

Progressive Growth of Fish Tumors after Transplantation into Thymus-aplastic (*nu/nu*) Mice¹

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ABSTRACT

The nude mouse does not reject xenografts of malignant and non-malignant tissues of mammalian or avian origin, due to a deficiency of functional T-lymphocytes. In this study, tissue from a cold-blooded vertebrate, a teleost fish, was for the first time successfully transplanted to Swiss albino *nu/nu* mice. Malignant melanotic melanoma of *Xiphophorus* transplanted to nude mice showed progressive growth and could be serially passaged. *In vitro* culture experiments revealed that the fish tumor cells adapt to the physiological conditions of the mammalian host, most obviously to the body temperature. On the other hand, fish-specific morphological characters and biochemical features, e.g., expression of a melanoma-associated antigen, were retained. This experiment demonstrates the enormous capacity of the melanoma cells to adapt to severe changes in their environment, which even enables them to overcome the physiological barriers between such taxonomically distant vertebrate groups as fish and mammals.

INTRODUCTION

Since the findings of Rygaard and Povlsen (1) that a human adenocarcinoma of the colon could be successfully transplanted to thymus-aplastic (*nu/nu*) (2) mice, the "nude-mouse system" has become a very useful tool for studies on tumor biology and tumor therapy with respect to the investigation of the malignant potential of tumor cells and to pathophysiological studies in clinical and experimental oncology. The melanoma system of the teleost fish *Xiphophorus* provides a unique model for studies on the genetic factors contributing to tumor formation in vertebrates (3). Specific crossings lead to formation of spontaneously arising malignant melanoma in the hybrid fish. Several oncogenes have been found to be considerably overexpressed in the tumors (Ref. 4; Footnote³), and the crossing-conditioned deregulation of a genetically defined primary melanoma oncogene is thought responsible for this event. At the histopathological and ultrastructural (5, 6) as well as biochemical levels (7, 8), the tumor is widely comparable to mammalian melanomas. In order to assess the malignant potential of the *Xiphophorus* tumor cells, and due to the fact that in general tumor cells show a high capacity to adapt to different environmental conditions, we investigated whether fish melanoma cells are capable of survival even in a mammalian organism. These studies may also help in evaluating the usefulness of nonmammalian tumor models and the applicability of nude mouse transplantations to these systems.

Several attempts have been made to transplant normal tissues from nonmammalian vertebrates, including birds, reptiles, and amphibians, to nude mice. Except for the avian tissues the

xenografts underwent rapid degeneration, and the physiological inadequacy between host and donor was held responsible for the failure of these experiments (for review, see Ref. 9).

In this paper we report on the successful transplantation of fish tissue to a mammalian host by using malignant melanomas of *Xiphophorus* and thymus-aplastic mice.

MATERIALS AND METHODS

Fish. All tissues used for transplantation were from *Xiphophorus* hybrids which were produced by crossing *Xiphophorus maculatus* (population, Rio Jamapa) carrying the X-chromosomal gene complex *Tu-Sd* with *Xiphophorus helleri* (population, Belize River) lacking this gene complex. The resulting F₁ hybrids were backcrossed to *Xiphophorus helleri*. The backcross generation segregates into 50% tumor-free animals, 25% carrying on the dorsal fin a benign melanotic lesion with only limited, two-dimensional growth, and 25% with highly malignant, invasively growing, melanotic melanomas. For the grafting experiments fish previously kept for 5 days in antibiotic-containing tap water were killed by caput dislocation, and the tissues were excised and immediately used for transplantation.

Mice. Only animals of the *nu/nu* Swiss albino strain were used throughout the transplantation experiments. All experimental animals were bred and kept in the special pathogen-free facilities of the Max-Planck-Institut in Martinsried. Control DNA was prepared from liver of BALB/c mice.

Transplantation Procedure. Recipient mice were anesthetized with ether, and a small incision was made in the skin of the flank. The fish tumor was trimmed to a piece approximately 3 to 4 mm in diameter. It was inserted in a s.c. pouch, which was generated by lifting the skin at the site of the incision with fine forceps. The wound was constricted with sterile metal clamps.

Light Microscopy. All specimens were fixed in Bouin's solution. Excess picric acid was eluted with 70% ethanol. The fixed specimens were dehydrated and embedded in paraffin; 5 μ m sections were cut with a Leitz base sledge microtome and stained with acid alizarine blue and aniline blue orange G in a modification of the azan staining method or with hematoxylin/eosin or using Lillie's melanin-specific stain (10).

Transmission Electron Microscopy. Small pieces of tissue were fixed in 3% glutaraldehyde for 3 h. After washing in phosphate-buffered saline with the osmolality of fish cells, the specimens were postfixed in 2% osmic acid. The tissue blocks were prestained with 2% uranyl acetate in 20% ethanol at 60°C for 3 h, and then they were dehydrated and embedded in ERL-4206. Ultrathin sections were cut with a diamond knife using a Reichert Om U 3 ultramicrotome and examined in a Zeiss EM 10 A electron microscope.

Southern Blot Analysis. High-molecular-weight DNA (>70 kilobases) was prepared according to the method of Blin and Stafford (11), digested to completion with restriction enzymes, run on 0.8% agarose gels, and transferred to a hybridization membrane (gene screen plus; New England Nuclear, Dreieich, Federal Republic of Germany) by the alkaline transfer procedure (12). The filters were hybridized to approximately 5×10^6 cpm of ³²P nick-translated probe (specific activity, usually 3 to 6×10^8 cpm/g of DNA). The hybridization was performed at 42°C in a buffer containing 5 \times standard saline citrate, 1% sodium dodecyl sulfate, and 50% formamide. Subsequent washings were performed in 0.1 \times standard saline citrate and 1% sodium dodecyl sulfate at 68°C. Probes used were the 1.2-kilobase *Pst*I fragment of clone λ 19-4 representing the carboxy-terminal part of the *Xiphophorus c-src* gene and a fragment from the mouse lysozyme M complementary DNA

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³ W. Mäuler, F. Raulf, and M. Scharl. Expression of protooncogenes in embryonic, adult, and transformed tissue of *Xiphophorus*, submitted for publication.

clone of a mouse macrophage complementary DNA library (pMclys-1).

Cell Culture. Tissue samples from nude mouse tumors were dissociated in a solution containing 0.125% trypsin, 0.06% collagenase (type IA; Sigma), and 2% bovine serum albumin in calcium- and magnesium-free Dulbecco's phosphate-buffered saline for 60 min at room temperature. Cells were cultured in F-12 medium supplemented with 10% fetal calf serum in an atmosphere containing 5% CO₂.

Immunofluorescence. Samples from freshly excised tumors were dissociated into single cells by passing the tissue through a mesh net and by repeated aspiration through a 21-gauge needle. The cells were reacted with monoclonal antibodies 21-7 and 4-7 as primary antibodies. Fluorescein isothiocyanate conjugated F(ab)₂ goat anti-mouse IgG (affinity purified; Jackson Immuno-Research) was used as secondary antibody. Specimens were observed in a Zeiss "Universal" photomicroscope equipped with phase-contrast and epifluorescence optics.

RESULTS

Thirty-four malignant melanomas from *Xiphophorus* hybrids were transplanted s.c. to 6- to 10-wk-old male Swiss-*nu/nu* mice. For controls, normal fish skin ($n = 6$), testes ($n = 2$), muscle ($n = 1$), and benign melanomas ($n = 2$) were transplanted either on the contralateral site of a mouse also receiving a malignant melanoma graft or on separate recipients. The fate of the pigmented grafts below the unpigmented skin of the albino mouse was easily followed by gross examination. All the control grafts degenerated and disappeared within 2 to 4 wk. Similarly, the tumor grafts underwent an immediate phase of degeneration. However, in eight cases,⁴ after a period of as early as 9 wk or as late as 6 mo following transplantation, a progressively growing, dark brown or black pigmented s.c. tumor was observed at the site of transplantation. These tumors grew either more or less two-dimensionally, finally occupying areas of up to 4 cm² ($n = 5$), or three-dimensionally, thus forming exophytic nodules of up to 1 cm in diameter ($n = 3$) (Fig. 1). In three cases a second unpigmented tumor was observed in the close vicinity of the pigmented tumor. It was not possible to decide whether these tumors might be considered as true metastases of the primary tumor or as secondary tumors, which arose from a different portion of the primary transplant. However, metastases of solid tumor grafts are a rare phenomenon in nude mouse transplantation (13).

From one of the melanized tumors growing rapidly in the nude mouse, a small portion was excised and immediately transplanted to a new host. Within 21 days the graft gave rise to a new melanotic tumor, which has now been successfully passed through nude mice for another three generations.

Histological analysis revealed that the black tumors consisted of heavily melanized cells with interspersed connective tissue (Fig. 2b). The unpigmented tumors that arose in the vicinity of the primary graft as revealed by Lillie's melanin-specific stain, were composed of poorly differentiated melanoma cells containing only small amounts of pigment, not visible by gross examination. All tumors were embedded in the mouse adipose tissue (Fig. 2c) and were vascularized by mouse blood vessels to varying degrees. Frequently, the melanotic cells followed the vessels draining off or going towards the tumors (Fig. 2d). Ultrastructural examination showed that the tumors consisted of intact cells densely packed with melanosomes and with the fish-specific type of premelanosomes (Fig. 2a). Nuclear pockets, well-developed endoplasmic reticulum, and Golgi complexes,

⁴ Due to the long latency period for some of the tumors and to the shortened life span of nude mice, the exact percentage of successful transplantations could not be determined. All mice that died before reaching an age of 9 mo without any sign of a developing tumor were not taken into consideration ($n = 12$).



Fig. 1. Recipient nude mouse 10 wk after transplantation with melanotic tumor.

as well as pinocytotic activity were further morphological features also characteristic for poorly differentiated, proliferating *Xiphophorus* melanoma cells *in situ* (14).

To prove conclusively the fish origin of the tumor cells, DNA was extracted and hybridized to a fragment of the *Xiphophorus* cellular *src* gene. In a Southern blot experiment (Fig. 3) under conditions of high hybridization stringency, the probe hybridized only to the tumor DNA and to control DNA from the donor fish. In both samples two identical bands of 4.2 and 3 kilobases were observed. No hybridization was detected with mouse DNA. This indicates that the tumor DNA is indeed of *Xiphophorus* origin. Reversely, using mouse lysozyme DNA sequences as a hybridization probe in a similar experiment, a strong signal was detected with the mouse DNA. The weak signal detected in the tumor sample obviously indicates the presence of some mouse connective and vascular tissue as already observed in the histological examination. The different pattern of fragment length in both samples is explained by a restriction fragment length polymorphism between different mouse strains because DNA from BALB/c mice was used for control in the Southern blot experiment.

Additional evidence for the fish origin of the melanomas in the nude mice comes from the fact that the tumor cells expressed a cell surface antigen that reacted with the *Xiphophorus* melanoma-specific monoclonal antibodies 21-7 and 4-7 as tested by immune fluorescence (data not shown). These monoclonal antibodies do not cross-react with normal cells from any mammalian tissue tested so far (15).

Melanoma cells taken directly from the primary fish tumor constantly failed to grow *in vitro* so far. However, when melanomas ($n = 3$) of the nude mice were put into cell culture using conventional tissue culture medium and fetal calf serum, the pigmented tumor cells commenced to propagate immediately. Interestingly, the optimum temperature for *in vitro* culture of the fish cells taken from the nude mouse tumors had changed. All the cultures showed optimal growth at 35°C and failed to propagate at temperatures below 30°C, while all established

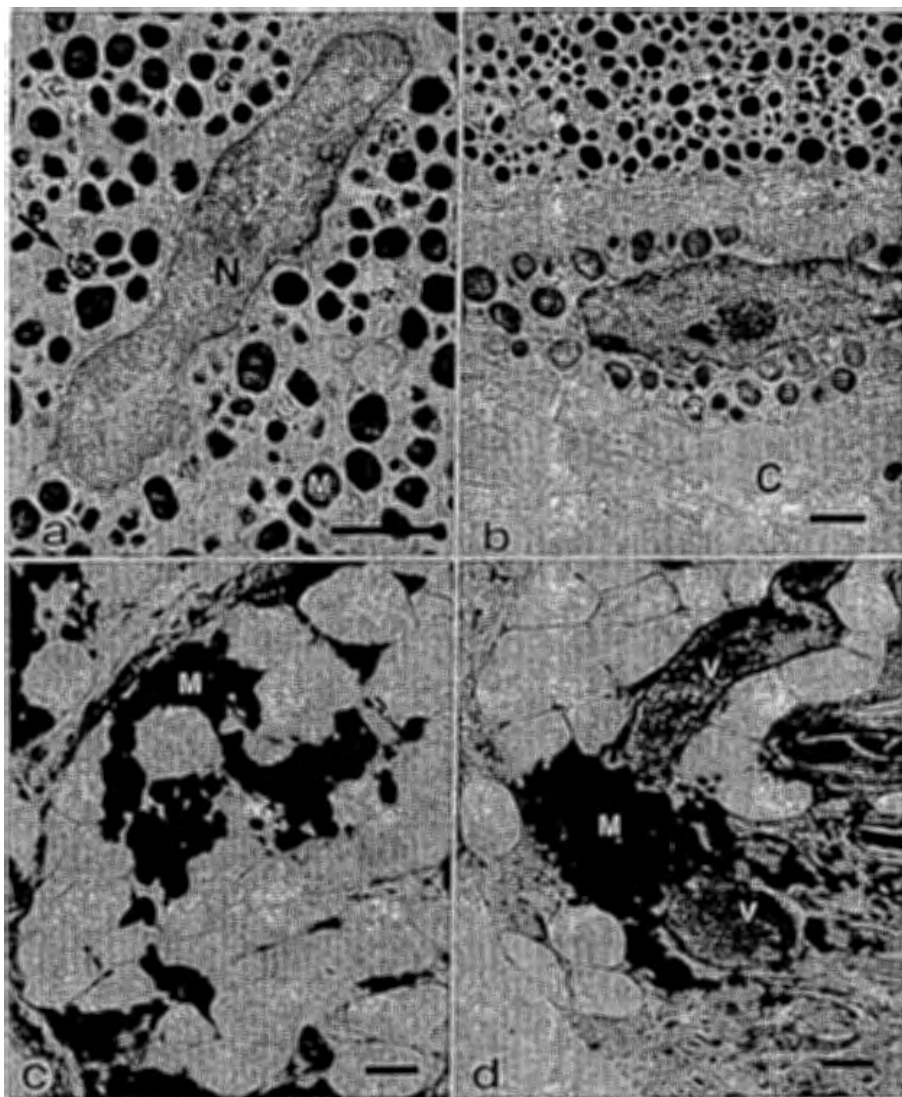


Fig. 2. Histological and ultrastructural appearance of the transplanted tumors. *a*, electron micrograph of a typical low-differentiated transformed pigment cell from the central portion of the tumor with the fish-specific type of premelanosomes (arrow); *M*, mature melanosomes; *N*, nucleus; bar, 1 μ m. *b*, section from the marginal area of the tumor with fish melanoma cell (upper) embedded in mouse connective tissue; *C*, collagen; bar, 1 μ m. *c*, histological section showing fish melanoma cells embedded in the mouse subcutaneous adipose tissue; *M*, melanoma cells; bar, 50 μ m. *d*, peripheral area of the tumor with fish melanoma cells accompanying a host blood vessel; *M*, melanoma cells; *V*, blood vessel; bar, 50 μ m. Note the intravascular erythrocytes without nuclei.

Xiphophorus cell lines as well as primary cultures grow preferentially at a temperature of 28°C and show heat-shock response at temperatures above 33°C. The morphology of the melanoma cells *in vitro* was very heterogenous (Fig. 4). Pigmentation varied from cells containing no visible melanin to cells which were totally filled with dark-red or black pigment. Also a difference in size was observed. The majority of the cells were small, while some cells, often arranged in groups, had diameters up to 5 to 10 times that of the smaller cells. The large cells always were heavily pigmented and are thus considered to represent a more advanced stage of melanophore differentiation. The small unpigmented cells obviously are the proliferating transformed melanoblasts/melanocytes (according to Ref. 16).

DISCUSSION

We have demonstrated that malignant melanoma from *Xiphophorus* fish can be grafted to nude mice and shows progressive growth in the host. To evaluate the factors that might have facilitated the successful transplantation of the fish melanoma, several aspects have to be taken into consideration. (a) Malignant

melanomas in general have one of the highest success rates for tumor xenografts in nude mice (17). (b) The adaptability of tumor cells to changes in the microenvironment is especially high in melanomas due to high mutation rates, leading to the high clonality of this tumor type (18). This might have helped in the selection and establishment of cells from the fish tumor which are able to tolerate the inadequate physiological conditions of the mammalian host. One of the important factors is the increased body temperature of the mouse compared to the fish. In this respect the eurythermia of cells from the poikilotherm teleost could have facilitated the adaptive process. For normal skin from cold-blooded vertebrates (reptiles, amphibians), however, the failure in temperature adaptation has been proposed as one of the primary causes for degeneration (8). Tumor grafts have the advantage that the site of transplantation is not as critical as for normal tissue. The s.c. application of the fish melanoma prevents drying of the graft and provides a suitable site for a cutaneous tumor.

Like human melanoma xenografts (19), the fish melanoma transplants exhibited varying degrees of vascularization. In any case, for vascularization of the tumor an angiogenetic factor is necessary (20). If a factor of this kind is produced by the fish

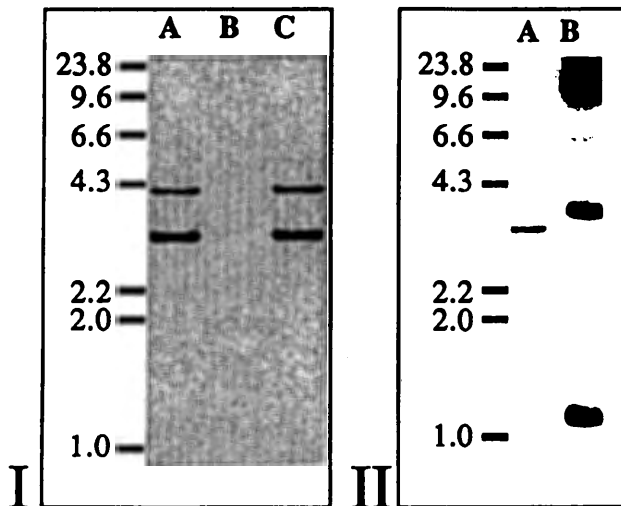


Fig. 3. Southern blot analysis of DNA from tumor (A), mouse (BALB/c) liver (B), and donor fish liver (C) probed with the fragment from the *Xiphophorus c-src* homologue (I) or with a mouse lysozyme clone from a macrophage complementary DNA library (II) showing strong hybridization of the tumor DNA with the fish gene and weak hybridization to the mouse gene, the latter indicating the presence of some mouse connective and vascular tissue in the tumor. In the mouse DNA, no cross-hybridization with the fish gene could be detected. For this analysis, equal genomic equivalents (4 μ g of fish DNA and 20 μ g of mouse DNA) were used in Gel I, while equal amounts of DNA were used in Gel II.

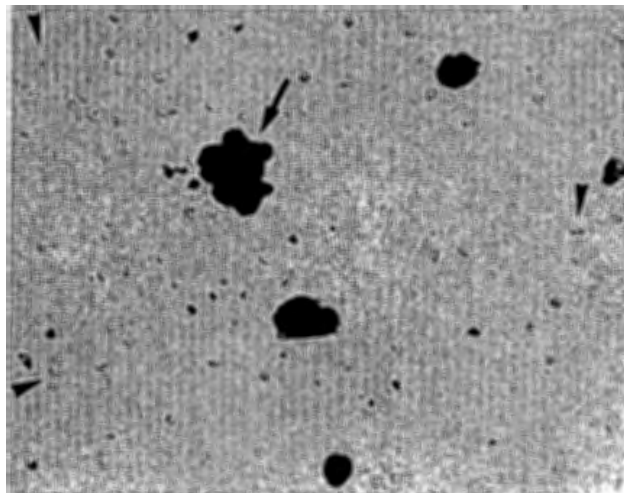


Fig. 4. Bright field image of melanoma cells *in vitro*. Note small unpigmented or scarcely pigmented melanoblasts/melanocytes (arrowheads) and large, heavily melanized melanophores (arrow).

melanoma, it must be reasonably conserved in order to provoke the appropriate reaction of the host blood vessels. This issue has to be clarified by further experiments. These future studies should also include investigation of the changes in oncogene expression during the adaptive process, attempts to monitor the clonality of the tumors by genetic markers using restriction fragment length polymorphisms, and further evaluation of the usefulness of tumor xenotransplantation for obtaining cell cul-

tures from nonmammalian vertebrates. In learning how fish xenografts are similar to or different from cells in culture and from tumors in fish, factors may be uncovered that are important in other xenograft experiments, but which are less obvious in heterotransplants between more closely related species, *e.g.*, mice and humans.

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REFERENCES

- Rygaard, R. J., and Povlsen, C. O. Heterotransplantation of a human malignant tumor to the mutant nude. *Acta Pathol. Microbiol. Scand.*, **77**: 758-764, 1969.
- Pantelouris, E. M. Absence of the thymus in a mouse mutant. *Nature (Lond.)*, **217**: 370-373, 1968.
- Anders, F., Scharl, M., Barnekow, A., and Anders, A. *Xiphophorus* as an *in vivo* model for studies on normal and defective control of oncogenes. *Adv. Cancer Res.*, **42**: 191-275, 1984.
- Scharl, M., Mäueler, W., Raulf, F., and Robertson, S. M. Molecular aspects of melanoma formation in *Xiphophorus*. In: J. Bagnara (ed.), *Pigment Cell*, Vol. 8. New York: Alan Liss, in press, 1987.
- Sobel, H. J., Marquet, E., Kallman, K. D., and Corley, G. J. Animal model of human disease: malignant melanoma. *Am. J. Pathol.*, **82**: 441-444, 1976.
- Riehl, R., Scharl, M., and Kollinger, G. Comparative studies on the ultrastructure of malignant melanoma in fish and human by freeze-etching and transmission electron microscopy. *J. Cancer Res. Clin. Oncol.*, **107**: 21-31, 1984.
- Mäueler, W., Eigenbrodt, E., Scharl, M., and Anders, F. Intermediary metabolism of normal and tumorous tissue of *Xiphophorus* (Teleostei: Poeciliidae). *J. Comp. Biochem. Physiol.*, **88**: 481-490, 1987.
- Barnekow, A., Paul, E., and Scharl, M. Expression of the *c-src* protooncogene in human skin tumors. *Cancer Res.*, **47**: 235-240, 1987.
- Reed, N. D., and Manning, D. D. Present status of xenotransplantation of non-malignant tissue to the nude mouse. In: J. Fogh and B. C. Giovanella (eds.), *The Nude Mouse in Experimental and Clinical Research*, Vol. 1, pp. 167-185. New York: Academic Press, 1978.
- Lillie, R. Ferrous ion uptake. *Arch. Pathol.*, **64**: 100-103, 1957.
- Blin, N., and Stafford, S. W. General method for isolation of high molecular weight DNA from eukaryotes. *Nucl. Acid Res.*, **3**: 2303-2309, 1976.
- Reed, K. C., and Mann, D. A. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucl. Acid Res.*, **13**: 7207-7214, 1985.
- Sordat, B. C. M., Ueyama, Y., and Fogh, J. Metastasis of tumor xenografts in the nude mouse. In: J. Fogh and B. C. Giovanella (eds.), *The Nude Mouse in Experimental and Clinical Research*, Vol. 2, pp. 95-147. New York: Academic Press, 1982.
- Vielkind, U. Genetic control of cell differentiation in platyfish-swordtail melanoma. *J. Exp. Zool.*, **196**: 197-203, 1976.
- Clauss, G., Lohmeyer, L., and Anders, A. Melanoma-associated antigens in *Xiphophorus* fish. *Cancer Res. Clin. Oncol.*, **113**: 25, 1987.
- Anders, F., Diehl, H., Schwab, M., and Anders, A. Contributions to an understanding of the cellular origin of melanoma in the Gordon-Kosswig xiphophorine fish tumor system. In: S. Klaus (ed.), *Pigment Cell*, Vol. 4, pp. 142-149. Basel: Karger, 1979.
- Fogh, J., Dracopoli, N., Loveless, J. D., and Fogh, H. Cultured human tumor cells for cancer research: assessment of variation and stability of cultural characteristics. *Prog. Clin. Biol. Res.*, **89**: 191-223, 1982.
- Fidler, I. J., and Hart, I. R. Biological diversity in metastatic neoplasms: origin and implications. *Science (Wash. DC)*, **217**: 998-1003, 1982.
- Solesvik, O. V., Rofstad, E. K., and Brustad, T. Vascular changes in human malignant melanoma xenografts following single dose irradiation. *Radiat. Res.*, **98**: 115-128, 1984.
- Giovanella, B. C., and Fogh, J. Present and future trends in investigations with the nude mouse as a recipient of human tumor transplants. In: J. Fogh and B. C. Giovanella (eds.), *The Nude Mouse in Experimental and Clinical Research*, pp. 282-312. New York: Academic Press, 1978.