
From Ectopic to Orthotopic Tumor Grafting Sites: Evidence for a Critical Role of the Host Tissue Microenvironment for the Actual Expression of the Malignant Phenotype

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How to search for something whose nature remains unknown and, suppose that you discover it, how will you recognize it as the thing you were looking for?

Plato, Meno 80d, 380 B.C.

*In memory of Jorgen Rygaard and Carl Povlsen
Initiators to the field and friends*

An Historical Account

From the initial and innovative 1969 report by Jorgen Rygaard and Carl Povlsen describing, for the first time, the heterotransplantation and successful growth of a human solid tumor, a surgical specimen from a colon carcinoma grafted within the subcutaneous space (s.c.) of immune-deficient athymic “nude” mice, it took about 12 years to move to additional and novel experimental grafting conditions which were presented to be more representative of the course and progression of tumors in humans and, potentially, more prone to provide a model with unbiased issues in targeted experimental therapeutic investigations. Following this seminal 1969 report [1] complemented in 1971 [2], abundant data were brought forward by many investigators along three “nude mice workshops” held in Scanticon (Denmark, October 1973) [3], Tokyo (October 1976), [4] and Bozeman (Montana, USA, September 1979) [5], respectively. They included a wealth of results obtained from tumor take and growth rates from a variety of s.c. grafted tumor types, either from *in vitro*

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cultured cell lines [6, 7] or patient-derived tumor fragments, mostly carcinomas. It was generally accepted that grafted tumors maintained their original histopathological characteristics such as differentiation grade or expression of some biomarkers during tumor *in vivo* passaging. Attempts were then made to transfer the *nu* allele to standard inbred strains of mice, and a particular attention was taken to improve mice breeding and maintenance in specific pathogen-free (spf) conditions. In brief, the nude mouse became a popular research tool, and interest was rapidly growing to explore and define new robust working protocols for exploiting attractive potentialities of this model in experimental cancer therapeutics.

In the meantime, evidence rapidly accumulated that the subcutaneous (s.c.) space grafting site, later denominated ectopic (from the Greek *ektos*, outside, *topos*, site), proved to be not fully representative of the growth and behavior characteristics observed along the natural history of most human carcinomas: typically slow growing, generally and clinically late symptomatic, micro-/macro-invasive and expressing an overt or discrete metastatic phenotype. In nude mice or in immune-repressed animals (so-called B mice), s.c. solid tumor grafts, such as those from colorectal carcinomas, were found to adopt in most cases, and in contrast to the original patient malignant tumor, an expansive type of growth (designated as “pseudo-benign”) progressing as multi-nodular tumor masses and clearly restrained at graft borders lined by multilayered fibroblastic-like host cells and usually by a lack of or few tumor inflammatory tumor infiltrating cells. As noted by many authors, a tendency of grafted solid tumors to grow more and more rapidly with s.c. passaging may preclude their use in interventional strategies targeting properties or behavior characteristics other than proliferative activity. In fact, solid s.c. tumor grafts rapidly develop hypoxic, pre-necrotic, and necrotic areas, likely due in part to the failure to be sustained by a sufficient and fully functional newly formed vasculature. Accordingly, tumor volume measurements may include a substantial proportion of cell debris affecting the issue of quantitative new drug testing.

Somehow, the s.c. grafting *in vivo* system is reminiscent of the 3-D multicellular tumor spheroids used as an *in vitro* model of nodular carcinoma, a method exposing cells in spinner culture flasks to gradients of oxygen and metabolite concentrations generating within tumor spheroid heterogeneous microenvironments with surface proliferating, underlying quiescent, hypoxic, and pre-necrotic micro-regions. The model exhibits similarities to tumors *in vivo* both in the patient and grown as xenografts in nude mice allowing for combinatorial drug-radiation therapy testing [8].

To initiate and to be able to accomplish the “deadly metastatic cascade,” it has been (over)repeatedly emphasized that malignant cells have to cross multiple and diverse host tissue structures, intravasate, adapt to the blood stream or to the lymphatic fluid, and survive to become a distant intra-vascular cell embolization and then extravasate and find a proper and specific “niche” environment amenable to survival, dormancy, or permissive to *in situ* proliferation. This “cascade” is also relevant to metastases occurring in cases of clinically-unidentified primaries or even metastases from metastases. The notion of accumulated cell-constraining events in the course of this cascade had led Leonard Weiss to elaborate the concept, somewhat paradoxical, of “metastatic inefficiency” [9].

It is perhaps historically valuable to mention here that a “closed” meeting (45 attendants!) was held on June 3–5, 1981, at the University of Saskatchewan, Saskatoon, Canada [10]. Organized by I. Carr, R.S. Kerbel, and P. Scholefield, it covered, for the first time in a meeting, to my knowledge, specific topics such as tumor heterogeneity and phenotypic cancer-cell instability, invasion, and cancer metastasis including tumor xenografting, now recurrent and compulsory themes in popular multi-annual clinical and basic- research workshops and symposia, and welcome in top cancer journals.

Initial micro-invasive processes are thus crucial steps for metastasis to occur. If rapid cell proliferation is privileged in s.c. tumor xenografts and, according to the notion “Go or Grow” issued from basic cell metabolic pathways regulating cycling, migratory, and invasive tumor cell-associated activities, such as proteolytic activities and cell surface integrin-ligand interactions, might be reduced or inhibited temporarily when grafted at ectopic sites, hence a pseudo-benign tumor behavior. Indeed, past work suggested that tumor cell proliferation and invasion were under separate controls.

The Popular Subcutaneous Route

Up until the 1980s, a majority of investigators active in the field of malignant xenografts would consider solid human tumors s.c. grafted in the nude mouse as noninvasive and non- or poorly metastatic. In this context, we contributed data along this line [11] and then reviewed the field in a book chapter published in 1982 [12]. Indeed, with some reported and notable exceptions, the s.c. tissue grafting site, if considered as an experimental primary tumor site in the nude mouse, appeared in most cases to provide no sufficient tissue structure conditions or no site or tumor matrix-associated, yet undefined, factors to restore actual tumor behavior, i.e., invasive and metastatic and eventually to be proposed *in fine* as a model amenable to improving the issues of experimental therapeutics.

Interest moved then to the search and understanding of the reason(s) for the inability of s.c. malignant tumor-grafted nu/nu mice to express a malignant phenotype. Questions were thus addressed to the potential role of the microbiological status of the recipient, the route of cancer- cell or patient-derived tumor fragment inoculation, as well as *a contrario* looking for a possible yet unknown link to some intrinsic properties expressed by defined and rare cell lines shown consistently to invade and disseminate in their host by overwhelming the nude mouse resistance. In brief, metastatic ability appeared dependent on both the microbiological and the genetic status of mice, i.e., for example, spf backcross-mated BALB/c athymic nude s.c.-grafted with a defined tumor showed more propensity to metastasize than conventionally- maintained outbred recipients grafted with the same tumor [13]. When transplanted early in life, nude mice proved to be more permissive for the growth and spreading from the s.c. site of both lymphomyelogenous and carcinoma cell lines, but from day 7 to day 12 after birth, fast-growing tumors stopped to disseminate [14]. Indeed, age, environmental, and genetic factors could determine the level

of natural killer (NK) activity, maturing around 3–5 weeks after birth in nude mice and highest in circulating blood. These and other factors were shown to affect the ability of grafted tumors to metastasize [15].

Regarding alternative routes of tumor cell inoculation [16], we and other investigators have demonstrated that intraperitoneal (i.p.) administration of cancer cells can generate a dose-dependent ascitic carcinosis with mesenteric invasion, mediastinal, and lung metastases. Ascites-forming tumors such as breast, stomach, larynx, and colorectal carcinomas were all diagnosed as poorly-differentiated or undifferentiated cell types. Moreover, it is generally agreed that a majority of carcinoma cells introduced i.v. in the blood compartment will embolize and rapidly be destroyed in the lungs of nude mice, as demonstrated histologically and monitored by ^{125}I UdR-labeled tumor cell death or survival [17]. More interesting perhaps, and in the perspective to better mimic the post-surgical status of a cancer patient, the removal of locally s.c.-grafted tumor xenografts has been used to follow the long-term tumor behavior in the presence or in the absence of local recurrence, both spontaneous and induced. In our hands, and using the colon carcinoma Co115 cell line, surgical excision of the s.c.-growing xenograft did not increase the presence of lung metastases unless recurrent tumors grew locally, suggesting that post-surgical healing and inflammatory processes at an s.c. site could contribute to extending cancer-cell dissemination from “minimal residual disease.” As reported [18], the extent of tumor deposits in the lungs varied among individual mice, and the number and size of tumor emboli at the time of graft excision might determine their subsequent survival, growth, or death, in the host lung tissue.

Questioning the structural and functional aspects of subcutaneous tissues in the nude mouse, it appears that this route of inoculation is merely a free space easily accessible for a needle containing small cell samples or for a microtrocar loaded with patient-derived solid or disaggregated tumor fragments. The s.c. compartment can develop from *in situ* axillary and inguinal vessels a newly formed and modified vasculature and is drained by regional axillary and inguinal lymph nodes. This space, covered by a hairless skin and easily expandable, is quite convenient for tumor-volume measurements and remains a popular route for pre-clinical drug evaluation despite its reported limited significance in terms of invasive and metastatic ability as an acceptable mimicry of natural tumor behavior. It should not be under-evaluated that, with passaging, the original tumor stroma is replaced by murine tissue supporting the initial establishment and growth of human tumor cells. As discussed above, it became rapidly evident that s.c. environmental tissue conditions presumably lack the proper factors or some stroma-associated cellular and/or structural components able to activate the process of host tissue invasion by malignant cells.

The Orthos Way

In the early days of metastasis research at least, it used to be imperative, perhaps fashionable, to introduce in a communication the historical hypothesis of Stephen Paget (1855–1926) published March 23, 1889, as an article in *The Lancet* (133,

3421, pp. 571–573) and entitled “The distribution of secondary growths in cancer of the breast.” Paget carefully analyzed 735 cases of breast cancer, reviewed the available literature, and went to the conclusion that the distribution of secondary growths cannot be due to chance and proposed the *seed and soil* theory of metastasis. He argued: “When a plant goes to seed, its seeds are carried in all directions but they can only live and growth if they fall on congenial soil.” Moreover, Paget honestly credited an Austrian ophthalmologist, Ernst Fuchs (1851–1930), for his theory of the relation between a cancer embolus and the recipient distant tissue *predisposed* to its specific growth. In addition to the study of the nature of the seeds, Paget, pioneering in the field, concluded “prophetically” that *observations of the properties of the soil may also be useful*.

Therefore, and in view of the well-established fact that the majority of human solid malignant tumors grafted s.c. to the nude mouse adopt a “pseudo-benign” behavior, it became evident that the “soil,” in this case the subcutaneous space and tissue, does not offer the “congenial” properties proper to the full expression by the “seeds” of their invasive and metastatic potential. However and in contrast with the behavior in the s.c. space, we showed, using the same colon Co115 tumor, that the peritoneal cavity of nude mice is permissive for the development of an ascitic carcinoma with free floating and aggregated colonic tumor cells able to adhere to the mesenterium, to grow as multiple foci. and to metastasize to regional lymph nodes and mediastinal and diaphragmatic areas. Furthermore, and in agreement with Paget’s theory, both clinical observations as well as mouse models strongly suggested that specific interactions can take place to promote preferential, non-random, metastatic development. Indeed in 1980, and using murine tumor models, Ian Hart and Isaiah Fidler were able to demonstrate experimentally that i.v. distributed cancer cells (melanoma) could only grow at particular “congenial” sites promoting in situ tissue selectivity for the malignant cells to expand [19].

Time had now come to test for a possible change in the phenotypic tumor behavior using a tissue site in the nude mouse corresponding to the one whereby the original carcinoma grew in the patient. Professor Wu-ru Wang, from the Department of Pathology, Harbin School of Medicine in China, on sabbatical leave at the Institute of Pathology, University of Lausanne, Switzerland, was interested in the project and joined the laboratory at the Cancer Institute (ISREC). Two colonic carcinoma lines established previously in the laboratory from xenografts were used: Coll2, a moderately differentiated, and Co115, a poorly differentiated type. To control for a potential site effect proper to colonic lines only, two melanoma lines were also used. Practice of tumor cell inoculation into the spf nude-mouse gut wall required careful microsurgical work under a laminar flow hood, anesthesia, and mouse body temperature control. Accurate tumorigenic-cell dose ($1-5 \times 10^6$ in a microliter volume) was difficult to ascertain using an inoculation procedure tangential to the gut wall. Observation time ranged from 20 to 92 days. In the first series of experiments, 11 out of 33 mice showed evidence, macroscopically, of growth within the gut wall. Each case was thus reported as drawings in laboratory notebooks as illustrated in the following Fig. 4.1 by two cases taken at 27 and 37 days after gut implantation.

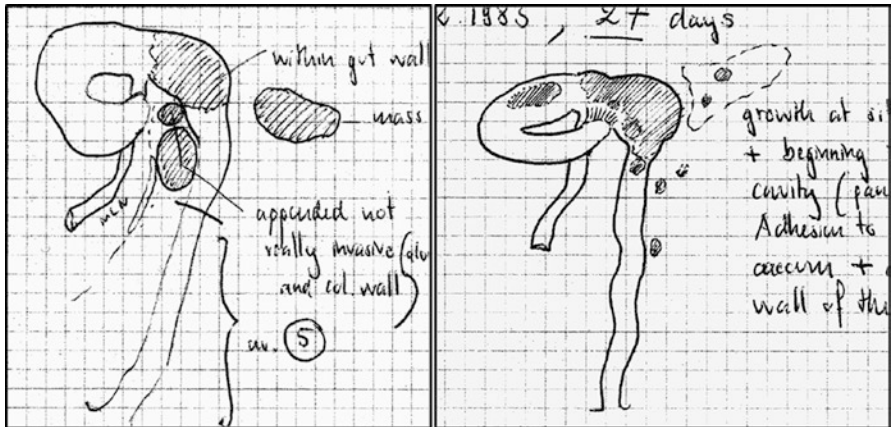


Fig. 4.1 Two cases illustrating the pattern of human tumor growth within the mouse large bowel wall and extending from implantation site to the caecum (magnif. 1:1 from the original drawings)

Light, electron microscopy and results from autoradiographic analyses were published in 1982 [20]: they were presented as a poster at the fourth International Workshop on Immune-Deficient Animals in Experimental Research that we organized and that was held October 31–November 3, 1982, in Chexbres, near Lausanne.

Clearly, both the gut-implanted Co115 and Co112 colon-cancer cell lines were now able to express a macro- and microinvasive behavior, proliferate, and infiltrate the different layers of the mouse large bowel walls, crossing the muscularis mucosae and the muscularis propria to dissociate smooth muscle layers. Both lymphatics and blood vessels were found to contain intravasated Co115 tumor cells. Moreover, at longer time intervals, tumor foci were found in the serosa and grew as nodular masses in the mesenterium of the peritoneal cavity and distant from the inoculation site. Metastases of Co115 cells only were found in mesenteric lymph nodes. Interestingly, and in contrast to s.c. grafted Co112 and Co115 xenografts, inflammatory cell types were regularly found associated with gut invasive tumors. Four out of seven mice implanted with melanoma cells showed serosal adhesion and implantation and grew preferentially as spheroid-like nodules on the surface of the large bowel wall with limited penetration of the underlying gut layers. These histopathological features obtained with tumor xenografts implanted in an orthotopic (*orthos*, right and straight) site of the nude mouse made them quite similar to those of the Co112 and Co115 original patient tumors.

The content of the poster attracted workshop attendants and abundant questions from invited experts in the metastasis research field: is Paget's "seed and soil" concept applicable to the ecto-orthotopic conversion from "benign" to invasive phenotypes and, for some tumor types, from "pseudo-benign" to invasive and metastatic phenotypes? Is the conversion dependent on structural component(s) of the soil, as suggested by Paget 93 years ago, such as specific constituents of basement membranes of epithelia or of smooth muscle layers? Or is the conversion dependent on

factors diffusible locally or trapped in the stroma or extracellular matrix specific constituents? Is the grade of tumor differentiation, the size of a stem cell population in a tumor of interest, influential since differentiated tumor types appear to need longer time intervals to establish metastatic deposits? Which role or roles do the diverse types of inflammatory cells regularly associated with invasive areas play? Do specific proteolytic activities take direct or indirect part in the process of phenotypic conversion?

As recently underlined by Robert Hoffman [21]: “subsequent studies from Fidler’s laboratory and others have shown that the implantation of many types of human tumours in the orthotopic sites of nude or other immunodeficient mice resulted in metastasis of human tumours.”

At that time and in the context of the interest in the laboratory for proteolytic activities involved in malignant tumor cell behavior, we took advantage of the model to characterize and quantitate plasminogen activators (PAs) expressed by primary colon carcinomas and their respective serial s.c.-grafted xenografts in the nude mouse. Activation of plasminogen to plasmin, a multipotent enzyme, can lead directly to the degradation of major stromal components or, indirectly, by activation of latent metalloproteinases. Patient primary invasive tumors showed high concentrations of PAs, while s.c. xenografts contained very low levels of activity not due to an increase in inhibitors of fibrinolytic activity [22]. A second series of experiments using the ecto-orthotopic conversion cancer-cell system demonstrated that only gut-implanted colonic tumors only could exhibit invasive growth and expressed high tumor cell-secreted PA activity, indicating that fibrinolysis could participate to the complex process of tumor invasion [23].

Another series of experiments allowed us to show that extracellular matrix-cancer cell interactions can regulate growth and migratory, invading, activities *in vivo*. Laminin isoforms are major constituents of basement membranes. The laminin-5 heterotrimer is composed of the alpha-3, beta-3, and gamma-2 subunits and is a primary adhesion protein in the skin and gastrointestinal mucosal epithelia. In colorectal carcinomas, gamma-2 and beta-3 subunits have been found in the cytoplasm of the so-called budding carcinoma cells sprouting out of the neoplastic tubules and invading the tumor stroma [24, 25]. Moreover, the budding phenotype proved to be of prognostic value in patients with colorectal cancer [26]. In our experiments [27], we found that laminin-5-associated budding activity was suppressed when colorectal carcinoma cells grew s.c. but was restored when gut-implanted in the nude mouse, as illustrated in Fig. 4.2.

Previous results using colorectal tumor xenografts support the role of the organ microenvironment in regulating expression of an array of gene products including degradative proteases and growth and inflammation-associated factors [22, 23]. In this context, and since pro-inflammatory cytokines have been shown in human epithelial non-malignant intestinal cells to modulate laminin-5 expression, we tested whether cytokine production *in situ* would affect the level of laminin-5 subunits in HT29 colonic carcinoma cells. Indeed, *in vitro*, preliminary results indicated that TNF-alpha (10 ng/mL) could increase, versus control, up to 5x the level of gamma-2 subunit transcripts and about 2x the levels of alpha3 and beta3 subunit transcripts.

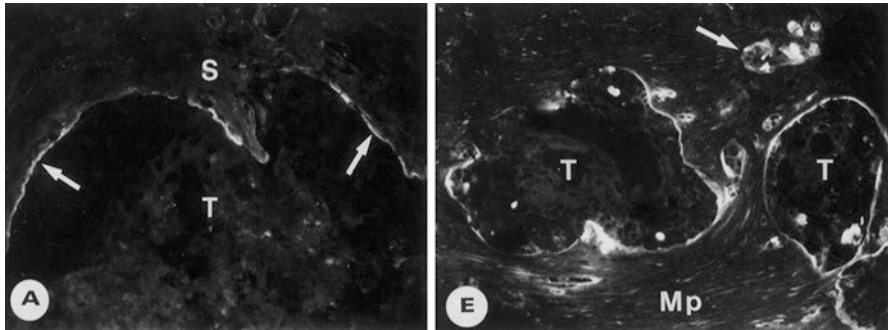


Fig. 4.2 In A, laminin-5 is extracellular and is confined (*arrows*) to the tumor (T, HT29 colon cells grafted s.c.)—host murine stroma (S) interface. In E, laminin-5 immunoreactivity is now both intra-cytoplasmic in invading HT29 carcinoma cells (*arrow*) and lining tumor nodules (T) infiltrating the mouse muscularis propria (Mp), following gut implantation of HT29 tumor cells (modified from reference [27])

HGF (50 ng/mL) increased 3x the level of the alpha3 subunit only. TGF- β (up to 10 ng/mL) did not alter significantly laminin-5 subunit expression under these conditions. A laminin-5-associated pathway could possibly contribute to initiate a process of cancer-cell invasion within the inflammatory context of the orthotopic site of colon carcinoma implantation (Sordat, I. unpublished, 2000).

Comments in Conclusion

As emphasized above, cancer-cell micro-invasion is a key initial step for metastases to develop in “predisposed” organ environments. Obviously, orthotopic tumor implantation routes in the nude mouse system, specifically the large-bowel gut implantation of human colorectal carcinoma cells (1982) [20], has opened a way from the first subcutaneous ectopic transplantation (1969) [1] to patient-derived orthotopic xenografts (PDOX models) using intact colorectal cancer tissue (1991) [28] where a greater extent of metastasis was observed and presumably due to intact tumor-stroma interactions allowing for malignant micro-invasion at the implantation site. Routes of cancer-cell initial implantation are among the critical factors for metastases to form and further develop non-randomly at distant sites [29]. A variety of tumor types has been established as PDOX models, and clinical relevance in cases of stomach cancer could be observed where a correlation was found between the development of liver metastases both in the patient and in the nude mice orthotopically transplanted with intact tumor tissue [30]. This book updates the present state of knowledge by pioneers in the field of tumor xenografting; see Chaps. 3, 5, 7, and 8. However, mainly due to the failure of pre-clinical mouse tumor models to be better predictive of clinical responses in the patients and as alternatives to human tumor xenografts, transgenic mouse cancer mice systems were developed, rapidly dominating the preclinical research area [21]. In the meantime, more immune-deficient mice strains such as SCID or NOD/SCID as tumor xenograft hosts became

available. In 2006, however, the ectopic s.c. and not the orthotopic human tumor xenograft model as a "back to the future" novelty was revived, and cancer heterotransplants from individual patients were now designated as "xenopatients" [21]!

Robert Kerbel reviewed recently an experience of 10 years of work in his laboratory developing pre-clinical models and pointing out the importance in innovative therapy studies of using and exploiting models of advanced late-stage tumors in mice with spontaneous metastases. A multi-model experimental approach was recommended including the surgical removal of orthotopic tumor grafting followed by *in vivo* selection of more metastatic variant lines [31]. For an update, see Kerbel's Chap. 8 in this book.

A perspective opinion article [32] (42 coauthors) published January 2017 representing a very large multi-institutional platform underlines the ability of patient-derived xenografts (PDXs) to contribute to increasing our knowledge on the role of tumor heterogeneity in the course of drug exposure as well as to plan novel therapeutic strategies or identify malignant biomarkers. PDXs as *in vivo* models are recognized to fulfill certain phenotypic and genotypic criteria but preclude immune-based investigations unless immune-deficient mice as human tumor-grafted hosts can be "humanized" with human specific immunologically- active subpopulations of cells. Surprisingly, the choice of PDXs and not of patient-derived orthotopic xenografts (PDOXs) was done, implying that a full expression of the malignant phenotype as a functional criterion of human tumor murine stroma microenvironmental interactions could not be considered in the study [32]. Certainly, orthotopic implantation routes according to tumor types to be investigated imply technical and financial constraints (microsurgery, imaging, quantitative analysis of drug application strategies, animal maintenance conditions) and necessitate similar protocols to be adopted between each member of the consortium. Moreover, in our experience, there exists a degree of heterogeneity in the success rate of both orthotopic implantation and in the temporal development of the tumor graft in the mouse imposing a relatively large number of tumor recipients. After all, mice (induced) and human beings (spontaneous) express a disease that requires a "personalized" treatment strategy for the potential benefit of one of the two! See citation by Plato under the title of this chapter.

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