

Murine Metastatic Tumour Models for Cancer Gene Therapy Research

HLKong,*^{FAMS, MRCP (UK), M Med (Int Med)}

Abstract

The appropriate use of animal tumour models has a pivotal role in the evaluation of any new anti-cancer therapy. Indeed, animal models of human diseases have been emphasised in the early development and evaluation of gene therapy. Given that most cancer treatment failures and cancer-related mortality are the direct results of metastatic cancers, the increasing use of clinically-relevant metastatic tumour models in the study of cancer therapeutics is both logical and necessary. Murine metastatic tumour models may be established either "experimentally" or "spontaneously". The yield and reproducibility of spontaneous metastasis models can be enhanced through manoeuvres such as using highly-metastatic sublines for primary tumour implantation and orthotopic transplantation. The use of immunodeficient rodents, although popular, suffers from the absence of T-cell responses in the host which may impact on therapeutic efficacy. While many gene therapy strategies today are capable of regressing primary tumours in experimental animals, only a limited number of approaches (viz. genetic immunotherapy and gene-mediated anti-angiogenesis) are designed to address the challenges posed by metastatic tumours. In extrapolating the results of gene therapy in animal models to humans, it is important to appreciate the heterogeneity of the latter populations, and anticipate greater variability in the treatment outcome.

Ann Acad Med Singapore 1999; 28:112-9

Key words: Antiangiogenesis, Immunotherapy, Nude, Severe combined immunodeficiency, Spontaneous

Introduction

In the evaluation of any new cancer treatment, the use of appropriate experimental animal tumour models to bridge the gap between *in vitro* discovery and its eventual clinical testing is an important step.^{1,2} *In vitro* experimentation does not take into consideration the complex biological interplay among the host tissues, the tumour cells and the therapeutic agents. As a result, demonstration of *in vitro* biological activities does not always predict *in vivo* treatment efficacy. In addition, there are important biological processes which cannot be reproduced in the test tubes, including anti-tumour immunity and tumour angiogenesis. Finally, the potential for the new therapies to cause untoward effects on the host can only be gleaned from *in vivo* experimentation. For these reasons, it is widely held that new anti-cancer treatment modalities, such as gene therapy, should be thoroughly tested in the appropriate animal tumour models before they are applied to the first human subjects. The importance of animal models of human diseases has been emphasised in the early development and evaluation of gene therapy. In the evaluation of gene therapy protocols for clinical trials by the United States Recombinant DNA Advisory Committee (RAC) of the National Institute of Health, investigators are advised to "provide results that demonstrate the safety, efficacy, and feasibility of

the proposed procedures using animal and/or cell culture model systems, and explain why the models chosen are the most appropriate".

A good animal tumour model should mimic a broad range of biological factors which influence, or are influenced by, the growing cancer cells in the host tissues in which the cancer cells operate.³ A very common experimental tumour model system involves the use of human cancer xenografts grown subcutaneously in the flanks (i.e. as primary tumours) in the athymic nude mice.^{4,5} This has proved to be rather useful as a crude *in vivo* screening system for novel anti-cancer therapies. However, with the realisation that most cancer treatment failures in humans occur in the setting of metastatic malignancy, the deficiency in such a primary tumour model system becomes apparent. The following sections in this review describe the application of metastatic murine tumour models to cancer research, especially as they relate to cancer gene therapy. The scope of discussion is confined to the relevance of animal tumour models in determining treatment efficacy; the usefulness of animal models in providing safety data is traditionally viewed in the context of toxicology, and shall not be further discussed here. Specific examples of certain tumour models will also be described in some details, particularly to illustrate their usefulness to cancer gene therapy.

* Assistant Professor

Department of Medicine (Medical Oncology)

National University of Singapore

Address for Reprints: Dr Kong Hwai Loong, Department of Medical Oncology, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074.

Special Role of Metastatic Tumour Models

While the primary subcutaneous flank tumours in rodents provide some idea of the *in vivo* efficacy of novel anti-cancer therapies, there are cogent reasons for testing them in the setting of disseminated tumours.^{4,5} Firstly, in most human cancers, the metastatic phase of the disease, rather than the primary malignancy, is the cause of death in the majority of patients. It would be erroneous to assume that a treatment that is efficacious against a primary tumour would also be similarly efficacious against disseminated tumours. Indeed, there are data to suggest that the biology of metastatic tumours is different from the parental primary tumours.⁶⁻⁹ As such, categorical testing of the treatment in the metastatic setting is necessary. Secondly, the mechanism of action of a therapeutic agent on distant tumours may be different from that on the primary tumours. This is particularly well illustrated in cancer gene therapy. For instance, the transduction of primary tumours with suicide genes followed by the systemic administration of the prodrugs leads not only to cytotoxic destruction of the primary tumours, but also suppression of distant metastases; the latter phenomenon has been attributed to the generation of tumour-specific immunity.¹⁰ Thirdly, even if the biology and treatment responses of metastatic tumours were similar to those of the primary tumours, disseminated tumours pose considerable physical challenges to the successful delivery of the therapeutic agents. The agents must have the capability to target tumour cells that are in circulation as well as those which have settled in viscera far remote from the primary tumours—in essence, a systemic anti-tumour response is needed to effectively control disseminated disease. From the above account, it is apparent that metastatic tumours present greater therapeutic challenges than localised malignancies.

Special Problems Presented by Cancer Gene Therapy

Gene therapy describes the process of transferring DNA or RNA to modify the genetic repertoire of target cells for therapeutic purposes.^{11,12} The process of gene transfer is generally accomplished with the use of gene carriers, referred to as “vectors”. Typically, the gene to be transferred is in the form of a complementary DNA (cDNA) coding for the therapeutic protein and is contained in an “expression cassette” that includes a constitutive or regulatable promoter driving the cDNA. Broadly, gene transfer vectors are classified as either viral or non-viral. The gene transfer process can be performed either *ex vivo* or *in vivo*, the latter approach being the more relevant one for the treatment of metastatic cancer.

The process of discovery and development necessary to make gene therapy a reality differs from that of traditional drug development in several important ways.

Firstly, the nature of gene therapy requires *in vivo* validation of efficacy in a biological system—testing in the test-tube alone is insufficient. Successful gene therapy entails a complex, multi-step process that involves the delivery of the therapeutic genes to the target cells *in situ*, followed by efficient gene entry into the cells, translocation of the foreign genes to the nuclear compartment, and finally, expression of the foreign genes using the host cellular machinery. In this cascade of events, there are many points of potential interactions between the host tissues and the transgenes, such as the stability of the gene vectors within the blood circulation if it is administered intravascularly, the negative influence of neutralising host immunity on gene expression, and so forth. These interactions cannot be reproduced in the test-tube, but can be adequately dissected only if gene therapy is tested in a living biological system. Secondly, it has become very clear in recent years that many gene therapy approaches provide only local therapy: they are not primarily designed to treat disseminated tumours (Table I). For example, the transfer of tumour suppressor genes and the use of antisense constructs would not be expected to treat non-transduced cells. To treat distant metastases using gene therapy, other approaches are necessary, such as the generation of tumour-specific immunity,^{10,13-16} and the induction of normal host cells to produce antiangiogenic proteins;^{17,18} both strategies will be discussed in greater details later in this review.

Establishment of Animal Metastatic Tumour Models

The application of animal tumour models is as diverse as the research disciplines themselves. Broadly speaking, there are two major ways by which metastatic tumour models are generated.¹⁹ Experimental metastasis involves the direct administration of tumour cells into the appropriate body compartments, with the intention of dispersing the tumour cells to specific target organs. For instance, intravenous administration of tumour cells in suspension predictably leads to the growth of multiple pulmonary metastatic nodules,^{1,16,18,20} whereas the intraperitoneal administration of tumour cells would be expected to generate peritoneal carcinomatosis. Spontaneous metastasis describes the appearance of metastases following the implantation and growth of primary tumours.^{3,5,21,22} For example, the growth of primary tumours in the mammary fat pads may be followed by the appearance of metastases in distant organs.²³

Each of the two models has its strengths and weaknesses. In experimental metastasis, the generation of tumour deposits in the distant organs is often reproducible with respect to the “tumour-take” rate and the pattern/number of metastases. The resultant metastatic tumour burden can also be varied according to the number of tumour cells in the suspension that is administered.²⁴ One theoretical shortcoming of such a method

TABLE I: CURRENT POTENTIAL OF CANCER GENE THERAPY STRATEGIES

Strategy	Examples	Potential to treat localised tumours	Potential to treat metastases
Gene replacement ⁷²⁻⁷⁴	p53, RB	+	0
Oncogene inactivation ⁷⁵⁻⁷⁸	Antisense, ribozymes	+	0
Suicide gene/prodrug activation ⁷⁹⁻⁸³	HSV-tk + GCV CD + 5FC	+	±*
Protection of bone marrow stem cells against chemotherapy effects ^{84,85}	MDR	N/A	N/A
Immunotherapy ^{15,16 20,86-89}	MHC Class I antigen, HLA B7, IL-2, 4, 6, 7, 12, GM-CSF, TNF- α , IFN- α , IFN- γ , tumour antigen, gene-modified dendritic cells	+	+
Antiangiogenesis ^{17,18,90-93}	sflt-1, sPF4, dominant-negative flk-1, angiostatin, thrombospondin-1	+	+

* Suicide gene treatment of primary tumour can induce an anti-tumour effect on distant metastases, partly due to the induction of a systemic tumour-specific immunity.

RB: retinoblastoma; HSV-tk + GCV: herpes simplex virus thymidine kinase gene with administration of ganciclovir; CD + 5FC: *E. coli* cytosine deaminase gene with administration of 5-fluorocytosine; MDR: multidrug resistance; N/A: not applicable; MHC: major histocompatibility complex; HLA: human leukocyte antigen; IL: interleukin; GM-CSF: granulocyte-macrophage colony stimulating factor; TNF: tumour necrosis factor; IFN: interferon; sflt-1: soluble flt-1 VEGF receptor; sPF4: soluble platelet factor 4 receptor

is the absence of the early events involved in spontaneous metastasis, namely, the evolution of metastatic clones within the primary tumour and their subsequent invasion into the blood stream. However, there are data to support the notion that the rate-limiting steps to successful metastasis are really the later events, namely, the growth of disseminated tumour cells in distant organs following their escape from the blood stream.^{3,25-31} On this basis, direct administration of tumour cells into the blood stream fairly closely mimics the actual metastatic process. Spontaneous metastases following successful establishment of the primary tumours most faithfully reproduce the full complement of biological processes that take place in human tumour metastasis.³ This method, however, suffers from a greater inconsistency in the tumour-take rate and the resultant metastatic tumour burden. Part of this inconsistency may be attributed to the different regional growth rates of the primary tumours following tumour cell inoculation.^{32,33} There

appears to be a significant predilection for tumour growth in the anterior (occipital) regional of the mouse trunk as opposed to the posterior (caudal) region. Another source of inconsistency in the establishment of spontaneous metastases is the faster growth rate of dorsally-implanted tumours as opposed to ventrally-implanted tumours. The reasons underlying these regional growth differences are unclear.

In an attempt to increase the chances of generating spontaneous metastases, several manoeuvres have been tried with variable success. These include the use of highly-metastatic clones derived from established metastases,^{4,6-9,19,21,22,30} deeper penetration of the body wall during growth of the primary tumour,^{21,22} selection of endothelial-adhesive tumour cell subpopulations,³⁴ transplantation of tumour blocks as opposed to tumour cell suspensions,³⁵ creation of a healing wound environment,³⁶ mixing the cells with Matrigel,³⁷ systemic administration of 17- β -estradiol to reduce endogenous NK cell activity,¹⁹ and orthotopic transplantation.^{23,30,35,37-44} Orthotopic transplantation involves the transplantation of histologically-intact cancer tissue to the biologically-relevant host organs (e.g. transplanting colon carcinoma cells to the intestines,^{30,35,38} breast cancer cells to the mammary fat pad,^{23,39} renal cell carcinoma to renal subcapsular space,⁴⁰ prostate cancer cells into the prostate,³⁷ small cell lung cancer cells into the lungs,⁴¹ or osteosarcoma cells to the bones⁴²). Orthotopic models are felt to recapitulate the entire metastatic cascade most closely, which comprises local tumour growth, vascular and lymphatic invasion at local site, entry of tumour cells into these vessels, and eventual extravasation at the metastatic organs.^{3,43,44} Besides these measures aimed at increasing the chances of forming spontaneous metastases, one other manoeuvre stands out as being the most dramatic and biologically intriguing: the removal of established primary tumours has been shown to dramatically increase the rate of occurrence and the number of distant metastases.^{5,45,46} This observation is, at least in part, explained by the decrease in the systemic level of endogenous inhibitors of angiogenesis (e.g. angiostatin and endostatin) following excision of the primary tumours. This laboratory phenomenon is reminiscent of patients whose primary tumours have been successfully removed, but who die soon after from fulminant distant metastases.

Specific Examples of Murine Metastatic Tumour Models

Metastasis is the most difficult tumour characteristic to simulate, mainly because the process is a complex sequence of biological interactions between the metastatic tumour cells and the host tissue milieu.³ A few metastatic tumour models will now be described to illustrate how they are generated.

Metastatic Lung Model

This has been traditionally established through intravenous administration of tumour cell suspension.^{1,8,14,16,18,23,47} The cells are trapped in microvessels within the pulmonary circulation, whereupon they egress and form discrete micrometastases within the lung parenchyma. The optimal number of tumour cells to be administered depends primarily on the cell types and the host species (perhaps reflecting the different nature of pulmonary circulation). Inadequate cell number in the tumour cell suspension lowers the metastatic yield, whereas excessively concentrated cell suspensions are apt to cause embolisation of the pulmonary circulation and instant death of the animals. As an example, a method whereby 3×10^5 CT26.CL25 murine carcinoma cells suspended in a volume of 500 μ l administered over a period of 30 seconds has been described with a high degree of reproducibility.¹⁸ Alternatively, spontaneous lung metastases have also been very well characterised for breast cancer, Lewis lung carcinoma, melanoma and osteosarcoma.

Metastatic Liver Model

A number of studies have described the administration of tumour cell suspension into the exteriorised spleen,^{8,9,18,19,23,30,35,48} from which the portal circulation carries the tumour cells distally into the liver where they are trapped in the microcirculation and then grow into larger metastatic deposits.⁴⁹ Some investigators remove the spleens immediately following administration, while others do not. The theoretical confounding effect of splenectomy on host immunity may be relevant to the treatment outcome, especially if immunotherapy is employed. Similar cell number and rate of administration as described in the metastatic lung model has been used with success in generating experimental liver metastases. Alternatively, orthotopic transplantation of colon carcinoma into the caecum has resulted in reproducible spontaneous liver metastases.³⁵

Other Metastatic Tumour Models

Peritoneal carcinomatosis can be reproducibly generated using intraperitoneal administration of ovarian carcinoma or colon carcinoma cells.⁴⁹ Bone metastases of prostate cancer can be generated by tail-vein injection of prostate cancer cells while the inferior vena cava is being occluded; this forces the tumour cells into the vertebral venous plexus.^{50,51} Finally, brain metastases can be created by injecting tumour cells into the carotid arteries.^{23,52,53}

In any chosen tumour model, it is important to maintain procedural consistency so that reproducible metastatic models can be generated. In this regard, limiting the number of operators in a particular study to a minimum will be helpful.

Choice of Animals

The use of human xenotransplantation in immunocompromised rodents, first described in 1968,^{54,55} has been very popular in cancer research.^{1,2,7-9,19,21,22,30,33,35,38,56} The athymic condition in nude mice is due to a recessive autosomal mutation, *nude*, the symbol for which is *nu*. Owing to a lack of T-cell responses, the nude mouse accepts grafts of allogeneic and xenogeneic tissue, including malignant tumours. Its use has contributed significantly to our present understanding of the metastatic process, and has helped us to test the *in vivo* efficacy of many novel anti-cancer therapeutic agents.^{1,2,57} However, the use of immunocompromised animals has its limitations, and a few comments on their use in relation to cancer gene therapy are warranted here. It is well known that human xenografts in athymic nude mice seldom metastasise, in contrast to their immunocompetent counterparts.⁵⁸⁻⁶⁰ The exact mechanism that accounts for this phenomenon is still a matter of some dispute. One hypothesis is that the presence of enhanced NK cell activity in nude mice effectively reduces the establishment of metastatic deposits.⁵⁹ This hypothesis is not universally accepted though.^{22,61} In contrast, severe combined immunodeficiency (SCID) mice, which lack both T and B-cell functions but retain their NK cell activity, develop spontaneous metastases more readily than nude mice although the primary tumour-take rates between the two species are similar.^{38,62} To explain this observation, it has been postulated that B cells may play an important role in modifying the metastatic behaviour of the xenografted tumours, through either unknown direct activity or the indirect action of antibodies, including antibody-dependent cell-mediated cytotoxicity. Regardless of the underlying mechanisms that account for the differences in metastatic potential between nude and SCID mice, some have argued that SCID mice are the more appropriate hosts for the study of metastatic tumours.^{38,60}

However, one may argue that both nude and SCID mice are unsuitable for the study of cancer treatment, especially in the context of cancer gene therapy. Firstly, these mice lack T-cell activity; the impact of this phenotype on the response of metastatic tumours to therapy is unclear, and may indeed be significant.³³ Secondly, gene therapy strategies that involve the generation of anti-tumour T-cell subsets could not be tested in T-cell immunodeficient hosts. Thirdly, the absence of T-cell-mediated immune responses against the gene vectors and/or the transgenes may spuriously enhance the extent and duration of the gene-mediated therapeutic effect. In this regard, it is noteworthy that the use of certain viral vectors in immunocompetent rodents predictably elicits a significant host immunity (both humoral and cellular) which tends to limit the usefulness of the gene therapy.⁶³⁻⁶⁶ With these considerations in mind,

it may be prudent to confirm the initial positive therapeutic results from nude mice in immunocompetent hosts as well.

Assessing Treatment Response: Establishing Therapeutic End-Points

Traditionally, experimental anti-cancer therapies in animals are assessed based on the following parameters:

- a. *Protection against tumour challenge.* This parameter is commonly employed in experimental immunotherapy, including genetic immunotherapy, in which naïve animals are pretreated with the therapeutic agent, followed by the inoculation of the animals with a bolus of tumour cells. The failure of tumour-take in the pretreated animals, in contrast to controls, provides evidence of efficacy. Such a system is artificial and does not resemble the real clinical scenario, but it does establish proof-of-principle.
- b. *Regression of-established tumours.* This parameter more closely mimics clinical scenario in which patients present with established cancers. In assessing primary tumour regression, two perpendicular diameters of the solitary tumours are measured (a and b, where a is the longer of the two); the tumour size is expressed as either the tumour surface area ($a \times b$) or the volume ($0.5 \times a \times b^2$).² The assessment of metastatic tumour regression is more complicated. Several methods have been used. The commonest one involves the manual quantification of the number of metastatic deposits on the surfaces of the organs of interest.^{14,16,18} The ease and accuracy of such a method can be significantly enhanced by staining with Indian ink and Fekete's solution, or by the use of tumour cells which have been genetically altered to expressed genes such as the LacZ. LacZ-expressing tumours would stain blue when immersed in X-gal solution.^{14,16,18} Other methods of metastatic tumour quantification involve computer-assisted image analysis,^{67,68} and the use of molecular probes for repetitive human DNA (e.g. human Alu) sequences if human xenografts were used.⁶⁹
- c. *Prolongation of survival.* One of the most reliable and clinically meaningful parameter to assess treatment efficacy in patients is the prolongation of survival in the treated patients as compared to controls. In murine metastatic tumour models, animals with progressive tumours often perish rather rapidly from ill-health and/or organ-specific complications such as respiratory failure in the metastatic lung model. Prolongation of survival in the treated animals is a robust indication of treatment efficacy.

Besides using the above end-points to evaluate treatment responses, there are other biological parameters that need to be assessed in cancer gene therapy as well.

These include the documentation of successful *in vivo* gene transfer, demonstration of relevant mRNA and/or protein production in transduced tissues, and the evaluation of vector- and transgene-specific host-mediated immunity which may significantly influence the extent and duration of gene expression.

An exciting development in the study of metastasis is the use of intravital videomicroscopy (IVVM) which provides "live" demonstration of the intricate interactions between the metastatic cells and their new local milieu. The advent of IVVM has allowed us to better understand the metastatic cascade, including the pivotal role of post-extravasation events in the successful engraftment of distant metastases.^{25-29,70}

Use of Murine Metastatic Tumour Models In Cancer Gene Therapy: Examples

A few examples of the use of gene therapy approaches to treat murine metastatic tumours will now follow to illustrate some of the principles discussed above.

Genetic Immunotherapy

Caruso et al¹⁵ described a model of colon cancer liver metastasis in which 5×10^4 MCA-26 colon carcinoma cells were directly implanted into the liver in syngeneic Balb/c mice. An adenovirus vector carrying the murine interleukin (IL)-2 gene and another vector carrying the IL-12 gene were separately injected directly into the growing liver tumours. Tumour regression and prolongation of survival in the treated animals were clearly documented with either gene.¹⁵ Wang et al¹⁴ described another genetic immunotherapy strategy in which a recombinant fowlpoxvirus gene vector encoding a model tumour antigen effectively immunised naïve Balb / c mice against subcutaneous and intravenous challenge by CT26.CL25 tumour cells which bore the model tumour antigen (β gal). Besides conferring protection against metastatic tumour challenge, the investigators showed that mice bearing established 3-day pulmonary metastases also showed tumour regression following *in vivo* gene therapy. Hurford et al⁴⁸ generated liver metastases by intrasplenic injection of tumour cells. Using the same route of administration, they showed that retroviral producer cell lines selectively transferred reporter genes to the liver metastases. Indeed, intrasplenic administration of retroviral producer cell lines encoding IL-2 and IL-4 led to reduction in liver metastases. Song et al¹⁶ demonstrated that genetically-modified dendritic cells induced the production of tumour antigen-specific cytotoxic T-lymphocytes in immunocompetent mice, and that the modified dendritic cells conferred potent protective and therapeutic anti-tumour effect. It is clear from these examples that genetic immunotherapy is capable of producing systemic treatment responses against widespread metastases.

Gene-mediated Tumour Antiangiogenesis

Besides genetic immunotherapy, another promising gene therapy strategy that may effectively target regional metastases is the generation of therapeutic proteins in transduced organs which inhibit the angiogenesis cascade.^{17,18} Using a replication-deficient adenovirus vector encoding a soluble form of the flt-1 vascular endothelial growth factor (VEGF) receptor, Kong et al¹⁸ demonstrated that established liver metastases could be effectively suppressed following intravenous administration of the vector. It has been shown previously that intravenous administration of adenovirus vectors predictably leads to hepatic localisation. Importantly, treated animals enjoyed a significant survival advantage. Further, the investigators showed that established pulmonary metastases could be suppressed in a regional fashion following intratracheal vector administration. One could, for instance, treat one lung and not the other by selective administration of the gene vector to the respective bronchus - a feat that cannot be achieved by systemic administration of conventional cytotoxic drugs.

Conclusions

With the recognition that metastatic tumours are the cause for most cancer treatment failures and cancer-related mortality, there have been recent efforts at establishing suitable animal metastatic tumour models which closely mimic the human disease in terms of their biology and responses to treatment.⁴ By virtue of its mechanism of action, gene therapy, perhaps more so than other forms of cancer therapeutics, relies heavily on its experimentation in animal tumour models. Although gene therapy has been used effectively to treat established murine metastatic tumour models, one must be very careful when extrapolating these results to the humans. Humans are not merely large mice-in contrast to the laboratory mice which are often genetically defined and uniform (inbreds), the humans to whom the results are extrapolated are highly variable outbreds, with striking genetic, environmental, social and behavioural differences. These aspects of the human population will be impossible to simulate in animal models. As a result, data (therapeutic or toxicologic) obtained from inbred animals will often be more consistent and predictable than will be observed in human populations. Therefore, while animal tumour models provide useful proof-of-principle and basic toxicological data, the real therapeutic efficacy and clinical utility can only be determined in appropriately designed human trials.

The ethical dilemmas surrounding animal experimentation have attracted passionate discussions from its proponents and opponents alike for decades. Numerous books and manuscripts have been written on this subject which, to date, remains a highly contentious issue. It is beyond the scope of this review to discuss this issue any

further. Suffices to state that the great potential benefits to humans of using animals in research provides a powerful reason for continuing to use them; yet, at the same time, the harms and suffering which can be caused to the animals provide an equally powerful reason for ceasing to do so. Thus, it was argued that we ought to regard the use of animals with "sentiments stronger than regret, if weaker than remorse" - a "tragic choice" of sort.⁷¹ It is the hope of this author that this review has provided some rationale for the use of metastatic tumour models in cancer gene therapy research, so that appropriate models will be chosen in future experiments to yield the most useful scientific information, with the least amount of animal sacrifice.

REFERENCES

1. Fine DL, Shoemaker R, Gazdar A, Mayo J G, Fodstad O, Boyd M R, et al. Metastasis models for human tumors in athymic mice: useful models for drug development. *Cancer Detect Prev Suppl* 1987;1:291-9.
2. Fodstad O, Aass N, Phil A. Assessment of tumour growth and of response to chemotherapy of human melanomas in athymic nude mice. *Br J Cancer* 1980; 41(Suppl IV):146-9.
3. Price J E. Analyzing the metastatic phenotype. *J Cell Biochem* 1994; 56:1-22.
4. Dore J-F, Bailly M, Bertrand S. Metastases of human tumors in experimental animals. *Anticancer Res* 1987; 7:997-1004.
5. Lundy J, Greiner J W, Colcher D. Development of a metastatic human colon cancer xenograft model in the nude mouse. *J Surg Oncol* 1986; 31:260-7.
6. Talmadge J E, Fidler I J. Enhanced metastatic potential of tumor cells harvested from spontaneous metastases of heterogenous murine tumors. *J Natl Cancer Inst* 1982; 69:975-80.
7. Neulat-Duga I, Sheppel A, Marty C, Lacroux F, Pourrat J, Caveriviere P, et al. Metastases of human tumor xenografts in nude mice. *Invasion Metastasis* 1984; 4:209-24.
8. Giavazzi R, Garofalo A. The nude mouse for the study of human colorectal carcinoma. *Adv Exp Med Biol* 1988; 233:39-47.
9. Giavazzi R, Campbell D E, Jessup J M, Cleary K, Fidler I J. Metastatic behaviour of tumor cells isolated from primary and metastatic human colorectal carcinomas implanted into different sites in nude mice. *Cancer Res* 1986; 46:1928-33.
10. Mullen C A, Coale M M, Lowe R, Blaese R M. Tumors expressing the cytosine deaminase suicide gene can be eliminated *in vivo* with 5-fluorocytosine and induce protective immunity to wild type tumor. *Cancer Res* 1994;1503-6.
11. Mulligan R C. The basic science of gene therapy. *Science* 1993; 260:926-32.
12. Crystal R G. Transfer of genes to humans: early lessons and obstacles to success. *Science* 1995; 270:404-10.
13. Chen S-H, Chen X H L, Wang Y, Kosai K-I, Finegold M J, Rich S S, et al. Combination gene therapy for liver metastasis of colon carcinoma *in vivo*. *Proc Natl Acad Sci USA* 1995; 92:2577-81.
14. Wang M, Bronte V, Chen P W, Gritz L, Panicali D, Rosenberg S A, et al. Active immunotherapy of cancer with a nonreplicating recombinant fowlpox virus encoding a model tumor-associated antigen. *J Immunol* 1995; 154:4685-92.
15. Caruso M, Pham-Nguyen K, Kwong Y-L, Xu B, Kosai K-I, Finegold M, et al. Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. *Proc Natl Acad Sci USA* 1996; 93:11302-6.
16. Song W, Kong H-L, Carpenter H, Torii H, Granstein R, Rafii S, et al. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor

- immunity. *J Exp Med* 1997; 186:1247-56.
17. Kong H-L, Crystal R G. Gene therapy strategies for tumor antiangiogenesis. *J Natl Cancer Inst* 1998; 90:273-86.
 18. Kong H-L, Hecht D, Song W, Kovesdi I, Hackett N R, Yayon A, et al. Regional suppression of tumor growth by *in vivo* transfer of a cDNA encoding a secreted form of the extracellular domain of the flt-1 vascular endothelial growth factor receptor. *Hum Gene Ther* 1998; 9:823-33.
 19. Kozlowski J M, Fidler I J, Campbell D, Xu Z L, Kaighn M E, Hart I R. Metastatic behavior of human tumor cell lines grown in the nude mouse. *Cancer Res* 1984; 44:3522-9.
 20. Chen P W, Wang M, Bronte V, Zhai Y, Rosenberg S A, Restifo N I. Therapeutic antitumor response after immunization with a recombinant adenovirus encoding a model tumor-associated antigen. *J Immunol* 1996; 156:224-31.
 21. Sharkey F E, Fogh J. Metastasis of human tumors in athymic nude mice. *Int J Cancer* 1979; 15:733-8.
 22. Sharkey F E, Fogh J. Incidence and pathological features of spontaneous tumors in athymic nude mice. *Cancer Res* 1979; 39:833-9.
 23. Price J E. Metastasis from human breast cancer cell lines. *Breast Cancer Res Treat* 1996; 39:93-102.
 24. Watts A M, Shearer M H, Pass H I, Kennedy R C. Development of an experimental murine pulmonary metastasis model incorporating a viral encoded tumor specific antigen. *J Virol Methods* 1997; 69:93-102.
 25. MacDonald I C, Schmidt E E, Morris V L, Chambers A F, Groom A C. Intravital videomicroscopy of the chorioallantoic microcirculation: a model system for studying metastasis. *Microvasc Res* 1992; 44:185-99.
 26. Morris V L, MacDonald I C, Koop S, Schmidt E E, Chambers A F, Groom A C. Early interactions of cancer cells with the microvasculature in mouse liver and muscle during hematogenous metastasis: videomicroscopic analysis. *Clin Exp Metastasis* 1993; 11:377-90.
 27. Chambers A F, MacDonald I C, Schmidt E E, Koop S, Morris V L, Khokha R, et al. Steps in tumor metastasis: new concepts from intravital videomicroscopy. *Cancer Metastasis Rev* 1995; 14:279-301.
 28. Koop S, MacDonald I C, Luzzi K, Schmidt E E, Morris V L, Grattan M, et al. Fate of melanoma cells entering the microcirculation: over 80% survive and extravasate. *Cancer Res* 1995; 55:2520-3.
 29. Koop S, Schmidt E E, MacDonald I C, Morris V L, Khokha R, Grattan M, et al. Independence of metastatic ability and extravasation: metastatic ras-transformed and control fibroblasts extravasate equally well. *Proc Natl Acad Sci USA* 1996; 93:11080-4.
 30. Giavazzi R, Jessup J M, Campbell D E, Walker S M, Fidler I J. Experimental nude mouse model of human colorectal cancer liver metastases. *J Natl Cancer Inst* 1986; 77:1303-8.
 31. Fodstad O, Kjonniksen I. Microenvironment revisited: time for reappraisal of some prevailing concepts of cancer metastasis. *J Cell Biochem* 1994; 56:23-8.
 32. Aeurbach R, Morrissey L W, Sidky Y A. Regional differences in the incidence and growth of mouse tumors following intradermal or subcutaneous inoculation. *Cancer Res* 1978; 38:1739-44.
 33. Sharkey F E. Considerations in the use of nude mice for cancer research. *Cancer Metastasis Rev* 1984; 3:341-60.
 34. Varki N M, Viswanathan B, Vu T. Endothelial cells enhance spontaneous metastasis of human lung carcinoma cells in athymic mice. *Cancer Lett* 1990; 51:251-7.
 35. Furukawa T, Kubota T, Watanabe M, Kuo T H, Nishibori H, Kase S, et al. A metastatic model of human colon cancer constructed using cecal implantation of cancer tissue in nude mice. *Surg Today* 1993; 23:420-3.
 36. Murthy M S, Scanlon E F, Jelachich M L, Klipstein S, Goldschmidt R A. Growth and metastasis of human breast cancers in athymic nude mice. *Clin Exp Metastasis* 1995; 13:3-15.
 37. Lee C, Shevrin D H, Kozlowski J M. *In vivo* and *in vitro* approaches to study metastasis in human prostate cancer. *Cancer Metastasis Rev* 1993; 12:21-8.
 38. Furukawa T, Kubota T, Watanabe M, Nishibori H, Kuo T-H, Saikawa Y, et al. A suitable model for experimental liver metastasis of human colon cancer xenografts using mice with severe combined immunodeficiency. *J Surg Oncol* 1993; 52:64-7.
 39. Price J E, Polyzos A, Zhang R D, Daniels L M. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res* 1990; 50:717-21.
 40. Naito S, Eschenbach A C, Giavazzi R, Fidler I J. Growth and metastasis of tumor cells isolated from a human renal cell carcinoma implanted into different organs of nude mice. *Cancer Res* 1986; 46:4109-15.
 41. Wang X, Fu X, Kubota T, Hoffman R M. A new patient-like metastatic model of human small-cell lung cancer constructed orthotopically with intact tissue via thoracotomy in nude mice. *Anticancer Res* 1992; 12:1403-6.
 42. Berlin O, Samid D, Donthineni-Rao R, Akeson W, Amiel D, Jr V L W. Development of a novel spontaneous metastasis model of human osteosarcoma transplanted orthotopically into bone of athymic mice. *Cancer Res* 1993; 53:4890-5.
 43. Kubota T. Metastatic models of human cancer xenografted in the nude mouse: the importance of orthotopic transplantation. *J Cell Biochem* 1994; 5:4-8.
 44. Manzotti C, Audisio R A, Pratesi G. Importance of orthotopic implantation for human tumors as model systems: relevance to metastasis and invasion. *Clin Exp Metastasis* 1993; 11:5-14.
 45. Wexler H, Chretien P B, Ketcham A S, Sindelar W F. Induction of pulmonary metastases in both immune and nonimmune mice. Effect of removal of a transplanted primary tumor. *Cancer* 1975; 36:2042-7.
 46. O'Reilly M S, Holmgren L, Shing Y, Chen C, Rosenthal R A, Moses M, et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis Lung Carcinoma. *Cell* 1994; 79:315-28.
 47. Kjonniksen I, Storeng R, Phil A, McLemore T L, Fodstad O. A human tumor lung metastasis model in athymic nude rats. *Cancer Res* 1989; 49:5148-52.
 48. Hurford R K, Dranoff G, Mulligan R C, Tepper R I. Gene therapy of metastatic cancer by *in vivo* retroviral gene targeting. *Nature Genet* 1995; 10:430-5.
 49. Naomoto Y, Kondo H, Tanaka N, Orita K. Novel experimental models of human cancer metastasis in nude mice: lung metastasis, intraabdominal carcinomatosis with ascites, and liver metastasis. *J Cancer Res Clin Oncol* 1987; 113:544-9.
 50. Shevrin D H, Kukreja S C, Ghosh L, Lad T E. Development of skeletal metastasis by human prostate cancer in athymic nude mice. *Clin Exp Metastasis* 1988; 6:401-9.
 51. Olden K. Human tumor bone metastasis model in athymic nude mice. *J Natl Cancer Inst* 1990; 82:340-1.
 52. Conley F K. Murine models of metastatic neoplasia to the central nervous system. *Cancer Metastasis Rev* 1982; 1:203-13.
 53. Schackert G, Fidler I J. Development of *in vivo* models for studies of brain metastasis. *Int J Cancer* 1988; 41:589-94.
 54. Pantelouris E M. Absence of thymus in a mouse mutant. *Nature* 1968; 217:370-1.
 55. Rygaard J, Povlsen C. Heterotransplantation of a human malignant tumour to "Nude" mice. *Acta Pathol Microbiol Scand* 1969; 77:758-60.
 56. Brunner N, Boysen B, Romer J, Spang-Thomsen M. The nude mouse as an *in vivo* model for human breast cancer invasion and metastasis. *Breast Cancer Res Treat* 1993; 24:257-64.
 57. Winograd B, Boven E, Lobbezoo M W, Pinedo H M. Human tumor xenografts in the nude mouse and their value as test models in anticancer drug development (review). *In vivo* 1987; 1:1-13.
 58. Sato N, Michaelides M C, Wallack M K. Characterization of tumorigenicity, mortality, metastasis, and splenomegaly of two cultured murine colon lines. *Cancer Res* 1981; 41:2267-72.
 59. Richie J P, McDonald J, Gittes R F. Resistance to intravenous tumor metastases in the nude mouse: paradoxical response. *Surgery* 1981; 90:214-20.
 60. Mueller B M, Reisfeld A. Potential of the scid mouse as a host for human tumors. *Cancer Metastasis Rev* 1991; 10:193-200.
 61. Naito S, Giavazzi R, Walker S M, Itoh K, Mayo J, Fidler I J. Growth and metastatic behavior of human tumor cells implanted into nude and beige nude mice. *Clin Exp Metastasis* 1987; 5:135-46.
 62. Xie X, Brunner N, Jensen G, Albrectsen J, Gotthardsen B, Rygaard J. Comparative studies between nude and scid mice on the growth and metastatic behavior of xenografted human tumors. *Clin Exp Metastasis* 1992; 10:201-10.

63. Yang Y, Li Q, Ertl H C, Wilson J M. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 1995; 69:2004-15.
64. Yang Y, Su Q, Wilson J M. Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. *J Virol* 1996; 70:7209-12.
65. Frey B M, Cannizzo S, Crystal R G, Moore M A S. Expression of human thrombopoietin transgene delivered by an adenovirus based strategy is dependent on host immune competence. *Exp Hematol* 1996;1056.
66. Song W, Kong H-L, Traktman P, Crystal R G. Cytotoxic T lymphocyte responses to proteins encoded by heterologous transgenes transfected *in vivo* by adenoviral vectors. *Hum Gene Ther* 1997; 8:1207-17.
67. Watts A M, Kennedy R C. Quantitation of tumor foci in an experimental murine tumor model using computer-assisted video imaging. *Anal Biochem* 1998; 256:217-9.
68. Iwahana M, Tohgo A, Tanaka N G, Osada Y. Quantification of experimental pulmonary metastases by image analyzer. *In vivo* 1991; 5:323-8.
69. Shoemaker R H, Smythe A M, Wu L, Balaschak MS, Boyd MR. Evaluation of metastatic human tumor burden and response to therapy in a nude mouse xenograft model using a molecular probe for repetitive human DNA sequences. *Cancer Res* 1992; 52:2791-6.
70. Chambers A F, Schmidt E E, MacDonald I C, Morris V L, Groom AC. Early steps in hematogenous metastasis of B16F1 melanoma cells in chick embryos studied by high-resolution intravital videomicroscopy. *J Natl Cancer Inst* 1992; 84:797-803.
71. Smith J A, Boyd KM. Ethics and laboratory animals: Can the use of animals in experiments be justified. In: Tuffery AA, editor. *Laboratory Animals: An Introduction for Experimenters*. West Sussex: John Wiley & Sons Ltd, 1995:1-15.
72. Cai D W, Mukhopadhyay T, Liu Y, Fujiwara T, Roth J A. Stable expression of the wild-type p53 gene in human lung cancer cells after retrovirus-mediated gene transfer. *Hum Gene Ther* 1993; 4:617-624.
73. Liu T J, Zhang W W, Taylor D L, Roth J A, Goepfert H, Clayman G L. Growth suppression of human head and neck cancer cells by the introduction of a wild-type p53 gene via a recombinant adenovirus. *Cancer Res* 1994; 54:3662-7.
74. Xu H J, Zhou Y, Seigne J, Perng G S, Mixon M, Zhang C, et al. Enhanced tumor suppressor gene therapy via replication-deficient adenovirus vectors expressing an N-terminal truncated retinoblastoma protein. *Cancer Res* 1996; 15:2245-9.
75. Askari F K, McDonnell W M. Antisense-oligonucleotide therapy. *N Engl J Med* 1996; 334:316-8.
76. Feng M, Cabrera G, Deshane J, Scanlon K J, Curiel D T. Neoplastic reversion accomplished by high efficiency adenoviral-mediated delivery of an anti-ras ribozyme. *Cancer Res* 1995; 55:2024-8.
77. Monia B I, Johnston J F, Geiger T, Muller M, Fabbro D. Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against C-raf kinase. *Nat Med* 1996; 2:668-75.
78. Mukhopadhyay T, Tainsky M, Cavender A C, Roth J A. Specific inhibition of K-ras expression and tumorigenicity of lung cancer cells by antisense RNA. *Cancer Res* 1991; 15:1744-8.
79. Huber B E, Austin E A, Richards C A, Davis S T, Good S S. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci USA* 1994; 91:8302-6.
80. Hirschowitz E A, Ohwada A, Pascal W R, Russi T J, Crystal R G. *In vivo* adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther* 1995; 6:1055-63.
81. Culver K W, Ram Z, Wallbridge S, Ishii H, Oldfield E H, Blaese R M. *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* 1992; 256:1550-2.
82. Bonini C, Ferrari G, Verzeletti S, Servida I, Zappone E, Ruggieri L, et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* 1997; 276:1719-24.
83. Deonarain M I, Spooner R A, Epenetos A A. Genetic delivery of enzymes for cancer therapy. *Gene Ther* 1995; 2:235-44.
84. Mickisch G H, Aksejtijevich I, Schoenlein P V, Goldstein L J, Galski H, Stahle C, et al. Transplantation of bone marrow cells from transgenic mice expressing the human MDR1 gene results in long-term protection against the myelosuppressive effect of chemotherapy in mice. *Blood* 1992; 79:1087-93.
85. Sorrentino B P, Brandt S J, Bodine D, Gottesma N M, Pasta N I, Cline A, et al. Selection of drug-resistant bone marrow cells *in vivo* after retroviral transfer of human MDR1. *Science* 1992; 257:99-103.
86. Rosenberg S A. Karnofsky Memorial Lecture. The immunotherapy and gene therapy of cancer. *J Clin Oncol* 1992; 10:180-99.
87. DeBruyne L. Treatment of malignancy by direct gene transfer of a foreign MHC class I molecule. *Cancer Immunol Immunother* 1996; 43:180-9.
88. Nabel G J, Gordon D, Bishop D K, Nickoloff B J, Yang Z Y, Aruga A, et al. Immune response in human melanoma after transfer of an allogeneic class I major histocompatibility complex gene with DNA-liposome complexes. *Proc Natl Acad Sci USA* 1996; 93:15388-93.
89. McDonnell M M, Askari F K. DNA vaccines. *N Engl J Med* 1996; 334:42-5.
90. Tanaka T, Manome Y, Wen P, Kufe D W, Fine H A. Viral vector-mediated transduction of a modified platelet factor 4 cDNA inhibits angiogenesis and tumor growth. *Nat Med* 1997; 3:437-42.
91. Tanaka T, Cao Y, Folkman J, Fine H A. Viral vector-targeted antiangiogenic gene therapy utilizing an angiostatin complementary DNA. *Cancer Res* 1998; 58:3362-9.
92. Millauer B, Longhi M I, Plate K H, Shawver L K, Risau W, Ullrich A, et al. Dominant-negative inhibition of Flk-1 suppresses the growth of many tumor types *in vivo*. *Cancer Res* 1996; 56:1615-20.
93. Goldman C K, Kendall R L, Cabrera G, Soroceanu L, Heike Y, Gillespie G Y, et al. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc Natl Acad Sci USA* 1998; 95:8795-800.