A Vascular Model of Tsc1 Deficiency Accelerates Renal Tumor Formation with Accompanying Hemangiosarcomas

Jarrett D. Leech1, Stephen H.T. Lammers1, Sam Goldman1, Neil Auricchio2, Roderick T. Bronson3, David J. Kwiatkowski2, and Mustafa Sahin1

Abstract

Tuberous sclerosis complex (TSC) is an autosomal disease caused by inactivating mutations in either of the tumor suppressor genes TSC1 or TSC2. TSC-associated tumor growth is present in multiple tissues and organs including brain, kidney, liver, heart, lungs, and skin. In the kidney, TSC angiomyolipomas have aberrant vascular structures with kidney, liver, heart, lungs, and skin. In the kidney, TSC is present in multiple tissues and organs including brain, heart, lungs, and skin. In the kidney, TSC is present in multiple tissues and organs including brain, heart, lungs, and skin. In the kidney, TSC is present in multiple tissues and organs including brain, heart, lungs, and skin.

Introduction

Tuberous sclerosis complex (TSC) is a genetic disorder due to an inactivating mutation in either TSC1 or TSC2. The protein products of TSC1 and TSC2, hamartin and tuberin, respectively, form a heterodimeric complex that inhibits the mTOR complex 1 (mTOR) pathway via RHEB-GTP modulation (1, 2). Activation of mTOR primarily regulates cell size and proliferation through promotion of protein translation (1). Germline TSC1 or TSC2 mutations are complemented by second-hit loss of the wild-type allele leading to hyperactive mTOR activity, which results in the growth of tumors. In brain (subependymal nodules and giant cell astrocytomas), kidney (renal angiomyolipoma), and lung (pulmonary lymphangioleiomyomatosis; refs. 3–5). In addition to tumor growth, a combination of intellectual disability, epilepsy, and autism occurs in a majority of individuals with TSC (2). Most tumors that arise due to TSC are of a benign phenotype (4). However, renal angiomyolipomas can proliferate, and subsequent renal complications are cited as the most common cause of death in adult patients with TSC. Thus, therapies that inhibit hyperactive mTOR pathway are candidates for treatment of TSC-related tumors.

Rapamycin and related compounds (rapalogues) have been studied with regard to the inhibition of mTOR and treatment of TSC-related tumor growth (3, 6). In multiple murine models of TSC, treatment of TSC-related tumors is effective in reducing tumor size (7–9). Clinically, rapalogue treatment has been shown to result in regression of both renal angiomyolipomas (10, 11) and subependymal giant cell astrocytomas (12, 13). However, rapalogue treatment does not lead to tumor elimination, and cessation of treatment results in re-growth of tumors (10, 13, 14). Other drawbacks to the use of rapamycin are side effects ranging from diarrhea, stomatitis, respiratory infection, and pyelonephritis (10). Hence, identification of more efficacious therapies is desirable to further the treatment of TSC-related tumor growth and subsequent clinical management.

Both cell-based and animal models have been used to identify therapeutics for treatment of TSC-related tumors (15). However, both Tsc1+/− and Tsc2+/− mice develop kidney cystadenomas and liver hemangiomas as their main phenotype, tumors which do not match the pathology of human TSC (16–18). Furthermore, the development of kidney and liver tumors in murine models can take from 4 to 15 months of age with varying incidence rates (17, 18). In the present study, by combining a conditional knockout allele of Tsc1 with a Darpp32-Cre allele, we created a mouse that develops kidney cystadenomas and hemangiomas in the...
extremities on an accelerated time scale. Furthermore, we tracked paw hemangiosarcoma development by simple observation during rapamycin treatment, demonstrating its use as an easily scor-able measure of tumor response. Our results demonstrate the usefulness of this model for preclinical testing of novel drugs for the treatment of TSC-related tumors.

Materials and Methods

Breeding strategy and phenotyping

All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) and Animal Resources at Children’s Hospital (ARCH). Mouse experiments were performed on a mixed strain background. Mice bearing the loxP-anked Tsc1 allele (Tsc1c/+; ref. 19) were bred with mice bearing the Darrpp-32 Cre+ allele, with further matings to generate Tsc1cu Darrpp-32 Cre+c male mice (20). All other non-mutant male genotypes were used as littermate controls (Tsc1cu and Tsc1cu+). Genotyping for lox-p sites for Tsc1 and for the Darrpp-32 Cre was performed with standard PCR. Primers F4530 (5′- AGGAGGCCCTTCTCGTACC-3′) and R4380 (5′-CAGCTCCGACCATGAGTG-3′) were used for Tsc1 and Cre F (5′-GGCATGTGTCAGGCGCCAGGG-3′) and Cre R (5′-GCATAACAGTGAAACAGCATTGCT-3′) were used for the detection of the Darrpp-32 Cre.

Tumor measurements

An observer (S. Goldman) blind to the genotype performed paw measurements. Hind paw diameter was measured using a 0.1-mm resolution caliper in the plantar to dorsal dimension. Mean paw diameters were determined by the average of both left and right paws. Paw diameters were measured biweekly until death. Individual mice were euthanized when paw diameter of ≥ 5 mm, weight loss of ≥ 20%, or greatly extreme lethargy was seen.

Treatment protocol

All mouse treatments commenced when the diameter of rear paw tumors averaged 4.0 mm. Mice treated with rapamycin (LC Laboratories, Cat. No: R-5000) were injected intraperitoneally at 6 mg/kg in 100 μL of vehicle solution (5% Tween 80, 5% Polyethylyl Glucol) every other day for 1 month. For statistical comparisons, 1- and 2-way ANOVAs with subsequent Bonferroni corrections, log-rank survival, and 2-tailed Student tests were performed using Prism (GraphPad Software, Inc., v5.0f). Alpha level for all analyses was equal to 0.05.

Histology

Standard hematoxylin and eosin (H&E) sections were prepared from mouse kidney and paws taken immediately after death of an animal. Sections were fixed in 10% formalin for 12 hours before staining. For cyst diameter measurements, an observer blind to the treatment and age statuses (S.H.T. Lammers) analyzed sections on 4× magnifications. Cyst diameter measurements were taken identifying the largest cross-sectional diameter of a defined, enclosed cyst. For cyst counts, a cyst was counted if it was enclosed and greater than 3.5 × 10^{-3} mm in diameter. All imaging was performed on an Olympus BX51 microscope with a Qimaging MicroPublisher 3.3 RTV camera and Qimaging QCapture software (Surrey).

Immunoblot analysis

Rear extremity tumor and kidney extracts were prepared in lysis buffer (20 mmol/L Tris-HCl, 140 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L Na2VO4, 1 mmol/L EDTA) and a Dounce homogeniz-
er. Protein concentrations were determined by Bradford Assay (BioRad Laboratories), equal concentrations were separated by electrophoresis on 6%–12% Bis-Tris acrylamide gels and then transferred onto Immobilon-P PVDF membranes (EMD Millipore). Membranes were blocked with 5% nonfat dry milk in 1× TBST (Tris-buffered saline and 1% Triton-X) for 1 hour at room temperature. Primary antibodies were diluted in 5% nonfat dry milk or 5% bovine serum albumin in 1× TBST and applied to the membranes overnight at 4°C. Primary antibodies included P-CAD (Cell Signaling), CAD (Cell Signaling), p-S6-240 (Cell Signaling), S6-240 (Cell Signaling), and anti-tubulin (Abcam). After primary antibody incubation and washing with 1× TBST, anti-mouse (Santa Cruz), or anti-rabbit (Rockland) horsepasth peroxidase (HRP)-conjugated secondary antibodies were applied for 1 hour at room temperature in 5% nonfat dry milk solution of 1× TBST. Supersignal West Pico and Dura Chemiluminescent (Thermo Fisher Scientific) signal solutions were used to detect antibody binding and signals were collected using an ImageQuant LAS 2000 imager (GE Healthcare).

Immunohistochemistry

Immunohistochemical analyses were performed on paraffin-embedded sections of kidney and paw after 4% paraformaldehyde fixation for 12 hours or on frozen OCT-embedded tissues following 4% paraformaldehyde fixation. Phospho-S6 Ser 240/244 (Cell Signaling, Cat. No: 2215) was used as a primary antibody at 1:500 dilution. Secondary antibodies included Alexa Fluor 488 anti-goat and anti-rabbit used at 1:500 dilution. For X-gal staining, tissue was harvested and fixed with 4% paraformaldehyde overnight, followed by standard sucrose cryopreservation and OCT embedding. Sections were sliced and then stained in X-gal staining solution overnight at 37°C followed by counterstaining with Nuclear Fast Red as previously described (21).

qPCR

All tissue samples from kidney and paws were extracted 1 week after the last day of treatment in a sterile fashion and immediately frozen in liquid nitrogen. RNA was precipitated by lysing tissue using TRizol (Invitrogen) and a 22-gauge syringe followed by chloroform extraction. RNA was then reverse transcribed to cDNA and amplified as previously described for qPCR (22). Amplification and quantification were run with VEGF-A, VEGF-C, VEGF-D, and GAPDH primers designed with Primer 3 and sequences were as follows: VEGF-A 5′-CGAGAACATCATGACTTCTGC-3′ (forward) and 5′-GACCTCTGCTCTGCTTGCTG-3′ (reverse), VEGF-C 5′-GAGGTCAAGGCTTTTGAAGGC-3′ (forward) and 5′-CTGCTCTTGTATGAGGTGG-3′ (reverse), VEGF-D 5′-TTAGGGCATACCCCGTGC-3′ (forward) and 5′-GGTGCATTGCTATGGCCAG-3′ (reverse), GAPDH 5′-GACATGC- CGCCTGGAGAAGC-3′ (forward) and 5′-AGGCCAGGATGCCCTTAGT-3′ (reverse).

Results

A new Tsc1 Darppp-32 Cre+ mouse model with rapid development of paw hemangiosarcoma

Our intent in creating a Tsc1cu Darrppp-32 Cre+ mice was to develop a unique TSC brain model with Tsc1 loss in striatal neurons (23). Although DARRP-32 (dopamine and cAMP-regulated neuronal phosphoprotein 322 or protein phosphatase 1 regulatory subunit 1B2, gene name PPP1R1B) is expressed at

www.aacrjournals.org Mol Cancer Res; 13(3) March 2015 549

Published OnlineFirst December 29, 2014; DOI: 10.1158/1541-7786.MCR-14-0178

Downloaded from mcr.aacrjournals.org on June 19, 2017. © 2015 American Association for Cancer Research.
highest levels in brain, it is also expressed in multiple other tissues, including pancreas, prostate, lung, thymus, and bone marrow (24). To confirm successful recombination and loss of expression of Tsc1, we used a LacZ reporter allele. X-gal staining of Tsc1cc Darpp-32-Cre+/– mouse tissues demonstrated expression of the Cre allele in the striatum and other cortical areas (data not shown). Separated PCR experiments revealed the animals that were mutant for Tsc1 and Darpp-32-Cre positive (Supplementary Fig. S1). X-gal staining (blue) was also seen in renal tubules of the outer medulla and cortex as well as the paws (Fig. 1A). Tsc1cc Darpp-32-Cre+/– mutant mice had significantly reduced weights compared with littermate controls (Tsc1cw and Tsc1cw Darpp-32-Cre+/–) beginning at P25, a trend that persisted throughout adulthood (Fig. 1B, *P < 0.05; **, P < 0.01).

Renal pathology

Pathologic evaluation demonstrated that renal cystic and tumor lesions were present in all (n = 11) mutant mice at the age of 8 weeks (Fig. 1D). However, the most striking phenotype seen in these mice was the development of expansile tumor lesions on all 4 paws beginning at post-natal day 21 (Fig. 1D). These progressive tumors distorted the normal anatomy of the paws. Histologically, these tumors were composed of pleomorphic spindle cells arranged in interlacing bundles and whorling patterns. The tumors are highly vascularized and with the spindle cell shape and architecture of these vascular structures, these tumors were identified as hemangiosarcomas (Fig. 2A–C and Supplementary Fig. S2A). Mitotic figures were rare. The edges of the tumors were well circumscribed but not encapsulated (Fig. 2A–C). By 6 weeks of age, all mutant mice (n = 11) developed visible swelling and abnormal growth on hind paws. Similar tumor formation also occurred in the forepaws but was of smaller extent (data not shown). Control mice (Tsc1cw and Tsc1cw Darpp-32-Cre+) showed no evidence of paw tumor formation (Fig. 2D–F).

Histopathologic examination of kidneys from all mutant mice showed polycystic kidney lesions at an early age (Fig. 3A). These lesions were cystadenomas (Fig. 3A–C), similar to those described in both Tsc1+/− and Tsc2+/− mouse models (17, 18). They consisted of pure cysts with hyperplastic lining epithelial cells, partially filled cysts with papillary fronds of adenomatous growth, and solid adenomas. These lesions grew rapidly and were extensive by age 4 months, suggesting that...
they were a major contributor to death in these mice (Fig. 3A–C, for high magnification imaging, see Supplementary Fig. S2B and S2C). Similar to the findings in the paws, there were no observed pathologic abnormalities in the renal tissue of control mice (Fig. 3D–F).

Paw hemangiosarcomas provide an easily assessable tumor model of TSC-related tumor growth

The paw hemangiosarcomas developed early in life (<4 months of age) and were easily assessed for size in a noninvasive, quantitative manner as shown by the difference in paw diameter between mutant and control mice (Fig. 4A, P < 0.01 between mutant and control mice at all time points from P40 onward, 2-way ANOVA). We thus saw the potential for using tumor growth to assess therapeutic effectiveness. As an initial proof-of-principle, we treated these mice with rapamycin. Rapamycin was given by intraperitoneal injection at 6 mg/kg 3 times per week for 30 days to a cohort of 11 mice beginning when the largest hemangiosarcoma reached a diameter of 4 mm (average age P102). We observed a significant reduction of 42.5% in hemangiosarcoma diameter after 1 month of therapy (Fig. 4B, P < 0.001, 1-way ANOVA). When rapamycin was discontinued after 30 days, there was significant regrowth of paw tumors (Fig. 4B, P < 0.001, 1-way ANOVA), consistent with observations in other TSC mouse models (25) and in patients with TSC (10, 13).

To confirm that activation of mTORC1 was occurring in these tumors and that rapamycin was inhibiting this pathway, we examined tumor lysates by immunoblot analysis. High levels of phospho-S6 Ser240 expression were seen in kidney and paw lysates from vehicle-treated mice (Fig. 4C). After 1 week of rapamycin treatment, a significant reduction in mTORC1 activation was indicated by reduced phospho-S6 Ser40 staining relative to total S6 Ser240 staining in both kidney (Fig. 4D, P < 0.01, 1-way ANOVA) and paw lysates (Fig. 4D, P < 0.05, 1-way ANOVA). We also examined levels of phosphorylated and total-CAD expression and found that phospho-CAD expression was significantly elevated in vehicle-treated kidney and paw lysates compared with both wild-type and rapamycin-treated lysates (Fig. 4C and D, P < 0.001, 1-way ANOVA). Histologic analyses of kidneys from mutant mice revealed that a significant reduction in mean cyst diameter was observed in mice treated with rapamycin (Fig. 5A, P < 0.001, 1-way ANOVA). In addition, there was a significant reduction in mean renal cyst count of rapamycin treated mice (Fig. 5B, P < 0.001, 1-way ANOVA). However, the mean renal cyst count in rapamycin-treated mice at 17 weeks was significantly greater than vehicle-treated mice at 14 weeks (Fig. 5B, P < 0.05, 1-way ANOVA)
Histologic sections of vehicle-treated mutant mice at 17 weeks (Fig. 5C) and rapamycin-treated mutant mice at 17 weeks (Fig. 5D) show these reductions in cyst size and count. Immunohistochemical analysis of paraffin-embedded tissue sections of kidneys and paws confirmed high levels of phospho-S6 in these tumors, consistent with mTORC1 activation due to loss of TSC1, and showed that rapamycin treatment eliminated phospho-S6 expression in both kidneys and paws (Fig. 6A and B, respectively).

VEGF mRNA expression and loss of TSC1
We had noted earlier that the paw hemangiosarcomas are deep red in appearance, suggesting that these tumors are highly vascularized. Prior findings showed that loss of Tsc1 or Tsc2 results in increased VEGF levels, which are associated with tumor development and responsive to rapamycin treatment (26, 27). Using qPCR, we observed significantly increased VEGF-A mRNA levels in mutant paws but not kidneys, compared with controls (Fig. 7A, P < 0.001, Student t test). In addition to VEGF-A mRNA levels, we also examined VEGF-C and VEGF-D mRNA levels. Compared with control mutant mice, paws from mutant mice expressed significantly higher amounts of both VEGF-C and VEGF-D mRNA expression (Fig. 7B and C, P < 0.001 and 0.05, respectively, Student t test). Furthermore, kidneys from mutant mice showed significantly elevated VEGF-D mRNA levels when compared with controls (Fig. 7C, P < 0.05, Student t test).

Discussion
In this study, we generated a new mouse tumor model in which targeted loss of Tsc1 in both the paws and the kidneys leads to rapid and progressive tumor development. In patients with TSC, hamartomas appear in multiple organs due to random genetic second hits events that lead to complete loss of TSC1 or TSC2 expression and subsequent downstream activation of mTORC1. However, there are still many aspects of tumor formation in TSC...
that are uncertain, including the cell of origin for most TSC hamartomas, the precise molecular mechanisms of tumor progression, and the exact role of mTORC1 activation in driving tumor growth. Multiple therapeutic strategies have been identified in cell-based or biochemical studies that may provide significant control in TSC tumor growth. In this set of experiments, we took advantage of the fortuitous rapid development of paw and renal tumors in a fully viable mouse model system, Tsc1cc Darpp-32-Cre mice. Kidney cystadenomas and paw hemangiosarcomas were seen by P42 and P21, respectively, and enabled rapid assessment of in vivo therapies for TSC tumors. Previous mouse models of TSC-related tumor growth report that extremity tumors (tail, paw, or lip) can take up to 12 months to develop (17). Furthermore, only 7% of the mutant mice in a previous model (17) developed these extremity tumors. Our conditional knockout mouse model demonstrates full penetrance of paw hemangiosarcomas by 6 weeks of life. Finally, another considerable advantage of this TSC model is that a noninvasive readout of therapeutic strategies can be performed without the need to sacrifice the mouse.

In a preliminary proof of concept drug trial, we demonstrated that rapamycin was an effective therapy for both paw hemangiosarcomas and renal cystadenomas. Most importantly, the progress of treatment could be followed through simple measurement of the paw hemangiosarcomas. Biochemically, we also provide evidence from phospho-S6 and phospho-CAD staining that this effect of rapamycin treatment is likely through inhibition of the hyperactive mTORC1 pathway in Tsc1cc Darpp-32-Cre+ mice. As it is well established that TSC tumor growth is reversed via this mechanism in both humans (10–13) and mice (7, 8), our model provides the specific development of TSC-related tumors that can be assessed for responsiveness to therapeutics. Noninvasive measurement of the paw hemangiosarcomas highlights the usefulness of this model, given that the renal phenotype is also responsive to rapamycin treatment, as
evidenced by both the biochemical data and reduction in mean cyst diameter and apparent cyst load.

Further support for the conclusion that Tsc1cre Darpp-32-Cre+ mice present a robust model for TSC-related tumor growth is evidenced by VEGF analyses. Beyond TSC-related tumors, VEGF-A expression has been shown to drive tumor angiogenesis and metastasis (28). Prior research in both TSC cell–based assays (27, 29, 30) and tumor tissue from patients (31) shows that hyperactivation of mTORC1 results in an increased production of hypoxia-inducible factor 1α (HIF1α), a transcription factor which results in an increased expression of HIF-responsive genes such as VEGF-A (29). Furthermore, mouse models have been reported to show increased serum VEGF-A expression in Tsc1−/− mice, which was also associated with the extent of tumor development (27, 31). Interestingly, a reduction in serum VEGF-A expression was observed as a result of short-term rapamycin treatment (4 days at 20 mg/kg), which also was associated with tumor reduction (27). In our model, we observed an increase in VEGF-A, C, and D mRNA expression in mutant mouse paws, likely due to mTORC1 hyperactivation and subsequent tumor formation. We also observed an increase in VEGF-D mRNA levels from kidneys as compared with kidneys from control mice.

Our findings with respect to VEGF mRNA levels support the conclusion that the Tsc1cre Darpp-32-Cre+ mouse is an accurate model of TSC-related tumor growth. As described, previous work has repeatedly shown that VEGF protein levels are elevated in TSC-deficient mice, a phenotype that is rescued by rapamycin treatment (27, 29). These results have been examined at the protein level, whereas the present findings begin to suggest differences in subtypes of VEGF expression at the mRNA transcript level. While we did not observe significantly increased levels of VEGF-A or C mRNA from kidneys, our analysis of VEGF-D showed the expected increase in VEGF levels from kidneys of mutant mice. VEGF-D has been proposed as a potential biomarker of lymphangioleiomyomatosis severity and treatment response (32). Recent work indicates that both VEGF-A and VEGF-D can be detected in the sera of patients with TSC (33). Taken together, these results indicate the similar mechanisms may be at play in tumorigenesis in Tsc1cre Darpp-32-Cre+ mouse as in patients with TSC.

The present evidence suggests that the Tsc1cre Darpp-32-Cre+ mouse can be an effective tool for the study of pharmaceutical treatments of TSC-related tumors. While there are uncertainties as to both the genetics of these tumors in terms of their abundance as well as their cell size/morphologic features when compared with human tumors, this model presents a valid, quantitative, in vivo model for the testing of potential therapeutic agents for the treatment of TSC-related tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J.D. Leech, D.J. Kwiatkowski, M. Sahin
Development of methodology: J.D. Leech, S.H.T. Lammers
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.D. Leech, S.H.T. Lammers, S. Goldman, N. Auricchio, R.T. Bronson, D.J. Kwiatkowski
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.D. Leech, S.H.T. Lammers, D.J. Kwiatkowski, M. Sahin
Writing, review, and/or revision of the manuscript: J.D. Leech, S.H.T. Lammers, N. Auricchio, D.J. Kwiatkowski, M. Sahin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.D. Leech, S. Goldman
Study supervision: D.J. Kwiatkowski, M. Sahin
Other (performed pathology): R.T. Bronson
Acknowledgments

The authors thank Dr. Elizabeth P. Henneke for assistance with treatment methods. They are grateful for the help of the Kwiatkowski lab members who aided with the use of equipment and the Sahin Laboratory members who contributed to the development of this project. They also thank Dr. Joanne Chan for her intellectual contribution. Finally, they thank the Rodent Histopathology Core of the Dana Farber/Harvard Cancer Center, mouse histology preparation, and interpretation.

Grant Support

This study was funded by NIH grants R37NS031535 and P01CA120964, the Tuberous Sclerosis Alliance, Boston Children’s Hospital Translational Research Program, and the Manston Family Foundation to M. Sahin. Dana-Farber/Harvard Cancer Center is supported in part by an NCI Cancer Center Support Grant # NIH S 5 P30 CA06516.

The costs of publication of this article were delayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

27. El-Hashemi N, Walker V, Zhang H, Kwiatkowski DJ. Loss of Tsc1 or Tsc2 induces vascular endothelial growth factor production through mamma

Received April 1, 2014; revised October 28, 2014; accepted December 10, 2014; published OnlineFirst December 29, 2014.
A Vascular Model of Tsc1 Deficiency Accelerates Renal Tumor Formation with Accompanying Hemangiosarcomas

Jarrett D. Leech, Stephen H.T. Lammers, Sam Goldman, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-14-0178

Supplementary Material
Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2015/01/06/1541-7786.MCR-14-0178.DC1

Cited articles
This article cites 33 articles, 10 of which you can access for free at:
http://mcr.aacrjournals.org/content/13/3/548.full.html#ref-list-1

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.