoptosis in various cell types (2, 25, 32, 33). Increased TRAIL sensitivity was also observed with 231-BrM #2 as well as with MCF7-BrM3 compared with the corresponding Par cells (Supplementary Fig. S6C and S6D). These data together suggest the synthetic lethal interaction between BrM-BCCs and TRAIL.

TRAIL-induced apoptosis is initiated by the binding of TRAIL to death receptors 4 and 5 (DR 4 and 5). This triggers activation of caspase-8 and the downstream effector caspases (caspase-3, 6, and 7) through cell type-specific mechanisms (34). In type I cells, direct activation of caspase-3/6/7 by caspase-8, a.k.a. extrinsic apoptotic pathway, is sufficient to execute apoptosis (35). On the other hand, apoptosis in type II cells requires activation of the mitochondrial (intrinsic) pathway. In this pathway, caspase-8-mediated cleavage of BID (BH3 interacting Domain Death Agonist) induces BAX/BAK oligomerization and subsequent release of cytochrome C from mitochondria. This is followed by activation of caspase-9 and caspases 3/6/7 (36).

Upon TRAIL treatment, 231-BrM exhibited faster cleavage of caspase-8 (CASP-8), indicating activation of an extrinsic pathway (Fig. 6B). 231-BrM also showed increased BID cleavage (Fig. 6B), faster oligomerization of BAX and BAK (Fig. 6C, Supplementary Fig. S6E), as well as accelerated cleavage of caspase-9 and caspase-3 (Fig. 6B). This suggests that increased TRAIL sensitivity in 231-BrM also involves activation of the intrinsic pathway.

On the basis of this finding, we next examined (i) whether c-MYC is responsible for the higher TRAIL sensitivity in BrM-BCCs and (2) if so, which apoptotic pathway is regulated by c-MYC.
c-MYC–depleted 231-BrM cells exhibited reduced TRAIL sensitivity, and restoring c-MYC expression resensitized them to TRAIL-induced apoptosis (Fig. 6D). Furthermore, overexpression of c-MYC in 231-Par cells increased their TRAIL sensitivity (Supplementary Fig. S6F). In 231-BrM cells, however, c-MYC overexpression had no effect on TRAIL sensitivity (Fig. 6D). This is probably because endogenous levels of c-MYC are sufficient to induce maximum TRAIL sensitivity. Consistent with results in 231-BrM cells, c-MYC knockdown in MCF7-BrM3 cells reduced their TRAIL sensitivity (Supplementary Fig. S6G). Taken together, this suggests the essential role of c-MYC in increased susceptibility of BrM-BCCs to TRAIL.

Next, we investigated molecular mechanisms underlying c-MYC–mediated increase in TRAIL sensitivity of BrM-BCCs. By monitoring activation of apoptotic pathways, we found that c-MYC knockdown in 231-BrM attenuated cleavage of caspase-8, BID, caspase-9, and caspase-3, which was restored upon c-MYC rescue (Fig. 6E). These indicate that c-MYC activates both extrinsic and intrinsic pathways. Further supporting this finding, DR5 and caspase-8 inhibitor FLIP was increased and decreased in 231-BrM compared with the 231-Par, respectively (Supplementary Fig. S6H), and DR5 increase was reversed by c-MYC knockdown in 231-BrM (Fig. 6F). Finally, accelerated BAX and BAK oligomerization observed in 231-BrM was impaired upon c-MYC knockdown and...
restored by c-MYC rescue (Fig. 6G; Supplementary Fig. S6I). This explains attenuated activation of caspase-9 in 231-BrM upon c-MYC knockdown which we showed in Fig. 6E. Therefore, these data together suggest that increased c-MYC in BrM-BCCs sensitizes them to TRAIL-induced apoptosis via activating both extrinsic and intrinsic pathways.

Finally, as our data indicate that TRAIL may be an effective death inducer of BrM-BCCs, we tested this possibility in vivo. To this, 231-BrM cells were introduced into the arterial circulation of immune-deficient mice, and mice were treated with either vehicle (water) or TRAIL. Brain metastases were monitored by using bioluminescence imaging. TRAIL treatment reduced the growth of brain metastases (Fig. 6H), suggesting TRAIL is effective in the treatment of brain-metastatic breast cancer.

Discussion

Evidence is accumulating that BCC subpopulations acquire brain-metastatic potential via the activation of distinct molecular pathways (21, 37). Despite significant advances in our understanding of the molecular mechanisms that govern breast cancer metastasis to the brain, currently no effective therapeutic agents are available. This necessitates new approaches to target this disease.

In this study, we demonstrate that BrM-BCCs exhibit increased c-MYC activity, which promotes brain metastasis, and this subsequently leads to increased susceptibility to TRAIL-induced apoptosis. To the best of our knowledge, this study is the first to report a link between c-MYC, brain-metastatic potential, and TRAIL sensitivity, thus providing evidence of a novel synthetic interaction in BrM-BCCs.

We found that c-MYC confers brain-metastatic potential on BCCs without affecting their general tumor-forming capability. This suggests c-MYC may function as a brain virulence gene—a gene that promotes BCC colonization within the brain—rather than providing BCCs with an advantage to expand within the primary tumor.

How, then, does c-MYC enable BCCs to form metastatic lesion within the brain? The brain contains a specialized extracellular matrix (ECM) and cell types including astrocytes (38). To colonize the brain, incoming BCCs must be able to cope with this unique brain microenvironment. Our data indicate c-MYC contributes to
the ability of BCCs to colonize the brain not only by promoting their invasive growth within the brain ECM, but also by modulating and exploiting stromal components such as macrophages and astrocytes. In this regard, c-MYC in BrM-BCCs enhances their ability to induce macrophage recruitment. This is consistent with previous reports that c-MYC promotes macrophage infiltration into pancreatic cancers (39, 40). Our results along with these published studies strongly suggest c-MYC modulates the tumor microenvironment, eventually leading to cancer cell survival and growth. We suspect this is accomplished via c-MYC–mediated changes in cytokines and chemokines. Future studies will provide more insight into the molecular mechanisms by which c-MYC–expressing BCCs induce macrophage influx.

We also discovered a novel role for c-MYC in the formation of gap junctions between BCCs and astrocytes, which is essential for the growth and chemoresistance of brain metastases (13). We
further showed that this c-MYC-mediated increase in gap junction formation is accomplished via an upregulation of Cx43. A previous study reported that Cx43 is upregulated in RAS-expressing NIH3T3 cells due to increased occupancy of the Hsp90 and c-MYC complex at the Cx43 promoter (41), which is consistent with our results.

Our clinical analyses indicate that MYC-BrMGS can be a prognostic marker for brain metastasis in breast cancer patients. We expect that this is due to the regulation of c-MYC at the posttranscriptional level (42–44), as we observed in our model system. Consistent with this, one study found elevated levels of c-MYC protein in brain metastases compared with the matched primary breast tumors (45).

Elevated c-MYC function in BrM-BCCs increases susceptibility of these cells to TRAIL-induced apoptosis. Our data demonstrate that the underlying mechanisms of elevated TRAIL sensitivity include c-MYC-regulated expression of DR5 as well as activation of the BAX and BAK oligomerization. Consistent with this result, previous studies reported that c-MYC exerts its proapoptotic function by upregulating DR5 in mammary epithelial cells (2), by activating BAX in rat fibroblasts and mammary epithelial cells (25, 46) and by promoting BAK oligomerization in MCF7 cells (25). Therefore, c-MYC induces TRAIL sensitivity in BrM-BCCs via a mechanism that is similar to the one used by mammary epithelial cells.

If c-MYC promotes brain metastasis but enhances TRAIL sensitivity as our data suggest, how do c-MYC-expressing BCCs survive within the brain in the first place? TRAIL can be secreted by the BCCs themselves and by various organs. Interestingly, TRAIL is downregulated in 231-BM compared with Par cells (21), suggesting BM-BCCs that overexpress c-MYC may protect themselves from apoptosis by suppressing the expression of TRAIL. In addition, the brain microenvironment produces less TRAIL than bone and lungs (31). This may help BCCs overexpressing c-MYC survive in the brain microenvironment.

The important clinical implication of our study is that TRAIL-mediated apoptosis may be used to target brain metastases. Despite the efficacy of TRAIL-based therapies in preclinical models, the clinical benefits of these therapies are not yet proven. This is typically blamed on TRAIL’s short half-life and rapid clearance from the body (47). Although recent studies have suggested solutions for increasing the efficacy of TRAIL-based therapies (48, 49), insufficient delivery of TRAIL to the brain due to the blood–brain barrier has remained as a major impediment for treatment of brain metastases (50). Several innovative approaches for resolving this problem have recently proven effective in preclinical models of glioblastoma and brain metastases of breast cancer. These include stem cell–mediated delivery of TRAIL and administration of a TRAIL-inducing compound (50). Thus, our findings together with these studies suggest breast cancer patients with brain metastases may benefit from TRAIL treatment.

Collectively, our results indicate that c-MYC plays an essential role in the successful formation of brain metastases and that, as a bystander effect, these cells become more susceptible to TRAIL-induced apoptosis.

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

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Writing, review, and/or revision of the manuscript: H.Y. Lee, J. Cha, P. Kim, M.-Y. Kim
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.Y. Lee
Study supervision: M.-Y. Kim

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References


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