Murine Endothelial Cell Lines as Models of Tumor Endothelial Cells

Jennifer Walter-Yohrling, Sharon Morgenbesser, Cecile Rouleau, Rebecca Bagley, Michelle Callahan, William Weber, and Beverly A. Teicher
Genzyme Corporation, Framingham, Massachusetts

ABSTRACT
Identification of appropriate models for in vivo and in vitro preclinical testing of inhibitors of tumor angiogenesis and progression is vital to the successful development of anticancer therapeutics. Although the focus is on human molecular targets, most preclinical in vivo efficacy testing occurs in mice. The goal of the current studies was to identify a murine endothelial cell line to model tumor endothelium for studying the antiangiogenic activity of therapeutic compounds in vitro. In situ hybridization was performed on three s.c. grown syngeneic murine tumors (B16 melanoma, Lewis lung carcinoma, and CT26 colon carcinoma) to assess expression of murine homologs of human tumor endothelial cell markers in the vasculature of these tumor models. Seven murine endothelial cell lines were characterized for expression of the murine homologs of recognized endothelial cell surface markers as well as for tumor endothelial cell surface markers. The seven murine endothelial cell lines had similar generation times and five of the seven lines were able to form tubes on Matrigel. Real-time-PCR and flow cytometry analysis were used to evaluate relative mRNA and protein expression of murine homologs of several recognized endothelial cell surface markers in the seven cell lines. The expression of the mRNA for the murine homologs of five tumor endothelial cell surface markers was also evaluated. The 2H11 cell line expressed all five of the tumor endothelial cell surface markers as well as several well-recognized endothelial cells markers. The 2H11 cell line responds to known and novel antiangiogenic agents by inhibition of proliferation and tube formation. These cells can be used in in vitro angiogenesis assays for evaluating the potential antiangiogenic properties and interspecies cross-reactivity of novel compounds.

INTRODUCTION
The mouse has become the critical host organism for the discovery and development of modern cancer therapeutics. However, it is important that we understand the strengths and limitations of mouse models of malignant disease (1). The most frequently used murine models are transplantable syngeneic or xenograft tumors and transgenic mice that develop autologous tumor nodules during their lifetimes. These models allow the study of biological processes associated with tumor growth, including the tumor-host interactions with murine stromal cells.

The abnormality of tumor vasculature has been studied extensively in mouse models. Modzelewski et al. (2) isolated and characterized fresh tumor-derived endothelial cells from the syngeneic RIF-1 fibrosarcoma. The dynamics of blood vessel growth, structure, cellular composition and gene expression have been followed using the murine syngeneic mammary MCaIV adenocarcinoma, the murine syngeneic Lewis lung carcinoma, and the murine transgenic RIP-Tag2 pancreatic islet cell tumor (3–5). The mobilization of bone marrow progenitor cells to circulation, the maturation of these cells into endothelial precursor cells, and the incorporation of the endothelial precursor cells from circulation into tumor vasculature has been elucidated largely in mice (6–14). These studies used murine syngeneic tumors, human tumor and bone marrow xenografts in immunodeficient mice and genetically engineered mice, and have been used to recapitulate the dynamics of processes observed in human patients.

Cancer therapeutics has moved away from general proliferation-related targets such as DNA and tubulin toward more nontraditional pathological processes including angiogenesis or immunomodulation, as well as more selective molecular targets. In most cases, efficacy in mouse models remains the critical determinant of whether a potential therapeutic moves into development in clinical trials. However, it has become evident that the homology between the murine and human proteins of specific molecular targets is, frequently, not sufficient to depend on efficacy of agents selected for the murine target to translate into highly effective therapy in the human clinic. This is most evident in the selection of monoclonal antibodies where it is has become necessary to develop monoclonal antibodies to the murine homologue of the human target (11). Therefore, mouse and human agents need to be developed in parallel, so that the tumor-bearing mouse remains the critical efficacy hurdle. Likewise, it is important to define cell-based models of murine tumor endothelial cells that can be used to evaluate potential therapeutics in cell culture to select those most appropriate for in vivo testing.

The isolation and maintenance of a pure population of primary murine endothelial cells has proven to be difficult and has restricted the use of these cells in angiogenesis assay systems. To overcome these barriers, several groups have generated murine endothelial cell lines from cells isolated from the axil-
Table 1
List of endothelial cell markers studied, the antibody used for FACS® analysis, and primer sequences for mRNA expression analysis

<table>
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<th>EC marker</th>
<th>Antibody</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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FACS, fluorescence-activated cell sorter; EC, endothelial cell; ENDRB, endothelin-B receptor; VCAM, vascular cellular adhesion molecule; VEGFR, vascular endothelial growth factor receptor; cat., catalogue number.

Materials and Methods

Chemicals. Murine endothelial cell lines, SVEC4–10, SVEC4–10EE2, SVEC4–10EHR1, 2F2B, 2H11, IP1B, and IP2-E4, were purchased from American Type Culture Collection (Manassas, VA). Human dermal neonatal microvascular endothelial cells (HMVEC) and EGM2-MV were obtained from Cambrex (East Rutherford, NJ). Cell culture media, serum, and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA). Primary antibodies against endothelial cell markers were purchased from PharMingen (San Diego, CA) and Chemicon (Temecula, CA; Table 1). Phycoerythrin and fluorescein-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). PCR primers that recognize the human and mouse gene were purchased from Integrated DNA Technologies (Coralville, IA). Trizol was purchased from Sigma (St. Louis, MO), and the RNA Extraction Kit was obtained from Qiagen (Valencia, CA). The High-Capacity cDNA Archive Kit, Taqman rRNA Control Reagents, Taqman Universal PCR Master Mix, and Sybr Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Cell Titer Glo was purchased from Promega (Madison, WI).

Cell Culture. The murine endothelial cell lines 2F2B, 2H11, 3B11, IP2-E4, SVEC4–10, SVEC4–10EER2, and SVEC4–10EHR1 were maintained in DMEM plus 10% fetal bovine serum in a humidified 10% CO2 environment. HMVECs were maintained in EGM2-MV that included 5% FBS, vascular endothelial growth factor (VEGF), basic fibroblast growth factor,
epidermal growth factor, and insulin-like growth factor, in a humidified 5% CO₂ atmosphere.

**Generation Time and Proliferation.** To determine cellular growth rates, cells from each of the seven murine endothelial cell lines (2 × 10⁵) were placed in each well of a 96-well plate in DMEM supplemented with 0, 2, 5 or 10% FBS. The cells were collected after 24, 48, 72, and 96 h using Cell Titer Glo and extrapolating cell number from a standard curve for each cell line. Generation time was calculated by applying an exponential curve fit to the cell number values and calculating the time required for the cell number to double. For proliferation experiments, cells were exposed to each agent for 48 h, and cell numbers were determined as described above.

**In Vitro Tube Formation.** Matrigel (BD Biosciences) was added to the wells of a 48-well plate in a volume of 120 µl and allowed to solidify at 37°C for 30 min. After the Matrigel solidified, cells from each of the seven murine endothelial cell lines (2 × 10⁴ cells) were added in 200 µl of DMEM supplemented with 10% FBS. The cells were incubated at 37°C with humidified 95% air/5% CO₂ for 5 h in the presence or absence of a potential inhibitor.

**Quantitative PCR.** A confluent T75 flask of cells was harvested and lysed using Trizol. RNA was isolated by phenol chloroform extraction followed by column isolation using the Qiagen RNA Extraction Kit. cDNA was generated using the High Capacity cDNA Archive Kit. Real-time-PCR for normal endothelial cell markers was performed with Sybr Green PCR Master Mix using the primers listed in Table 1 on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative mRNA expression of normal endothelial cell markers was determined by the 2⁻ⁿ≤nth delta Ct method where the cycle threshold (Ct) of the sample is subtracted by the Ct of 18S and compared to the reference expression by 2H11 cells.

**Flow Cytometry Analysis.** Cells were suspended by exposure to versene and 0.005% trypsin and then washed with PBS containing 5% FBS. Cells from each of the seven murine endothelial lines (2 × 10⁵) were incubated with 1 µg of primary antibody diluted in PBS containing 5% FBS in 100 µl total volume for 1 h. The cells were washed twice in PBS containing 5% FBS and incubated with a 1:50 dilution of the appropriate fluorescently conjugated secondary antibody for 1 h. Samples were analyzed using an FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The results were compared with appropriate isotype controls. Positive expression was determined by dividing the number of cells stained with the antibody by the total number of cells assayed multiplied by 100 (percent-positive cells). Each determination was based on 10,000 events.

**In Situ Hybridization.** Briefly, cDNA fragments were generated by PCR amplification of fragments ranging from 200 to 650 bp, using primers with T7 promoters incorporated into the antisense primers (19, 20). Digoxigenin riboprobes were generated by in vitro transcription in the presence of digoxigenin, according to manufacturer’s instructions (Roche, Indianapolis, IN). Murine syngeneic tumors, B16 melanoma, Lewis lung carcinoma, and CT-26 colon carcinoma grown s.c. were harvested and prepared as formalin-fixed, paraffin-embedded 3–5 µm sections on slides. All treatments were carried out at room temperature, unless otherwise stated. Sections were deparaffinized in xylene, washed in 100% ethanol, then hydrated in 85, 75, and 50% ethanol in distilled water. After incubation in diethyl pyrocarbonate-treated water (Quality Biological, Inc., Gaithersburg, MD), sections were permeabilized by treatment with pepsin in 0.2 N hydrochloric acid, washed briefly in PBS, then fixed in 4% paraformaldehyde. Sections were acetylated in acetic anhydride/0.1 M triethanolamine (pH 8.0), equilibrated for 10 min in 5 × SSC [3 M sodium chloride, 0.3 M sodium citrate (pH 7.0); Invitrogen], and prehybridized for 1 to 2 h at 55°C in mRNA hybridization buffer (DAKO, Carpinteria, CA). Sections were hybridized with digoxigenin riboprobes (100–200 ng/ml) in mRNA hybridization buffer (DAKO) overnight at 55°C.

After removing unbound riboprobes by washing, sections were incubated with RNase (Ambion, Austin, TX) to remove any nonspecific-bound riboprobe. Sections were treated with peroxidase block (DAKO) to eliminate any endogenous peroxidase, then blocked with a 1% blocking reagent (DIG nucleic acid detection kit; Roche), containing rabbit immunoglobulin fraction (DAKO), in Tris buffered saline. Rabbit antidigoxigenin-horseradish peroxidase (DAKO) was used to detect the riboprobes and served to catalyze the deposition of biotinylated tyramide (Gen-Point, DAKO) according to manufacturer’s instructions. Additional amplification was accomplished through additional rounds of strept-av-horseradish peroxidase (Gen-Point, DAKO) and biotinylated tyramide. Final detection was accomplished through rabbit antibiotin conjugated to alkaline phosphatase (DAKO). Alkaline phosphatase was detected with Fast Red (DAKO) for 10–60 min at RT, then counterstained in hematoxylin. The nuclei were blued with ammonium hydroxide for 30 s, then mounted with crystal-mount (BioMeda, Foster City, CA). For the negative control, sense riboprobes were used to detect any nonspecific sequences. Additionally, a slide that was exposed to RNase to destroy the mRNA was hybridized with the antisense riboprobe to detect any nonspecific hybridization.

**RESULTS**

*In situ* hybridization was performed on tissue sections of three murine syngeneic tumors, B16 melanoma, Lewis lung carcinoma, and CT-26 colon carcinoma grown s.c. in the appropriate hosts to determine the mRNA expression of the murine homologs of the known endothelial cell marker, VEGFR2, and the murine homologs of several tumor endothelial cell markers in the intratumoral vessels. The tumor endothelial cells were found to express VEGFR2 as well as murine (m)TEM1, mTEM3, mTEM5, and mTEM8 in all three murine syngeneic tumors, B16 melanoma, Lewis lung carcinoma, and the CT26 colon carcinoma. The confirmation that the murine homologs of several markers identified in endothelial cells isolated from human tumors are expressed in these transplantable murine syngeneic tumor models validates their use in the study of tumor endothelial biology and
Fig. 1  In situ hybridization of the following s.c. grown syngeneic mouse tumors: B16 melanoma, Lewis lung carcinoma, and CT-26 colon carcinoma. Tissue sections (5 μm) of each tumor were stained with hematoxylin and exposed to riboprobes for mouse vascular endothelial growth factor receptor 2 (VEGFR2), murine tumor endothelial cell (mTEM) 1, mTEM 7 (A), and mTEM 8, mTEM 9, and mTEM 3 (B) and visualized by amplification with fast red chromagen. Images were taken at ×20 magnification. LLC, Lewis lung carcinoma.
provides guidance for selection of murine endothelial cell lines that would be appropriate models for tumor angiogenesis in cell culture.

The generation times for each of the seven murine endothelial cell lines 2H11, 2F2B, 3B11, IP2E4, SVEC4-10, SVEC4-10EE2, and SVEC4-10EHR1 were determined (Fig. 2). The effect of serum on the proliferation of each cell line was assessed at concentrations of 2, 5, and 10% FBS. Cell number was determined at 24, 48, 72, and 96 h using standard curves derived from a metabolic luminescent end point. The effect of serum concentration on the cellular proliferation was small except with the slower growing SVEC4-10EHR1 cells with generation time increased 1.8-fold when the cells were grown at the lowest serum concentration. In general the primary derivative cell lines, SVEC4-10EE2 and SVEC4-10EHR1, from the parental SVEC4-10 line were slower growing than the secondary derivative lines, 2F2B, 2H11, 3B11, and IP2E4. The cell line with the shortest doubling time was the 2H11 cells with a generation time of 18.7 h at a serum concentration of 10% FBS and a generation time of 23.8 h at a serum concentration of 2% FBS.

The assembly of cells into tubes/networks on a layer of extracellular matrix components is characteristic of endothelial cells in culture. The capability of each of the seven murine endothelial cell lines to assemble into tubes/networks was assessed in a tube formation assay on a layer of Matrigel over a 5 h period (Fig. 3). The parental cell line SVEC4-10 was capable of tube formation. Neither one of the initial derivative lines, SVEC4-10EE2 or SVEC4-10EHR1, were able to form tubes on Matrigel. The second derivative line, 2F2B, that was derived from the SVEC4-10EE2 was able to form tubes on Matrigel, and each of the lines derived from the SVEC4-10EHR1 cells were also active in the tube formation assay.

The mRNA expression of the murine homologs of recognized cell surface endothelial cell markers in each of the seven murine endothelial cell lines was compared with the expression of the same marker in primary HMVEC using primers that recognized both murine and human mRNAs (Fig. 4). The mRNA expression of each marker in the murine endothelial cell lines is represented as the expression relative to HMVECs. The expression of VEGFR2, VEGFR1, and Tie1 by the murine endothelial cells was similar to the expression in HMVEC. The mRNA for endoglin/CD105 and endothelin receptor B was found at much lower levels in the murine cell lines than in HMVEC, and the mRNA for sialomucin/CD34 was found at much higher levels in the murine endothelial cells than in HMVEC. The SVEC4-10 parent cell line expressed VEGFR1, VEGFR2, endothelin receptor B, and Tie1 but not endoglin/CD105 or sialomucin/CD34. The first derivative cell lines, SVEC4-10EE2 and SVEC4-10EHR1, had similar mRNA expression patterns to the parental cell line except that expression of VEGFR1 is markedly decreased, and expression of endoglin/CD105 is increased in the SVEC4-10EE2 cells. The 2F2B cell line that was derived from the SVEC4-10EE2 cells also has decreased expression of VEGFR1 mRNA relative to the other murine endothelial cell lines. Only the 2H11 cell line and the two lines derived from ascites, 3B11 and IP2E4, expressed all six of the cell surface endothelial cell markers.

Because the goal is to identify a murine endothelial cell line that could be useful as a model for tumor endothelial cells, the mRNA expression of cell surface markers predicted to be selective for tumor endothelial cells was evaluated (Fig. 5). The expression of the mouse homologs of five tumor endothelial markers identified in endothelial cells isolated from human colon carcinoma was determined in six murine endothelial cell lines, and values are relative to the expression in 2H11 cells.
Overall, the 2H11 cell line was the highest expressor of these markers. The two lines derived from ascites, 3B11 and IP2E4, were also good expressors of all of the markers. The related lines SVEC4-10EE2 and 2F2B had variable expression of the markers and were particularly low expressors of mTEM7 and mTEM1. On the basis of this analysis, the murine 2H11 endothelial cell line appeared to be the most promising model for tumor endothelial cells.

Antibodies specific for the murine homologs for several recognized endothelial cell surface proteins are available. The cell surface expression of five endothelial cell markers was assessed for the seven murine endothelial cell lines and, using human specific antibodies, for HMVEC, by flow cytometry (Table 2). As might be expected from the mRNA expression results, none of the seven murine endothelial cell lines expressed endoglin/CD105, although HMVEC had strong expression of the endoglin protein. Three of the murine endothelial cell lines, 2H11, 3B11 and IP2E4, expressed sialomucin/CD34 protein as was reflected by the mRNA expression in these same three cell lines. All of the murine endothelial cells had some expression of GPIIIB/CD36. Only the 2H11 cell line expressed P1H12/CD146. Although all of the murine endothelial cell lines expressed vascular cellular adhesion molecule 1 (VCAM1)/CD106, the expression level for VCAM1 by the 2H11 and SVEC4-10EHR1 cell lines was most similar to that of the HMVEC.

The 2H11 mouse endothelial cell line has been tested in standard endothelial cell functional assays. In a proliferation assay, 2H11 cells were exposed to a control anti-2,4-dinitrophenol antibody or to an anti-TEM antibody at a concentration of 100 μg/ml or were exposed to SU5416 (10 or 50 μM) for 48 h (Fig. 6). Although neither antibody altered the proliferation of the 2H11 cells, SU5416 inhibited proliferation in a concentration-dependent manner. The 2H11 cells form tubes in 4 to 5 h on a Matrigel surface. Exposure of the cells to SU5416 (50 μM) during that time was able to disrupt tube formation (Fig. 6). Exposure of the cells to anti-TEM antibody also disrupted tube formation although exposure to the control anti-2,4-dinitrophenol antibody did not.

DISCUSSION

The identification of a murine endothelial cell line that has tumor endothelial marker expression is important for the appropriate analysis of tumor angiogenesis and potential antiangiogenic anticancer strategies in vitro. These experiments have identified the 2H11 immortalized murine endothelial cells to be a relevant murine model for tumor endothelial cells because 2H11 cells express many of the murine homologs of standard endothelial cell markers, including sialomucin/CD34, GPIIIB/CD36, endoglin/CD105, P1H12/CD146, and VCAM1/CD106, as well as several murine homologs of tumor endothelial markers. Like normal endothelial cells, the 2H11 cells will form a cellular network when grown on extracellular matrices.
The variety of endothelial cell markers that were studied represent many aspects of endothelial cell function, but not all are specific for endothelial cells. Platelet endothelial cell adhesion molecule-1/CD31 is also expressed by platelets, monocytes, neutrophils and selected T-cell subsets. The platelet endothelial cell adhesion molecule-1/CD31 protein plays a major role in the cell-cell interactions of endothelial cells and is widely accepted as a pan-endothelial marker of all types of endothelial cells (21, 22). Sialomucin/CD34 also participates in cell-cell interactions by playing a role in adherens junction formation and is primarily expressed by the tumor neovasculature (23). GPIIIB/CD36 expression has been identified on human dermal microvascular endothelial cells as well as other nonendothelial cell types, including platelets and monocytes (24). The GPIIIB glycoprotein binds to extracellular matrix proteins including thrombospondin and collagen (25, 26) and is believed to play a role in the vascular complications associated with malaria (27). Endoglin/CD105 is related to the transforming growth factor-β type III receptor and has been found to be expressed by endothelial cells (28). It has been shown that endoglin/CD105 plays a role in normal vascular architecture and has been found to be elevated in tumor endothelial cells in some systems (29, 30). Using antibodies selective for endothelin receptor B, Shetty et al. (31) found this receptor on the surface of vascular endothelial and smooth muscle cells and defined its role in mediating vasoregulatory activity. PIH12/CD146 is involved in calcium-independent homotypic microvascular endothelial cell adhesion and has become a widely used marker for microvascular endothelial cells (19, 32, 33). Tie1 and Tie2 are tyrosine kinase receptors for angiopoietin 1 and 2 believed to be specifically expressed on endothelial cells. The angiopoietin/Tie1 and Tie2 pathways are involved in embryonic and tumor angiogenesis mediating endothelial cell motility and recruitment of periendothelial cells (34–37). The adhesion molecule, VCAM-1/CD106, has been used as a marker for endothelial cells. In some systems, levels of VCAM-1 correlate with vascular injury and tumor progression (33, 38–40). The VEGF pathway including VEGF and its receptors, VEGFR1 (flt-1) and VEGFR2 (kinase insert domain-containing receptor/flk-1), is critical in embryonic, normal, and tumor angiogenesis (41). The VEGF receptors are expressed on varied cells, including monocytes (VEGFR1), neuronal precursor cells (VEGFR1 and VEGFR2), and podocytes (VEGFR2), but are most highly expressed on resting and active endothelial cells (42–44).

St. Croix et al. (19) identified genes that were up-regulated in endothelial cells isolated from a human colon carcinoma as
compared with endothelial cells isolated from normal colon mucosa from the same patient. The genes expressed by the colon tumor endothelial cells differed significantly from the genes expressed by HMVEC and human umbilical vein endothelial cell, the cells traditionally used when studying angiogenesis in vitro. Therefore, the identification of cells or cell lines with a gene expression profile more relevant to malignant disease is critical. Carson-Walter et al. (20) identified several human tumor endothelial markers predicted to be associated with the cell surface and verified the differential expression pattern of the homologs, mTEM1, mTEM5, and mTEM8 in murine tumor endothelial cells. The immortalized murine endothelial cell line, mTEM, murine tumor endothelial cell.

Table 2 Flow cytometry detection of murine homologs recognized endothelial cell surface proteins.

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<tr>
<th>Cell type</th>
<th>Sialomucin CD34</th>
<th>GPIIIB CD36</th>
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*HMVEC, human microvascular endothelial cell; VCAM, vascular cellular adhesion molecule.*
2H11, expresses relatively high levels of mTEM1, mTEM5, mTEM7, and mTEM8, suggesting that these cells may be a useful model of tumor endothelial cells for cell-based assays.

The mouse remains the model species of choice in cancer experimental therapeutics. It is critical to the selection of potential therapeutics to be aware of the similarities and differences between the human and murine molecular targets. Because of the inter-species differences in specific molecular targets, it is often necessary to develop potential therapeutic agents directed toward the murine protein. Whether syngeneic murine tumors or human tumor xenograft models are used, the stromal compartment of the tumors is murine. Several strategies have been used to transplant functional human endothelial cells into immunodeficient SCID mice such as s.c. implantation of a Matrigel matrix, collagen/fibronectin matrices, or polylactic acid sponges containing genetically altered human umbilical vein endothelial cell or HMVEC (45–49). Alternatively, the SCID mouse has been successfully engrafted with human stem cells (50). Raychaudhuri et al. (51) developed a model that involves the transplantation of human psoriasis plaques into SCID mice that maintain the hyperproliferative characteristics of the psoriasis as well as a functional human vasculature. These methods allow human angiogenesis in a murine host but are not models of angiogenesis associated with malignant disease.

Animal models including genetically engineered mice and xenotransplanted mice are being developed to facilitate the assessment of therapies directed toward human angiogenesis targets in the mouse. However, the mouse remains important in early preclinical development of potential antiangiogenic therapies. Cell-based models, including endothelial cell proliferation, migration, and tube formation, are well-established as the primary screen for potential antiangiogenic activity. Performing these assays in both human and murine endothelial cells that have characteristics of human tumor endothelial cells from patients is the ideal. The immortalized murine 2H11 endothelial cell line appears to be an appropriate murine endothelial cell model of tumor angiogenesis. Continuing use of these murine cells in cell-based angiogenesis assays will be needed to establish their usefulness in understanding endothelial cell biology and drug discovery.
REFERENCES


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