Surgically-Induced Multi-organ Metastasis in an Orthotopic Syngeneic Imageable Model of 4T1 Murine Breast Cancer

YONG ZHANG[1], NAN ZHANG[1], ROBERT M. HOFFMAN[1,2] and MING ZHAO[1]

[1]*AntiCancer, Inc., San Diego, CA, U.S.A.;*
[2]*Department of Surgery, University of California, San Diego, CA, U.S.A.*

Abstract. *Background/Aim: Murine models of breast cancer with a metastatic pattern similar to clinical breast cancer in humans would be useful for drug discovery and mechanistic studies. The 4T1 mouse breast cancer cell line was developed by Miller et al. in the early 1980s to study tumor metastatic heterogeneity. The aim of the present study was to develop a multi-organ-metastasis imageable model of 4T1. Materials and Methods: A stable 4T1 clone highly-expressing red fluorescent protein (RFP) was injected orthotopically into the right second mammary fat pad of BALB/c mice. The primary tumor was resected on day 18 after tumor implantation, when the average tumor volume reached approximately 500-600 mm^3. Results: When the post-surgical mice were sacrificed 6-8 weeks after cell implantation, metastases were found in the lung in 91%; in the lymph nodes in 100%, including axillary nodes; in the brain in 25%; and in bone in 42% of the mice. The metastases were readily visualized by fluorescence imaging. Detailed fluorescence analysis visualized extensive metastasis in the thoracic cavity and the lymphatic system. Large metastatic nodules in the lung involved most of the pulmonary parenchyma in all lobes. In the liver, fluorescent macroscopic metastatic nodules were found under the capsule. Bone metastases were found mainly in the spine and thigh bone. Conclusion: Metastasis appeared to be enhanced by resection of the primary tumor. The metastatic pattern in the model thus reflected the clinical metastatic pattern of breast cancer and should be of use for discovery and evaluation of novel therapeutics.*

The 4T1 murine breast cell line was originally developed by Miller *et al.* (1, 2) to study metastatic tumor heterogeneity. When implanted orthotopically, 4T1 has been shown to metastasize to organs similarly to clinical breast cancer in humans, including to lungs, liver, brain and bone (3-6). Tao *et al.* transformed the 4T1 cell line to express luciferase for longitudinal detection of primary growth and metastases (7). In their study, metastasis at high rates, including the lungs, liver and bone, occurred in most animals within six weeks with lower frequency of metastasis to brain and other sites. This imageable model is limited by the weak signal of luciferase which requires photon-counting of anesthetized animals. The weak signal of luciferase cannot produce a true image. Pseudo images overlain on mice are used to indicate the numbers of photons emitted (7).

We previously developed a red fluorescent protein (RFP)-expressing subline of 4T1. We examined the effects of mesenchymal stromal cells (MSCs) on the tumorigenicity of 4T1-RFP cells. When co-injected with MSCs into the mouse mammary fat pad, 4T1-RFP cells exhibited enhanced primary tumor growth and increased spontaneous lung metastasis. Using *in vivo* fluorescence color-coded imaging, the interaction between green fluorescent protein (GFP)-expressing MSCs and RFP-expressing 4T1 cells was visualized. As few as five 4T1 cells successfully gave rise to tumor formation when co-injected with MSCs into the mouse mammary fat pad. However, no tumor was formed when five or 10 4T1 cells were implanted alone. Moreover, *in vivo* longitudinal fluorescence imaging demonstrated that MSCs created a vascularized environment which enhanced the ability of 4T1 cells to colonize and proliferate (8).

In the present study, we demonstrate the surgically-enhanced metastatic potential of 4T1-RFP cells orthotopically implanted in immunocompetent BALB/C mice in which metastasis can be readily detected by fluorescence imaging.

Materials and Methods

Mice. A total of 12 female normal BALB/c mice (AntiCancer, Inc., San Diego, CA), 5-6 weeks old, were used in the study. Test animals were bred and maintained at AntiCancer Inc. in a high-efficiency particulate arrestance (HEPA)-filtered environment for the

This paper is dedicated to the memory of A. R. Moossa, M.D.

Correspondence to: Robert M. Hoffman, Ph.D., AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111, U.S.A. Tel: +1 8586542555, Fax: +1 8582684175, e-mail: all@anticancer.com

Key Words: Breast cancer, Balb-c mice, syngeneic, orthotopic, tumor resection, metastasis, RFP, imaging.

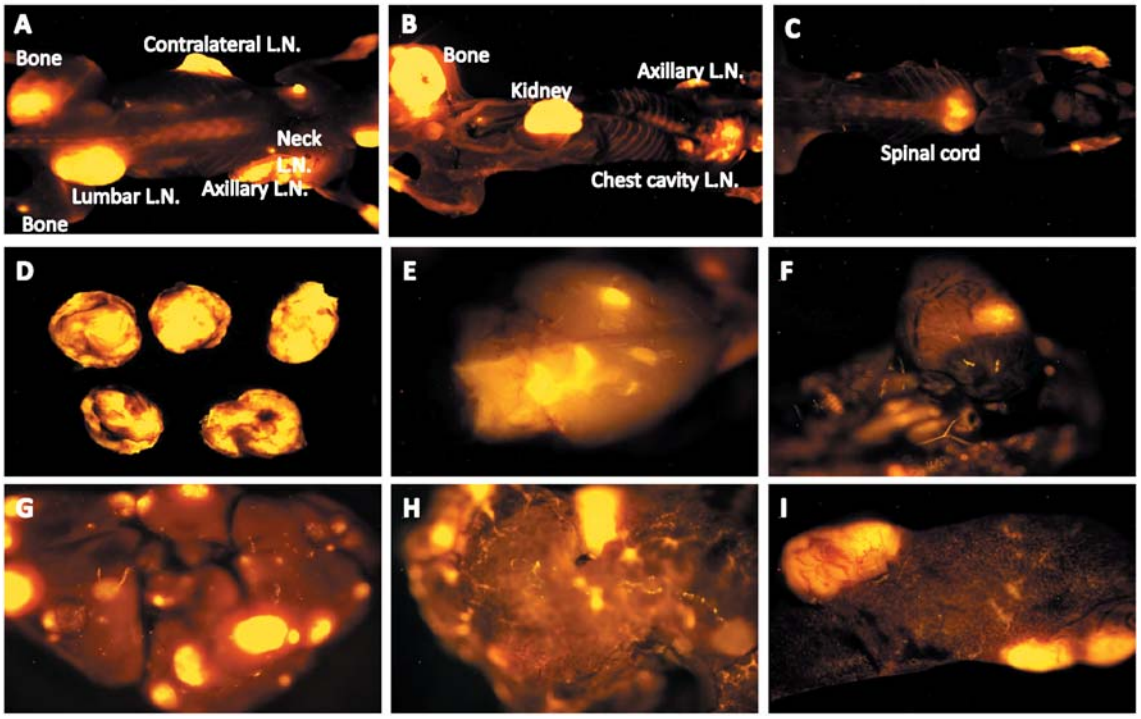

Figure 1. *Multiorgan metastasis of 4T1 cells visualized by red fluorescent protein (RFP) expression occurs after resection of the primary tumor. 4T1-RFP breast cancer cells (5.0×10^6/100 μl) were injected into the right second mammary gland. The primary tumor was removed on day 18 after tumor implantation, when the average tumor volume reached approximate 500-600 mm^3. After tumor progression in the animals post-surgery, the performance status of the mice began to decrease, at which time the animals were sacrificed and autopsied. The whole animal and organs were imaged as described in the Materials and Methods. All images were obtained with the Olympus OV100 Small Animal Fluorescence Imaging System. A-C: Whole-body images; D: removed primary tumor; E: removed brain; F: removed heart; G: removed lungs; H: removed liver; I: removed spleen. LN: Lymph node.*

experiment. Cages, food and bedding were autoclaved. The animal diets were obtained from PMI Nutrition International Inc. (Brentwood, MO, USA). Animals were housed in a barrier facility on a HEPA-filtered rack under standard conditions of a 12-h light/dark cycle. Animals were housed with no more than five per cage. The animals were fed an autoclaved laboratory rodent diet.

All animal studies were conducted with an AntiCancer Institutional Animal Care and Use Committee protocol specifically approved for this study and in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1. In order to minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments. Animals were anesthetized by intramuscular injection of a 0.02 ml solution of 20 mg/kg ketamine, 15.2 mg/kg xylazine, and 0.48 mg/kg acepromazine maleate. The response of animals during surgery was monitored to ensure adequate depth of anesthesia. Ibuprofen (7.5 mg/kg orally in drinking water every 24 h for 7 days post-surgery) was used in order to provide analgesia postoperatively. The animals were observed on a daily basis and humanely sacrificed by CO_2 inhalation when they met the following endpoint criteria: prostration, skin lesions, significant body weight loss, difficulty in breathing, epistaxis, rotational motion or body temperature drop.

Preparation of RFP-expressing 4T1 cells. For RFP gene transduction, 70% confluent 4T1 cells (8) were incubated with a 1:1 mixture of retroviral supernatants of RFP-PT67 packaging cells (Clontech, Mountain View, CA, USA) and RPMI-1640 for 72 h. Fresh medium was replenished at this time. 4T1 cells were harvested by trypsin-EDTA 72 h post transduction and subcultured at a ratio of 1:15 into selective medium that contained 200 μg/ml G418. The level of G418 was increased to 1000 μg/ml stepwise in order to select for high expression of RFP. The brightest 4T1 cell clones expressing RFP were selected, combined, and then amplified and transferred by conventional culture methods (9-11).

Mammary fat pad injection of 4T1-RFP cells. Nude mice were anesthetized i.m. with the ketamine mixture. 4T1-RFP cells (5.0×10^6) in 100 μl serum-free solution were injected slowly into the right second mammary gland (underneath the nipple). The needle holes were pressed in order to prevent any cancer cells from overflowing and seeding at the incision site (12).

Surgical removal of orthotopic primary tumor. The primary tumor was removed on day 18 after tumor implantation, when the average tumor volume reached approximate 500-600 mm^3. The animals were anesthetized with the ketamine mixture, and the tumors were resected. The surgical area was sterilized using iodine

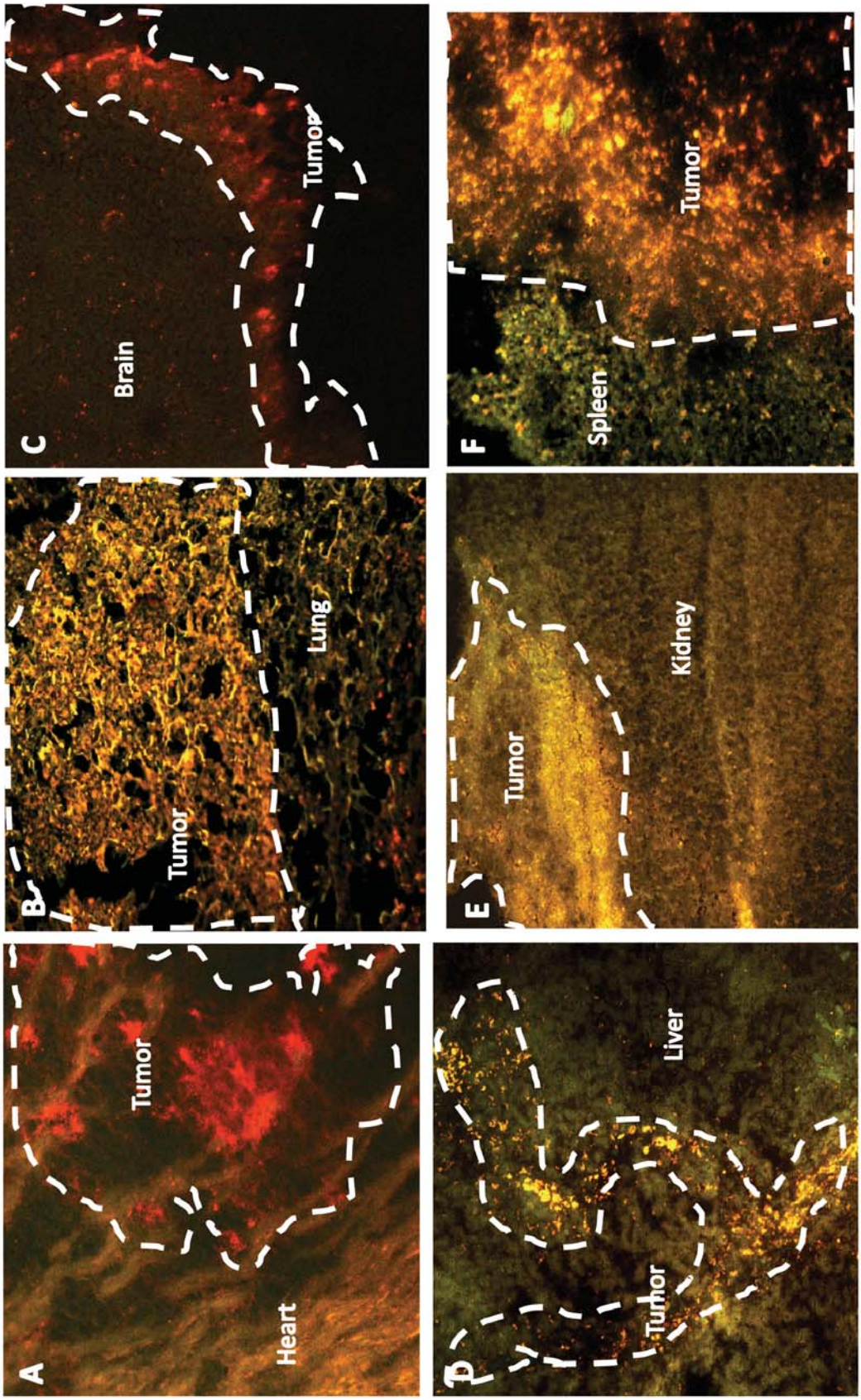

Figure 2. *Visualization of 4T1 metastases by red fluorescent protein (RFP) in fresh BALB/c mouse tissue occurring after resection of primary tumor when the mice were sacrificed 6-8 weeks after cell implantation. Metastasis: heart (A); lung (B); brain (C); liver (D); kidney (E); and spleen (F). All images were obtained with the Olympus IV100 laser scanning imaging system.*

Table I. *Metastatic pattern of 4T1 cells after surgical resection of the primary tumor. Twelve BALB/C mice were each injected orthotopically with 5×10^6 cells and the tumor was resected on day-18 after cell injection. The mice were sacrificed at 6-8 weeks after cell injection, at the time of significant decline in performance status.*

Mouse no.	Site of tumor spread								
	Brain	Spinal cord	Lung	Heart	Liver	Spleen	Kidney	Lymph node	Bone
1			+		+			+	
2		+			+			+	+
3			+		+			+	
4			+		+			+	
5			+		+			+	
6	+		+		+			+	+
7	+		+	+	+		+	+	+
8		+	+	+	+	+		+	+
9	+		+		+			+	
10			+		+			+	
11			+		+			+	
12			+			+	+	+	+

and alcohol. After proper exposure of the tumor following the right higher-chest skin incision, the artery and vein connecting the tumor were ligated and cut off. The tumor was then removed from the right higher-chest wall. The wound was closed with a 6-0 surgical suture (silk).

Imaging. The Olympus OV100 Small Animal Imaging System (Olympus, Tokyo, Japan) containing an MT-20 light source (Olympus) and DP70 CCD camera (Olympus) was used. The instrument combines high numerical aperture and long working distance. Four individually optimized objective lenses, parcentered and parfocal, provide a 105-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 lenses are mounted on an automated turret with a high magnification range of ×1.6 to ×16 and a field of view ranging from 6.9 to 0.69 mm. Images were captured on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and CellR (Olympus Biosystems) (13).

An IV100 scanning laser microscope with a 488 nm argon laser (Olympus) was also used. The novel stick objective (UMPLANFLN, ×10/030W) (Olympus) delivers very high-resolution images. All images were recorded and stored as proprietary multilayer 16-bit Tagged Image File Format files (14).

Frozen sections and hematoxylin & eosin (H & E) staining. All fresh sample specimens were immediately frozen in liquid nitrogen, embedded in tissue-freezing embedding medium (Triangle Biomedical Sciences, Durham, NC, USA) and stored at −80˚C until further processing. Frozen sample sections, 10 μm-thick, were cut with a Leica CM1850 cryostat (Leica Biosystems, Nussloch, Germany) and air dried. The frozen sample sections were directly observed with the IV100. Subsequently, the frozen sections were used for H & E staining.

Analysis of metastases. When the performance status of the mice began to decrease, the animals were sacrificed and autopsied. BALB/c mice were sacrificed 6-8 weeks after injection of 4T1-RFP cells. Metastases were visualized with RFP imaging and confirmed by histology. All major organs were explored. The fresh samples were sliced at 1 mm thickness and observed with the IV100.

Results and Discussion

Twelve BALB/C normal mice were used for orthotopic transplantation of the 4T1-RFP breast cancer cells. All 12 mice had primary tumor growth after transplantation. The primary tumors were encapsulated, with no local invasion or distal organ metastasis observed upon removal of the primary tumor. After surgery, major organs involved with metastasis included lung and liver metastasis (eleven of twelve mice); lymph node metastasis (twelve of twelve mice); brain metastasis (three of twelve mice); spine, heart, spleen and kidney metastasis (two of twelve mice each); and bone metastasis (five out of 12 mice) (Figures 1-4; Table I).

Multi-organ metastasis of 4T1-RFP mammary carcinoma cells occurred after surgical resection of the primary orthotopic tumor. The 4T1 model is syngeneic in BALB/c mice and can be used to study the role of the immune system in tumor growth. Tao *et al*. observed biphasic growth of 4T1 where the primary tumor became necrotic and then grew again, with metastasis occurring during the second growth phase (7). It is possible that resection of the tumor in our study enhanced metastatic growth. Both Tao *et al*.'s study (7) and ours suggest the presence of primary tumor may suppress metastasis. Further studies are necessary to investigate the role of the primary tumor on

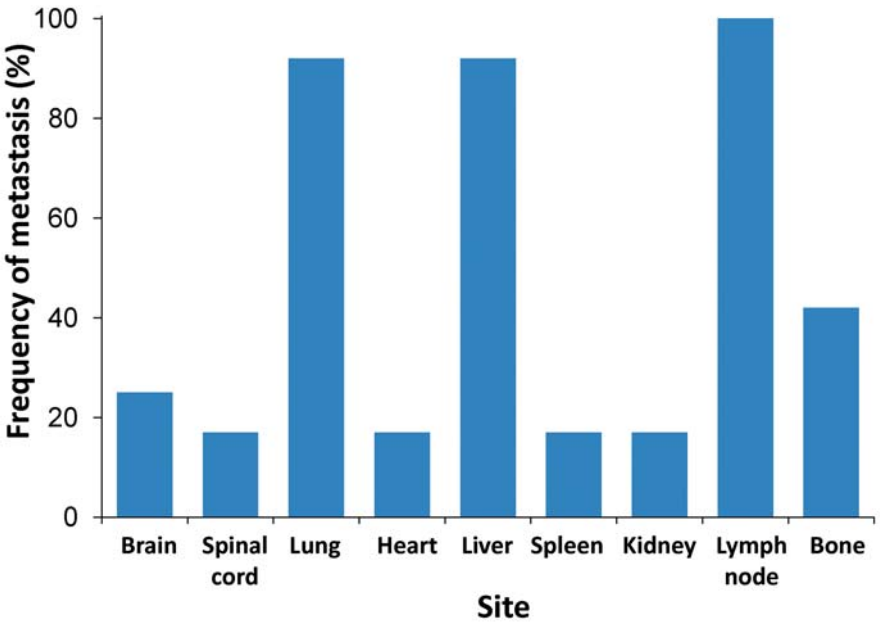

Figure 3. *Frequency of 4T1 metastasis at different sites. Please see the Material and Methods for details.*

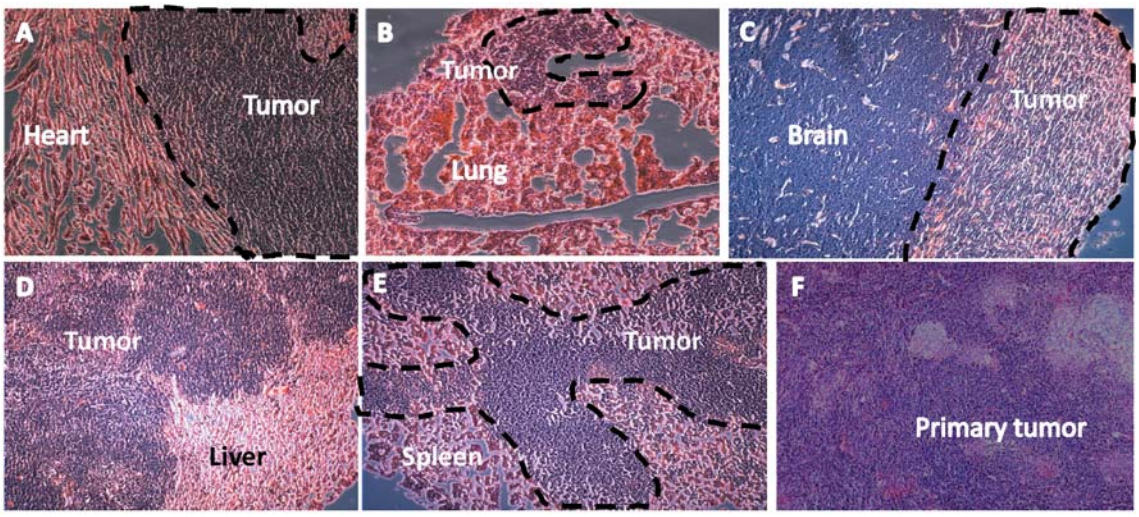

Figure 4. *Hematoxylin & eosin (H & E) staining of sections from heart (A), lung (B), brain (C), liver (D) and spleen (E) with metastases, and the primary tumor (F).*

metastasis. In another study, laparotomy increased metastatic growth, reduced tumor cell apoptosis, increased tumor cell proliferation, increased microvessel density and circulating vascular endothelial growth factor (VEGF) (15). Surgery may also increase metastasis by impairing circulating natural killer and lymphocyte-activated killer cells (15-17). The possibility of surgery-induced metastasis (18-25) in the 4T1 model will be further investigated in future studies.

The 4T1-RFP model has important advantages over the luciferase 4T1-RFP model described by Tao *et al*. (7) in that the strong signal of RFP, which is about 1000 times stronger than that of luciferase (26), allows true imaging of metastasis in any organ.

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