Cancer and Metastasis Reviews 5: 29–49, 1986
Martinus Nijhoff Publishers, Boston, Printed in the Netherlands

Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis

Isaiah J. Fidler
Department of Cell Biology, University of Texas, M. D. Anderson Hospital and Tumor Institute, Houston, USA

Key words: nude mice, human tumors, model, metastasis therapy

Summary

Human neoplasms are biologically heterogeneous. The extensive cellular diversity found in malignant neoplasms is generated by the rapid emergence of clonal subpopulations of tumor cells with different properties that include invasion, metastasis and responsiveness to treatment. Studies in rodent systems have indicated that cancer metastases can be clonal in their origin and that different metastases can originate from different progenitor cells from the primary tumor. This metastatic heterogeneity of tumor cells has many ramifications for studies of tumor biology, in general, and studies of therapy, in particular.

The heterogeneous nature of metastatic human neoplasms can now be studied under defined conditions in healthy athymic nude mice. The neoplasms must be free of mouse pathogens and the mice must be kept in specific-pathogen-free conditions. Careful consideration must be given to the intimate tumor-host relationship for each tumor system studied, because the metastatic potential of human neoplasms can vary with the site of implantation into nude mice.

Several methods for studying the biology of human neoplasms in the nude mouse are described as well as techniques to assure the success of these studies. The data show that the healthy young nude mouse can be a useful *in vivo* model for ascertaining the metastatic potential of human neoplasms, for selecting and maintaining cell variants of high metastatic potential from heterogeneous human tumors, and for studying therapeutic agents directed against metastatic cells proliferating in visceral organs.

Introduction

During the last decade, the realization that neoplasms are biologically heterogeneous has gained wide acceptance (reviews, 1–5). In reality, the concept is not new. In 1889, Paget (6) analyzed autopsies of a large number of patients with breast cancer and concluded that the nonrandom pattern of metastasis was not due to chance, but that some tumor cells ('seeds') traveling by vascular routes had affinity for growth in the environment provided by certain organs ('soil'). Only when the 'seed and soil' were matched did metastases develop. In the last few years Paget's hypothesis has received considerable experimental and clinical support. Site-specific metastasis occurs with many transplantable experimental tumors (7–11) and has been reported recently in autochthonous human tumors in patients with peritoneovenous shunts (12).

Address for offprints: Isaiah J. Fidler, DVM, Ph.D, Department of Cell Biology, University of Texas, M. D. Anderson Hospital and Tumor Institute at Houston, 6723 Bertner Avenue, Houston, TX 77030, USA

A modern definition of the 'seed and soil' hypothesis would consist of three principles. First, the process of metastasis is not random. Rather, the process consists of a series of linked, sequential steps that must be completed by tumor cells if a metastasis is to develop. Thus, a metastatic cell must succeed in invasion, embolization, survival in the circulation, arrest in a distant capillary bed, and extravasation into and multiplication in organ parenchyma. Interruption of the metastatic sequence at any of these steps can prevent the production of grossly visible, clinical metastases. Indeed, a large body of data suggests that at every stage of the process of metastasis the rules of the survival of the fittest apply with regard to the interplay of metastatic tumor cells with their host. Thus, metastasis generally represents a highly selective, nonrandom event (2, 13–18) favoring the survival of a minor subpopulation of metastatic cells that preexist within the parent neoplasm (14).

Second, neoplasms are not uniform entities, but rather consist of cells exhibiting heterogeneous biologic and metastatic properties. The term heterogeneous is defined as 'consisting of, or composed of, dissimilar elements or ingredients; not having a uniform quality throughout' (19) and there is now considerable evidence that, at the time of diagnosis, most human and animal neoplasms consist of different populations of cells with diverse biological characteristics (1–5, 15). Cells obtained from individual tumors have been shown to differ with respect to many phenotypes, including immunogenicity, growth rates, karyotypes, pigment production, cell-surface receptors for lectins, hormone receptors, susceptibility to cytotoxic drugs, and, perhaps most important, propensity for invasion and metastasis (1–5, 15). Biological heterogeneity is not confined to cells in primary tumors: it is equally true of the cells populating metastases (2, 4, 15, 20, 21). Indeed, many clinical observations now suggest that multiple metastases proliferating in different organs or even in the same organ of cancer patients can exhibit diversity in many biological characteristics such as hormone receptors, antigenicity-immunogenicity, and sensitivity to various chemotherapeutic drugs (3, 13).

Third, the outcome of metastasis depends on the properties of both tumor cells and host factors, and the balance of these contributions may vary in tumors arising in different tissues and in tumors of similar histologic origin in different patients (9, 15, 22). The complexity of the pathogenesis of metastasis explains, in part, why the process is deemed to be inefficient insofar as tumor cells are concerned (23, 24). For example, the presence of tumor cells in the circulation does not predict that metastasis will occur. Most tumor cells that enter the blood stream are rapidly eliminated (25). Using radiolabeled tumor cells, we have observed that by 24 h after entry into the circulation, $<1\%$ of the cells are still viable, and $<0.1\%$ of tumor cells placed into the circulation survived to produce metastases (25). Observations such as this are compatible with the 'seed' hypothesis (6) and prompted us to question whether the 0.1% of the circulating cells responsible for the development of metastases survived at random, or whether the cells represented preexistent subpopulations of cells endowed with special properties.

The first experimental proof for metastatic heterogeneity in neoplasms was provided by Fidler and Kripke in 1977, who, in working with the murine B16 melanoma (14), used a modified fluctuation assay of Luria and Delbruck (26). The finding that preexisting tumor cell subpopulations growing in the same tumor exhibit heterogeneous metastatic potential has since been confirmed in numerous laboratories using a wide range of experimental animal tumors of different histories and histological origins (8, 9, 15). The data demonstrating metastatic heterogeneity in neoplasms ('seed') and those showing that the outcome of metastasis is also dependent on host factors ('soil') support the concept that metastasis is selective, not random (17, 18, 27).

The majority of the above-mentioned studies have been carried out with rodent tumor systems. Whether or not human tumors are heterogeneous for invasion and metastasis remained unanswered. Animal models have proved to be invaluable for the elucidation of various host factors and tumor properties involved in the patho-

genesis of metastasis (28). The use of such models could enhance our knowledge of the biology of metastasis and thus contribute to the development of new approaches to the therapy of metastasis. However, adequate animal models for *in vivo* studies of human neoplasia in general, and of metastasis in particular, have been lacking. The discovery of the athymic T-cell-deficient nude mouse and its availability for studies of heterotransplantation of tissues have provided a most valuable tool for examining some aspects of human tumors *in vivo*.

This review provides a summary of our current understanding of metastatic heterogeneity of human neoplasms. I shall also review the methods we routinely employ to isolate metastatic cells from human neoplasms and to implant human tumor cells into athymic nude mice. The data strongly suggest that under appropriate conditions nude mice are a valuable model for the study of the biology and therapy of human cancer metastasis.

The growth of xenografted tumors in nude mice

A. Transplantation and natural killer cell system

Since the observation by Rygaard and Povlsen (29) that human tumors could progressively grow in athymic nude mice, intensive use of this animal model has been made in studies of the growth characteristics and metastatic potential of xenogeneic neoplasms (30, 31) and in determinations of the tumorigenic potential of transformed cells (32, 33). Progressive growth of xenogeneic tumors in nude mice is dependent on tumor-related properties, such as the origin and type of tumor, and on the route of inoculation into the mice. Human melanomas, carcinomas of soft tissues, and sarcomas can be transplanted successfully into nude mice (33, 34). In contrast, carcinomas of the breast (34), stomach, and prostate (35) are more difficult to establish as xenografts. To succesfully grow lymphomas and leukemias, the recipient nude mouse must be immunosuppressed or the tumor must be injected intracranially; sometimes both are required (36).

In addition to tumor cell properties, nude mouse-related factors, such as the age, strain, and state of health of the recipient animals, also influence the growth of tumors (31, 33). For example, nude mice infected with mouse hepatitis virus exhibit great resistance to transplanted xenogeneic human neoplasms. This could be due, in part, to the fact that although the athymic nude mouse lacks functionally mature T lymphocytes, it is not totally immunodeficient. Nude mice show a near-normal response to T-cell-independent antigens and have high titers of natural antibodies that can react with tumor cells (37). Tumoricidal macrophages can be isolated from nude mice, and their activity can be enhanced after *in vivo* stimulation with bacterial adjuvants (38). Most important, however, nude mice consistently exhibit a high level of natural killer (NK) cell activity (39). All three T-cell-independent effector mechanisms may thus play a significant role in host-tumor interaction and contribute to the natural resistance of nude mice to transplanted neoplasms.

The usefulness of the athymic nude mouse as an *in vivo* model for studying the biology of neoplasms has also been limited by the fact that malignant neoplasms only rarely metastasize when transplanted into adult nude mice (39). The expression of metastatic potential of tumor cells in nude mice depends on the experimental protocols, the nature of the tumor cells, and the natural defense mechanisms of the recipient mice. The observations that immunosuppressed (35), young (39), or newborn (40) nude mice are more susceptible to tumor growth and metastasis suggests that active T-cell-independent defense mechanisms may be responsible, at least in part, for the low incidence of tumor metastasis in adult nude mice.

Unlike tumor cells growing in a primary neoplasm, the progenitors of metastases usually circulate in the blood stream as single cells or small cell clumps (homotypic or heterotypic aggregates) and are, therefore, vulnerable to the destructive effects of nonimmune and immune defense mechanisms (41). The initial studies of Hanna and co-workers (31, 39, 42–46) suggest

that NK cells (high levels are found in nude mice) are particularly efficient in destroying tumor emboli in the circulation. Hanna and co-workers demonstrated an inverse correlation between the levels of NK cell activity and the incidence of experimental metastasis of rodent tumors. The incidence of cancer metastasis is higher in mice with low NK cell activity and lower in mice with high NK cell activity; e.g., there is a high incidence of metastasis in 3-week-old syngeneic mice and in beige mutant (bg^J/bg^J) mice derived from C57BL/6 mice (44, 47). Moreover, the *in vivo* depletion of NK cells by pretreatment of mice with cyclophosphamide (43), beta-estradiol (46) or rabbit serum rich in antiasialo GM_1 activity (48) is associated with enhancement of pulmonary and extrapulmonary metastasis of mouse tumors. Finally, direct evidence that NK cells are responsible for inhibiting experimental mouse tumor metastasis *in vivo* was provided by adoptive transfer experiments in which the reconstitutive antimetastatic activity of normal spleen cells was abolished by pretreatment of the cells with specific anti-NK 1.2 antibodies and complement (39).

Collectively, the data demonstrate that high levels of NK cell activity can interfere with hematogenous tumor spread and thus inhibit the subsequent development of metastases of transplanted allogeneic mouse neoplasms injected into nude mice.

B. Environmental and genetic factors influencing the suitability of nude mice for studies of metastasis

The findings that the level of NK cell activity can influence the outcome of experimental metastasis prompted Nabil Hanna and co-workers to investigate whether the animal's age, genetic background, and environmental condition could also affect the sensitivity of the nude mouse to the development of experimental metastases (31, 39, 49). BALB/cAnN nude mice exhibited lower levels of NK-cell-mediated cytotoxicity than age-matched N:NIH(S) nude mice maintained under similar housing conditions. Likewise, nude mice maintained under barrier conditions exhibited weaker NK cell activity than mice maintained under coventional housing conditions. The incidence of experimental pulmonary metastasis of allogeneic tumors injected into nude mice was inversely correlated with the levels of NK-cell-mediated cytotoxicity (see below): 3-week-old BALB/cAnN nude mice raised under barrier conditions were more sensitive to development of experimental metastases than age-matched N:NIH(S) nude mice maintained under barrier conditions or nude mice of either strain maintained under conventional housing conditions. In both strains, however, the incidence of metastasis of allogeneic tumors was strikingly similar to that observed in syngeneic recipients (31). The low NK cell activity and high incidence of experimental metastasis observed in 3-week-old BALB/c nude mice reared under barrier conditions suggested that these animals could serve as a model for studies of the *in vivo* (metastatic) behavior of neoplasms and for assessment of antimetastatic activity of agents to be used in cancer therapy.

C. The use of nude mice to ascertain the malignant potential of rodent neoplasms

Much like normal mice, 3-week-old nude mice exhibit a lower level of NK cell activity than do adult 6- to 8-week-old mice. This finding prompted us to investigate whether young nude mice devoid of NK cell activity could serve as a model for studying metastasis of allogeneic mouse or xenogeneic rat tumors (45). Young (3-weeks-old) and adult (6- to 8-week-old) nude mice were injected subcutaneously or intravenously with single-cell suspensions prepared from various mouse and rat tumors with known metastatic behavior under syngeneic conditions. All the tumors grew subcutaneously. However, only those tumors that were capable of producing metastases in syngeneic animals produced experimental metastases in young 3-week-old nude mice. Virtually no comparable metastasis was observed in adult nude mice injected with the identical cell preparation.

The patterns of metastasis of the tumors stud-

ied were similar both in the syngeneic hosts and in young nude mice. All metastatic neoplasms, irrespective of their degree of antigenicity, produced lung tumor colonies in young nude mice, whereas nonmetastatic tumors did not.

In the next set of studies, we examined whether tumor cells populating spontaneous metastases produced from local tumors exhibited greater colonizing potential than cells populating the parent tumor (17), after implantation into nude mice. In both syngeneic mice and allogeneic nude mice, cells form spontaneous metastases produced significantly more experimental pulmonary metastases than did cells isolated from the heterogeneous and unselected parent population.

D. The use of nude mice for selection of metastatic cells from heterogeneous mouse neoplasms

Data from our laboratory and many others indicate that metastases may have a clonal origin and that different metastases can be produced by different progenitor cells (20). These findings may partly explain the clinical observation that different metastases can exhibit different growth rates, antigenicities, and responses to treatment (1–4, 15, 20, 21). The existence in heterogeneous neoplasms of minor but specialized subpopulations of cells that can give rise to metastases has profound implications for testing agents designed to treat disseminated disease. Because differences in the response of primary and metastatic lesions or of various metastases to therapeutic agents are well documented in clinical practice (review, 4), procedures that use heterogeneous tumors to identify effective agents for treatment of metastases may miss a beneficial response against the minor but fatal subpopulation of metastatic cells.

Several methods have been described to isolate subpopulations of cells with increased metastatic potential from a heterogeneous malignant neoplasm. Perhaps the most widely used procedure is the one we initially described in studies on the B16 melanoma. This procedures involves the isolation and propagation of variant lines from metastatic foci (3, 28) and has been successfully used with diverse rodent tumor systems such as melanoma, fibrosarcoma, 3-methylocholanthrene-induced sarcoma, mammary tumors, liver tumors, lung carcinoma, colon carcinoma, lymphoma, and lymphosarcoma (9, 13, 15, 16). Essential to the success of such a procedure is the availability of normal syngeneic recipients for both the selection and the testing of the isolated, metastatic cells. Because we found that young nude mice can be used for quantitation of the metastatic potential of neoplasms, we were encouraged to use their animals for the selection of metastatic subpopulations from the allogeneic heterogeneous B16 melanoma syngeneic to the C57BL/6 mouse and the K-1735 melanoma syngeneic to the C3H/HeN mouse (49). Groups of 3-week-old BALB/cAnN nude mice were injected i.v. with viable cells of either the parental B16 melanoma or the parental K-1735 melanoma. About 4 weeks later, the mice were killed, and solitary lung tumor colonies harvested from different mice were transplanted by trocar implantation into the subcutis of nude mice (1 metastasis/mouse) to expand the populations. Single-cell suspensions of viable tumor cells were prepared from each subcuteaneous tumor mass by dissociation with collagenase. The metastatic potential of tumor lines (parent and metastases) was tested by monitoring the production of experimental pulmonary metastasis subsequent to i.v. injection into the lateral tail veins of unanesthetized syngeneic mice or 3-week-old nude mice. The results were expressed as the median and range of lung tumor counts, and differences in metastatic incidence were analyzed with the Mann-Whitney U-test. In both tumor systems (B16, K-1735), cells isolated from lung tumor colonies produced a significantly higher incidence of metastasis ($P<0.001$) than did cells of the unselected parent tumor (Table 1). This enhanced potential was also expressed in the respective syngeneic recipients (49).

These data suggested that young nude mice can be used to select populations of tumor cells with increased metastatic potential. However, the use of specific pathogen-free nude mice is mandatory to the success of these procedures. Nude mice infected with murine hepatitis virus or

Table 1. Metastatic potential of cells from parent tumors and experimental metastases produced in allogeneic nude mice[a]

Strain	Source of cells	Median number (range) of lung metastases[b]	
		Nude mice (3-wk-old)	Syngeneic mice (6-wk-old)
C57BL/6	B16 parent	0.5 (0–7)	1.0 (0–11)
	Nu.Met-1	9.5 (1–39)	8.0 (1–16)
	Nu.Met-3	40.0 (3-101)	27.0 (7–40)
	Nu.Met-4	87.0 (37–298)	68.0 (2–98)
	Nu.Met-5	25.0 (4–56)	11.0 (3–23)
C3H/HeN	K-1735 parent	19 (3–56)	44 (10–148)
	Nu.Met-1	>500 (all >500)	>500 (all >500)
	Nu.Met-2	406 (184–>500)	>500 (all >500)
	Nu.Met-3	>500 (all >500)	>500 (all >500)
	Nu.Met-4	124 (57–186)	200 (134–245)

[a] From Reference 49.
[b] Ten mice/group. The median number of lung tumor colonies differed significantly between groups injected with cells isolated from metastases or parental tumors ($P<0.001$).

other pathogenic microorganisms exhibit increased NK activity (50) and thus become resistant to the production of metastases (39) or even growth of tumor allografts or xenografts at subcutaneous sites (50, 51).

The use of nude mice for studying the biology of human neoplasms

A. Metastatic propensity of human tumor cells in nude mice

One reason that the nude mouse can serve as a useful *in vivo* model for the study of human neoplasms is that it does not disrupt the cellular characteristics of transplanted tumors. Many investigators report that human tumors grown in nude mice maintain their karyotype (29, 33, 52), morphological, and histological appearance, and the production of specific enzymes, antigens, and hormones (29, 33, 52–58). Some human colon tumor cells xenografted into nude mice manifest increased tumorigenicity upon repeated passages. The acquisition of xenotropic murine C-type viruses by human tumors may accont for this phenomenon (60). The induction of murine stromal neoplasms in the presence of transplanted human tumors has also been reported. This process has been designated horizontal oncogenesis and is thought to represent a form of genetic transfer from human to murine cells (61, 62). Although apparently a rare event, the implications of this finding warrant the intensive investigation currently being conducted in many laboratories. Although human neoplasms have been shown to be heterogeneous with regard to many biologic characteristics, there has been little experimental evidence that these tumors contain populations of cells with different metastatic properties (63).

B. Studies with the A375 melanoma cell line

The success of studying the metastatic behavior of rodent neoplasms in nude mice (63) has prompted us to investigate the metastatic heterogeneity of the human A375 melanoma line (64). The tumor line, which was established from a metastatic lesion, was grown in culture as an adherent monolayer. The parental tumor was cloned in a 0.3% mixture of Noble agar. Tumor colonies derived from single cells were propagated an serially transferred to vessels of increasing size. The parent cell line and established clones were examined for and were found to be free of mycoplasma and pathogenic murine viruses.

C. Assay for experimental pulmonary metastasis

Tumor cells were harvested from subconfluent cultures by overlaying of the monolayers with a solution of 0.25% trypsin and 0.02% EDTA. After 1 min the flasks were tapped sharply to dislodge the cells, which were then washed in complete medium and resuspended in Hanks' balanced salt solution (HBSS) for inoculation. We used only single-cell suspensions with greater than 95% viability as determined by trypan blue exclusion. The final suspension consisted of 2.5 x 10^6 cells/ml. Unanesthetized mice received i.v. injections of 0.2 ml of this tumor cell suspension via the lateral tail vein (Fig. 1). Eight weeks later, the mice were killed, and the lungs were removed, rinsed briefly in water, and fixed in Bouin's

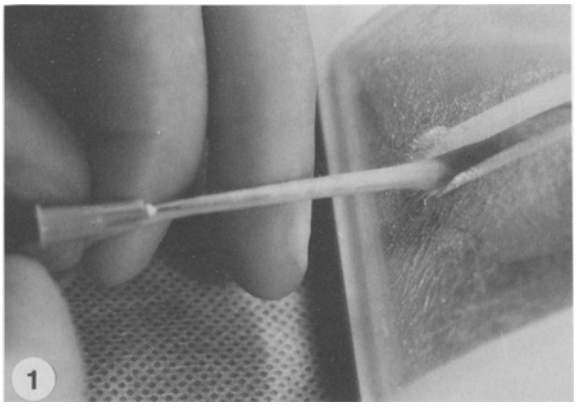


Fig. 1. Intravenous injection of tumor cells into the lateral tail vein of unanesthetized nude mouse. Note the use of a ¾ inch 27-gauge needle and stabilization of the tail for the injection.

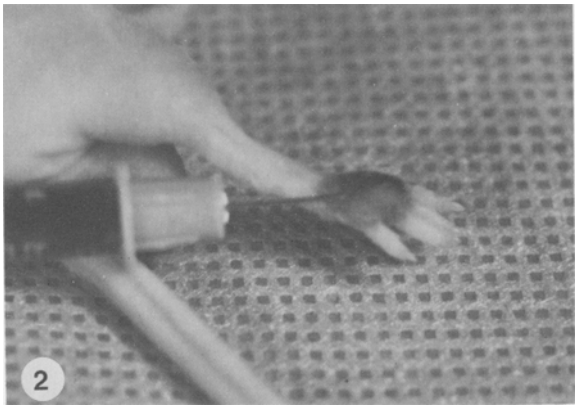


Fig. 2. Intra-footpad injection of tumor cells. Grasp the footpad of an anesthetized mouse. Insert an ½ inch 27-gauge needle into the dorsal aspect of the foot and inject a volume of 0.05 ml. The formation of a bleb is the criterion for a succesful injection.

solution. After 24 h, lungs were examined under a dissecting microscope and the number of gross peripheral lung tumor nodules was determined. The neoplastic nature of these lung nodules was confirmed by histologic examination.

D. Assay for spontaneous metastasis

Tumor cells were harvested exactly as described above. Groups of mice were inoculated in the right hind footpad with viable cells in 0.05 ml inoculum volume (Fig. 2). When the tumor growing at this site reached a 1 x 1 cm size, the hind limb was amputated while the animal was under methoxyflurane anesthesia (Metofane; Pitman-Moore, Inc., Washington Crossing, NJ). Eight weeks after the amputation, the mice were killed, the lungs were removed and fixed in Bouin's solution, and multiple histologic sections obtained from these lungs were examined for the presence of metastases.

E. Selection of tumor lines from lung tumor nodules

Animals were killed by cervical dislocation and immersed first in an iodine solution and then in 70% alcohol. Under a laminar flow hood, sterile instruments were used to open the thoracic cavity and to remove the lungs. The lungs were placed in HBSS to which gentamicin had been added. Discrete, isolated lung colonies were removed with the tip of a 19-gauge needle and individual lesions were placed in separate culture wells. After dissociation of each metastasis with sterile iris scissors, 1—2 ml medium containing 10% fetal calf serum was added to each well. The plates were incubated at 37°C in a 5% CO_2 and air atmosphere. After a confluent monolayer was established, 1 ml of 0.25% trypsin-0.02% EDTA was added and cells transferred to increasingly larger vessels. A number of the cell lines derived from the individual lung tumor colonies were pooled to form the A375-Met-Mix variant, and this line has been maintained in culture.

F. Metastatic heterogeneity of A375 – cloned cell lines

The A375 melanoma cell line is heterogeneous and contains cells with different metastatic capacities. This conclusion is based on the results summarized in Table 2. Relatively few lung tumor nodules were obtained when cells from the parent cell lines were injected i.v. into recipient mice (median number of nodules, 0; range, 0–13). In contrast, four of the cloned cell lines were significantly more metastatic at the same cell dose, as determined by the Mann-Whitney U-test (clones 1 and 4, $P \leq .0001$; clones 3 and 7, $P \leq .005$). The

Table 2. Long tumor colonies produced by the intravenous injection of parent A375 cells and its *in vitro* cloned sublines[a]

Cell line[b]	Tumor nodules/mouse[c] Median	(Range)
Parent A375	0	(0–13)
Clone 1	30	(0–>250)
Clone 2	0	(0–1)
Clone 3	2	(0–5)
Clone 4	>250	(0–>250)
Clone 5	0	(0–1)
Clone 6	0	(0–2)
Clone 7	4	(0–20)
Clone 8	0	(0–8)
Clone 9	0	(0)
Clone 10	0	(0–1)

[a] From Reference 64.
[b] All clones formed progressively growing tumors at a subcutaneous site by 28 days after injection of 5×10^5 cells.
[c] 15 mice/group.

human origin of each of the clones was confirmed by karyotype or isozyme analysis, and the tumorigenicity of each clone, including those that produced no lung tumor nodules after i.v. injection, was demonstrated subsequent to s.c. injection.

The term experimental metastases refers to tumor colonies produced after the i.v. injection of cells. Although these tumor cells do not go through the initial steps of metastasis (separation from primary neoplasm and invasion into blood vessels), all the subsequent steps in the metastatic process must occur for metastases to become established. Elimination of the initial steps of the process introduces the possibility that noninvasive tumor cells might form metastases when injected i.v., but might be unable to metastasize spontaneously when implanted s.c. However, studies have shown that this is not the case. A good correlation between the ability of tumor cells to produce metastases after i.v. (experimental) and s.c. (spontaneous) implantation has been reported for many heterogeneous murine tumor systems confirming that the development of experimental metastases (after i.v. injection of tumor cells) can be used as an assay for the metastatic potential of malignant neoplasms (15, 17).

G. Selection for increased metastatic capacity of A375 melanoma cells

In the next set of experiments, Kozlowski and coworkers (64) determined whether metastasis-derived A375 melanoma variants had increased metastatic capacity in nude mice, like the B16 and K-1735 melanomas of mouse origin do (49). As shown in Table 3 tumor cell lines initiated from the lung tumor nodules were consistently more metastatic than the parent line, regardless of whether they had been passaged and expanded in tissue culture or in the s.c. site. A dose of 8×10^5 parent cells produced a median of 3 lung nodules (range 0–21), whereas 1×10^5 viable cells from each of 7 individual lung metastases that had been expanded *in vivo* produced medians of more than 70 tumor nodules ($P \leq 0.001$). The results were not due to adaptation to growth in the nude mouse, since A375 parental cells expanded s.c. in nude mice were no more metastatic than A375 parental cells grown in culture.

H. Metastasis from tumors growing subcutaneously

Differences in metastatic capacity between the

Table 3. Production of experimental lung metastases by A375 parental cells and metastasis-derived variantes[a]

Cell line	Cell dose ($\times 10^{-5}$)	Lung tumor nodules Median (range)[b]
In vivo passaged cells		
A375 parent	1	0 (0)
A375 parent	8	3 (0–21)
Lung M-1	1	135 (6–160)
Lung M-2	1	>250 (>250)
Lung M-5	1	138 (16–182)
Lung M-6	1	71 (12–93)
Lung M-7	1	>250 (>250)
Lung M-8	1	>250 (62–>250)
Lung M-9	1	84 (65–115)
In Vitro passaged cells		
A375 parent	1	0 (0)
A375 parent	8	2 (0–12)
Lung M-1	0.5	>250 (179–>250)
Lung M-1	1	All>250
Lung M-2	1	87 (53–185)
Lung M-4	1	3 (0–25)
Lung M-4	0.5	153 (49–>250)
Lung M-4	1	>250 (73–>250)

[a] From Reference 64.
[b] Mice killed 56 days after i.v. injection of tumor cells (at least 10 mice/group).

parent line and the metastasis-derived lines were not restricted to experimental metastasis alone but were obtained also with spontaneous metastasis from a s.c. site. Three-week-old nude mice were given s.c. injections of either 5 x10^5 A375 parent cells or A375-Met-Mix cells (pooled A375 lung metastases). Mice were given injections either in the footpad (Fig. 2) or s.c. in the lateral aspect of the anterior thoracic wall (termed 'cranial subcutaneous site'). When tumors reached a size of 1 cm x 1 cm, they were removed surgically. After 8 weeks, the mice were killed and the presence of lung metastases was determined by examination of multiple histologic sections from each animal. Lung tumor foci (from 1 to 15/mouse) were detected in all of the nude mice given injections of the A375-Met-Mix line, whereas none were detected in nude mice given s.c. injections of the parent cells (Table 4).

I. Metastasis from tumors growing in the external ear

The injection of tumor cells into the external ear produces s.c. tumors whose growth and vascularization can be easily monitored. Cells from the parental and Met-Mix A375 lines were implanted into the ear (Fig. 3). Tumors could be observed within 4 weeks after the implantation of 2 x 10^5 cells in 0.05 ml of HBSS (Fig. 4). Six weeks later, the ears with the tumors were amputated at the base. Four to six weeks after the surgical resection, the mice were killed. Once again, cells of the A375 Met-Mix line produced significantly more lung metastases than did the A375 parental line (Fig. 5). Histologic examination of the lungs confirmed the melanoma nature of nonmelanotic lesions.

Collectively, the studies with the human melanoma cell lines showed that, like rodent neoplasms, the A375 melanoma was heterogeneous and contained cells with different metastatic properties. *In vivo* selection techniques developed in rodent tumor systems could be used to isolate human melanoma cells with high capacity to produce experimental and spontaneous metastasis subsequent to implantation into nude mice.

Table 4. Spontaneous metastasis produced by A375 parent cells and A375 cells derived from pooled metastases[a]

Cell line	No. of mice with microscopic lung metastases[b]	
	Injected in cranial subcutaneous site	Injected in footpad
A375	0/10	0/20
A375-Met-Mix	10/10	19/20

[a] From Reference 64.

[b] The number of individual lung tumor nodules obtained with the A375-Met-Mix line varied between 1 and 15.

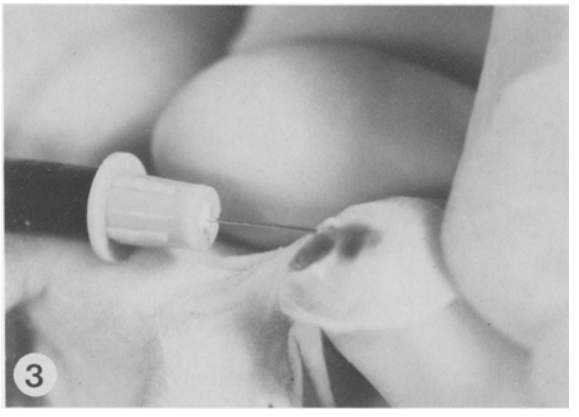


Fig. 3. Injection into the external ear. Mice are anesthetized with methoxyflurane and placed in a lateral position. Grasp the external ear and stabilize it by wrapping the medial side around your index finger. Use a 1-ml syringe with an ½ inch 27-gauge needle. Insert the needle with bevel side up through the base of the ear on the medial side to a depth of ¼ inch. Inject a volume of 0.05-0.1 ml.

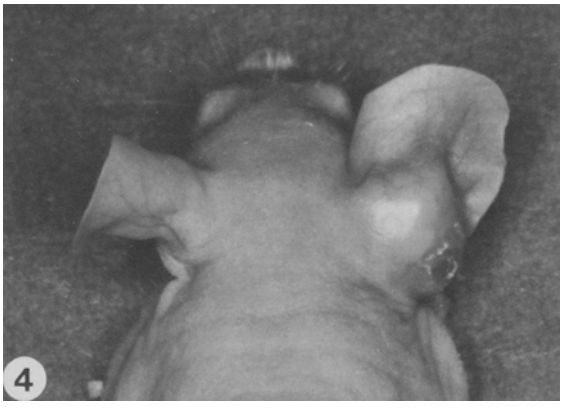


Fig. 4. A375 melanoma growing in the external ear of a nude mouse 4 weeks after the injection of 2 x 10^5 cells.

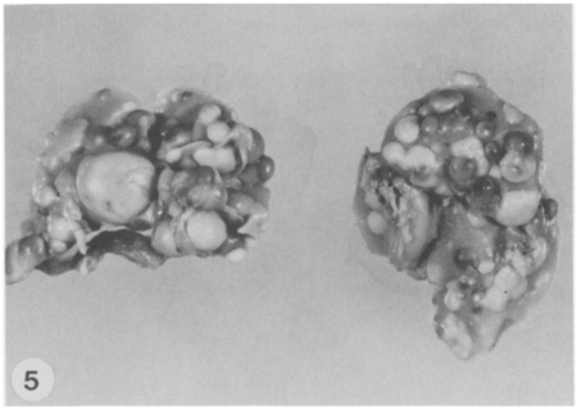


Fig. 5. Grossly visible lung metastases produced by A375 Met-Mix cells.

The metastatic behavior of human tumor cell lines in nude mice

The successful demonstration that the human A375 melanoma can produce experimental and spontaneous metastases in athymic nude mice prompted Kozlowski, Hart, and co-workers to investigate the metastatic behavior of seven other human tumor cell lines consisting of two lines derived from prostate adenocarcinoma, two from renal adenocarcinomas, two from malignant melanoma, and one from a colon adenocarcinoma (65). All the cell lines were implanted into young (3-week-old) nude mice with low NK cell activity. Although the state of activation of the NK cell system may be an important determinant in regulation of the spread of human tumors in the nude mouse, other factors have also been cited as obstacles to tumor spread in the model (42, 50). The anatomical location of the primary tumor implant can have a profound effect on the subsequent incidence of metastasis (7–8, 15, 28, 66–76). A fibrous sheath or pseudocapsule frequently forms around xenografted human tumors (72, 76); to circumvent this barrier, some investigators have injected human tumor cells into the peritoneal cavity of recipient mice (72, 75, 76). Multifocal metastases and widespread carcinomatosis resulting from the i.p. route of tumor cell injection have been attributed to improved tumor vascularity and the absence of the restrictive fibrous sheath (71, 73). Recently, Witte and Ber (77) reported the improved growth of hybridoma cells injected into the spleens of recipient mice, and consideration of these findings led Kozlowski et al. (65) to inject human tumor cells into nude mice by different routes, including the spleen. The splenic route of tumor inoculation was very successful in allowing metastatic spread to occur. In addition, many of the cells lines produced lung tumor colonies after i.v. injection and after growth in the subcutaneous space.

Interestingly, of the seven lines tested only two renal cell carcinomas failed to metastasize from spleen implantation sites. Metastasis to the liver was particularly common from this site. The sinusoidal network of the spleen provides ready access to the portal venous system, and the relatively high survival of injected cells at 24 h, compared with i.v. injected cells (14, 28), may reflect less turbulent, and less damaging conditions in this vein. More surprising than the incidence of hepatic metastasis was the increased incidence of pulmonary metastasis, a phenomenon not as easily explained by simple anatomical distribution of tumor cells (6). It is possible, however, that the continued growth of the injected tumor in the spleen allowed consistent shedding of many tumor cells into the circulation and that greater numbers of cells traversed the initial capillary bed of the liver than were introduced into the tail vein by a single i.v. injection (65). These studies allowed us to conclude that metastasis of human tumor cell lines in nude mice not an uncommon event and that the incidence of metastasis is dependent ultimately on the nature of the tumor cells under study and the organ site of tumor cell implantation.

Metastatic behavior of tumor cells isolated from primary and metastatic human colorectal carcinomas

Subsequent to studying the metastatic behavior of human tumor cell lines in nude mice, our laboratory began to investigate the metastatic behavior in nude mice of human colorectal carcinoma

lines established from primary neoplasms and from metastases (78). The purpose of these studies was to examine the growth characteristics and metastatic behavior of freshly isolated human colorectal carcinomas in athymic nude mice. Four tumor lines were derived from primary colorectal carcinomas, three lines from hepatic metastases, and one line from a metastasis to the mesenteric lymph node. To determine the suitability of the nude mouse to serve as a model for studies of human colorectal carcinoma metastasis, the human tumor cells from primary neoplasms and from metastases were injected into different organ sites.

The studies with fresh specimens of human colorectal carcinomas required specialized techniques, and some will now be described.

A. Establishment of tumor lines

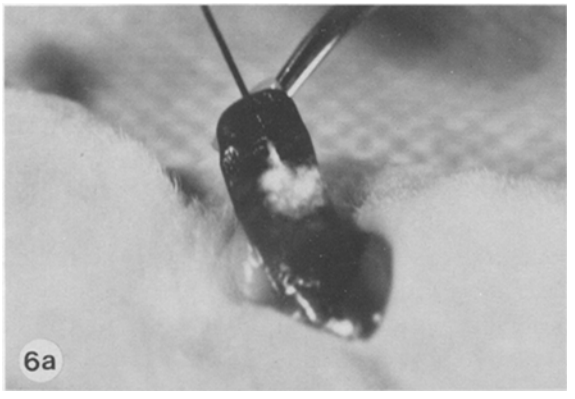
Tumor specimens from patients with primary colorectal cancers or from their metastases were obtained immediately after surgery and processed as follows: tumor tissue was dissected free of necrotic areas, connective tissue, and blood clots, and rinsed several times in cold (4°C) medium containing 1% human serum albumin and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; amphotericin B, 3 μg/ml; and gentamycin, 50 μg/ml). The tissue was then cut into 1-3 mm^3 fragments using a sterile scalpel blade. The fragments were subjected to a sequential enzymatic digestion of 20 min at 37°C in medium containing collagenase type I (200 units/ml) and DNase (270 units/ml). To complete the dissociation process, hyaluronidase type IV (35 NF U/ml) was added to the enzyme mixture. After enzymatic dissociation, the cells were maintained at 4°C. The cell suspension was filtered through a four-layer sterile gauze, washed three times in serum/free medium, and resuspended in HBSS. For *in vivo* implantation, 2–4 x 10^6 tumor cells, shown viable by trypan blue exclusion, in a 0.1 ml volume were injected i.m. in the hind thighs of nude mice.

The experiments examined the growth and metastatic potential of cells subsequent to injection into multiple organ sites. Because the enzymatic dissociation of solid tumors may introduce variabilities in the cell suspension preparation, we always used a group of mice injected s.c. as controls. This practice allows for comparisons among multiple experiments. Only experiments in which the s.c. injection of human tumor cells yields a positive result can be considered appropriate in such studies.

Because nude mice are highly susceptible to pathogenic viruses, we routinely examined the growing tumors and found these to be free of reovirus type 3, pneumonia, virus of mice, K-virus, Theiler's virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus in assays performed by Microbiological Associates (Bethesda, MD). To certify the human origin of the tumors, representative samples of growing tumors were harvested, and established in culture, and their karyotype and isoenzyme (Authentikit, Corning Medical, Corning, NY) were determined.

B. Intrasplenic injection

Groups of mice were anesthetized with methoxyflurane and placed in the right lateral decubitus position. A transverse incision was made in the left flank through the skin and peritoneum, exposing the medial aspect of the sleen. Gentle retraction of the tail of the pancreas was used to stabilize the spleen. Separate groups of mice received tumor cells suspended in 0.05 ml of HBSS injection into the medial splenic tip with a 27-gauge needle so as to raise a visible pale wheal. No significant bleeding or extravasation was encountered (Fig. 6). The spleen was returned to the abdominal cavity, and the wound was closed by one layer with wound clips. Six to 8 weeks after the injection of tumor cells, the animals were killed by cervical dislocation, autopsies were performed, and organs were removed and placed in Bouin's solution. Further examination was performed after the organs were fixed for 24 h. This examination consisted of inspection under the dissecting microscope and submission of appro-



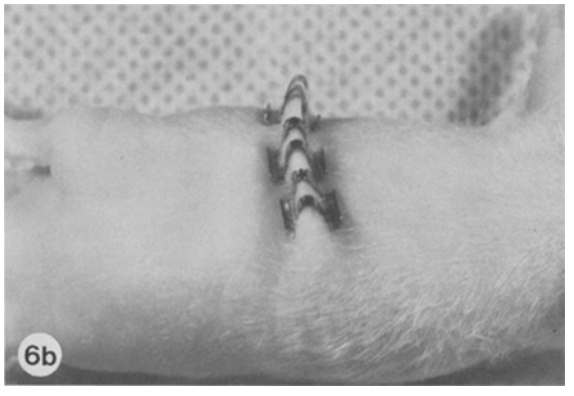


Fig. 6. Injection of tumor cells into the spleen. Mice were anesthetized with methoxyflurane and placed in the right lateral decubitus position. A transverse incision is made in the left flank through the skin and peritoneum, exposing the medial aspect of the spleen. Gentle retraction of the tail of the pancreas is used to stabilize the spleen. Inject tumor cells in volume of 0.05 ml into the medial splenic tip with a 27-gauge needle so as to raise a visible pale wheal. No significant bleeding or extravasation should to be encountered (a). The spleen is returned to the abdominal cavity, and the wound is closed in one layer with wound clips (6).

priate tissue for histologic examiniation.

The implantation of metastatic human colorectal carcinoma cells into the spleen of athymic nude mice was followed by growth of the tumors in the spleen and liver and the death of the recipient animal. In contrast, the injection of the same population of cells into the subcutis or musculature of nude mice produced only local tumors without systemic metastases (Table 5). We studied over 200 mice with colorectal cancers growing s.c., and in only one did we find macroscopic evidence of visceral metastases at time of autopsy. Growth of colorectal cancers in the hind thigh with or without amputation of the local tumor also did not produce frequent metastases. Only in a very few mice did we find lung metastases at 6 months after tumor implantation, resection, and local tumor recurrence. Whether these metastases were due to a growth of tumor cells already present in the lung at the time of the primary tumor excision, to a successive dissemination of cells from the recurrent tumor, or to the shedding of tumor cells into blood vessels consequent to surgical manipulation is unclear (33, 76, 78, 81–84).

The rarity of metastasis production by human tumors implanted s.c. or i.m. into nude mice is well documented (30, 33, 63, 85). The relatively brief life span of nude mice compared with human beings may explain why. However, many other factors may influence this process in a more profound manner. First, the majority of human neoplasms xenografted into nude mice have been implanted s.c., an anatomical site that bears little relevance to the organ of origin for the neoplasm, e.g. colorectal carcinomas. In this regard, it is interesting to note that the implantation of a human colon carcinoma line into the gut of nude mice was followed by extensive invasion and lymph node metastasis, but this phenomenon was not found for gut-implanted melanoma (86). Second, the metastatic capacity of human tumor cells implanted s.c. in nude mice has been correlated with invasion of the body wall (33, 70, 87). Thus, the lack of invasion and metastasis by human tumor cells has been often associated with the presence of a fibrous capsule of mouse origin surrounding the locally growing human tumor (72).

We questioned the validity of implanting human colorectal carcinomas into the subcutis or muscularis of nude mice. Implantation at these sites may be relatively easy to accomplish, but its relevance to the biology of colorectal carcinoma is open to question. The injection of tumor cells into the spleen of mice has been shown to produce extensive liver and lung metastases (64). We confirmed these earlier results and extended the findings by the use of recently isolated human colorectal carcinoma cells.

The implantation of human colorectal carcinoma cells into the spleen of athymic nude mice

Table 5. The metastatic behavior of human colorectal carcinoma cells injected intravenously or into the spleen of nude mice

Tumor source	Dukes' classification	Experimental lung metastases		Liver metastasis after intrasplenic injection			
		No. mice with lung colonies	Median (range)	Time of autopsy (day)	No. mice with spleen tumor	No. mice with liver tumors	Liver tumor foci
Primary							
Rectal	B1	9/ 9	3 (1–26)	90	8/ 9	4/ 9	<10
Rectal	B2	6/ 6	15 (5–29)	90	9/10	10/10	<10
Rectal	B3	3/10	0 (0–8)	90	8/10	7/10	<10
Rectal	B3	8/ 8	48 (7–150)	90	5/ 5	5/ 5	<10
Metastasis							
Lymph node	C2	7/10	1 (0–12)	90	6/ 6	1/ 6	<10
Liver	D	0/ 6	0	90	6/ 6	2/ 6	<10
Liver	D	6/10	2 (0–21)	40	6/ 6	6/ 6	>200
Liver	D	8/ 9	51 (0–161)	30	9/ 9	9/ 9	>200

Mice were injected with 1×10^6 viable cells intravenously and with 2×10^6 viable cells into the spleen.

allowed us to distinguish between cells isolated from primary neoplasms and cells isolated from liver metastases. Specifically, by days 30–40 after injection of cells isolated from patients' metastases the nude mice became moribund (Table 5). Autopsy revealed that their livers were totally replaced by neoplastic growth (Fig. 7), which was ascertained to be human colorectal carcinoma by morphology, and isozyme and karyotypic analysis. No gross liver metastases were found in nude mice killed 40 days after the intrasplenic injection of cells from primary carcinomas. Thus, if the assay was terminated after 30–40 days, qualitative differences between metastatic and nonmetastatic cells were observed. If nude mice injected with primary colon carcinoma cells were allowed to survive for up to 90 days, few liver metastases were found.

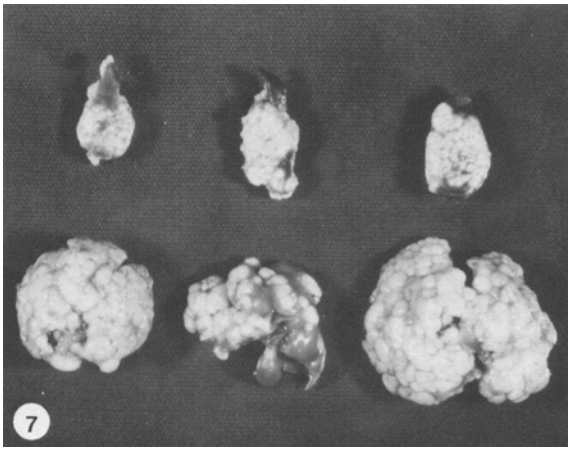


Fig. 7. Human colorectal carcinoma tumors growing in the spleen and liver of nude mice after intrasplenic implantation of viable cells. The extensive tumor burden in the livers occurred at 28 days after implantation of 1×10^6 cells isolated from a liver metastasis.

Model for human colorectal carcinoma hepatic metastasis

The most formidable problem to the clinical oncologist treating colon carcinoma is the production of systemic metastasis. By the time many colorectal cancers are diagnosed and surgically excised, micrometastases are already present in lymph nodes and the liver (88). The prognosis of a patient with colorectal cancer metastasis is generally poor because no effective systemic therapy is currently available (88). In order to develop new therapeutic approaches for this disease, suitable models for *in vivo* studies of the biology and therapy of colorectal cancer must be developed (89). Our previous studies demonstrated that under specific transplantation procedures, human colorectal carcinoma cells injected into nude mice could exhibit different metastatic capacities and that the injection of carcinoma cells into the spleen of nude mice represent an advantageous route of injection for determining the metastatic potential of human colorectal carcinomas. In the next set of experiments, Giavazzi and co-workers (89) wished to determine whether the nude mouse could be used as a reliable model for in-

vestigating the production of hepatic metastases by human colorectal carcinoma cells. To do so, we studied the malignant potentials of three different human colorectal carcinomas, one derived from a primary tumor, one from a lymph node metastasis, and one from a hepatic metastasis, implantated into the spleens of athymic nude mice.

The intrasplenic injection of cells isolated from the liver metastasis produced rapid and consistent growth of the human cells in the liver. Cells derived from a primary colon cancer produced tumors in the spleen of injected nude mice but exhibited a lower malignant potential as measured by the ability to produce liver tumor foci. Once again, the human origin of all tumors growing in the spleens and livers of injected mice was repeatedly ascertained by both isoenzyme and karyotype and analysis. The results confirmed that human colorectal carcinomas can produce tumor foci in livers of nude mice and that this ability is related to their malignant nature in patients (Table 6).

Collectively, the results suggest that the implantation of malignant human colorectal carcinoma cells into the spleens of athymic nude mice can produce tumor growth in the liver, an organ most often involved by metastasis from these neoplasms. For this reason, this animal model can now be used to study some aspects of liver metastasis such as invasion and growth in the liver environment and the therapy of colorectal carcinoma cells proliferating in a relevant metastatic site, the liver (89).

Studies with a human renal cell carcinoma

Human renal cell carcinoma (HRCC) is not a very common cancer. The prognosis of a patient with this cancer is poor because no effective therapy has been established for advanced stages of this disease. Although several investigators have reported the successful transplantation of HRCC cells into nude mice, (90–96) the usefulness of this model has been limited. Like other human tumor cells, transplantated HRCC cells rarely metastasize in nude mice, regardless of their degree of malignancy in the patient (33, 63, 83). In the main, HRCC cells have only been implanted in the subcutis of nude mice (90, 96). The finding that the growth rate and incidence of cancer metastasis in nude mice can be increased by manipulation of the route of tumor cell implantation and organ implantation sites prompted Naito and co-workers (97) to investigate whether the implantation of HRCC into the kidney of nude mice would allow the expression of malignant potential.

The purpose of the initial study was to determine whether the methods for isolating cells from a surgical specimen of a HRCC influence the biologic behavior of the cancer cells. HRCC obtained from a surgical specimen was dissociated by enzymatic treatment (78, 79) and cells were plated into culture dishes or injected s.c. and into the kidney of BALB/c nude mice. The resultant kidney tumor produced liver metastases and ascites. All tumors growing in nude mice (s.c., kidney, liver, ascites) were also established in cul-

Table 6. Production of liver tumors by human colorectal carcinoma cells injected into spleens of nude mice.

Source of cells	Autopsy day	No. of mice with spleen tumors/ total mice	No. of mice with liver tumors/ total mice	Median liver tumor foci/ mouse (range)
Primary colorectal cancer	50	5/ 5	4/ 5	9 (0–11)
(Duke B1)	90	6/ 6	5/ 6	11 (0–20)
Lymph node metastasis	40	6/ 6	4/ 6	5 (0–10)
(Duke C)	90	5/ 5	3/ 5	0 (0–2)
Hepatic metastasis (Duke D)	30	13/13	13/13	all>300

Mice were injected with 1–2 × 10^6 cells into their spleen.

ture. The human origin of all five lines was ascertained by karyotypic and isoenzyme analyses. Because the five cell lines of the HRCC were derived from five different isolation conditions (culture, s.c. tumor, renal tumor, liver metastasis from the renal tumor, ascites from the renal tumor), we asked whether the cell lines exhibited biologic heterogeneity, including differences in metastatic potential (97), and whether different implantation sites in nude mice would influence such behavior.

Cells from all lines were injected s.c., i.p., i.v., intrasplenically, and beneath the renal capsule (Fig. 8) of nude mice. All the lines were tumorigenic after s.c. or renal subcapsule injection, although the rate of tumor growth varied among the five lines. The metastatic behavior of the HRCC cells as influenced by both the nature of the tumor cells and by the route of injection. The biologic behavior of HRCC cells isolated by direct culture technique (SN12C) and the cells isolated from HRCC tumors and grown first in the nude mice and then established in culture differed (Table 7). This difference raises a question about which method should be routinely employed if human tumor cell lines are to be isolated either for studies of biology or for predictive sensitivity for therapeutic agents.

Cell lines derived from HRCC tumors produced by the original HRCC cells injected into the skin (SN12S1) or into the kidney of nude mice (SN12K1) differed in their biologic properties. Moreover, cell lines derived from a liver metastasis (SN12L1) and ascites (SN12A1) produced by

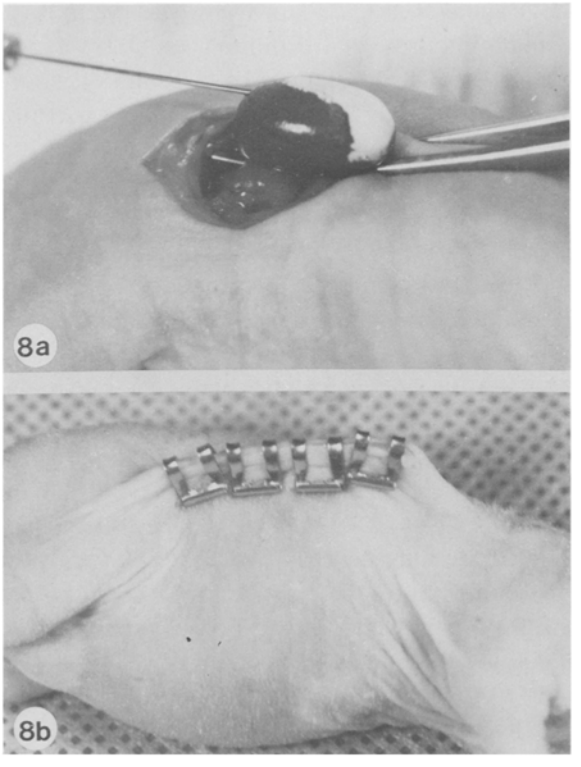


Fig. 8. Renal subcapsule injection. Mice are anesthetized with methoxyflurane and placed in the left lateral decubitis position. A vertical incision is made through the right flank through the skin and peritoneum exposing the lateral aspect of the kidney. The kidney is stabilized and lifted outside the peritoneal cavity. Tumor cells in a volume of 0.05 ml are injected via a 1 inch 27-gauge needle, which is inserted into the renal parenchyma from the lower pole of the kidney and advanced until its point reaches just below the renal capsule. A visible bulla formed between the renal parenchyma and capsule and lack of bleeding or leaking are the criteria for successful injection. The material injected was white in color (a). After injection the kidney is returned to the abdominal cavity and the wound is closed in one layer with metal wound clips (b).

Table 7. The growth and spread of human renal cell carcinoma cells after subcutaneous, intravenous or intraperitoneal implantation into nude mice

Cell line	S.C growth			Intravenous injection:		Intraperitoneal injection		
	Incidence at s.c. site	Lung metastasis		Experimental lung metastasis		Incidence of	Pulmonary metastasis ascites	
		Incidence	Median (range)	Incidence	Median (range)		Incidence	Median (range)
SN12C	5/6	4/5	7 (0–40)	1/6	0 (0–2)	2/6	1/6	0 (0–8)
SN12S1	5/6	5/5	28 (6–99)	6/6	1 (1–2)	4/6	2/6	0 (0–12)
SN12K1	5/6	3/5	4 (0–21)	4/6	2 (0–13)	3/5	4/5	2 (0–21)
SN12L1	7/7	6/7	5 (0–14)	4/5	4 (0–12)	2/6	1/6	0 (0–2)
SN12A1	5/6	4/5	1 (0–19)	0/6	0 (0)	5/6	2/6	0 (0–3)

Mice were injected with 1×10^6 viable cells. All lung tumor nodules were larger than 0.1 mm.

the original HRCC cells growing in the kidney of a nude mouse also exhibited unique biologic properties. This finding raises a second question: which organ site of nude mice should be used for the transplantation of freshly isolated human tumors?

The growth rate of cells from the five lines varied under both *in vitro* and *in vivo* (s.c. or kidney implantation) conditions (Tables 7, 8). No direct correlation among growth *in vitro,* in the s.c. site, or in the kidney was found. The growth rate of tumor cells *in vitro* can be regulated by manipulation of the environment (i.e., by serum, media, temperature). Similarly, different organ environments influence the growth of some, not all, tumor cells (7–9, 12, 63–69, 74, 75, 78, 89). Indeed, this specific interaction of tumor cells with host environment was the basis for the original 'seed and soil' hypothesis by Paget (6) discussed in the beginning of this chapter.

The growth of the HRCC in the skin, kidney, and liver metastasis and in the ascites form in nude mice must have selected for cells with different biologic properties. This conclusion is based in part on the following data. First, SN12L1 cells exhibited invasive and metastatic properties regardless of organ site for their implantation (s.c., i.v., i.p., or kidney). In contrast, cells of the SN12A1 line were poorly metastatic regardless of the site of implantation. Second, the metastatic properties, in nude mice, of the cells were stable despite 8-week assays. Third, the highest incidence of metastasis by some cell lines was produced by tumors growing in the kidney, but cells of SN12C and SN12A1 lines produced few metastases (Table 8). In contrast, cells of SN12L1 produced extensive visceral metastasis that included the lung (Fig. 9). The results reemphasize that both tumor cell properties and host factors determine the outcome of metastasis (97).

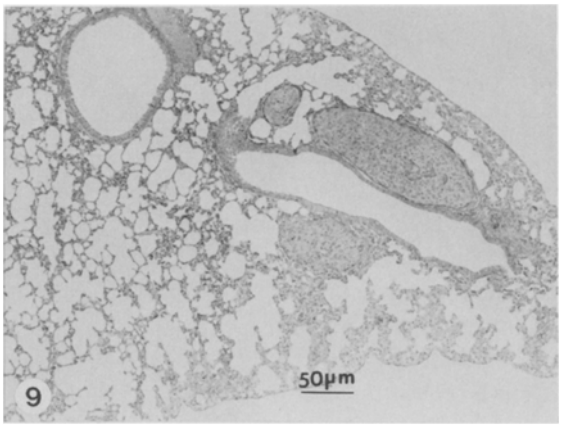


Fig. 9. Lung metastases produced by human renal cell carcinoma cells injected into the kidney of nude mice. *x 180.*

Contrasting pattern of growth of HRCC in the skin and kidney of nude mice

A. Histopathology

The injection of HRCC cells into the subcutis or renal subcapsule produced tumors in all the mice. The tumors in the kidney, however, grew more rapidly than did the s.c. tumors. For this reason, nude mice injected with cells into the kidney became moribund well before mice injected s.c. The differences in the size of tumors growing in the kidney or the skin were also significant.

Table 8. Growth and metastasis of human renal cell carcinoma cells after intrasplenic or renal subcapsule implantation

Cell line	Intrasplenic injection			Renal subcapsule injection			
	Incidence of growth in the spleen	Pulmonary metastasis		Time of autopsy (wk)	Incidence of growth in the kidney	Pulmonary metastases	
		Incidence	Median nodules/ mouse (range)			Incience	Median nodules/ mouse (range)
SN12C	5/6	0/6	0 (0)	8	6/6	5/6	7 (0–21)
SN12S1	4/8	3/8	0 (0–37)	7	5/5	4/5	13 (0–158)
SN12K1	4/6	1/6	0 (0–3)	7	5/5	5/5	4 (2–61)
SN12L1	1/6	2/6	0 (0–1)	5	6/6	6/6	32 (17–64)
SN12A1	0/5	1/5	0 (0–1)	8	8/8	3/8	0 (0–7)

Mice were injected with 1×10^6 viable cells. Lung metastases were larger than 0.1 mm.

Two weeks after injection of tumor cells, small tumor nodules, averaging 4 mm in diameter, were found at the s.c. site. The tumors were well vascularized, had small areas of central necrosis, and were surrounded by a fibrous capsule (Fig. 10). At this time, the tumors in the injected kidney were localized between the renal parenchyma and the capsule with prominent areas of invasive growth into the renal parenchyma (Fig. 11). The kidney tumors were free of necrosis and were not encapsulated.

By 6 weeks after injection, the s.c. tumors exceeded 10 mm in diameter. The tumors were well circumscribed and encapsulated by a fibrous connective tissue. Some invasion into the muscle was found. Greater than 70% of the tumor was necotic, yet the periphery of the tumor mass was well vascularized. At this time, tumor cells injected into kidney showed extensive growth both inside and outside the kidney. Most of the renal parenchyma was destroyed by the growing tumor with only a part of renal medulla left uninvolved. The tumor adhered to the liver and peritoneum, and the diaphragm, and mesenteric and omental lymph nodes contained growing HRCC. Grossly apparent pulmonary metastases were also found in the mice.

B. Implications of histopathological findings to design of model for therapy of HRCC

Implantation of tumor cells into the kidney has been used to propagate human xenografts in nude mice. Indeed, the high rate of take for tumor implanted into the renal subcapsule has been used to detect responsiveness to drug therapy (98, 99). However, growth in the kidney does not assure that metastasis will occur. For example, we have observed that although human colorectal carcinoma cells readily proliferate in the spleen and kidney of nude mice, these cells frequently metastasize from the spleen but not from the kidney. Clinical observations have suggested that the response of human cancer metastases to anticancer drugs is influenced by the anatomical location of the lesions. With few exceptions in women with breast carcinoma, the superficial metastases

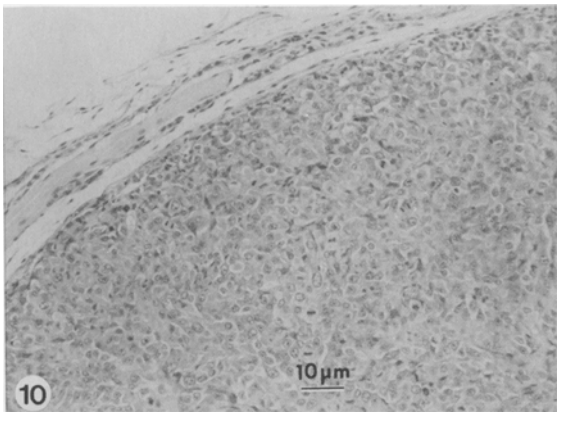


Fig. 10. Human renal cell carcinoma cells proliferating in the subcutis of nude mice 2 weeks after implantation. Note the development of an extensive fibrous capsule. *x 720.*

in lymph nodes and skin responded better than skeletal or pulmonary metastates (100–102). Similar results for differential response of metastatic lesions in different organs to different cytotoxic drugs have also been reported. Subcutaneously transplanted mouse neoplasms were sensitive to three cytotoxic agents whereas the same tumor cells implanted intracerebrally were not (103–104). Although it is tempting to attribute the difference in the response of metastases to chemotherapy to heterogeneity in cell population, the influence of the organ environment must not be ignored.

The viability and growth of tumor cells depends on an adequate source of nutrients. The

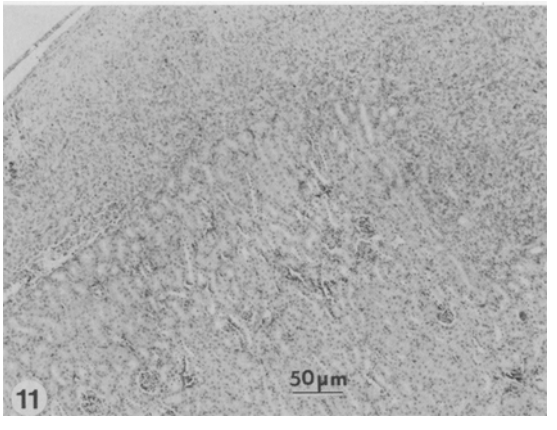


Fig. 11. Human renal cell carcinoma cells proliferating in the kidney of nude mice 2 weeks after implantation. Note the invasive growth into the kidney. *x 120.*

degree of tumor vascularity can control the delivery of nutrients, clearance of metabolities, and the delivery of cytotoxic drugs to a lesion (104). Measurements of blood flow in subcutaneous tumors of rats reveal a lack of autoregulation in response to infusions of angiotensin II. This selective increase in tumor blood flow, with no corresponding increase in normal tissue blood flow, can enhance chemotherapeutic drug delivery (105). Finally, the extent of tumor angiogenesis, a host response to a growing neoplasm, differs among different organs, and this obviously could contribute to differential response of metastases to anticancer drugs.

Conclusions

With the recent interest in understanding the origin and pathogenesis of neoplastic heterogeneity, a great deal of effort has been given to elucidating the various properties associated with the malignant phenotype. Less emphasis, however, has been given to the various host-organ environmental factors that can influence the metastatic process. The majority of studies on the biology of metastasis were carried out with transplantable rodent tumors. Little data are available regarding the malignant phenotype and tumor cell-organ interactions of human tumor cells. To a large extent, this has been due to the unavailability of *in vivo* models for human cancer metastasis. In this chapter I have summarized our work and that of many other laboratories on the use of athymic nude mice for *in vivo* studies of human neoplasms. Several points need emphasis.

1. It is imperative that all metastasis studies be carried out in healthy young nude mice. Thus, the nude mice must be specific pathogen free and maintained under barrier conditions. Moreover, all tumors to be implanted into the nude mice must be free of pathogenic mouse viruses and mycoplasma infections. Failure to strictly adhere to these conditions will assure failure of the metastasis studies.
2. The method used to isolate tumor cells from fresh human neoplasms could greatly influence their biologic behavior upon implantation into nude mice.
3. The nude mouse can be used to ascertain the metastatic potential of human neoplasms and to isolate metastatic tumor cells from heterogeneous human neoplasms.
4. The success of all these studies is dependent upon correct experimental procedures, such as maintaining anatomical compatibility of the tumor cells ('seed') and the recipient mouse organ environment ('soil'). Specifically, although the s.c. implantation of tumor cells is easily accomplished, it is doubtful whether this environment is relevant to such tumors as colorectal, lung, or renal carcinomas.

The proper use of athymic nude mice can now allow us to investigate in more detail the biologic behavior of many metastatic human neoplasms. This opportunity will no doubt lead to a significant increase in our knowledge about the most devastating aspect of cancer: cancer metastasis.

Aknowledgments

This work was supported in part by funds from the R. E. 'Bob' Smith Chair in Cell Biology. I thank Dr. Corazon Bucana for excellent photography and Miss Emily Rondon for help in preparation of the manuscript.

References

1. Fidler IJ, Poste G: The heterogeneity of metastatic properties in malignant tumor cells and regulation of the metastatic phenotype. In: Owen A, Coffey DS, Baylin SB (eds) Tumor cell heterogeneity. New York, Academic Press, New York, 1982, pp 127–142.
2. Fidler IJ, Hart IR: Biological diversity in metastasis and metastatic neoplasms: Origins and implications. Science 217:998–1003, 1982.
3. Heppner G: Tumor heterogeneity. Cancer Res 214:2259–2265, 1984.
4. Fidler IJ, Poste G: The cellular heterogeneity of malignant neoplasms: Implications for adjuvant chemotherapy. Semin Oncol 12:207–222, 1985.
5. Dexter DL, Leith JT: Tumor heterogeneity and drug resistance. J Clin Oncol 4:244–257, 1986.
6. Paget S: The distribution of secondary growths in can-

cer of the breast. Lancet 1:571–573, 1889.
7. Hart IR: 'Soil and soil' revisited: Mechanisms of site-specific metastasis. Cancer Met Rev 1:5–17, 1982.
8. Poste G: Experimental systems for analysis of the malignant phenotype. Cancer Met Rev 1:141–200, 1982.
9. Poste G, Fidler IJ: The pathogenesis of cancer metastasis. Nature 283:139–146, 1980,
10. Nicolson GL: Organ colonization and the cell surface properties of malignant cells. Biochim Biophys Acta 695:113–176, 1982.
11. Nicolson GL, Custead SE: Tumor metastasis is not due to adaptation of cells to a new organ environment. Science 215:176–178, 1982.
12. Tarin D, Price JE, Kettlewell MGW, Souter RG, Vass ACR, Crossley H: Mechanisms of human tumor metastasis studied in patients with peritoneovenous shunts. Cancer Res 44:3584–3592, 1984.
13. Fidler IJ: Selection of successive tumor lines for metastasis. Nature 242:148–149, 1973.
14. Fidler IJ, Kripke ML: Metastasis results from preexisting variant cells within a malignant tumor. Science 197:893–895, 1977.
15. Fidler IJ: The evolution of biological heterogeneity in metastatic neoplasms. In: Nicolson GL, Milas L (eds) Cancer invasion and metastasis: Biologic and therapeutic aspects, Raven Press, New York, 1984, pp 5–27.
16. Poste G, Fidler IJ: The pathogenesis of cancer metastasis. Nature 283:139-145, 1980.
17. Talmadge JE, Fidler IJ: Cancer metastasis is selective or random depending on the parent tumor population. Nature 27:593–594, 1982.
18. Talmadge JE, Fidler IJ: Enhanced metastatic potential of tumor cells harvested from spontaneous metastases of heterogeneous murine tumors. JNCI 69:975–980.
19. Dorland WA: Dorland's Illustrated Medical Dictionary: Philadelphia, W. B. Saunders & Co., 24th ed, 1965.
20. Talmadge JE, Wolman SR, Fidler IJ: Evidence for the clonal origin of spontaneous metastases. Science 217:361–363, 1982.
21. Talmadge JE, Benedict K, Madsen J, Fidler IJ: Development of biological diversity and susceptibility to chemotherapy in murine cancer metastases. Cancer Res 44:3801–3805, 1984.
22. Fidler IJ, Gersten DM, Hart IR: The biology of cancer invasion and metastasis. Adv Cancer Res 28:149–250, 1978.
23. Weiss L: A pathobiologic overview of metastasis. Semin Oncol 4:5–17, 1977.
24. Weiss L: Metastasis: Differences between cancer cells in primary and secondary tumors. Pathobiol Annu 10:51–81, 1980.
25. Fidler IJ: Metastasis: quantitative analysis of distribution and fate of tumor cell emboli labeled with ^{125}I-5-iodoxyuridine. JNCI 45:773–782, 1970.
26. Luria SE, Delbruck M: Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28:491–511, 1943.
27. Talmadge JE: The selective nature of metastasis. Cancer Met Rev 2:25–41, 1983.
28. Fidler IJ: General considerations for studies of experimental cancer metastasis. In: Bush H ed Methods in cancer research, Academic Press, New York, 1978, pp 399–439.
29. Rygaard J, Povlsen CO: Heterotransplantation of human malignant tumor to nude mice. Acta Pathol Microbiol Scand [A] 77:758–760, 1969.
30. Fogh J, Giovanella BC: The nude mouse in experimental and clinical research. Academic Press, New York, 1978.
31. Hanna N, Davis TW, Fidler IJ: Environmental and genetic factors determine the level of NK activity of nude mice and affect their suitability as models for experimental metastasis. Int J Cancer 30:371–376, 1982.
32. Stiles CD, Desmond W, Chuman LM, Sato G, Saier HJ Jr: Relationship of cell growth behavior *in vitro* to tumorigenicity in athymic nude mice. Cancer Res 36:3300–3305, 1976.
33. Sharkey FE, Fogh J: Considerations in the use of nude mice for cancer research. Cancer Met Rev 3:341–360, 1984.
34. Outzen HC, Custer RP: Growth of human normal and neoplastic mammary tissues in the cleared mammary fat pad of the nude mouse. J Natl Cancer Inst 55:1461–1463, 1975.
35. Reid J.C, Shin SI: Transplantation of heterologous endocrine tumor cells in nude mice. In: Fogh J, Giovanella BC (eds) The nude mouse in experimental and clinical research. Academic Press, New York, 1978, pp 313–351.
36. Epstein AL, Herman MM, Kim H, Dorfman RF, Kaplan HS: Biology of the human malignant lymphomas III. Intracranial heterotransplantation in the nude, athymic mouse. Cancer 37:2158–2176, 1976.
37. Manning JK, Reed ND, Jutila JW: Antibody response to *Escherichia coli* lipopolysaccharide and type III pneumococcal polysacchaaride by congenitally thymusless (nude) mice. J Immunol 108:1470–1472, 1972.
38. Johnson WJ, Balish E: Macrophage function in germfree, athymic (nu/nu), and conventional-flora (nu/+) mice. J Reticuloendothel Soc 28:55–66, 1980.
39. Hanna N: Role of natural killer cell in control of cancer metastasis. Cancer Met Rev 1:45—65, 1982.
40. Sordat B, Merenda C, Carrel S: Invasive growth and dissemination of human solid tumors and malignant cell lines grafted subcutaneously to newborn nude mice. In: Nomura T, Ohsawa N, Tamaoki N, Fujiwara K (eds) Proceedings of the Second International Workshop on Nude Mice. University of Tokyo Press, Tokyo, 1977, pp 313–326.
41. Fidler IJ: Kripke ML: Tumor cell antigenicity, host immunity and cancer metastasis. Cancer Immunol Immunother 7:201–205, 1980.
42. Hanna N: Expression of metastatic potential of tumor cells in young nude mice is correlated with low levels of natural killer cell-mediated cytotoxicity. Int J Cancer 26:675–680, 1980.
43. Hanna N, Fidler IJ: The role of natural killer cells in the destruction of circulating tumor emboli. JNCI 65:801–809, 1980.
44. Hanna N, Fidler IJ: Relationship between metastatic potential and resistance to NK cell-mediated cytotoxicity in three murine tumor systems. JNCI 66:1183–1190, 1981.
45. Hanna N, Fidler IJ: Expression of metastatic potential of allogeneic and xenogeneic neoplasms in young nude

mice. Cancer Res 41:438–444, 1981.
46. Hanna N, Schneider M: Enhancement of tumor metastasis and suppression of natural killer cell activity by B-estradiol treatment. J Immunol 130:974–980, 1983.
47. Talmadge JE, Meyers KM, Prieur DJ, Starkey JR: Role of NK cells in tumor growth and metastasis in beige mice. Nature 284:622–624, 1980.
48. Urdal DL, Kawase I, Henney CS: NK cell-target interactions: approaches toward definition of recognition structures. Cancer Met Rev 1:65–83, 1982.
49. Pollack VA, Fidler IJ: Use of young nude mice to select subpopulations of tumor cells with increased metastatic potential from nonsyngeneic neoplasms. JNCI 69:137–141, 1982.
50. Kyriazis A, DiPersio L, Michael JG, Pesce AJ: Influence of the mouse hepatitis virus (MHV) infection on the growth of human tumors in the athymic mouse. Int J Cancer 23:402–409, 1979.
51. Kindred B: Nude mice in immunobiology. Prog Allergy 26:137–238, 1979.
52. Povlsen CO, Fialkow PJ, Klein E, Klein G, Rygaard J, Wiener F: Growth and antigenic properties of a biopsy-derived Burkitt's lymphoma in thymusless (nude) mice. Int J Cancer 11:30–36, 1973.
53. Giovanella BC, Yim SO, Morgan AC, Stehlin JS, Williams IJ: Metastasis of human melanoma transplanted in 'nude' mice. J Natl Cancer Inst 50:1051–1060, 1973.
54. Povlsen CO: Heterotransplantation of human malignant melanomas to the mouse mutant nude. Acta Pathol Microbiol Scan [A] 84:9–17, 1976.
55. Kameya T, Shimosato Y, Tumuraya M, Ohsawa N, Nomura T: Human gastric choriocarcinoma serially transplanted in nude mice. J Natl Cancer Inst 56:325–333, 1976.
56. Nomura T, Ohsawa N, Tamaoki N, Fujiwara K (eds) Proceedings of the Second International Workshop on Nude Mice. University of Tokyo Press, Tokyo, 1977.
57. Shimosato Y, Kameya T, Nagai K, Hirohaski S, Koide T, Hayashi H, Normura T: Transplantation of human tumors in nude mice. J Natl Cancer Inst 56:1251–1259, 1976.
58. Kanzaki T, Hashimoto K, Bath DW: Heterotransplantation of human malignant melanoma cell lines in athymic nude mice. JNCI 62:1151–1157, 1979.
59. Fogh J, Hajdu SI: The nude mouse as a diagnostic tool in human tumor cell research. J Cell Biol 67:117–125, 1975.
60. Tompkins MB, Rao GV, Tompkins WAE: Increased tumorigenicity and resistance to antibody lysis of human colon tumor cells xenografted in congenitally athymic mice. Cancer Res 39:2160–2166, 1979.
61. Goldenberg D, Pavia R: *In vivo* horizontal oncogenesis by a human tumor in nude mice. Proc Natl Acad Sci USA 79:2389–2394, 1982.
62. Goldenberg D, Pavia R: Malignant potential of murine stromal cells after transplantation of human tumors into nude mice. Science 212:65–67, 1981.
63. Fidler IJ, Pollack VA, Hanna N: The use of nude mice for studies of cancer metastasis. In: Sordat B (ed) Immune deficient animals. Karger AG, Basel, 1984, pp 328–338.
64. Kozlowski JM, Hart Ir, Fidler IJ, Hanna N: A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice. JNCI 72:913–917, 1984.
65. Kozlowski JM, Fidler IJ, Campbell DE, Xu Z, Kaighn ME, Hart IR: Metastatic behavior of human tumor cell lines grown in the nude mouse. Cancer Res 44:3522–3529, 1984.
66. Morrissey LW, Sidky YA, Auerbach R: Regional differences in the growth of tumor cells injected intraperitoneally into syngeneic adult mice. Cancer Res 40:2197–2201, 1980.
67. Auerbach R, Auerbach W: Regional differences in the growth of normal and neoplastic cells. Science 215:127–134, 1982.
68. DeWys WD: Studies correlating the growth rate of a tumor and its metastases and providing evidence for tumor-related systemic growth retarding factors. Cancer Res 32:374–379, 1972.
69. Kyriazis AA, Kyriazis AP: Preferential sites of growth of human tumors in nude mice following subcutaneous transplantation. Cancer Res 40:4509–4511, 1980.
70. Kyriazis AP, DiPersio L, Michael GJ, Pesce AJ, Stinnett JD: Growth patterns and metastatic behavior of human tumors growing in athymic mice. Cancer Res 38:3186–3190, 1978.
71. Auerbach R, Morissay, Sidkey YA: Gradients in tumor growth: Nature 274:697–699, 1978.
72. DeVore DP, Houches DP, Overjera AA, Dill GS, Hutson TB: Collagenase inhibitors retarding invasion of a human tumor in nude mice. Exp Cell Biol 48:367–373, 1980.
73. Takahashi S, Konishi Y, Nakatoni K, Inui S, Kojima K, Shiraton T: Conversion of a poorly differentiated human adenocarcinoma to ascites form with invasion and metastasis in nude mice. J Natl Cancer Inst 60:925–929, 1978.
74. Tarin D: Investigations of the mechanisms of metastatic spread of naturally occurring neoplasms. Cancer Met Rev 1:215–225, 1982.
75. Tarin D, Price JE: Influence of microenvironment and vascular anatomy on 'metastatic' colonization potential of mammary tumors. Cancer Res 41:3604–3609, 1981.
76. Sharkey FE, Fogh J: Metastasis of human tumors in athymic nude mice. Int J Cancer 24:733–738, 1979.
77. Witte PL, Ber R: Improved efficiency of hybridoma ascites production by intrasplenic inoculation in mice. J Natl Cancer Inst 70:575–577, 1983.
78. Giavazzi R, Campbell DE, Jessup JM, Cleary K, Fidler IJ: Metastatic behavior of tumor cells isolated from primary and metastatic human colorectal carcinoma implanted in different sites of nude mice. Cancer Res 46: 1928–1933, 1986.
79. Peters, LC, Brandhorst, JS, Hanna MG, Jr: Preparation of immunotherapeutic autologous tumor cell vaccines from tumors. Cancer Res 39:1353–1360, 1979.
80. Vose BM: Separation of tumor and host cell populations from human neoplasms. In: Reid E, Cook GMW, Moore DY (eds) Cancer cell organelles Horwood Ltd Publishers, Chichester 1981:, pp 45–56.
81. Sordat B, Ueyama Y, Fogh J: Metastasis of tumor xenografts in the nude mouse. In: Fogh J, Giovanella BC (eds) The nude mouse in experimental and clinical research Academic Press, New York, 1982, pp 95–147.

82. Sordat B, Wang WR: Human colorectal tumor xenografts in nude mice: Expression of malignancy. Behring Inst Mitt 74:291–300, 1984.
83. Keller R: Elicitation of macroscopic metastases via surgery: Various forms of surgical intervention differ in their induction of metastatic outgrowth. Invasion Metastasis 3:183–192, 1983.
84. Stackpole CW: Distinct lung colonizing and lung metastasizing cell populations in B16 mouse melanoma. Nature 289:798–800, 1981.
85. Povlsen C, Rygaard J, Fogh J: Long-term growth of human tumors in nude mice: Evaluation of stability. In: Fogh J, Giovanella BC (eds) The nude mouse in experimental and clinical research Academic Press, New York, 1982, pp 79–93.
86. Wang WR, Sordat B, Piguet D, Sordat M: Human colon tumors in nude mice: Implantation site and expression of the invasive phenotype. In: Sordat B (eds) Immune-deficient animals Karger, Basel, 1984, pp 239–245.
87. Neulat-Duga I, Sheppel A, Marty C, Lacroux F, Pourrat J, Caveriviere P, Delsol G: Metastases of human tumor xenografts in nude mice. Invasion Metastasis 4:209–224, 1984.
88. August DA, Ottow RT, Sugarbaker PH: Clinical perspectives of human colorectal cancer metastasis. Cancer Met Rev 3:303–325, 1984.
89. Giavazzi R, Jessup JM, Campbell DE, Walker SM, Fidler IJ: Experimental nude mouse model of human colorectal cancer liver metastases, JNCI In Press, 1986.
90. Katsuoka Y, Baba S, Hata M, Tazaki H: Transplantation of human renal cell carcinoma to the nude mice as an intermediate of in vivo and in vitro studies. J Urol 115:373, 1976.
91. Hoehn W, Schroede FH: Renal cell carcinoma: Two new cell lines and a serially transplantable nude mouse tumor (NC 65). Invest Urol 16:106–114, 1978.
92. Otto U, Kollermann MW, Kloppel W, Dimegen J, Linden W, Rudiger H: Transplantation von menschlichem Nierenadenokarzinomgewebe auf die nackte maus. Urol Int 36:110–116, 1981.
93. Naito S, Kanamuri T, Hisano S, Tanaka K, Momose S, Kamata N: Human renal cell carcinoma: Establishment and characterization of two new cell lines. J Urol 128:1117–1122, 1982.
94. Otto U, Kloppel G, Baisch H: Transplantation of human renal cell carcinoma into NMRI nu/nu mice. I. Reliability of an experimental tumor model J Urol 131:130–141, 1984.
95. Otto U, Huland H, Baisch H, Kloppel G: Transplantation of human renal cell carcinoma into NMR I nu/nu mice. II. Evaluation of response to vinblastine sulfate monotherapy. J Urol 131:134–140, 1984.
96. Clayman RV, Figenshau RS, Bear A, Limas C: Transplantation of human renal carcinomas into athymic mice. Cancer Res 45:2650–2656, 1985.
97. Naito S, Von Eschenbach AC, Giavazzi R, and Fidler IJ: Growth and metastasis of tumor cells isolated from a surgical specimen of a human renal cell carcinoma subsequent to implantation into different organs of nude mice. Cancer Res: in press, 1986.
98. Bogden AE, Ketton DE, Cobb WR, Esber HJ: A rapid screening method for testing chemotherapeutic agents against human tumor xenografts. In: Houchens DP, Ovejera AA (eds) Proceeding of the symposium on the use of athymic (nude) mice in cancer research. Gustav Fischer Inc, New York, 1978, pp 231–250.
99. Bogden AE, Hoff DD: Comparison of the human tumor cloning and subrenal capsule assays. Cancer Res 44:1087–1090, 1984.
100. Canellos GP, Devita VT, Gold GL, Chabner BA, Schein PS, Young RC: Cyclical combination chemotherapy for advanced breast carcinoma. Br Med J 1:218–220, 1974.
101. Brambilla C, Delena M, Rossi A, Valagussa P, Bonadonna G: Response and survival in advanced breast cancer after two non-cross-resistant combinations. Br Med J 1:801–804, 1976.
102. Slack NH, Bross JDJ: The influence of site of metastasis on tumor growth and response to chemotherapy. Br J Cancer 32:78–86, 1975.
103. Donelli MG, Rosso R, Garattini S: Selective chemotherapy in relation to the site of tumor transplantation. Int J Cancer 2:421–424, 1967.
104. Donelli MG, Colombo T, Broggini M, Garattini S: Differential distribution of antitumor agents in primary and secondary tumors. Cancer Treat Rep 61:1319–1324, 1977.
105. Suzuki M, Hori K, Abe I, Saito S, Sato H: A new approach to cancer chemotherapy: Selective enhancement of tumor blood flow with angiotensin II. JNCI 67:663–669, 1981.