TUSC4 Functions as a Tumor Suppressor by Regulating BRCA1 Stability
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Abstract
BRCA1 expression is lost frequently in breast cancers in which it promotes malignant development. In the present study, we performed a global expression analysis of breast cancer cells in which the tumor-suppressor candidate gene TUSC4 was silenced to gain insights into its function. TUSC4 silencing affected genes involved in cell cycle and cell death, which have broad reaching influence on cancer development. Most importantly, we found a cluster pattern of gene-expression profiles in TUSC4-silenced cells that defined a homologous recombination (HR) repair defect signature. Mechanistic investigations indicated that TUSC4 protein could physically interact with the E3 ligase Herc2, which prevents BRCA1 degradation through the ubiquitination pathway. TUSC4 silencing enhanced BRCA1 polyubiquitination, leading to its degradation and a marked reduction in HR repair efficiency. Notably, ectopic expression of TUSC4 suppressed the proliferation, invasion, and colony formation of breast cancer cells in vitro and tumorigenesis in vivo. Furthermore, TUSC4 silencing was sufficient to transform normal mammary epithelial cells and to enhance sensitivity to PARP inhibitors. Our results provide a set of genetic and biologic proofs that TUSC4 functions as a bona fide tumor suppressor by regulating the protein stability and function of BRCA1 in breast cancer. Cancer Res; 75(2); 378–86. ©2014 AACR.

Introduction
Loss of tumor-suppressor gene function leading to genomic instability is a well-known prerequisite toward cancer development (1, 2). Through genome-wide sequencing, many tumor suppressors were found frequently deleted or mutated in various cancers (3). Tumor-suppressor candidate 4 (TUSC4); also known as nitrogen permease regulator-like 2 (NPRL2), was first identified in a human lung cancer homozygous deletion region on chromosome 3p21.3. NPRL2/TUSC4 is highly conserved in species ranging from yeasts to humans (4). Sequence analysis demonstrated that NPRL2/TUSC4 possesses a protein-binding domain other than the nitrogen permease domain and that overexpression of NPRL2/TUSC4 reduces lung cancer proliferation both in vitro and in vivo (5). Homozygous deletions in chromosome 3p21.3 were also frequently detected in breast cancers (6). Indeed, we confirm through microarray and pathway analyses that loss of NPRL2/TUSC4 was associated with cancer development and multiple molecular functions such as cell cycle and cell death. Interestingly, we have identified that loss of NPRL2/TUSC4 expression resulted in a similar pattern to homologous recombination (HR) repair defects when clustered with our previously established HR defect gene signature profile (7).

HR repair maintains genomic stability by mediating high-fidelity error-free repair of DNA double-strand breaks (DSB; refs. 8, 9). HR repair deficiency (HRD) has been reported to promote cancer development and sensitize cancer cells to DNA damage–inducing therapy (10). The tumor-suppressor protein BRCA1 is considered one of the most important safeguards against breast cancer, by maintaining genome integrity through regulation of DNA damage repair, particularly HR repair (11, 12). BRCA1 germline mutation carriers are more likely than noncarriers to have basal-like breast cancer features with widespread genomic instability at both morphologic and immunohistochemical levels (13). BRCA1-deficient cells exhibit enhanced sensitivity to irradiation and chemotherapy that induces DNA single- or DSBs (14, 15), largely owing to their impaired capacity for HR repair (16). Therefore, the similar gene-expression profiles between NPRL2/TUSC4 deficiency and HR repair defect raise the possibility that NPRL2/TUSC4 regulates HR repair through BRCA1. As expected, we have shown that BRCA1 stability and foci formation in response to DNA damage was markedly reduced upon knockdown of NPRL2/TUSC4 expression. Although BRCA1 expression is tightly regulated at both the transcriptional and protein level throughout the different stages of the cell cycle (17, 18), knowledge of how BRCA1 protein stability is regulated is still very limited. Our data suggest that one mechanism of regulation occurs through direct binding between NPRL2/TUSC4 and HERC2, the E3 ligase of BRCA1 (19), thus inhibiting HERC2-mediated BRCA1 degradation. Together, we have demonstrated that NPRL2/TUSC4 is a bona fide tumor-suppressor gene in breast cancer, and its function in suppressing genomic instability and cancer development may be at least in part through protecting the protein stability of BRCA1.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell culture and plasmids
The U2OS, MDA-MB-231, and MCF-10A cell lines were purchased from the ATCC. McCoy’s 5A medium (Cellgro; 10-050-CV) supplemented with 10% FBS was used to maintain U2OS cells, RPMI-1640 medium (Corning; 10-104-CV) supplemented with 10% FBS was used to culture MDA-MB-231 cells, and serum-free mammary epithelial growth medium (Clonetics; CC-3051) containing insulin, hydrocortisone, EGF, and bovine pituitary extract was used to maintain MCF-10A cells. All cells were incubated under humidified conditions in 5% CO₂. The pCMV5-3 Flag vector plasmid was kindly provided by Dr. Maria Jasin (Memorial Sloan-Kettering Cancer Center, New York, NY). U2OS cells were transfected with an empty vector or FLAG-NPRL2/TUSC4 plasmids. Seventy-two hours after transfection, G418 was added to the medium for selection purposes. After stable clones were isolated from the pool, whole cellular extracts were incubated with RIPA buffer as described previously (20), and the products were immunoprecipitated with anti-FLAG M2 affinity gel for 8 hours at 4°C. After washing, the complexes were eluted with 3×FLAG peptide and evaluated using SDS-PAGE. For immunoprecipitation of the binding between HERC2 and BRCA1, cell lysate was pre cleared with A/G Agarose beads (Santa Cruz Biotechnology; SC-2003) and incubated with 1 μg of antibody at 4°C overnight. Precipitate was then washed and eluted in 5× SDS buffer and subjected to SDS-PAGE and immunoblotting. For immunoblotting, after samples were separated using electrophoresis, membranes were blocked with 5% milk diluted in Tris buffer with 0.1% Tween 20 for 1 hour at room temperature. The primary antibody was diluted in 5% BSA in PBS with sodium azide (Sigma; S227), and then incubated with the membranes for 2 hours at room temperature. Subsequently, membranes were washed with PBS with 0.1% Tween 20 and incubated with secondary antibody. Finally, signals of the bound antibody were detected using enhanced chemiluminesence (GE Healthcare; RPN2232).

In vitro proliferation and PARP inhibition assays
MTT (Sigma; M5655) was used to evaluate the proliferation of cells. Briefly, cells were counted and seeded in a 96-well flat-bottomed plate. After 96 hours, cells were incubated with MTT substrate (Sigma; 20 mg/mL) for 4 hours, and the cultures were removed and replaced with DMSO. The optical density was measured spectrophotometrically at 570 nm. The colony formation assay was performed by seeding 200 cells in 6-well plates. Olaparib and rucaparib were added to the culture medium, and the cells were compared with untreated control cells. Colonies were scored after 3 weeks. All experiments were repeated three times.

Microarray analysis
The mirVana RNA Isolation Kit (Ambion) was used to isolate total RNA. A 500 ng of total RNA was used with a Sentrix Human-6 Expression Bead Chip (Illumina) for labeling and hybridization. BeadArray Reader was used for chip scanning (Illumina). As described previously (7), the gene-expression profile was subjected to normalization and log transformation. The NextGenEx software program was used to identify genes whose expression differed in two clusters, and a t test was used to separate genes with significantly different expression (P < 0.001). The Ingenuity Pathway Analysis system was used for gene enrichment analysis.

Immunoprecipitation and immunoblotting
For immunoprecipitation of FLAG-NPRL2/TUSC4, U2OS cells were first transfected with an empty vector or FLAG-NPRL2/TUSC4 plasmids. Seventy-two hours after transfection, G418 was added to the medium for selection purposes. After stable clones were isolated from the pool, whole cellular extracts were incubated with RIPA buffer as described previously (20), and the products were immunoprecipitated with anti-FLAG M2 affinity gel for 8 hours at 4°C. After washing, the complexes were eluted with 3×FLAG peptide and evaluated using SDS-PAGE. For immunoprecipitation of the binding between HERC2 and BRCA1, cell lysate was pre cleared with A/G Agarose beads (Santa Cruz Biotechnology; SC-2003) and incubated with 1 μg of antibody at 4°C overnight. Precipitate was then washed and eluted in 5× SDS buffer and subjected to SDS-PAGE and immunoblotting. For immunoblotting, after samples were separated using electrophoresis, membranes were blocked with 5% milk diluted in Tris buffer with 0.1% Tween 20 for 1 hour at room temperature. The primary antibody was diluted in 5% BSA in PBS with sodium azide (Sigma; S227), and then incubated with the membranes for 2 hours at room temperature. Subsequently, membranes were washed with PBS with 0.1% Tween 20 and incubated with secondary antibody. Finally, signals of the bound antibody were detected using enhanced chemiluminesence (GE Healthcare; RPN2232).
using a FACSCalibur and the CellQuest software program (Becton Dickinson). Three independent experiments were performed to obtain mean values and their SDs. Cell-cycle analysis was performed at the MD Anderson Cancer Center Flow Cytometry and Cellular Imaging Facility.

Tumor growth in nude mice and immunohistochemistry
Six-week-old female athymic nu/nu mice (NCI, average weight 25 g) were used in this study. The MD Anderson Institutional Animal Care and Use Committee approved the animal protocol. A total of 5 x 10⁶ MDA-MB-231 cells with and without NPRL2/TUSC4 overexpression or 1 x 10⁷ MCF-10A cells with and without knockdown of NPRL2/TUSC4 expression were injected to the mammary fatpads of mice. Tumors were measured from 1 week after MDA-MB-231 cells injection and monitored weekly, whereas MCF-10A tumors were observed 1 month after cell injection. At least 5 nude mice were used for each group. Human breast tissue samples (Biomax) were embedded in Xylene and 100%, 95%, 70%, 50% ethanol, respectively, for deparaffinization, slides were then incubated with NPRL2/TUSC4 antibody at 4°C overnight followed by antigen retrieval. Then samples were processed and evaluated immunohistochemically under microscope after being dehydrated and stabilized.

Transfection and ubiquitination assay
U2OS cell transfection was conducted using Oligofectamine (Life Technologies; 12252-001). Plasmids encoding HA-tagged ubiquitin were transfected in U2OS cells with and without knockdown of NPRL2/TUSC4 expression. Forty-eight hours after transfection, cells were harvested and lysed with RIPA buffer. Precipitated BRCA1 protein was isolated using SDS-PAGE and detected using an antibubiquitin antibody (Cell Signaling Technology; 3933).

Statistical analysis
All statistical analyses were performed with a two-tailed Student t test.

Results
NPRL2/TUSC4 expression is reduced in breast cancer and correlates with breast cancer progression
To characterize whether NPRL2/TUSC4 expression is associated with breast cancer, we performed Western blotting to measure the expression of NPRL2/TUSC4 in nontransformed breast cell lines, including HMEC, MCF-10A, and MCF-12A, and breast cancer cell lines with both luminal and basal-like subtypes (Fig. 1A). We observed that NPRL2/TUSC4 expression was markedly higher in the nontransformed cell lines than that in breast cancer cell lines. We also evaluated NPRL2/TUSC4 expression in normal breast tissue and breast carcinomas using immunohistochemical staining, and found that NPRL2/TUSC4 expression was lower in tumor tissue than in the matched adjacent normal breast tissue (Fig. 1B). Furthermore, The Cancer Genome Atlas–based analysis of mRNA expression in invasive breast carcinomas demonstrated a significant difference between the survival rates in patients with unaltered NPRL2/TUSC4 expression and those with downregulated expression level.
Here, we have identified an association between the expression profile of NPRL2/TUSC4 knockdown and BRCA1-deficient HRD gene signature. We suspected that loss of TUSC4 will also affect the foci formation of BRCA1, so we carried out phenotypic examination to test whether TUSC4 is required for BRCA1 foci formation by immunostaining. We performed BRCA1 foci staining followed by IR and UV irradiation. TUSC4 knockdown significantly abolished the BRCA1 foci formation after irradiation, whereas control siRNA did not affect the formation of BRCA1 foci (Fig. 2A and B). We further evaluated HR repair efficiency by a standard HR repair analysis system (20, 25) with NPRL2/TUSC4-deficient U2OS model cells. We found that NPRL2/TUSC4-knockdown cells had a significant decrease in HR reporter activity compared with control cells, which suggested impaired HR repair efficiency (Fig. 2C). We used BRCA1-knockdown cells as a positive indicator of HR repair to confirm HR repair efficiency. NPRL2/TUSC4-knockdown cells presented comparative reduction in HR repair efficiency with BRCA1-knockdown cells, which were around 40% to 50% lower than that in control cells. To confirm that the defective HR repair efficiency was not caused by transfection efficiency or inaccurate efficiency from I-Scel, we reintroduced BRCA1 expression into the NPRL2/TUSC4-knockdown cells and observed a significant increase in HR repair efficiency.

Surprisingly, we found that knockdown of NPRL2/TUSC4 expression reduced BRCA1 protein expression (Fig. 2D), indicating that the decrease in HR repair efficiency in the NPRL2/TUSC4-knockdown cells may have resulted from abnormal BRCA1 protein expression. These results are consistent with our above findings that NPRL2/TUSC4-knockdown cells have HRD gene expression patterns similar to those in BRCA1-deficient cells, which were used to generate our HRD gene signatures. These results revealed for the first time a novel function of NPRL2/TUSC4 in that disruption of its expression decreases BRCA1 expression and functions.

NPRL2/TUSC4 regulates BRCA1 protein stability

We next sought to determine how NPRL2/TUSC4 affects BRCA1 protein expression. To that end, we first sought to determine whether reduced BRCA1 protein expression after NPRL2/TUSC4 knockdown was caused by altered cell-cycle distribution because BRCA1 expression is known to be cell-cycle dependent. We carried out a cell-cycle analysis and did not observe a significant difference in G1, S, or G2/M, or S-phase distribution between control and NPRL2/TUSC4-knockdown cells (Fig. 3A; Supplementary Fig. S4), indicating that decreased BRCA1 expression into the NPRL2/TUSC4-knockdown cells was not due to shifts in the cell cycle. To determine whether such changes occur through transcriptional regulation, we performed quantitative reverse transcriptase PCR to measure BRCA1 mRNA expression in control cells and cells with NPRL2/TUSC4 knockdown. We identified no significant BRCA1 mRNA differences after NPRL2/TUSC4 knockdown, whereas there is a significant drop in NPRL2/TUSC4 mRNA (Fig. 3B), thus ruling out the possibility that BRCA1 expression by NPRL2/TUSC4 was regulated at the mRNA level.

Next, we sought to determine whether NPRL2/TUSC4 regulates BRCA1 protein stability. To answer this question, we conducted cycloheximide chase experiments to determine BRCA1 stability in control and NPRL2/TUSC4-knockdown U2OS cells. As shown in Fig. 3C, NPRL2/TUSC4 knockdown reduced the half-life of BRCA1 from about 20 hours to less than 6 hours, suggesting that NPRL2/TUSC4 plays an essential role in stabilizing BRCA1 at the protein level (Fig. 3D and E). In addition, to determine whether BRCA1 protein stability is regulated by NPRL2/TUSC4 via the proteasome pathway, we treated control and NPRL2/TUSC4-knockdown U2OS cells with the proteasome inhibitor MG132. As shown in Fig. 3F, MG132 restored BRCA1 expression by
NPRL2/TUSC4 regulates BRCA1 protein stability via the ubiquitination pathway

It has been previously reported that HERC2 is an E3 ligase that targets BRCA1 for degradation (18). To determine whether NPRL2/TUSC4 regulates BRCA1 stability via HERC2, we examined BRCA1 ubiquitination under normal versus NPRL2/TUSC4 knockdown conditions. Surprisingly, we observed no signs of ubiquitination regardless of the NPRL2/TUSC4 knockdown status. However, after treatment with MG132, Western blotting shows that BRCA1 is highly ubiquitinated upon NPRL2/TUSC4 knockdown, whereas control cells exhibited comparatively reduced BRCA1 ubiquitination (Fig. 4A), indicating that knockdown of NPRL2/TUSC4 expression caused a robust increase in BRCA1 protein polyubiquitination. We also confirmed previous findings that downregulation of HERC2 expression leads to increased expression of BRCA1 regardless of the presence of NPRL2/TUSC4 (Fig. 4B and C). Under both conditions, BRCA1 expression was markedly upregulated after depletion of HERC2.

The next question to be answered was whether NPRL2/TUSC4 participates in ubiquitination of BRCA1 via HERC2 indirectly or stabilizes BRCA1 directly by binding. We performed immunoprecipitation with established NPRL2/TUSC4-overexpressing U2OS cell lines to determine the relationships among NPRL2/TUSC4, BRCA1, and HERC2. We found that NPRL2/TUSC4 physically interacts with HERC2 but not with BRCA1 (Fig. 4D), which suggests that NPRL2/TUSC4 regulates BRCA1 stability via interaction with HERC2. Considering previous reports on NPRL2/TUSC4 interactions (26, 27), we suspected that NPRL2/TUSC4 may prevent physical interaction between BRCA1 and HERC2. The binding between these two proteins has been previously described in Hela cell lines (19), so we further performed immunoprecipitation to determine whether overexpression of NPRL2/TUSC4 weakens this binding. As shown in Fig. 4E, although endogenous HERC2 physically binds BRCA1, overexpression of NPRL2/TUSC4 interrupts the binding, indicating that NPRL2/TUSC4 may regulate BRCA1 stability by preventing physical interaction between BRCA1 and HERC2.

NPRL2/TUSC4 suppresses the tumorigenicity of human breast cancer cells

Our data so far suggest that NPRL2/TUSC4 functions as a breast tumor suppressor in part through positive regulation of BRCA1 stability. We postulated that overexpression of NPRL2/TUSC4 would, therefore, suppress breast tumor proliferation in vitro and in vivo. To test this, we compared the proliferation rate of the breast cancer cell line MDA-MB-231 with or without NPRL2/TUSC4 overexpression. Colony formation assays indicated markedly reduced proliferation of NPRL2/TUSC4-overexpressing cells
NPRL2/TUSC4 deficiency effectively inhibits breast cancer cell growth in vitro, we further examined the effect of NPRL2/TUSC4 overexpression in a xenograft model of breast cancer. We injected control or NPRL2/TUSC4-knockdown MDA-MB-231 cells into the mammary fatpads of female nude mice. We then monitored and measured tumor growth weekly. Six weeks after injection, 5 of 10 mice injected with NPRL2/TUSC4-knockdown cells remained tumor free, whereas all 5 mice injected with control 231 cells had large tumors (Fig. 5A and B; Table 1).

NPRL2/TUSC4 deficiency transforms normal mammary epithelial cells

Previous reports indicate that HR repair defects sensitizes cancer cells to DNA-damaging drugs (14, 15) and the PARP inhibitors (10, 11). Thus, NPRL2/TUSC4-deficient cells with HR repair defects are likely to be more sensitive to the treatment of PARP inhibitors, which can effectively inhibit the repair of single-strand DNA breaks. To test this hypothesis, we examined PARP inhibitor sensitivity in cells with NPRL2/TUSC4 deficiency. We performed colony formation assay in U2OS cells with NPRL2/TUSC4 knockdown after PARP inhibitor olaparib and rucaparib treatment, as well as control cells. As expected, both drugs significantly reduced colony formation in NPRL2/TUSC4 knockdown cells compared with control cells (Fig. 5C and D). In addition, we examined whether NPRL2/TUSC4 depletion initiates breast tumor development in a xenograft mouse model. We injected stable NPRL2/TUSC4-knockdown MCF-10A cells and control cells into the mammary fatpads of female nude mice, and monitored tumor formation in the mice. Notably, tumors began to form in 3 of 10 mice injected with NPRL2/TUSC4-knockdown cells after 3 weeks, whereas no tumors formed in the control groups (Table 2). These results demonstrated that loss of NPRL2/TUSC4 expression alone is sufficient to initiate malignant transformation of immortalized nontransformed mammary epithelial cells, which is consistent with our hypothesis that NPRL2/TUSC4 functions as a bona fide tumor suppressor in breast cancer.

Discussion

BRCA1 plays a key role in DNA damage responses, including HR repair. BRCA1 executes cellular functions in multiple
complexes to safeguard genomic stability, and therefore loss of BRCA1 expression is associated with aggressive phenotypes of breast carcinoma (13, 28). However, very little is known about the mechanism of how BRCA1 expression is regulated during different stages of cancer development. Several previous studies demonstrated that BRCA1’s stability is regulated via ubiquitination pathways (19, 29), but the network underlining this regulation on BRCA1's stability is regulated via ubiquitination across tumor types (7). We surprisingly identified 230 genes that predict HRD deficiency across breast cancer. Indeed, we found that NPRL2/TUSC4 physically interacts with HERC2 but not BRCA1. E, NPRL2/TUSC4 overexpression reduced the binding between BRCA1 and HERC2 in Hela cell lines. A 2% input was used to indicate the level of HERC2, IgG control antibody and BCRA1 antibody were used to perform immunoprecipitation (IP) in both control and NPRL2/TUSC4 overexpression cells. A total of 4 mg of protein were used to perform in each condition.

Figure 4. NPRL2/TUSC4 regulates BRCA1 stability via the ubiquitination pathway by blocking the binding of HERC2 to BRCA1. A, NPRL2/TUSC4 knockdown increased ubiquitination level of BRCA1 compared with control cells (after MG132 enrichment for ubiquitination). B, HERC2 knockdown rescued BRCA1 expression level, with and without the presences of NPRL2/TUSC4. C, bar graph, a significant BRCA1 reduction after NPRL2/TUSC4 knockdown (P < 0.05). BRCA1 increases after HERC2 knockdown with NPRL2/TUSC4 presence (P < 0.05), or without NPRL2/TUSC4 (P < 0.001). All values were compared with control cells. D, NPRL2/TUSC4 physically interacts with HERC2 but not BRCA1. E, NPRL2/TUSC4 overexpression reduced the binding between BRCA1 and HERC2. A, BRCA1 expression ratio of control si TUSC4 and NPRL2/TUSC4 KD cells. B, Western blotting. C, quantitation of Western blotting. D, immunoprecipitation. E, co-immunoprecipitation.
expression as well as reduced recruitment of BRCA1 to DNA damage foci. Furthermore, double knockdown of NPRL2/TUSC4 and BRCA1 in an HR repair assay reduced the HR repair efficiency more than knockdown of NPRL2/TUSC4 or BRCA1 expression alone, suggesting that in addition to positive regulation for BRCA1 protein stability, NPRL2/TUSC4 may regulate HR function through BRCA1-independent pathways (data not shown).

Table 1. Tumorigenicity of orthotopically implanted control and TUSC4 overexpression MDA-MB-231 cells

<table>
<thead>
<tr>
<th>Number of mice with tumors (%)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
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<tr>
<td>231 Control</td>
<td>3/5 (60)</td>
<td>5/5 (100)</td>
<td>5/5 (100)</td>
<td>5/5 (100)</td>
<td>5/5 (100)</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>TUSC4-7</td>
<td>15/20 (75)</td>
<td>25/50 (50)</td>
<td>25/50 (50)</td>
<td>25/50 (50)</td>
<td>25/50 (50)</td>
<td>25/50 (50)</td>
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<tr>
<td>TUSC4-13</td>
<td>25/50 (50)</td>
<td>25/50 (50)</td>
<td>25/50 (50)</td>
<td>25/50 (50)</td>
<td>25/50 (50)</td>
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</table>
| NOTE: A total of 5 x 10^6 cells from MDA-AB-231 control and two independent NPRL2/TUSC4-overexpressing MDA-AB-231 cell lines (NPRL2/TUSC4 #7 and NPRL2/TUSC4 #13) were injected per mouse into mammary fat pads glands of 6-week-old female nude mice. Each cell line was injected in 5 different mice, and tumor sizes were analyzed.

Table 2. Tumorigenicity of orthotopically implanted control and TUSC4-knockdown MCF10A cells

<table>
<thead>
<tr>
<th>Number of mice with tumors (%)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>Week 7</th>
<th>Week 8</th>
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<tr>
<td>MCF10A control</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
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</tr>
<tr>
<td>TUSC4-1</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
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<tr>
<td>TUSC4-4</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
<td>2/5 (40)</td>
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</table>
| NOTE: A total of 1 x 10^6 cells from MCF-10A control and two independent NPRL2/TUSC4-knockdown MCF-10A cell lines (NPRL2/TUSC4 #1 and NPRL2/TUSC4 #4) were injected per mouse into mammary fat pads glands of 6-week-old female nude mice. Each cell line was injected in 5 different mice, and tumor sizes were analyzed.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Peng, G. Peng, S.-Y. Lin
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Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): Y. Peng, E. Wang, G. Peng, S.-Y. Lin
Writing, review, and/or revision of the manuscript: Y. Peng, C.-C. Lin, W. Mo, S.-Y. Lin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Mo
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