

An Orthotopic Mouse Model of Remetastasis of Human Colon Cancer Liver Metastasis

Babak Rashidi, Reza Gamagami, Aaron Sasson, Fang Xian Sun, Jack Geller, A. R. Moossa, and Robert M. Hoffman¹

AntiCancer, Inc., San Diego, California 92111 [B. R., F. X. S., J. G., R. M. H.], and Department of Surgery, University of California, San Diego, California 92103-8220 [B. R., R. G., A. S., A. R. M., R. M. H.]

ABSTRACT

Whether liver metastases from colon cancer are capable of metastasizing to other sites is an important question in surgical oncology. To answer this question, we have developed a highly metastatic orthotopic transplant model of a liver metastasis from a human colon cancer patient in nude mice that targets the liver and lymph nodes. The metastatic human tumor was transplanted in athymic nude mice by surgical orthotopic implantation (SOI) of a liver metastasis from a colon cancer patient. The human colon tumor was then subsequently implanted in the colon by SOI or, in an additional series of nude mice, in the liver by surgical hepatic implantation (SHI). The mice were then explored over time for lymph node involvement beginning 10 days after implantation. After SOI, 100% of the animals had liver metastasis within 10 days, and subsequently, 19 days after SOI, all lymph nodes draining the liver were involved with metastasis without any retroperitoneal or lung tissue involvement. After SHI, all sites of lymphatic drainage of the liver, including portal, celiac, and mediastinal lymph nodes, were massively involved by metastasis in 100% of the animals as early as 10 days after tumor implantation on the liver. The results of this study demonstrate that liver metastases from colon cancer are capable of remetastasing to other sites. This study thus suggests that in colon cancer patients with liver metastasis, mediastinal, celiac, and portal lymph node metastases originate from the liver metastasis and not, as previously thought, from primary colon cancer.

INTRODUCTION

There are over 130,000 new cases of colorectal cancer diagnosed per year in the United States (1). Lymph node and liver metastasis are the two major obstacles for successful treatment of colon cancer. At presentation with colorectal car-

cinoma, 26–44% of patients have positive lymph nodes (2). The survival time of these patients is related to the number of lymph nodes involved (3). Approximately 50% of patients with colorectal carcinoma develop recurrence within five years after treatment of their primary colorectal cancer. The liver is the site of recurrence in 40–80% of the recurrent cases (4–8).

Liver resection for metastatic colon cancer disease is the only effective therapy. However, liver resection has several contraindications: the presence of hepatic metastatic lymph nodes, the presence of extrahepatic metastasis (even if resectable), or the presence of four or more liver metastases (9). Lymphatic spread of hepatic lesions has not been a fully accepted concept despite the fact that 25–50% of lymph reaching the thoracic duct is of hepatic origin (10).

There are only few case reports in the literature that attempt to explain the source of hepatic, celiac, and mediastinal lymph node metastasis in patients with liver metastasis from colon cancer (10, 11). In patients with hepatic metastases from colorectal cancer, it has been postulated that involved nodes in the porta hepatis are derived from hepatic colorectal metastases metastasizing to that site (12, 13). Because portal-associated lymphatic drainage of the colon does occur to a certain extent, it is difficult to prove this hypothesis (3, 14). In addition, the occurrence of remetastasis is not a fully accepted concept (3).

Although the literature does suggest that metastatic tumors can produce further dissemination of cancer cells, reports are sparse, and the data are inconclusive. The knowledge of remetastasis in cancer may critically influence treatment options. According to Weiss (15), if metastasis from metastasis were to occur, it could alter the spatial and temporal patterns of disease and could therefore make the surgical removal of primary cancers only a palliative procedure.

SOI,² which involves the orthotopic transplantation of histologically intact tumor fragments, has allowed the development of models of human cancer in nude mice that demonstrate the variety of clinical behavior that occurs in human patients (16–25). Models developed with SOI exhibiting patient-like metastasis include colon cancer (18, 19), lung cancer (20), bladder cancer (21), pancreatic cancer (22), prostate cancer (23), ovarian cancer (24), and stomach cancer (25).

In previous studies, a liver metastasis of a patient's colon cancer (AC3488) that metastasized to the liver and lymph nodes in 100% of the animals after SOI was established in nude mice (18, 19). In this model, the routes of metastatization from the liver in mice were also discussed (18).

In the present study, we additionally performed SHI of AC3488 directly on the liver simulating liver metastasis from colon cancer. This model bypasses the colon lymph drainage

Received 7/19/99; revised 2/18/00; accepted 3/7/00.

The costs of publication of this article were defrayed 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.

¹ To whom requests for reprints should be addressed, at AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111. Phone: (858) 654-2555; Fax: (858) 268-4175; E-mail: all@anticancer.com.

² The abbreviations used are: SOI, surgical orthotopic implantation; SHI, surgical hepatic implantation.

Table 1 Comparison of lymph nodes involved by metastasis after SOI and SHI

Lymph nodes (LN) draining the liver included portal, celiac, and mediastinal. They were involved with metastases in both the SOI group and the SHI group. Tumor was implanted on the colon for SOI or on the liver for SHI. See "Materials and Methods" for details.

Group	Hepatic LN	Celiac LN	Mediastinal LN	Retroperitoneal LN
Lymph nodes involved with metastases in SOI group (%)	100	100	100	0
Lymph nodes involved with metastases in SHI group (%)	100	100	100	0

Table 2 Comparison of organs involved by metastasis after SOI and SHI

Various organs involved with metastases in the SOI group were liver, colon, spleen, kidney, and suprarenal gland. Liver and spleen were involved with metastases in the SHI group. See "Results" for details.

Group	Liver	Colon	Spleen	Kidney	Other ^a	Lung
Organs involved with metastases in SOI group (%)	100	100	50	10	10	0
Organs involved with metastases in SHI group (%)	100	0	80	0	0	0

^a Suprarenal gland.

system to the porta hepatis. Although SOI and SHI are models, they closely resemble primary and metastatic tumors, respectively. We demonstrate in this study that the SOI and SHI models give rise to similar lymph node metastatic patterns. These results indicate that in animals with liver metastasis from colon cancer, metastatic involvement of hepatic, celiac, and mediastinal lymph nodes is secondary to the liver metastasis and is not related to the colon lymph drainage system. This study demonstrates that remetastasis is a real biological phenomenon and that it may change our approach to treatment of secondary tumor sites because metastatic sites themselves are capable of spreading tumor cells.

MATERIALS AND METHODS

Animals. Male and female athymic BALB/c nude mice between 4 and 6 weeks of age were used in this study. The animals were bred and maintained in a HEPA-filtered environment. Cages, food, and bedding were sterilized by autoclaving. The breeding pairs were obtained from Charles River Laboratories (Wilmington, MA). The animal diets were obtained from Harlan Teklad (Madison, WI). All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals under assurance number A3873-1.

Surgical Specimen. The fresh surgical specimen was obtained from a resected liver metastasis of a patient with colon cancer at the Department of Surgery, School of Medicine, University of California, San Diego (19). The tumor, termed AC3488, was derived from a 46-year-old man with poorly differentiated adenocarcinoma of the colon, stage IV with liver metastasis (18, 19). The specimen was obtained from the right lobe of the liver after resection. The patient died of metastasis in the liver 7 months after surgery. The surgical specimen was kept at 4°C in Earl's MEM. The specimen was transplanted using SOI in nude mice within 24 h of surgery (19).

Implantation Procedure. Before implantation, specimens were washed twice with antibiotic-containing Earl's MEM, at least 10 min each time, to prevent possible infection

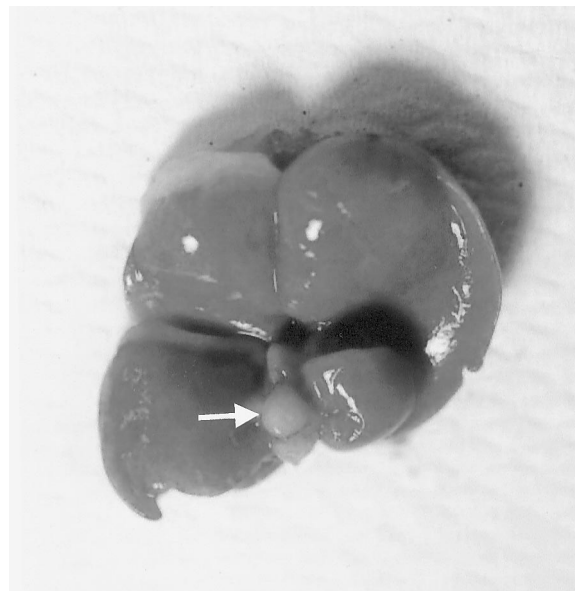


Fig. 1 Inferior surface of the liver of nude mouse and portal lymph node remetastasis 18 days after SHI (arrow).

(19). After necrotic tissue and noncancerous tissue of the specimen were removed, the remaining cancerous tissue was divided into small pieces, approximately 1 mm in diameter. Nude mice were anesthetized with isoflurane (Forane) inhalation. The abdomen was sterilized with iodine and alcohol swabs. For SHI, a subcostal incision was made, and the left lobe and the middle lobe of the liver were exteriorized. The serosa of the liver, corresponding of the implantation site, was removed, and one or two tumor fragments of 1 mm³ each were implanted on the left lateral lobe and on the right lateral portion of the middle lobe. An 8-0 nylon surgical suture was used to penetrate these small tumor pieces and suture them on the liver. The liver was returned to the abdominal cavity, and the abdominal wall was closed with a 6-0 silk surgical suture.

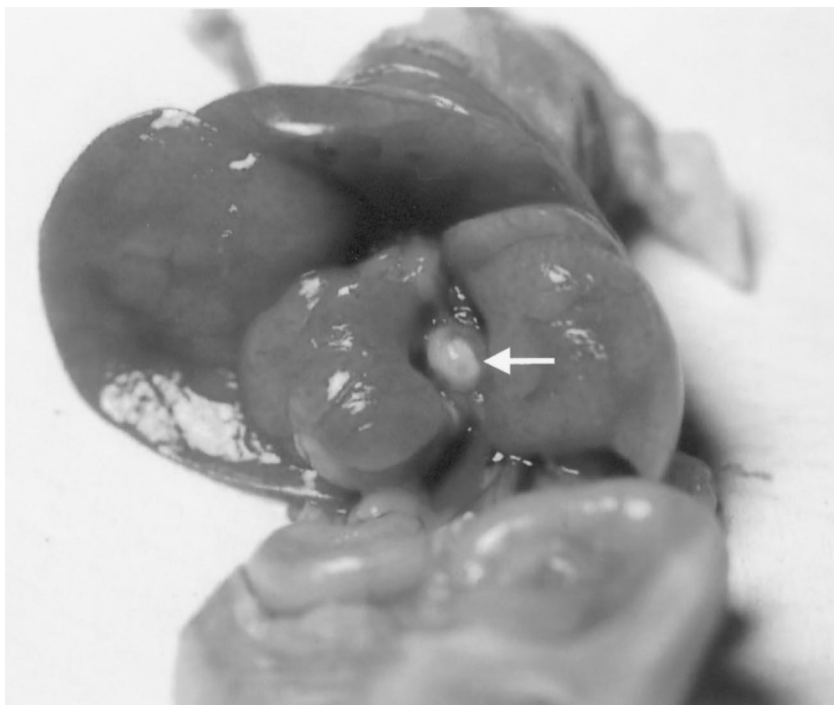


Fig. 2 Inferior surface of the liver of nude mouse and portal lymph node remetastasis 23 days after SOI (arrow). All lobes of liver are involved with metastatic tumor.

For colon SOI (18), a small midline incision was made, and the colocecal part of the intestine was exteriorized. A small area of the serosa of the colon was removed, and one or two tumor fragments of 1 mm³ each per mouse were implanted. An 8-0 nylon surgical suture was used to penetrate tumor pieces and suture them to the intestine wall. The intestine was returned to the abdominal cavity, and the abdominal wall was closed with a 6-0 silk surgical suture. Animals were kept in a barrier facility under HEPA filtration.

Evaluation of Growth and Metastases. The SOI-colon mice were sacrificed if they developed signs of distress. The SHI-liver mice were sacrificed every 4 days starting from day 10 after tumor implantation. At autopsy, the liver, lymph nodes, lung, kidney, spleen, and other organs were resected and processed for routine gross and microscopic examination. Metastasis was considered to have occurred if at least one microscopic metastatic lesion was found in any of the mice.

Human DNA Detected by *In Situ* Hybridization. The human origin of the tumor growing in nude mice was confirmed by *in situ* hybridization of human DNA (19). A human DNA probe (P5080-B.5) and hybridization kit (S1340-KIT) were purchased from Oncor (Gaithersburg, MD). Fresh tumor tissues were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Four sections were layered on silanized slides that were assayed for human total DNA by *in situ* hybridization. Briefly, tumor tissue sections on slides were baked at 65°C for 14 h, deparaffinized in xylene, hydrated in graded ethanol, and placed in preheated protein digestion solution at 45°C for 20 min (19). Slides were dehydrated in graded ethanol for hybridization. Probe preparation was as follows: the human DNA probe was prewarmed at 37°C for 5 min, denatured for 5 min in a 70°C water bath for 5 min, and quickly chilled on ice

and centrifuged for 2–3 s. Slide preparation was as follows: slides were placed in 70% formamide/2 × SSC at 20°C for 2 min and dehydrated in cold (–20°C) graded ethanol for 10 min. The hybridization procedure was as follows: The DNA probe was added to slides that were prewarmed to 37°C. Glass coverslips were sealed with rubber cement. The slides were transferred to 50% formamide/2 × SSC and 1 × PBS. The slides were then incubated with FITC-labeled avidin for 25 min at 37°C. Cells were counterstained with propidium iodide (0.3 μg/ml). Slides were observed under a microscope equipped with epifluorescence (19, 26).

RESULTS

After SOI, AC3488 involved 100% of the transplanted animals with massive liver metastasis and metastasis in the lymph nodes draining the liver, including the portal, celiac, and mediastinal nodes, without any retroperitoneal lymph node involvement (Table 1).

The spleen was involved in 50% of the animals. The kidney and adrenal gland were involved in 10% of the animals (Table 2).

In the SOI-colon group, all mice on postoperative day 10 developed massive multilobe liver metastasis. All lymph nodes draining the liver, including the portal, celiac, and mediastinal lymph nodes, were involved with metastasis by postoperative day 19. In the mouse, the abdominal lymphatics are divided into anterior and posterior routes draining the liver, including the hepatic hilum lymph nodes and celiac lymph nodes, as well as through the cephalad route to the posterior mediastinal lymph nodes (27, 28). The data thus suggest that metastatic involvement of lymph nodes draining the liver occurs after the appear-

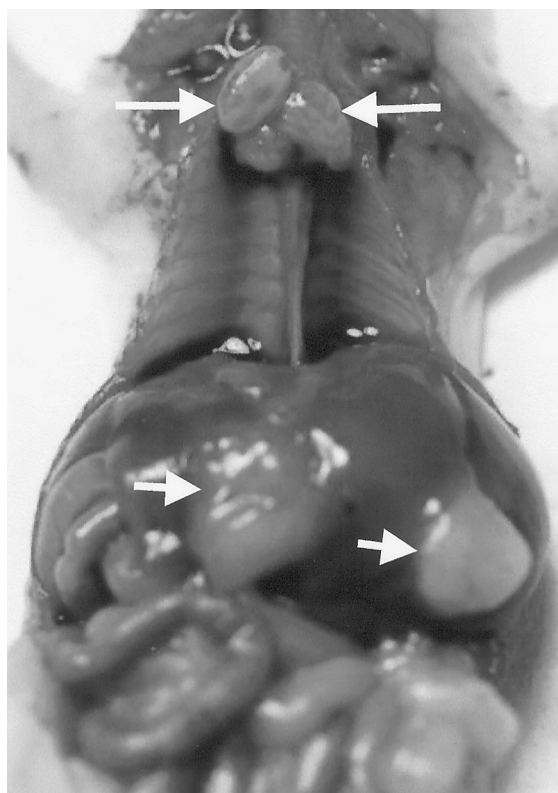


Fig. 3 Mediastinal lymph node remetastasis (long arrows) 18 days after SHI. Short arrows, hepatic tumor implantation on the left lateral and right middle lobes of the nude mouse liver.



Fig. 4 Mediastinal lymph nodes remetastasis 23 days after SOI (arrows). Liver is massively involved with metastatic tumor.

ance of liver metastasis from colon cancer and thus secondary to liver metastasis and not from the primary colon tumor.

To substantiate this hypothesis, AC3488 was implanted on the liver in a second group of animals, thus bypassing all lymphatic routes connecting the colon and the liver. In this group, beginning 10 days after tumor implantation, two mice were sacrificed every 4 days. In the first two mice, sacrificed 10 days after tumor implantation on the liver, all lymph nodes draining the liver, including portal, celiac, and mediastinal, had already been involved by metastasis (Figs. 1–4). These results confirmed that metastatic lymph nodes draining the liver are secondary to the liver metastasis. Table 1 demonstrates that the patterns of lymph node involvement in the SOI and SHI groups are similar.

Autopsy did not show any involvement of the colon, lung, mesenteric lymph nodes, or retroperitoneal lymph nodes in the SHI group. The spleen was the only organ that was involved with massive metastasis at a higher rate in the SHI group (80%) than in the SOI group (50%). This high rate of spleen involvement by metastasis suggests that spleen involvement can be considered another site of remetastasis from liver metastasis. Table 2 compares metastatic organ involvement in the SHI and SOI groups.

Histological Characteristics. Histological findings of AC3488 cells demonstrated poorly differentiated adenocarcinoma at all sites (19). Histological examination of the liver

revealed massive liver metastatic involvement and tissue edema surrounding the tumor (19). Lymph nodes at histological examination were massively infiltrated by metastatic cells.

Human DNA *In Situ* Hybridization. *In situ* hybridization previously tested positive for human DNA in all AC3488 tumor cells and indicated the human origin of AC3488 tumor grown in nude mice (19).

DISCUSSION

Many studies have demonstrated the importance of liver metastasis for the prognosis of patients with metastatic colon cancer (2–4, 9). In particular, metastasis of lymph nodes draining the metastatic liver, including their number and their site, as well as the liver metastasis, can influence survival and treatment strategies (3, 9).

There are few case reports in the literature of colon cancer on the origin of metastatic involvement of lymph nodes draining the liver (10, 11). These studies suggest that in patients with liver metastasis originating from colon cancer, lymph nodes draining the liver are involved by metastasis secondary to liver metastasis and not through the primary colon tumor. These events are considered unrecognized rather than rare (10). However, because some portal-associated lymphatic drainage of the colon does occur, it has been difficult to prove this hypothesis (3, 14). In addition, remetastasis is not a fully accepted concept.

The concept of metastasis of metastasis is not new. Weiss (15) noted that Travers, in 1829, discussed the “successive

appearance" of metastases; in 1887, Paget asked whether cancers would grow and spread distantly with the distant metastases resulting in further metastases; and in 1883, Eve noted dissemination of lung metastases to the bone. However, the data in the literature are inconclusive. Sugarbaker's study of a 3-methylcholanthrene-induced tumor suggested metastases from metastasis do not occur (29). The study by Hoover and Ketcham (12) using a technique of parabiosis demonstrated metastases from metastasis does occur.

Bross and Blumenson (13) emphasized that from the knowledge of the origins of the metastatic pattern and the sequence of target organ involvement, it is possible to focus on key disseminating sites and to rationalize therapy. Therefore, remetastasis of liver metastasis to the lymph nodes draining the liver, when it occurs, has significant clinical implications (9).

In our study, using a highly metastatic colon cancer model that resembles the natural history of highly metastatic colon cancer in humans, remetastasis occurred from the liver metastasis to the lymph nodes draining the liver.

After SOI of colon tumor AC3488 on the colon of nude mice, by postoperative day 10, all mice developed multilobar liver metastasis, and by postoperative day 19, all lymph nodes draining the liver were involved by metastasis. These data indicate that metastatic lymph nodes are secondary to liver metastasis. Our model resembles the colon cancer patients in case reports from Vetto and Cohen (10) and August *et al.* (11), in which liver metastasis from colon cancer was thought to be remetastasizing to the lymph nodes draining the liver. This source of spread may, in part, explain the all too frequent occurrence of distal failure after an apparently successful liver resection.

To conclusively demonstrate that metastatic lymph nodes are secondary to the liver metastasis and are not caused by a lymphatic connection between colon and liver or by a retroperitoneal lymphatic connection between mesenteric lymphatic routes and the mediastinum, animals received an implantation directly on the liver of colon cancer AC3488. All possible lymphatic connections between colon and lymphatic drainage of liver were thus bypassed. In the intrahepatic-transplanted animals, all lymph nodes draining the liver (portal, celiac, and mediastinal lymph nodes) became involved with metastasis as early as 10 days after tumor implantation. The pattern of the disease was similar to that in the animals that had AC3488 transplanted on the colon by SOI, as was survival after SHI, thereby demonstrating the liver-metastasis origin of the lymph node metastases. The fact that survival after SOI and SHI was almost identical suggests a similar cause of death in the two groups, most probably liver failure but perhaps also massive lymph node involvement, such as in the mediastinum (Figs. 3 and 4).

Thus, from the data presented in this report, we can conclude that metastatic involvement of portal, celiac, and mediastinal lymph nodes in animals with liver metastasis from colon cancer originates from remetastasizing liver tumors and not from primary colon cancer. These results are important for development of new treatment strategies of metastatic colon cancer.

REFERENCES

- Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1997. *CA Cancer J. Clin.*, 47: 5-27, 1997.
- August, D. A., Ottow, R. T., and Sugarbaker, P. H. Clinical perspective of human colon cancer metastasis. *Cancer Metastasis Rev.*, 3: 303-324, 1984.
- Copeland, E. M., Miller, L. D., and Jones, R. S. Prognostic factors in carcinoma of the colon and rectum. *Am. J. Surg.*, 116: 875-880, 1968.
- Pestana, C., Reitemeier, R. J., Moertel, C. G., Judd, E. S., and Dockerty, M. B. The natural history of carcinoma of the colon and rectum. *Am. J. Surg.*, 108: 826-829, 1964.
- Bross, I. D. J., Viadana, E., and Pickern, J. Do generalized metastases occur directly from the primary? *J. Chron. Dis.*, 28: 149-159, 1975.
- Welch, J. P., and Donaldson, G. A. The clinical correlation of an autopsy study of recurrent colorectal cancer. *Ann. Surg.*, 189: 496-502, 1979.
- Clarke, D. N., Jones, P. F., and Needham, C. D. Outcome in colorectal carcinoma: seven year study of a population. *Br. Med. J.*, 280: 431-435, 1980.
- Povoski, S. P., Fong, Y., Shouros, S. C., Kemeny, N. E., Downey, R. J., and Blumgart, L. H. Role of chest CT in patients with negative chest X-rays referred for hepatic colorectal metastasis. *Ann. Surg. Oncol.*, 5: 9-15, 1997.
- Hughes, K. S., Simone, R., Songorabodi, S., Adson, M. S., Ilstrup, D. M., Fortner, J. G., Maclean, B. J., Foster, J. H., Daly, J. M., and Fitzherbert, D. Resection of the liver for colorectal carcinoma metastases: a multi-institutional study of patterns of recurrence. *Surgery (St. Louis)*, 100: 278-284, 1988.
- Vetto, J. T., and Cohen, A. M. Isolated spread of hepatic metastatic disease to a mediastinal lymph node. *Dis. Colon Rectum*, 34: 1128-1130, 1991.
- August, D. A., Sugarbaker, P. H., and Schneider, P. D. Lymphatic dissemination of hepatic metastases, implication for the follow-up and treatment of patients with colorectal cancer. *Cancer (Phila.)*, 55: 1490-1494, 1985.
- Hoover, H. C., and Ketcham, A. S. Metastasis of metastases. *Am. J. Surg.*, 130: 405-410, 1975.
- Bross, I. D. J., and Blumenson, L. E. Metastatic sites that produce generalized cancer: identification and kinetics of generalizing sites. *In: L. Weiss (Ed.), Fundamental Aspects of Metastasis*, pp. 359-375. New York: North Holland Publishing Co., 1976.
- Rosenberg, S. A. *Surgical Treatment of Metastatic Cancer*. Philadelphia: J. B. Lippincott, 1987.
- Weiss, L. *Principles of Metastasis*. Orlando, FL: Academic Press, Inc., 1985.
- Hoffman, R. M. Orthotopic is orthodox. Why are orthotopic-transplant metastatic models different from all other models? *J. Cell. Biochem.*, 56: 1-3, 1994.
- Fu, X., Besterman, J. M., Monosov, A., and Hoffman, R. M. Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically-intact patient specimens. *Proc. Natl. Acad. Sci. USA*, 88: 9345-9349, 1991.
- Rashidi, B., Sun, F. X., Jiang, P., An, Z., Gamagami, Moossa, A. R., and Hoffman, R. M. A nude mouse model of massive liver and lymph node metastasis of human colon cancer. *Anticancer Res.*, in press, 2000.
- Sun, F.-X., Sasson, A. R., Jiang, P., An, Z., Gamagami, R., Li, L., Moossa, A. R., and Hoffman, R. M. An ultra-metastatic model of human colon cancer in nude mice. *Clin. Exp. Metastasis.*, 17: 41-48, 1999.
- Wang, X., Fu, X., and Hoffman, R. M. A new patient-like metastatic model of human lung cancer constructed orthotopically with intact tissue via thoracotomy in immunodeficient mice. *Int. J. Cancer*, 51: 992-995, 1992.

21. Fu, X., Theodorescu, D., Kerbel, R. S., and Hoffman, R. M. Extensive multi-organ metastasis following orthotopic implantation of histologically-intact human bladder carcinoma tissue in nude mice. *Int. J. Cancer*, *49*: 938–939, 1991.
22. Fu, X., Guadagni, F., and Hoffman, R. M. A metastatic nude-mouse model of human pancreatic cancer constructed orthotopically from histologically-intact patient specimens. *Proc. Natl. Acad. Sci. USA*, *89*: 5645–5649, 1992.
23. Fu, X., Herrera, H., and Hoffman, R. M. Orthotopic growth and metastasis human prostate carcinoma in nude mice after transplantation of histologically-intact tissue. *Int. J. Cancer*, *52*: 987–990, 1992.
24. Fu, X., and Hoffman, R. M. Human ovarian carcinoma metastatic models constructed in nude mice by orthotopic transplantation of histologically-intact patient specimens *Anticancer Res.*, *13*: 283–286, 1993.
25. Furukawa, T., Fu, X., Kubota, T., Watanabe, M., Kitajima, M., and Hoffman, R. M. Nude mouse metastatic models of human stomach cancer constructed using orthotopic implantation of histologically intact tissue. *Cancer Res.*, *53*: 1204–1208, 1993.
26. Chromosome *In Situ* Hybridization Manual. Gaithersburg, MD: Oncor, 1996.
27. Hebel, R., and Stromberg, M. W. Anatomy and Embryology of the Laboratory Rat. Wörthsee, Federal Republic of Germany: Biomed Verlag, 1986.
28. Lambert, R. Surgery of the Digestive System in the Rat. Springfield, IL: Charles C. Thomas Publishers, 1965.
29. Sugarbaker, E. V., Cohen, A. M., and Ketman, A. S. Do metastases metastasize? *Ann Surg.*, *174*: 161–166, 1971.