TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Wasf3 Deficiency Reveals Involvement in Metastasis in a Mouse Model of Breast Cancer

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The *WASF3* gene has been implicated in cancer cell movement, invasion, and metastasis by regulating genetic pathways important in these processes. Invasion and metastasis assays, however, are largely centered on xenograft models in immune-compromised mice. To facilitate analysis of the role of *WASF3* in the spontaneous development of cancer cell metastasis, we generated a *Wasf3* null strain by deleting exons 4 and 5, which encode essential motifs for Wasf3 function. On exposure to cre-recombinase a stop codon is generated immediately downstream in exon 6. Using a cytomegalovirus (CMV)-cre strain, *Wasf3* constitutively was inactivated, which led to viable mice with no visible morphologic or behavioral abnormalities. There was no abnormal development or function of the mouse mammary gland in the *Wasf3* null mice and brain development was normal. In the mouse mammary tumor virus (MMTV)-driven polyoma middle-T oncogene strain, which shows early onset breast cancer development and metastasis, Wiskott-Aldrich syndrome protein family member 3 (Wasf3) is up-regulated in metastatic lesions. When this oncogene was introduced onto the *Wasf3*-null background, although metastasis was observed in these mice, there was a reduction in the number and size of metastatic lesions in the lungs. These data provide evidence for a role in *WASF3* in the development of metastasis in a spontaneous model of breast cancer. (*Am J Pathol* 2019, 189: 2450–2458; <https://doi.org/10.1016/j.ajpath.2019.08.012>)

The Wiskott-Aldrich syndrome protein family member 3 (*WASF3*/*WAVE3*)¹ is part of a three-member gene family that orchestrates actin polymerization through specific domains that bind monomeric actin and actin related protein (ARP)2/3 proteins.^{2,3} WASF proteins normally are auto-inhibited through a protein complex that binds to the WASF regulatory region.^{4–6} In response to extracellular stimuli, the regulatory complex is released and verprolin/cofilin/acidic domains in the WASF proteins are exposed, allowing recruitment of actin monomers for polymerization.^{7,8} Each of the WASF proteins has been implicated in various aspects of cell movement involving reorganization of membrane structures such as ruffles, invadopodia, and lamellipodia.^{9,10} The same actin polymerization process is involved in cancer cell invasion and metastasis, but only *WASF3* has been shown to have a central role in this process because inactivation of *WASF3* in highly metastatic breast cancer cells leads to suppression of invasion and metastasis but inactivation of *WASF1* and *WASF2* does not.¹¹ The *in vitro* invasion phenotype has been correlated

with metastasis *in vivo* and loss of *WASF3* function in human cancer cells suppresses metastasis *in vivo*.^{12–14} Overexpression of *WASF3* in nonmetastatic cells leads to increased invasion and metastasis in *in vivo* experimental metastasis models.¹⁵ These *in vitro* and mouse xenograft studies, therefore, have clearly implicated *WASF3* in the promotion of metastasis and targeting its function suppresses metastasis in xenograft models.¹⁴ Gene expression studies in human cancers have further implicated *WASF3* in metastasis development with positive correlations between expression levels and unfavorable outcomes in breast cancer patients.¹⁶ The majority of functional analyses, however, have been performed in mouse model systems, where cells are manipulated *in vitro* and then metastasis is evaluated in xenograft studies *in vivo* in immunocompromised mice.

To investigate the effects of deleting *WASF3* in spontaneous models of breast cancer metastasis, a

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conditional *Wasf3* null mouse strain was generated and it was shown that homozygous inactivation of *Wasf3* leads to viable mice with no observable morphologic or behavioral abnormalities. To investigate the role of *Wasf3* in spontaneous metastasis, an oncogene was introduced into the *Wasf3* null mouse. The majority of analyses of *Wasf3* function in metastasis have centered on breast cancer; therefore, a mouse model of spontaneous metastasis was considered as follows: i) in which the incidence of the primary tumor was completely penetrant, ii) that showed a small latency period, iii) in which metastases developed in at least 90% of animals, iv) in which metastases developed with a short latency period, and v) in which all mice carried the transgene. The mouse mammary tumor virus–driven polyoma middle-T oncogene (PyMT) model¹⁷ develops palpable tumors by approximately 5 weeks of age in all animals and shows metastasis by approximately 4 to 6 months of age.¹⁷ Although human breast cancers do not typically express PyMT, this oncoprotein is responsible for dysregulation of many of the genes that are hallmarks of breast cancer including *MYCC*, *SRC*, *ERBB2*, integrins, cyclin D, *RAS*, and *PI3K*, as well as loss of estrogen receptor and progesterone receptor during progression.¹⁸ This wholesale dysregulation of key breast cancer–associated genes likely is responsible for the early onset and complete penetrance of breast cancer in this model. The tumors that develop in these mice also go through the same histologically defined changes seen in human tumors from benign hyperplasia to carcinoma.^{17,18} Here, the PyMT oncogene was introduced onto a *Wasf3* null background and a significant reduction in metastatic lung lesions, without elimination of metastasis, was shown in the *Wasf3* null mice.

Materials and Methods

Generation of the *Wasf3* Conditional Knockout Mouse Strain

The *Wasf3* conditional knockout vector was generated using homologous recombination strategies.¹⁹ Homology arms flippase recognition target were isolated from the RP23-134N18 bacterial artificial chromosome (BAC) genomic clone using Red recombination as described previously.²⁰ After homologous recombination, the vector contained exons 4, 5, and 6 of the *Wasf3* gene with approximately 5 kb of flanking sequence on either side. Two loxP sites and a chloramphenicol resistance gene (*Cat*) were inserted into the vector using PCR. Chloramphenicol-resistant clones were isolated carrying the pStart-WASF3-loxp/loxP vector. Flippase recognition target (FRT) and the neomycin resistance gene (*neo*), together with its phosphoglycerate kinase (PGK) promoter, were inserted into the vector as described previously.²⁰ A herpes simplex virus thymidine kinase (HSV-tk) cassette also was inserted as a negative selection marker using Gateway recombination.²⁰ The sequence-

verified targeting vector then was electroporated into C57BL/6 embryonic stem (ES) cells (at the Emory University Core facility) and clones subsequently were selected using G418 and fialuridine (FIAU) and sequence-verified for the homologous recombination event. ES cells were microinjected into C57BL/6-derived blastocysts to generate the targeted mouse. Subsequently, the blastocysts were surgically transferred to C57BL/6 pseudopregnant foster mothers to allow the embryos to come to term. Chimeric males with the high ES cell contribution were crossed with C57BL/6 females. Deletion of *Wasf3* was achieved by crossing to CMV-cre–expressing mice.

The conditional *Wasf3*^{fllox/fllox} mice are available from the Jackson Laboratory (stock 033353; Bar Harbor, ME).

Molecular Studies

DNA preparation and PCR amplification were performed using standard procedures.

Western blot was performed as described previously.¹⁰ Cells were lysed in ice-cold RIPA buffer [25 mol/L Tris-HCl, pH 7.5, 150 m NaCl, 1 m EDTA, 1% Triton X-100 (Fischer, Fair Lawn, NJ), and a mixture of proteases inhibitors] and analyzed by SDS-PAGE, followed by immunoblotting. Briefly, after blocking with 5% milk, the membranes were probed with primary antibodies at 4°C overnight. The membrane was washed and then incubated with secondary antibodies at room temperature for 1 hour. Immunoreactive proteins were developed with enhanced chemiluminescence reagents (Pierce, Rockford, IL). The following antibodies were used: mouse monoclonal anti- β -actin antibody (A5316; Sigma, St. Louis, MO) and WASF3 antibody (2806S; Cell Signaling, Danvers, MA). Horseradish-peroxidase–conjugated anti-mouse and anti-rabbit were used as secondary antibodies. Signal was detected and developed using the SuperSignal West Pico Chemiluminescent Substrate system (Thermo Scientific, Waltham, MA).

Histopathology

Whole Mount Analysis

The fourth inguinal glands were dissected at the indicated development times and spread on a glass slide. After fixation for 2 to 4 hours in Carnoy's fixative, the tissues were hydrated and stained in carmine alum overnight.²¹ Slides then were dehydrated, cleared in xylene, and mounted.

Tissue Sections

Tissues were fixed in 4% neutral formalin, dehydrated in alcohol, and embedded in paraffin using standard procedures. Six-micrometer sections then were prepared, dewaxed, stained with hematoxylin and eosin, and mounted on microscope slides.

Statistical Analysis

Individual data points were plotted using Prism8 GraphPad software (Graphpad Software Inc., San Diego, CA) in which the means and SDs were calculated. Significance was determined using a two-tailed *t*-test using Excel version 16.25 (Microsoft, Redmond, WA).

Results

Generation of a *Wasf3* Conditional Knockout Vector

The *Wasf3* start codon is located in exon 3 and, by deleting exons 4 and 5, a stop codon is generated at the beginning of exon 6 (Figure 1A), leading either to loss of the resultant mRNA through nonsense-mediated decay pathways or a severely truncated (52 amino acid) protein lacking both the important WASF regulatory domain and the verprolin/cofilin/acidic domains essential for WASF3 function in actin polymerization. The targeting construct (Figure 1B) was generated in the pSTART-K vector¹⁹ following a previously described approach.²⁰

The targeting vector was analyzed extensively using PCR and restriction enzyme digestion where the expected size-specific fragments were confirmed. Finally, the various essential regions of the vectors were sequenced individually to confirm the correct vectors had been produced. The sequence-verified targeting vectors then were electroporated into C57BL/6 ES cells and clones were selected using G418 and FIAU. These cells were microinjected into B6-derived blastocysts, which were transferred surgically to B6 pseudopregnant foster mothers to allow the embryos to come to term. Chimeric progeny were identified by PCR genotyping and males estimated to contain >80% ES cells were used to generate the F1 generation.

Generation of *Wasf3* Null Mice

Wasf3 heterozygous flox/+ mice were bred to generate homozygous conditional (flox/flox) null mice, which were verified through PCR analysis using tail-derived DNA. These mice then were bred to a strain expressing cre recombinase driven by the CMV promoter,²² which provides global knockout of the *Wasf3* gene. Genotyping of offspring (Figure 1, C and D) showed that the ratio of null and heterozygous null mice followed a Mendelian distribution and that the *Wasf3* null mice survived to term. Over the first 2 months of observation there was no obvious change in structural or behavioral phenotypes and the mice were the same size as normal mice. Because *Wasf3* is expressed strongly in neurons in the brain,²³ attention was provided to their general psychomotor activity and the ability to feed, grip, and walk, for which there was no difference compared with either heterozygous littermates or wild-type mice. High-resolution histopathologic analysis of the cortex of the null mice also was performed, which

showed no abnormalities in cortical structure (Supplemental Figure S1).

Because *Wasf3* is expressed highly in the brain, Western blot was used to analyze the presence of Wasf3 in the isolated cortex from the parental and knockout mice. Western blot analysis of the knockout mice showed an absence of the protein, confirming that targeted deletion of exons 4 and 5 led to loss of Wasf3 function as predicted (Figure 1E).

Wasf3 Loss Does Not Affect Mammary Gland Development

Because the aim of this project was to evaluate the role of *Wasf3* in spontaneous development of metastases in the mouse mammary tumor virus—PyMT mouse model of breast cancer, we first examined whether loss of Wasf3 function had any effect of normal mammary gland development. It was clear from the breeding studies, however, that the *Wasf3* null female mice gave birth to normal litter sizes (8 to 10 pups) and nursed the pups in the same way normal mice would. The fact that the pups survived and grew to adulthood indicated that the mammary glands were functioning normally. Histopathologic analysis of the gland in normal virgin mice is shown in Figure 2, showing normal development within the gland generating a normal ductal tree with multiple tertiary branches. Pregnant females showed normal secondary and tertiary ductal development with alveologenesis and branching of the ducts and lactating animals showed typical ductal expansion (Figure 2). Thus, there was no evidence of any abnormal development or function of the mammary gland as a result of loss of *Wasf3*.

Loss of *Wasf3* Reduces Metastatic Lesions in the Lung

The mouse mammary tumor virus—PyMT mice develop tumors spontaneously in all animals after 3 to 4 months and metastasis occurs in the vast majority of these animals within 6 to 8 months. This metastatic ability of the primary tumor cells in the mammary gland was shown by analysis of vimentin and E-cadherin, two markers of the epithelium-to-mesenchyme transition. Mammary gland tumors showed down-regulation of E-cadherin and increased levels of vimentin, consistent with the epithelium-to-mesenchyme transition phenotype (Supplemental Figure S2).

To determine whether loss of *Wasf3* affected spontaneous metastasis of breast cancer tumor cells, the mouse mammary tumor virus—PyMT oncogene was introduced onto the *Wasf3* null background, using the breeding protocol described in *Materials and Methods*. Mice were genotyped for PyMT+WASF3^{−/−}, and parallel cohorts with parental PyMT mice were maintained to compare the effect of loss of Wasf3 function on metastasis. The number and size of tumors in PyMT and PyMT/*Wasf3*^{−/−} mice was studied. The lungs from the same two cohorts of mice were removed at sacrifice to evaluate metastatic load. Macroscopic analysis of the unfixed lungs from the PyMT mice frequently

Figure 1 Generation of the *Wasf3* null mouse strain. **A:** Schematic of the exon structure of the *Wasf3* gene showing the consequences of deleting exons 4 and 5, leading to a stop codon in exon 6. **B:** The targeting construct to generate the deletion through homologous recombination. **C:** Genotyping for the wild-type (WT) allele involves using primers (WTF1) located in exon 5 and WTF2 located downstream in intron 5 (WT forward: 5'-TCTAAGGCTTGGACCTTGACACTGGCTGGA-3'; WT reverse: 5'-TGACTTTGACAGCAAGCCGATCGA-3'), which produces a 226-bp fragment only in the WT mice. When exons 4 and 5 are deleted, no PCR product is generated with these primers. Using primers WASF3F and WASF3R, designed from within introns 3 and 5 (forward: 5'-AGCAGTAA-CATCTTGACTGACAGCTCAGGCA-3'; reverse: 5'-TGACACATGCAACCAGCATAGCAA-3'), a 266-bp fragment is generated across the deletion site. **D:** Analysis of mice after expression of Cre recombinase using the primers described in **C** shows the WT PCR product in wild-type mice but not the PCR fragment across the deletion site. Instead, a 2214-bp fragment is generated including exons 4 and 5. Using the WASF3F and WASF3R PCR primers, the 266-bp fragment is present only in the null mice. **E:** Western blot analysis of proteins from the brains of *Wasf3* null mice (-/-) shows the absence of the Wasf3-specific product compared with that seen in heterozygous floxed mice (+/-) or WT mice. The Lgi1 protein, which is highly expressed in the mouse cortex, was used as the loading control. E, exon; F, forward; FRT, flippase recognition target; KO, knockout; NC, negative control with no template; PC, positive control from the original founder mice; R, reverse.

Figure 2 Development of the mammary gland in *Wasf3* null mice. **A** and **B**: Whole mount, carmine alum–stained mammary glands from *Wasf3* null virgin mice show the typical ductal structure. **C** and **D**: Ten-day-old pregnant females show ductal expansion (**C**), which is even more accentuated in day 5 lactating females (**D**). d, day.

showed the presence of large surface nodules (Supplemental Figure S3). None of the lungs from the PyMT-*W3*^{-/-} mice showed these macroscopic lesions, suggesting, at least at this level of resolution, that the loss of *Wasf3* could suppress metastatic dissemination. The lungs then were fixed in formalin and subjected to histologic analysis. For each lung, two sections were prepared, with the first cut through the center of the lung sample and the second section was prepared 100 μ m deeper into the lung. Each section was stained with hematoxylin and eosin and the presence and size of tumors were evaluated.

The variation in tumor incidence seen on the lung surface (Supplemental Figure S3) was reflected in the histologic analysis, which showed large tumors (Figure 3A) distributed throughout the lungs in 40% of the PyMT mice. In other animals, the lungs showed variation in both the size and number of tumors (Figure 3A), although all mice showed at least one metastatic lesion, in one case, only a single small lesion was detected. Histology of the lungs from the PyMT/*Wasf3*^{-/-} mice, however, showed no metastatic lesions in the lungs in 5 of 13 (38%) mice. In the other mice, there were generally fewer, smaller tumors in the lungs (Figure 3B). Because the extent of metastasis likely is dependent on the tumor load at the primary site in the breast, it was shown that although there was some heterogeneity in the latency with which the primary tumors arose in the mammary fat pads, typically the parental PyMT mice developed large tumors within 120 to 140 days in the majority of mammary fat pads. The same was true for the PyMT/*Wasf3*^{-/-} mice and there was no significant difference in the number of primary tumors in the two cohorts (Figure 4A). When the number of individual tumor foci was counted in the sections from the two different depths in the lung, there was no significant difference within each genotype (Figure 3B). When the total number of tumors identified in the tissue sections was compared between genotypes, there was a significant reduction ($P = 0.0255$) in the number of metastatic lesions in the PyMT/*Wasf3*^{-/-} mice (Figure 4B). Next, the area of each lung occupied by tumor metastases was measured. ImageJ software version 1.48 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) was used to measure the total area in each tissue section and was expressed as a percentage of the total area of the lung present in the section. In the PyMT mice there was clearly a bimodal distribution in the extent of the tumor area in the lungs, with several mice showing >90% of the lungs occupied by metastases, which skewed the data. When these mice were excluded from the analysis, there was a marginally significant ($P = 0.057$) reduction in the overall area of the lungs occupied by tumor in the PyMT/*Wasf3*^{-/-} mice (Figure 4C).

Analysis of *WASF3* in primary human breast cancers has suggested an association with more aggressive tumors such as the claudin-low subgroup, which has the poorest survival.¹⁶ To determine whether *Wasf3* is up-regulated in the metastatic lesions in the mouse models described here, *Wasf3* expression levels were analyzed in primary breast tumors from the PyMT and the PyMT-*Wasf3*^{-/-} mice and their metastases. None of the primary breast tumors that developed in either genotype ($n = 5$) showed detectable *Wasf3* expression (Figure 4D). Analysis of *Wasf3* expression levels in the metastatic lung lesions in the PyMT mice ($n = 3$), compared with the primary breast tumors, showed up-regulation of *WASF3* (Figure 4D), supporting the suggestion seen in human studies that increased *WASF3* expression correlates with metastatic progression. As expected, neither the primary tumors nor the metastatic lesions

Figure 3 Histologic analysis of lungs from PyMT and PyMT/*WASF3* null mice. **A:** Hematoxylin and eosin–stained sections from representative PyMT mice show the variation in tumor number and size of metastatic lesions in the lungs. The tumors in these mice are typically large (MT3, MT8, MT12), although smaller tumors also are present (arrows). **B:** Analysis of lungs from PyMT/*WASF3* null mice typically show only fewer, small tumors (arrows), with the exception of the single large tumor seen in mouse KO10 and the absence of tumors in mouse KO11.

in the lungs of the PyMT/*WASF3*^{−/−} mice expressed *WASF3* (Figure 4E).

Discussion

Evidence suggesting a role of *WASF3* in the development of metastasis has been established through the following: i) correlative studies in human cancers,¹⁶ ii) *in vitro* manipulation of *WASF3* expression levels,^{10,13,24} and iii) pharmacologic studies targeting *WASF3* function *in vivo*.¹⁴ The *in vitro* studies provide a more robust model to evaluate the molecular mechanisms behind *WASF3*

regulation of invasion and motility, which is supported consistently in xenograft studies using the genetically manipulated cells. Together, these studies have shown that *WASF3* function has a significant role in suppressing metastasis. These studies, however, consistently showed that loss of *WASF3* function does not lead to total elimination of invasion and metastasis, possibly because shRNA knockdown used in these studies could not totally eliminate expression. The absence of *WASF3* expression in mammary glands, although not affecting normal breast development or restructuring ductal expansion during pregnancy, leads to a significant suppression of the number and size of the metastases, although cannot totally prevent metastatic dissemination to the lungs. The mechanism of metastasis typically begins with intravasation into blood vessels and/or the lymphatic system. A zebrafish model of human breast cancer cells showed that metastasis²⁵ is suppressed as a result of *WASF3* knockdown. This is likely owing to the regulation of metastasis-promoting genes such as various matrix metalloproteinases (MMPs), which are regulated by *WASF3*.^{26,27} This may provide one explanation for the reduced number of metastases, possibly through delays in the release of metastasizing cells as a result of their reduced ability to intravasate, and why the tumors in the lungs are generally smaller or absent. Successful development of metastatic lesion in sites such as the lungs also depends on extravasation from the blood vessels into the distant organ, which is facilitated by the same mechanisms leading to intravasation. In experimental models of metastasis in which the cells are injected directly into the blood vessels and therefore bypass intravasation, there has been a reduction in the number and size of metastatic lesions in animals receiving cells in which *WASF3* has been suppressed,¹² again possibly explaining why primary breast cancer cells in our PyMT model show smaller and fewer metastatic lesions in the lungs. Once established in the lungs, however, these metastatic lesions can expand because loss of *WASF3* does not have a significant effect on cell proliferation.^{28,29} Therefore, the number of metastatic lesions, rather than their size, is the best indication of metastatic potential. It is also possible that, even though *WASF3* exerts a significant effect on the metastasis process, other genetic changes in cancer cells can compensate for this loss and use other pathways to promote metastasis. The apparently random nature of the acquisition of these compensatory events also could contribute to the heterogeneity in latency and hence size of the metastatic lesions.

Although the involvement of *WASF3* in the suppression of breast cancer metastasis has been established using model cell systems, it is clear from the studies presented here that loss of *WASF3* does not prevent metastasis in the PyMT model of breast cancer. There is a major difference, however, in investigating the role of *WASF3* in highly evolved cell lines compared with primary breast epithelial cells. The up-regulation and activation of *WASF3*

Figure 4 Molecular analysis of tumors and metastases from PyMT and PyMT/*Wasf3* null mice. **A:** Analysis of the number of primary tumors that arise in the mammary glands of the two cohorts showed no significant difference comparing PyMT and PyMT/*Wasf3* null mice. **B:** The number of individual metastatic lesions was scored from each of the two different lung sections from each mouse. There was no significant difference between sections A and B from the PyMT mice or from sections C and D from the PyMT/*Wasf3* null mice. When duplicate data from each cohort were combined, there were statistically fewer lesions in the lungs from the PyMT/*WASF3* null mice. **C:** Analysis of the sum area of the lung occupied by tumor, relative to the overall size of the lung in the section, shows a marginally significant reduction in tumor volume in the *Wasf3* null mice compared with the pyMT mice. **D:** Western blot analysis of paired tumors and lungs from three randomly selected PyMT mice (MT) shows *Wasf3* expression only in the lungs of these animals. In a similar comparison between *Wasf3* null mice (W3) there is no *Wasf3* expression in either tissue. **E:** Analysis of *Wasf3* in the brains of *Wasf3* null mice (W3KO) compared with brains from wild-type mice (BL6) and MDA231 shows no expression of *Wasf3*. Primary tumors from MT and W3KO mice show no *Wasf3* expression. Similarly, embryonic fibroblasts (B6MEF) derived from B6 mice and the *Wasf3* null mice (W3KOMEF) do not express *Wasf3*. $n = 10$ (**B**); $n = 3$ (**D** and **E**). $P = 0.0255$ (**B**), $P = 0.057$ (**C**). ACTB, beta-actin; KO, knockout; W, *Wasf*.

promotes metastasis and this can be achieved through activation of receptors such as human epidermal growth factor receptor 2,³⁰ interleukin 6 signaling through the Janus kinase/signal transducers and activators of transcription pathway,³¹ and other growth factors (such as *PDGFR*²⁶). As a result, one of the major consequential effects was on gene expression levels, particularly the down-regulation of *KISS1*, which is involved in NF- κ B signaling through *ZEB1*.^{15,27} It therefore seems that deregulation of a number of pathways is required for *WASF3* to function as a metastasis suppressor and, although beyond the scope of this study, whether the same conducive genetic environment is present in the PyMT tumors, remains to be determined. Clearly, from the study of primary human breast cancers, however, up-regulation of *WASF3* is a late event in the transformation process,¹⁶ which is also the case in the PyMT model.

Although the majority of early studies focused on breast cancer, in our case, because of the availability of convenient *in vivo* models to evaluate molecular mechanisms,¹² there is evidence that *WASF3* also can influence metastasis and outcome in other cancer types. We have shown, for example, that suppression of *WASF3* in

prostate cancer cells also leads to reduced invasion and metastasis¹³ as well as in colon cancer cells²⁹ and melanoma (unpublished). Beyond these studies, there are reports comparing *WASF3* expression levels and metastasis/outcome in, for example, hepatocellular carcinoma³² and small cell lung cancer,³³ but in these largely correlative studies it was not possible to determine the specific role of *WASF3*. Functional relationships between *WASF3* and metastasis, however, have been established in ovarian cancer,³⁴ gastric cancer,³⁵ pancreatic cancer,³⁶ and osteosarcoma.³⁷ The availability of the *Wasf3*^{flx/flx} strain, therefore, provides the opportunity to investigate the role of *WASF3* in other cancer models such as the transgenic adenocarcinoma of the mouse prostate (TRAMP) model for prostate cancer³⁸ or the multiple intestine neoplasia (MIN) mouse for colon cancer.³⁹

The *WASF* family of genes comprises three members that share homologous regulatory and verprolin/cofilin/acidic domains related to actin polymerization dynamics. Knockdown of *WASF1* and *WASF2* in highly metastatic breast cancer cells does not affect invasion, whereas knockdown of *WASF3* has a profound influence on invasion and metastasis.¹¹ It appears, therefore, that the other members of this

gene family determine overlapping, but nonredundant, functions, which is reflected in the phenotypes of mice null for these genes. *Wasf3* null mice appear to have normal development. In contrast, the *WASF1* null mice survive to term but show neurologic defects that affect brain development and die in the first month of life.^{40,41} *Wasf2* null mice are embryonic lethal, showing distinct phenotypes associated with abnormal brain development.⁴² Brain development in *Wasf3* null mice, however, is apparently normal, suggesting it has a more restricted role in development.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.08.012>.

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