Tumor Endothelium-specific Transgene Expression Directed by Vascular Endothelial Growth Factor Receptor-2 (Flk-1) Promoter/Enhancer Sequences\textsuperscript{1,2}

Regina Heidenreich, Andreas Kappel,\textsuperscript{3} and Georg Breier\textsuperscript{4}

Max-Planck-Institute for Physiological and Clinical Research, 61231 Bad Nauheim, Germany

ABSTRACT

The receptor tyrosine kinase Flk-1 plays a pivotal role in the development of the vascular system and in the vascularization of a wide variety of tumors. We have investigated the activity of cis-acting sequences of the murine Flk-1 gene in the tumor endothelium of experimental tumor models in vivo. B16 melanoma, BFS-1 fibrosarcoma, and polyoma middle T-induced mammary adenocarcinoma were grown in transgenic mice that express the LacZ reporter gene under the control of a 939-bp Flk-1 promoter fragment and an enhancer element located in a 2.3-kb fragment of the first intron. In all experimental tumor models examined, strong endothelium-specific reporter gene expression was observed while being absent from most blood vessels in normal adult tissue. The expression patterns of the LacZ reporter gene correlate well between established tumors grown in Flk1-LacZ transgenic mice and tumors grown in Flk-1+/LacZ knock-in mice that express the LacZ reporter gene from the endogenous Flk-1 locus. The endothelium-specific activity of the Flk-1 promoter/enhancer sequences in three different experimental tumor models demonstrates that the regulatory sequences that mediate the up-regulation of Flk-1 in the tumor endothelium are contained in the Flk-1 promoter/enhancer sequences used, and that these elements function relatively independently of the tumor type. The Flk-1 promoter/enhancer sequences should allow the analysis of the signaling pathways that lead to the up-regulation of Flk-1 in the tumor endothelium and to specifically target therapeutic genes to the endothelium of tumors for antiangiogenic tumor therapy.

INTRODUCTION

Angiogenesis, the formation of new capillaries from preexisting blood vessels, plays an important role in the development of the vascular system and in certain physiological processes in the adult organism, e.g., the female reproductive cycle, and during wound healing. Angiogenesis is also crucially involved in several pathological processes, including solid tumor growth. The inhibition of angiogenesis in tumors is considered to be a promising alternative to conventional tumor therapy, because the expansion of tumors beyond a minimum size requires the formation of new blood vessels to supply the tumor tissue with oxygen and nutrients (1).

VEGF\textsuperscript{5} and its high-affinity tyrosine kinase receptor Flk-1/KDR (VEGFR-2) are central regulators of both physiological and pathological angiogenesis. Gene targeting experiments showed that early embryonic vascular development is dependent on VEGF function (2, 3). Flk-1 is expressed in endothelial cells or their precursors and is essential for endothelial cell differentiation, vasculogenesis, and hematopoiesis (4). Consistent with a signaling function in angiogenesis, Flk-1 is down-regulated in most adult vascular beds. However, VEGF and Flk-1 are up-regulated in a variety of human tumors, including glioma and carcinoma of several tissues (5, 6). The expression of VEGF in the tumor cells and Flk-1 in the tumor endothelium indicates that this signal transduction system stimulates the proliferation and the survival of tumor vessels by a paracrine mechanism (5–7). Direct evidence for this hypothesis was provided by the inhibition of tumor growth in animal models by the application of VEGF neutralizing antibodies (8, 9) or by the gene transfer of dominant-negative Flk-1 receptor mutants (10, 11). In the latter experiments, the cotransplantation of retrovirus producer cells encoding a signaling-defective Flk-1 mutant with tumor cells resulted in the suppression of tumor growth and angiogenesis in intracerebral implants of rat gliosarcoma cells or in xenografts of these and various other tumors. These studies revealed the Flk-1/KDR receptor as central target for antiangiogenic tumor therapy and encouraged the development of low molecular weight inhibitors of Flk-1 signaling that prevented tumor growth in experimental systems (12). Moreover, these results support the validity of gene therapy approaches for the antiangiogenic treatment of tumors. To minimize the risk of inherent side effects, however, it is highly desirable to restrict the expression of therapeutic genes to the tumor endothelium by using transcriptional control elements of endothelium-specific genes. Although evidence for endothelium-specific gene expression after retrovirus-mediated gene transfer in vivo has been presented (13), the promoter sequences used in these studies are not sufficient to direct selective expression of the transgene to the tumor endothelium in vivo. In contrast to other endothelial genes that are expressed constitutively, gene regulatory sequences of the Flk-1/KDR gene are ideally suited for the delivery of therapeutic genes to the tumor endothelium because Flk-1 is absent from most vascular beds of the adult organism but strongly and specifically up-regulated in tumor endothelium (14).

We have recently established transgenic mouse lines that express the LacZ reporter gene under the control of a 939-bp fragment of the 5'-flanking region of the Flk-1 gene, in combination with an enhancer element located in a 2.3-kb fragment of the first intron (15). In these mice, high-level, endothelium-specific expression of the LacZ reporter gene is observed throughout vascular development but is down-regulated in most adult vessels. Here, we have analyzed whether the Flk-1 promoter/enhancer elements are also active during tumor angiogenesis in three different tumor models in vivo. Transplants of B16 melanoma and BFS-1 fibrosarcoma in syngeneic mice and polyoma middle T-induced mammary adenocarcinoma were analyzed in the transgenic reporter mice. In all three tumor types, endothelium-specific transgene expression was detected, indicating that the gene regulatory sequences sufficient for up-regulating the Flk-1 expression in tumor endothelium are localized in the characterized promoter/enhancer fragments. This should allow the study of the transcriptional control mechanisms involved in the induction of Flk-1 expression in vascular development and tumor angiogenesis. Moreover, these Flk-1 regulatory elements should represent a powerful tool to target the expression...
of therapeutic genes specifically to the tumor vasculature, opening new strategies for an antiangiogenic tumor therapy.

MATERIALS AND METHODS

Transgenic Mice. Three different reporter mouse lines were analyzed. Transgenic lines 2603 and 2610 carry the LacZ reporter gene (three and two copies of the transgene, respectively) under the control of Flk-1 promoter/enhancer sequences (~640 bp+/+299 bp+/+1677/+3947 bp, relative to the transcriptional start site; Ref. 15). The heterozygous Flk-1+/+LacZ knock-in mice express the LacZ reporter gene from the endogenous Flk-1 locus (4). The heterozygous Flk-1+/+LacZ knock-in mice express the polyoma middle T oncogene, controlled by the Flk-1 promoter/enhancer sequences (~640 bp+/+299 bp+/+1677/+3947 bp, relative to the transcriptional start site; Ref. 15). The heterozygous Flk-1+/+LacZ knock-in mice express the polyoma middle T oncogene, controlled by the Flk-1 promoter/enhancer sequences (~640 bp+/+299 bp+/+1677/+3947 bp, relative to the transcriptional start site; Ref. 15). The heterozygous Flk-1+/+LacZ knock-in mice express the polyoma middle T oncogene.

Cell Culture and Generation of Tumors. Media and supplements were obtained from Life Technologies, Inc. (Eggenstein, Germany). B16 melanoma cells (American Type Culture Collection) were cultured in DMEM containing 10% FCS (PAA Laboratories, Linz, Austria), 1% penicillin/streptomycin, and 2% glutamine. B16 fibroblasts (Clonetics, Eggenstein, Germany) were cultured in RPMI containing 10% FCS, 1% penicillin/streptomycin, 1% pyruvate, and 2% glutamine. Confluent monolayers were washed two times with PBS and trypsinized, and the cell suspension was collected by centrifugation (1200 rpm for 5 min at room temperature). The cells were resuspended in DMEM containing 10% FCS, and 1.5×10^6 cells/50 μl were injected s.c. into adult mice. Tumors were harvested at 4, 7, or 12 days p.i. for histological analysis.

Double transgenic animals, which express the LacZ reporter gene and the polyoma middle T oncogene, were generated by crossing male MMTV-PyVT transgenic mice with female mice harboring the Flk-1+/+LacZ knock-in mice, respectively. Mammary tumors developed between 11 and 16 weeks after birth. Genotyping was performed by PCR analysis as described (18) using the primer pairs LacZP1/LacZP2 and PyVTforw/PyVTrev: LacZP1, 5′-ATCCTCTGCAGTGTCAGGTC-3′; LacZP2, 5′-GCGGCATTGATGGTTC-3′; PymTfor, 5′-CAGGAGATATCAGCGCATC-3′; and PymTrev, 5′-TGGCTTGTTGTCCTACAGT-3′.

Histological Analysis. Tumor-bearing mice were perfused with 1% PFA. The tumors were removed and postfixed for 4 h at 4°C in 1% PFA, incubated overnight in 18% sucrose in PBS, embedded in Tissue Tek (Sakura), and stored at −80°C. For immunohistochemical analysis, 14-μm frozen sections were prepared, air dried, and stained. Double staining for β-galactosidase and PECAM-1 and staining for Flk-1 was performed on serial sections. For the β-gal/PECAM-1 double staining, sections were first incubated with a monoclonal rat-anti-PECAM-1 antibody (19), rinsed two times in PBS, fixed in 1% PFA, and stained for β-gal as described (20). Afterward, the slides were washed in PBS, fixed in 1% PFA, and rinsed two times with PBS. The sections were then incubated with a biotinylated rabbit-antibody secondary antibody (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, VT) that was subsequently detected with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit). The color was developed with an AEC kit (Sigma, Deisenhofen, Germany). The Flk-1 immunostaining was performed using a rat monoclonal anti-Flk-1 antibody (21).

RESULTS

Flk-1 Regulatory Sequences Confer Endothelium-specific Transgene Expression to the Vasculature of B16 Melanoma. B16 melanoma cells were injected s.c. into transgenic mice that express the LacZ reporter gene under the control of regulatory Flk-1 gene sequences (line 2603; Ref. 15). The resulting tumors were harvested 4, 7, or 12 days p.i. (Table 1A). Frozen sections of all tumors were prepared and stained in parallel for β-gal expression and for the endothelial marker PECAM-1 (19) to visualize the blood vessels. To analyze the endogenous Flk-1 expression, we performed immunohistological staining with a monoclonal anti-Flk-1 antibody (21) on adjacent tumor sections. Endothelium-specific reporter gene expression was detected at all three time points investigated (Fig. 1, B–D; Table 1A; and data not shown). Tumors isolated after 4 days were already palpable (~1 mm in diameter) but were mostly avascular. The LacZ reporter gene was weakly expressed in ~50% of the capillaries in the tumor periphery, which showed Flk-1 expression (data not shown). Tumors harvested at day 7 p.i. were highly vascularized, and uniform LacZ reporter gene expression was seen in the majority of the tumor blood vessels (Fig. 1B; Table 1A). No obvious differences in the patterns of endogenous Flk-1 expression (Fig. 1E; Table 1A) and of LacZ reporter gene expression was detectable. Tumors that had developed central necrosis showed an accumulation of small, Flk-1-positive vessels in viable tumor tissue at the borderline of necrosis (Fig. 1, C and F). Strong reporter gene expression was detected in most if not all of these vascular structures (Fig. 1C). In the majority of the melanomas examined at 12 days p.i., the Flk-1 expression corresponded well with the reporter gene expression (Fig. 1, D and G; Table 1A).

In comparison, we analyzed tumors grown in heterozygous Flk-1+/+LacZ knock-in mice that express the LacZ reporter gene from the endogenous Flk-1 locus (4). Therefore, LacZ expression in this mouse line reproduces the endogenous Flk-1 expression pattern (4, 15). B16 melanomas were grown and analyzed 4, 7, and 12 days p.i. as described above. Vascular reporter gene expression was detected at all time points investigated (Fig. 1, H–J; Table 1A; and data not shown). In contrast to the 4-day tumors in the transgenic line 2603, all melanomas in the Flk-1+/+LacZ knock-in mice analyzed were already vascularized. This difference might be attributable to differences in the genetic background of the reporter mice. In three of four 4-day p.i. tumors, as well as in 7-day p.i. and 12-day p.i. tumors, the majority of the blood vessels was LacZ positive (Fig. 1H–J; Table 1A), corresponding to the Flk-1 expression pattern (Table 1A). Necrotic areas that had developed in half of the tumors at day 7 p.i. were surrounded by blood vessels expressing the LacZ reporter gene (Fig. 1J) and Flk-1 (Table 1A), similar to the melanomas grown in line 2603.

These results indicate that Flk-1 regulatory sequences are capable of inducing endothelium-specific reporter gene expression in the vasculature of B16 melanoma are contained in the characterized Flk-1 promoter/enhancer sequences. Moreover, transgene expression was induced in parallel with Flk-1 in blood vessels in the periphery of prevascular tumors, which are recruited by the growing tumor.

Flk-1 Regulatory Sequences Are Active in BFS-1 Fibrosarcoma. We next investigated whether the Flk-1 promoter/enhancer sequences are also functional in other tumor types. BFS-1 fibrosarcoma cells
were injected s.c. into transgenic mice of line 2603, and tumors were harvested 4 and 7 days p.i. and analyzed as described above. We observed strong reporter gene expression in the tumor vasculature at both time points (Fig. 2, A and B). At day 4 p.i. (B) and day 12 p.i. (D), most vessels showed strong β-gal staining. C, blood vessels at the borderline of necrotic areas. Most if not all capillaries were LacZ positive. E-G, staining for Flk-1 expression on adjacent tumor sections. Most of the vessels showed Flk-1 staining. H-J, LacZ reporter gene and PECAM-1 expression in the vasculature of B16 melanoma grown in Flk-1 +/LacZ knock-in mice. Reporter gene expression was confined to endothelial cells. At all time points investigated, most of the blood vessels showed strong β-gal staining (H and I, day 7 p.i.; J, day 12 p.i.). I, blood vessels at the borderline of necrotic areas in B16 melanoma. Most if not all capillaries were LacZ positive. n, necrotic area; arrows in B and I, LacZ-positive vessels; arrows in E and G, Flk-1-positive vessels; bar, 40 μm.

indicating that the LacZ reporter gene expression is not dependent on a specific integration site of the transgene (data not shown). In comparison, we analyzed fibrosarcomas grown in the Flk-1 +/LacZ knock-in mice. Most of the tumors examined at day 4 p.i. and day 7 p.i. were highly vascularized and showed a strong LacZ reporter gene expression in the majority of the tumor blood vessels (Fig. 2, E and F; Table 1B), corresponding to the Flk-1 expression (Table 1B). Thus, in experimental BFS-1 fibrosarcoma, the Flk-1 expression pattern corresponded well with the LacZ reporter gene expression in both the transgenic mouse line 2603 and the Flk-1 +/LacZ knock-in mice. These data demonstrate that the Flk-1 promoter/enhancer sequences are sufficient to confer a strong endothelium-specific LacZ reporter gene expression also in experimental fibrosarcoma.
The MMTV-PyVT/Flk-1/LacZ double-transgenic females developed mammary adenocarcinoma by 11–15 weeks of age. We harvested 10 tumors of four double-transgenic females and performed whole-mount β-gal staining. All of these tumors showed LacZ reporter gene expression in vessel-like structures (data not shown). Three additional tumors were sectioned, stained in parallel for LacZ and PECAM-1, and for Flk-1 expression on adjacent tumor sections. Endothelium-specific LacZ reporter gene expression was detected in all three well-vascularized mammary adenocarcinomas (Fig. 3A). The tumors differed in size, measuring 3, 4, and 10 mm in diameter, respectively. Although the two smaller tumors showed more Flk-1-positive than LacZ-positive capillaries, the majority of tumor blood vessels in the largest tumor was LacZ positive, and no significant difference in the Flk-1 and the LacZ reporter gene expression was detectable. In whole-mount-stained tumors of Flk-1 LacZ/+ knock-in mice, the LacZ reporter gene was also expressed in vessel-like structures (data not shown). Immunohistological analysis of three tumors revealed that the Flk-1 expression corresponded well with the LacZ reporter gene expression (Fig. 3B) in all tumors investigated.

These results show that the Flk-1 promoter/enhancer sequences are active in an endothelium-specific manner in the vasculature of transgenic experimental mammary adenocarcinoma. The lower number of LacZ-positive endothelial cells in the two smaller tumors of the line 2603 suggests that the reporter gene expression is active in established tumors but only at relatively low levels in nascent mammary adenocarcinomas.

**DISCUSSION**

One major problem of the conventional chemotherapeutic treatment of tumors is the development of drug resistance by tumor cells because of the genetic instability and high mutational rate of tumor cells. In contrast, endothelial cells are normal diploid cells, which are genetically stable and have a low mutational rate. Therapeutic approaches targeting the tumor endothelium should therefore lead to no or only little drug resistance. The treatment of tumors with substances that inhibit angiogenesis or induce endothelial cell apoptosis may therefore be a promising alternative to conventional chemotherapy. Direct evidence for this hypothesis was provided by the growth inhibition of different animal tumors in vivo by the administration of the antiangiogenic compound Endostatin (22). Highly specific antiangiogenic therapy can also be achieved by the targeting of central regulators of tumor angiogenesis, such as the VEGFR-2, Flk-1. The inhibition of VEGF activity by the administration of neutralizing

**Flk-1 Regulatory Sequences Are Functional in Mammary Adenocarcinoma.** In the experimental B16 melanoma and BFS-1 fibrosarcoma models used, tumors develop from established, rapidly growing tumor cell lines. In contrast, mammary adenocarcinomas in transgenic line MMTV-PyVT develop in situ as a consequence of malignant transformation of epithelial cells with the polyoma middle T oncogene (16). All female litters develop multifocal mammary adenocarcinoma. To analyze the Flk-1 regulatory sequences in mammary adenocarcinoma, female mice of the transgenic Flk-1/LacZ line 2603 were mated with male mice of the transgenic line MMTV-PyVT.
anti-VEGF antibodies or soluble receptors, or the inhibition of Flk-1-mediated signal transduction by the retrovirus-mediated gene transfer of dominant-negative receptor mutants, was shown to prevent tumor neovascularization in various animal tumor models, including glioma and carcinoma (8, 10, 11, 23).

Although the involvement of the VEGF/VEGFR signal transduction system in tumor angiogenesis is well established, the mechanisms of Flk-1 up-regulation in tumor endothelial cells is still to be elucidated. In a cerebral slice culture system, it has been shown that Flk-1 expression is up-regulated indirectly in response to hypoxia via its ligand VEGF (24). VEGF itself is strongly up-regulated in hypoxic regions in several tumor types (25, 26) and perhaps also by tumor-associated cells and stromal cells surrounding the tumor (27). To gain more insight into the transcriptional mechanisms of Flk-1 up-regulation in the tumor endothelium, we analyzed the activity of Flk-1 gene regulatory elements in experimental melanoma, fibrosarcoma, and mammary adenocarcinoma. The tumors were grown in transgenic mice that express the LacZ reporter gene under control of Flk-1 promoter/enhancer elements (line 2603). In these mice, a 939-bp fragment of the Flk-1 promoter, in combination with an enhancer element located in the first intron, mediated endothelium-specific LacZ reporter gene expression in transgenic mouse embryos (15). As described for the endogenous Flk-1 gene (28), transgene expression was down-regulated in most vascular beds of adult mice (15). In this study, we demonstrate that the analyzed Flk-1 promoter/enhancer sequences are also active during tumor angiogenesis in vivo. To our knowledge, this is the first description of gene regulatory sequences that specifically target gene expression to the tumor endothelium. The Flk-1 gene regulatory sequences were activated relatively independently of the tumor type. Endothelium-specific reporter gene expression was also observed in fibrosarcomas grown in a second independent transgenic line (line 2610), demonstrating that the transgene expression is not dependent from a specific integration site. Whether the copy number of the transgene has an influence on the strength of reporter gene expression remains to be determined.

Interestingly, prevacular melanomas in line 2603 at day 4 p.i. showed LacZ reporter gene expression and Flk-1 staining in blood vessels surrounding the tumor tissue. This observation is consistent with the idea that tumor neovascularization is initiated in the periphery of the tumor, through the up-regulation and activation of Flk-1 in endothelial cells of normal vessels. Later on, the melanomas of line 2603 and of the Flk-1+/+LacZ knock-in mice showed similar vascularization and a similar LacZ staining pattern. These data demonstrate that the Flk-1 promoter/enhancer elements used are sufficient for a strong, endothelium-specific LacZ reporter gene expression during B16 melanoma angiogenesis. Because fibrosarcomas also grown in line 2603 showed endothelium-specific LacZ reporter gene expression, the activity of the regulatory Flk-1 gene sequences is not restricted to the endothelium in a single tumor type.

In polyoma middle T oncogene-induced mammary adenocarcinoma, strong endothelium-specific LacZ reporter gene expression was detectable in all tumors analyzed, demonstrating that the Flk-1 gene sequences are not only active in transplanted tumors but also in a tumor that arises in vivo. The incomplete reporter gene expression in the two smaller carcinomas of line 2603 suggests that additional Flk-1 regulatory sequences might be required to induce early angiogenesis in this tumor model. However, because the more advanced tumor showed an almost homogeneous vascular LacZ staining, the analyzed Flk-1 gene sequences appear to be sufficient for a strong reporter gene expression in the vasculature of expanding adenocarcinoma.

The Flk-1 promoter, which contributes to a strong gene expression, is activated in vitro by Sp-1 (29) and by hypoxia-inducible factor-2α (15), a basic helix-loop-helix/PAS-domain transcription factor that is prominently expressed in the embryonic endothelium (30) and in hemangioblastoma (31). On the basis of these observations, it seems likely that hypoxia-inducible factor-2α stimulates transcription of Flk-1 during embryonic vascular development and in hemangioblastoma. The endothelial-specific promoter/enhancer sequences of the Flk-1 gene used in this study also contain binding sites for transcription factors of the Tal1, GATA, and c-ets families (15). Members of these families are expressed in endothelial cells or their precursors (30, 32–37) and are likely to mediate the endothelium specificity of Flk-1 expression during embryonic development in a combinatorial fashion. During neovascularization of benign and malignant tumors, endothelial cells express c-ets1, whereas no expression is detected in mature capillaries and larger vessels without angiogenic activity (33). Our recent observation that c-Ets1 stimulates the Flk-1 promoter supports the hypothesis that c-Ets1 regulates Flk-1 expression in tumors. The nature of transcription factors that up-regulate Flk-1 in tumor endothelium, however, remains to be determined.

On the basis of the results of this study, the further functional analysis of the Flk-1 promoter/enhancer sequences allows an unraveling of the signaling pathways that lead to the up-regulation of Flk-1 in the endothelium of tumor blood vessels. Insights into the mechanisms that control Flk-1 transcription might open up new strategies to modulate Flk-1 expression during tumor angiogenesis and endothelial cell survival. Previous gene therapy protocols relied mainly on rather unspecific expression systems (10, 11, 38). In contrast, the isolated regulatory elements could be used to develop vectors that allow targeting of the expression of therapeutic genes specifically to tumor endothelium. For example, the thymidine kinase gene of the herpes simplex virus that activates the produg gancyclovir was recently used successfully to target tumor cells in vivo (38). The inhibition of the VEGF/VEGFR signal transduction system by a dominant-negative Flk-1 mutant that is specifically targeted to the tumor endothelium would be an alternative strategy for the antiangiogenic tumor therapy. Flk-1 promoter/enhancer sequences may thus turn out to become a powerful tool for antiangiogenic tumor therapy and vascular targeting.

ACKNOWLEDGMENTS

We are grateful to Dr. Werner Risau for continuous support of our studies. We thank Dr. Daniela Männel for providing the BFS-1 fibrosarcoma cells; Drs. Shin-Ichi Nishikawa and Hiroshi Kataoka (Kyoto, Japan) for the generous gift of the monoclonal Flk-1 antibody; Drs. Karl Plate, Urban Deutsch, Britta Engelhardt, Ingo Flamme, and Thorsten Schlaeger for helpful discussions; and Andreas Hilbig for technical advice.

REFERENCES


Tumor Endothelium-specific Transgene Expression Directed by Vascular Endothelial Growth Factor Receptor-2 (Flk-1) Promoter/Enhancer Sequences 1,2

Regina Heidenreich, Andreas Kappel and Georg Breier

Cancer Res 2000;60:6142-6147.

Updated version  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/60/21/6142

Cited articles  This article cites 38 articles, 14 of which you can access for free at:  
http://cancerres.aacrjournals.org/content/60/21/6142.full#ref-list-1

Citing articles  This article has been cited by 5 HighWire-hosted articles. Access the articles at:  
http://cancerres.aacrjournals.org/content/60/21/6142.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.