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lacZ Transduced Human Breast Cancer Xenografts as an *in vivo* Model for the Study of Invasion and Metastasis

Nils Brünner, Erik W. Thompson, Mogens Spang-Thomsen, Jørgen Rygaard, Keld Danø and James A. Zwiebel

A number of human cancer cell lines have been described as being invasive and metastatic in immune incompetent animals. However, it is difficult to assess metastatic spread of a subcutaneously injected or inoculated cell line, since an exact detection of all microfoci of human tumour cells in the animals by usual histological procedures would require extensive sectioning of the whole animal. To overcome this problem, we transduced human breast cancer cells with a replication-defective Moloney murine leukaemia retroviral vector (M-MuLV) containing both *neo*^{R} (neomycin resistance) and *lacZ* genes. The resulting cell lines were selected for antibiotic (G418) resistance, and cell-sorted for *lacZ* expression. *lacZ* continued to be expressed in cultured cells for at least 20 passages without further G418 selection. The *lacZ* gene codes for β-D-galactosidase, and cells expressing this gene stain blue with the chromogenic substrate X-gal. The *lacZ*-expressing cells retained the pre-transduction ability to traverse Matrigel *in vitro*, to form subcutaneous tumours in nude mice, and to grow invasively with the formation of metastases. X-gal staining showed high specificity, staining the tumour cells but not the surrounding mouse tissue on either whole tissue blocks or histological sections. The staining procedure was highly sensitive, allowing detection of microfoci of human cancer cells, and quantitative estimation of the metastatic capacity of the cells. These results indicate that *lacZ* transduction of human tumour cells is a powerful means of studying human cancer cell invasion and metastases *in vivo*.
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INTRODUCTION

DISSEMINATION OF cancer involves escape of cells from the primary tumour, degradation of the normal tissue and migration, intravasation, homing, extravasation and colonisation in an environment potentially different from the original location. Although cancer cells are known to produce proteolytic enzymes, adhesion molecules and integrins which may all contribute to the invasive and metastatic phenotype, the mechanisms involved in the metastatic process are not fully understood.

Human cancer cell invasion studies have mainly been confined to *in vitro* conditions [1, 2]. A major limitation of *in vitro* models for this type of study, however, is the lack of the many host factors which participate in the physiological and pathological mechanisms contributing to the malignant phenotype. Important interactions that occur between the cancer cells and the surrounding non-malignant tissue include the interaction of the cancer cells with extracellular matrix and stromal cells, i.e. fibroblasts, endothelial cells, parenchymal cells and other host cells capable of non-immune host reactions [3, 4]. In addition, the host immune system may be a barrier which the cancer cells must overcome before they can disseminate into various host tissues. Thus, the cancer cells exist in a competitive microenvironment with surrounding normal cells as well as non-malignant tumour-infiltrating cells, and the ability of the cancer cells to dominate and evade host non-immune and immune responses determines their invasive/metastatic capacity.

Only a few experimental models are available for *in vivo* investigations of human cancer cell invasion and metastasis [5, 6]. One model is the athymic nude mouse [7, 8]. However, most studies in this model have been based on injections of tumour cells intravenously, and this approach involves only the later steps of the metastatic process. Subcutaneously inoculated or serially transplanted tumours demonstrating local invasion and spontaneous metastatic spread are a more adequate model for the invasive and metastatic behaviour of cancer cells. However, not only has there been a limited success in establishing human tumour cell lines that reproducibly spread in these animals after subcutaneous inoculation, but difficulties in identifying small metastases throughout the mouse have also been a significant problem [9]. A number of human melanoma cell lines have been found to metastasise in nude mice [10, 11], and the expression of melanin in some of these melanoma cell lines facilitated the identification of metastasis in the nude mouse. Unfortunately, melanin production is restricted to certain malignancies, and therefore cannot be used as a general marker for the identification of human cancer cells in the nude mouse.

Recently, Lin *et al.* [12, 13] demonstrated that metastases in mice injected with *ras*-transformed mouse 3T3 cells transfected with the *E. Coli lacZ* gene were easily detected following X-gal staining. The *lacZ* gene codes for β-D-galactosidase, the activity of which can be detected by staining with the chromogenic substrate 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), which gives a dark blue reaction product. In order to identify human cancer cells after their dissemination in the nude mouse, we adopted the method used by Lin *et al.* by genetically labelling human breast cancer cells with the bacterial *lacZ* gene.

Correspondence to N. Brünner.
N. Brünner and K. Danø are at the Finsen Laboratory, Strandboulevarden 49, 2100 Copenhagen, Denmark; E.W. Thompson and J.A. Zwiebel are at the Lombardi Cancer Center, Georgetown University, Washington DC, U.S.A.; M. Spang-Thomsen is at the University Institute of Pathological Anatomy, University of Copenhagen, Copenhagen; and J. Rygaard is at the Bartholin Institute, Kommunehospitalet, Copenhagen, Denmark.
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Two human breast cancer cell lines, MDA-MB-231 and MDA-MB-435, were used in the study. These cells are invasive when injected subcutaneously into nude mice [14]. Both cell lines were transduced with the BAG Moloney murine leukaemia retroviral vector encoding for both β-galactosidase and *neo*^{R} genes. The latter gene confers resistance to the antibiotic G418. Stable *lacZ* expressing cell lines were established by G418 selection followed by Fluorescence Activated Cell Sorting (FACS) using a fluorescein-conjugated substrate for β-galactosidase. The cells were then inoculated subcutaneously into athymic nude mice and tested for tumorigenicity, growth rate, invasiveness, metastatic capacity, and for a number of phenotypic characteristics *in vitro*.

The results demonstrate that X-gal staining of whole mouse organs allowed visual detection of even very small metastatic foci. Subsequent histologic examination of the stained areas confirmed the presence of human cancer cells. X-gal did not stain normal mouse tissue. Furthermore, *lacZ* transduction did not alter the neoplastic cells phenotype, including invasive and metastatic growth mode in nude mice. We, therefore, conclude that *lacZ* transduction of human cancer cells is a highly specific and sensitive method for quantitative detection of invasive and metastatic human tumour cells in the nude mouse. The model is useful both for examining interactions between tumour cells and host tissue in relation to the invasive and malignant phenotype, and for quantitativly evaluating the effect of drugs with may interfere with biological events involved in the invasive and metastatic process.

MATERIALS AND METHODS

Cell lines

The MDA-MB-231 human breast cancer cell line was originally obtained from ATCC, Maryland, USA, and the MDA-MB-435 human breast cancer cell line was kindly provided by Dr Janet Price, MD Anderson Hospital, Houston, Texas, USA. The cells were routinely propagated in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, UK) supplemented with 10% fetal calf serum (D10). Cells for nude mouse experiments were harvested with a cell scraper. All cell lines were tested and found free from mycoplasma contamination.

Nude mice

In all experiments described, 6–8-week-old intact female nu/nu-META/Bom (Bomholtgaard, Denmark) nude mice were used. The mice were kept in sterile laminar flow clean benches at 25°C and 50% humidity. At the end of the experiment, the mice were killed by cervical dislocation.

Tumour growth

Tumours were measured twice weekly in two dimensions and tumour growth curves were constructed on the basis of a transformed Gompertz function [15].

Retroviral transduction

Viral stocks of the BAG vector (Fig. 1) [16] packaged in PA317 cells [17] (kindly provided by Constance Cepko) were used to transduce the breast cancer cell lines. MDA-MB-231 and MDA-MB-435 cells were plated 1 day before infection at a 1:10 split ratio. To infect the cells, the culture medium was replaced with 5 ml of viral supernatant containing 4 μg/ml of polybrene and incubated for 2 h at 37°C in a 5% CO_2 incubator. An additional 5 ml of D10 was then added and the cells were returned to the incubator. The medium was replaced with fresh D10 the next day. The resulting cell lines were named MDA-MB-231 BAG and MDA-MB-435 BAG, respectively.

Fig. 1. Schematic illustration of the BAG vector [12]. The BAG vector consists of the *lacZ* gene under the transcriptional control of the Moloney murine leukemia long terminal repeat (LTR) promotor and the SV40 promoted *neo*^{R} gene.

Selection procedure

To increase the number of cells containing the BAG vector, the infected cell populations were grown in medium containing 500 μg/ml G418. The selected cells, however, did not all express β-galactosidase as determined by X-gal staining (see below). Therefore, cells were subjected to fluorescein-di-β-D-galactopyranoside (FDG)-FACS selection, as described by Nolan *et al.* [18]. In this procedure, the release of fluorescein from a non-fluorescent substrate within cells expressing the *lacZ* gene product, β-galactosidase, allows cell separation in a flow cytometer. A confluent 100 mm dish was trypsinised and the single cell suspension was adjusted to 1–5 × 10^7 cells/ml in D10. 100 μl of the cell suspension was incubated at 37°C in a Falcon 2058 tube. Uptake of the substrate FDG by the cells was accomplished by hypotonic shock with the addition of 100 μl of 2 mmol/l FDG in dH_2O. Following a 1 min incubation at 37°C, the substrate was trapped within the cells by the addition of 1.8 ml ice-cold D10. The cells were incubated on ice for 1 h and sorted on a Becton Dickinson dual laser FACStar Plus flow cytometer set to a 488 nm wavelength.

Cell inoculation

For all experiments, 2 × 10^6 tumour cells were inoculated subcutaneously bilaterally into the flanks of the animals. The cells were placed below the thoracic wall. The mice were killed 6 or 8 weeks after cell inoculation.

X-gal staining of cells in culture

Cells in culture were placed on glass slides and allowed to grow for 2 days. They were then fixed with 0.5% (vol/vol) glutaraldehyde in PBS for 5 min, washed three times with PBS, and incubated overnight at 37°C in X-gal staining solution (1 mg X-gal/ml; 35 mmol/l potassium ferricyanide; 35 mmol/l potassium ferrocyanide; 2 mmol/l $MgCl_2$ in PBS).

X-gal staining of tumours and whole organs

Primary tumours and whole organs (liver, spleen, pancreas, intestine and lungs) were dissected from the animals and placed in a mixture of 2% (vol/vol) paraformaldehyde and 0.2% (vol/vol) glutaraldehyde in PBS for 2–3 h at 4°C. After fixation, the tissue blocks were rinsed three times with PBS and then incubated for 24 h at 4°C in 1 mg X-gal/ml; 35 mmol/l potassium ferricyanide; 35 mmol/l potassium ferrocyanide; 2 mmol/l $MgCl_2$; 0.02% (vol/vol) Nonidet P-40; 0.01% (wt/vol) sodium deoxycholate in PBS. The tissue blocks were then rinsed, first with 3% (vol/vol) dimethyl sulphate in PBS and then with PBS only. Until photographs were taken, the tissues were stored at 4°C in 0.02% sodium azide in PBS. Photographs were taken through an inverted dissecting microscope.

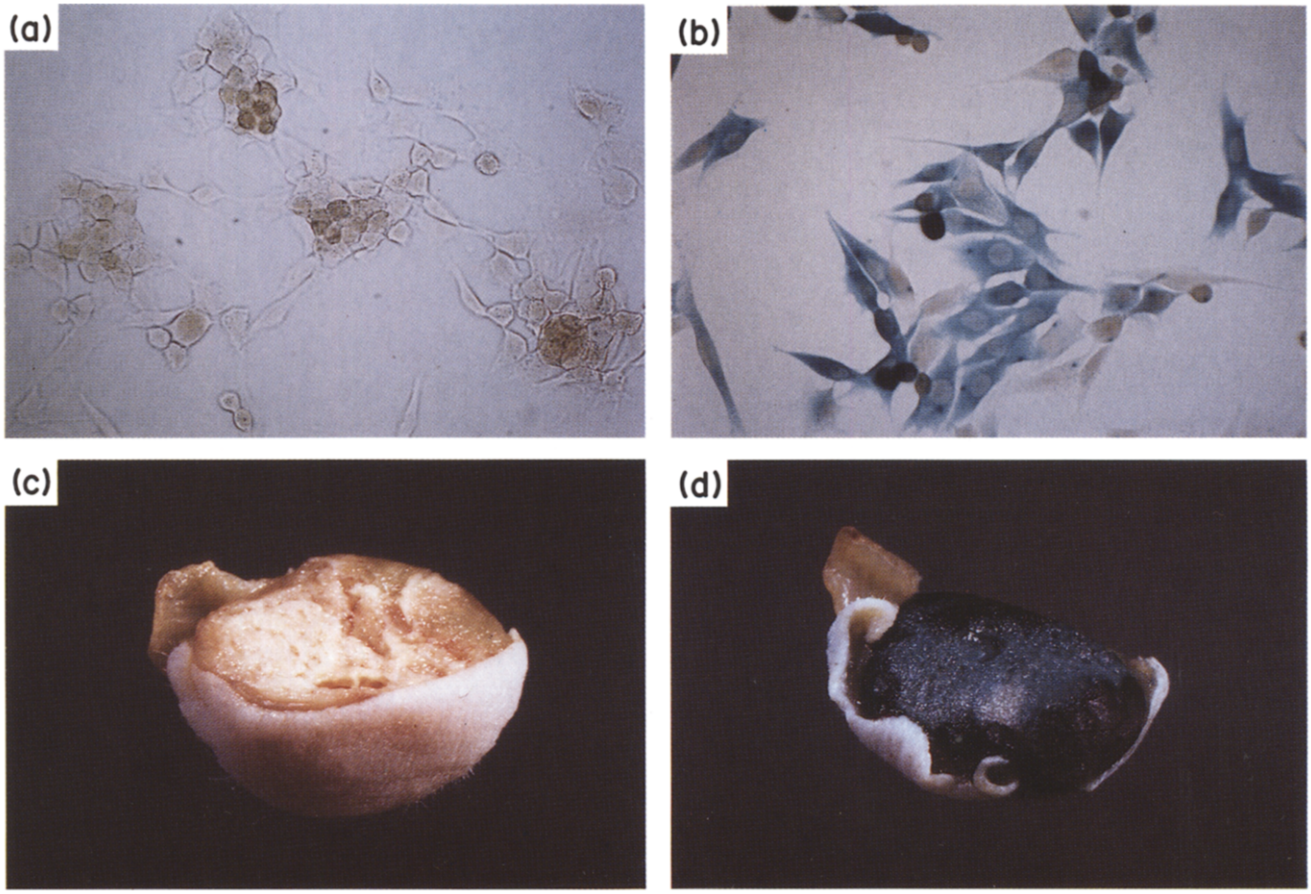

Fig. 2. X-gal staining of tumour cells grown *in vitro* and *in vivo*. Cells and tumours were fixed and processed for X-gal staining (see Materials and Methods). (a) MDA-MB-435 cells; (b): MDA-MB-435 BAG cells at passage 22 after transduction; (c) subcutaneous MDA-MB-231 xenograft; (d) subcutaneous MDA-MB-231 BAG xenograft passage 2 in nude mice.

Frozen sections

Organs were fixed at 4°C for 2 h in 2% paraformaldehyde in 0.1 mol/l PBS, pH 7.4, rinsed with 0.1 mol/l PBS and dehydrated in 0.1 mol/l PBS with 7% sucrose for 2 h and 15% sucrose for additional 2 h, and finally processed for cryo-sections. The cryo-sections were then stained with X-gal following the procedure used for the cell lines.

X-gal staining of ascites

Smears of ascites fluid were produced on glass slides from the intraperitoneal cavity. The slides were dried and subsequently fixed and stained as described for cells in culture.

Histology

After paraffin embedding and haematoxylin–eosin (HE) staining, selected areas from primary tumours and from secondary foci identified by X-gal staining were processed for routine histological examination.

Matrigel assay

The Boyden chamber chemoinvasion assay was performed as described previously [1]. Matrigel, a mixture of basement membrane components, was kindly provided by Dr Hynda Kleinman, NIH. 25 μg/filter was dried onto polycarbonate filters (12 μ pore, PVP free, Nucleopore, Pleasonton, California) and then reconstituted at 37°C to give an even layer over the filter surface. Fibroblast-conditioned medium, obtained by incubating NIH-3T3 cells for 24 h with IMEM, was used as the chemoattractant. Cells for assay were harvested with trypsin, washed twice with BSA/IMEM, and added to the top chamber (300 000 cells/chamber). Three chambers were used for each of the cell lines. The chambers were then incubated at 37°C in 5% CO_2/95% air for 6 h. The filters were stained with Diff-Quick (American Scientific Products, McGaw Park, Illinois) and the number of cells that had spread on the lower surface of the filter were counted using the Optimax V image analyser. In a parallel experiment, the polycarbonate filters were stained with X-gal, following the same procedure as described above for cell lines.

RESULTS

lacZ Transduction

MDA-MB-231 and MDA-MB-435 cells were infected with the BAG vector (Fig. 1). These cells do not stain with X-gal unless they have been infected with the BAG vector (Fig. 2a). Because of a low viral titre (5×10^4 cfu/ml), only about 1% of either cell line was initially found to stain positive with X-gal. Following G418 selection, approximately 60–70% of the G418-resistant cells expressed the *lacZ* gene as based upon X-gal staining. In order to enrich for *lacZ*-expressing cells, both breast cancer cell lines were subjected to FDG-FACS selection. This enriched both cell populations to > 95% X-gal positivity. In order to determine the stability of the *lacZ* transduction, cells were passaged without G418 15 times following FDG-FACS selection and stained with X-gal. As seen in Fig. 2b, MDA-MB-435 BAG cells showed only a slight loss in the relative number of *lacZ* expressing cells. However, heterogeneity in

expression level was noted. Similar results were obtained with MDA-MB-231 BAG cells (not shown).

Detection of human tumour cells in vivo

The purpose of transducing the human tumour cells with the *lacZ* gene was to be able to find the cells after their dissemination in the nude mouse. Primary subcutaneous tumours of each transduced cell line demonstrated highly specific X-gal staining, whereas tumours from non-transduced cells did not stain blue. This is shown for MDA-MB-231 and MDA-MB-231 BAG tumours in Fig. 2c and d, respectively. Identical results were seen following staining of MDA-MB-435 and MDA-MB-435 BAG primary xenotransplants. Both BAG lines were serially passaged in nude mice, and they retained *lacZ* expression after at least four passages. In cryo-sections of the primary tumours, the X-gal staining was also confined to the BAG-transduced human cancer cells (Fig. 3a). Both untransduced and transduced tumours were locally invasive, penetrating the peritoneal wall of the animals (not shown). X-gal staining of mouse liver, spleen, pancreas, intestine and lungs from mice with transduced subcutaneous tumours of either tumour line demonstrated blue staining of secondary tumour formation within organs in the peritoneal cavity (Fig. 3b–d). Untransduced and *lacZ* transduced secondary tumours were locally invasive in various intraperitoneal organs: MDA-MB-231 BAG preferentially spread to pancreas and the hepatic portal tract, whereas secondary MDA-MB-435 BAG tumours most often were on the omentum of the intestine, and in the spleen, pancreas and hepatic portal tract. Lung metastases were found constantly in mice with either tumour (Fig. 3e, f). Histological examination of the blue stained areas in lungs confirmed the presence of micrometastases (Fig. 3g). We did not find positive X-gal staining in mice inoculated with uninfected tumour cells or in organs without tumour metastases, with the exception of weakly positive staining of the gastro-intestinal tract in some animals. The percentage of mice with intraperitoneal spread and lung metastases following either 6 or 8 weeks of subcutaneous tumour growth, is shown in Table 1.

We next asked whether *lacZ* expression would allow the detection of clinically undetectable ascites. In approximately 50% of mice inoculated subcutaneously with MDA-MB-435 or MDA-MB-435 BAG cells, ascites formation was evident at autopsy. X-gal staining of recovered ascites fluid from animals with MDA-MB-435 BAG revealed X-gal staining of transduced tumour cells only (Fig. 3h). Although no macroscopic ascites was apparent in the remaining animals with MDA-MB-435 BAG tumours, X-gal staining of peritoneal imprints from these animals revealed blue stained tumour cells. This suggests that some ascites formation frequently occurs in MDA-MB-435 BAG cells inoculated subcutaneously in nude mice. In contrast, we never observed ascites formation in mice carrying MDA-MB-231 BAG cells.

Comparative studies between non-transduced and transduced tumour cells

The aim of our study is to obtain a model for studying the invasive and metastatic phenotype of human cancer cells. We wished to determined if retroviral transduction had an effect on the transformed phenotype of the tumour cells. First, we observed that the transduction altered neither the tumorigenicity nor the lag period or growth rate of the tumours (not shown). Second, we compared *in vitro* and *in vivo* invasion of the cells before and after transduction. Since the invasion process of tumour cells includes infiltration of the basement membrane, we studied the cells ability to cross an artificial basement membrane (Matrigel) [1]. Table 2 shows the average number (SEM) of cells which crossed the Matrigel over 6 h. In this assay, no significant activity difference was found between non-transduced and transduced cells. By X-gal staining of cells which had crossed the Matrigel (cells located on the lower part of the polycarbonate filter), *lacZ* expressing cells were shown to be capable of crossing the Matrigel (Fig. 4). In nude mice, transduction did not interfere with the cells ability to locally invade the peritoneal wall or to invade into the peritoneal cavity and into internal organs. However, a reliable *in vivo* comparison between the invasive and metastatic activity of wild-type cells vs. transduced cells were not possible, since micrometastases from wild-type tumours could not be readily identified. With this reservation, transduced and non-transduced cells seem to behave similarly in both *in vitro* and *in vivo* invasion assays, indicating that the retroviral integration of the *lacZ* gene did not significantly change the cells invasive behavior.

DISCUSSION

This study shows that transduction of human cancer cells with the *lacZ* gene is a highly specific and sensitive method for detection of microfoci of human cancer cells growing in the nude mouse. X-gal staining of xenotransplants of primary and secondary tumours demonstrated specific staining of the transduced tumour cells; i.e. neither non-transduced human tumour cells nor the surrounding normal mouse tissue stained positive with X-gal, while very small metastases could easily be detected.

These data also demonstrate the usefulness of retroviral transduction with a double expression vector directing G418 antibiotic resistance and β-galactosidase production of human cancer cells. Stable cell lines were isolated by G418 selection, followed by sterile FDG-FACS for *lacZ* expression. The use of a vector that encodes both *lacZ* and *neo*^{R} genes provided two different selectable markers as well as a histochemical marker. Following BAG vector transduction and five passages *in vitro* under G418 selection, a significant proportion of the cells still stained negative by X-gal. A discordance of *lacZ* expression in G418-resistant cells following BAG transduction has also been observed in other cells that have been transduced with the BAG vector, including canine endothelial cells and mouse fibroblasts (James Zwiebel, unpublished observation). While the cause for this is not known, it may involve inactivation of the *lacZ* gene due to gene deletion [19] or promotor interference [20]. The latter phenomenon has been observed to occur when two promotors are present in close proximity and one selects for expression of one gene and not the other [19]. Subsequent cell sorting for *lacZ* expression resulted in an enriched population of cells staining positive with X-gal. After the cell sorting, G418 selection pressure was stopped and the cells were propagated for an additional 15 passages under normal cell culture conditions. During this time, an approximately 10% decrease in X-gal positive cells was observed. This drop-off in *lacZ* gene expression may also be due to gene deletion, although other mechanisms may be involved. In a recent study of recombinant adenosine deaminase (ADA) gene expression in rat fibroblasts, a complete shutdown of ADA expression was seen following fibroblast implantation into animals [20]. DNA and RNA analysis indicated that the inactivation was occurring at the level of transcription, although no obvious explanation for this phenomenon was apparent. In our own studies, the transduced cells have been

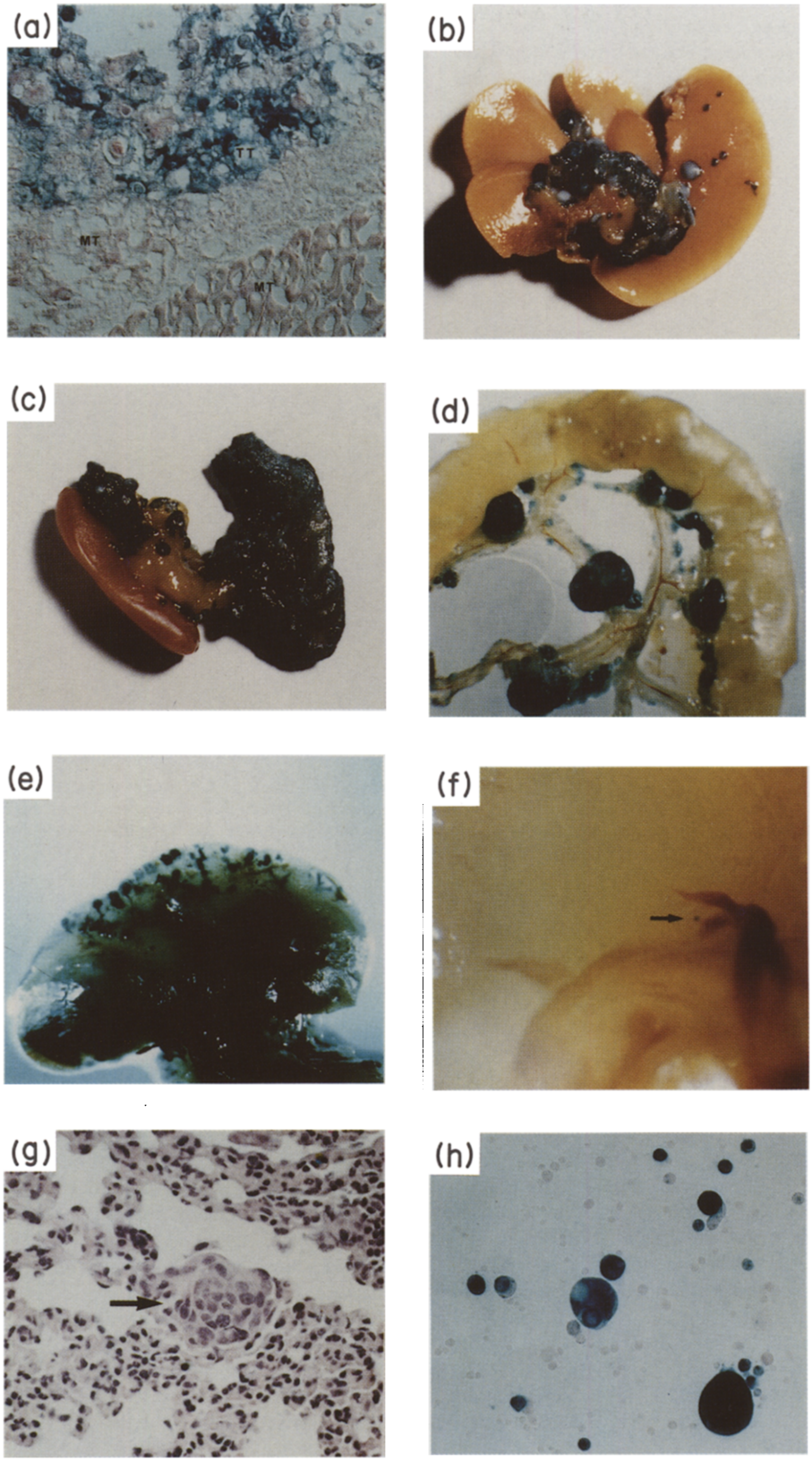

Fig. 3. (a) Cryosection of a primary MDA-MB-435 BAG tumour. Tumour tissue was processed for cryo-sectioning and stained with X-gal (see Materials and Methods). Only the tumour cells stained positive with X-gal. TT: Tumour tissue; MT: Mouse tissue. (b–d) Macroscopic appearance of secondary MDA-MB-435 BAG tumours. Whole organ staining with X-gal. (b) Liver; (c) Spleen and pancreas; (d) Intestine. (e–g) Metastatic spread of MDA-MB-435 BAG tumour cells to mouse lung. (e) Macroscopic appearance of lung metastases; (f) single lung metastasis (arrow); (g) histological section of the lung metastasis (arrow) seen in 3f. Haematoxylin and eosin staining, 40 X. (h) X-gal staining of ascites from a mouse inoculated subcutaneously with MDA-MB-435 BAG tumour cells.

Table 1. Intraperitoneal tumour spread and lung metastases as determined by X-gal staining at 6 and 8 weeks, respectively, following subcutaneous inoculation of 2 × 10^6 tumour cells

	Intraperitoneal spread	Lung metastases
MDA-MB-231 BAG		
6 weeks	10/18	17/18
8 weeks	5/5	5/5
MDA-MB-435 BAG		
6 weeks	4/19	18/19
8 weeks	5/5	5/5

Table 2. Average number (SEM) of cells which crossed the Matrigel during a 6 h period

	Number of cells (SEM)
MDA-MB-231	917 (11)
MDA-MB-21 BAG	1033 (15)
MDA-MB-435	470 (18)
MDA-MB-435 BAG	525 (24)

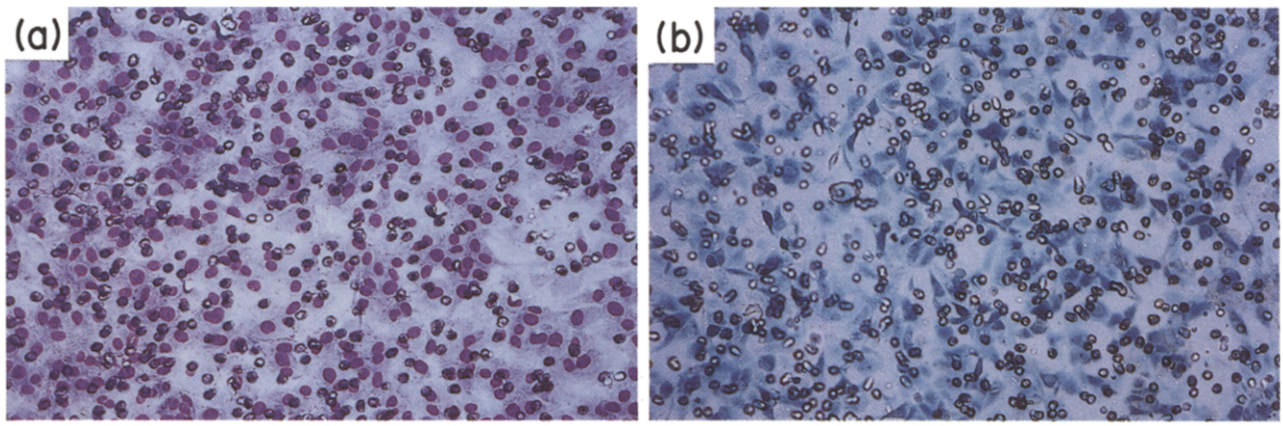

Fig. 4. *In vitro* invasion assay of untransduced and transduced MDA-MB-231 human breast cancer cells. The assay was performed as described in Materials and Methods. (a) MDA-MB-231 human breast cancer cells located on the lower side of the filter were stained with Diff-Quick. (b) MDA-MB-231 BAG human breast cancer cells located on the lower side of the filter were stained with X-gal.

serially passaged in nude mice four times (7 months) without loss of *lacZ* expression. Thus, the cell lines have maintained stable *lacZ* expression without the need for subcloning. If *lacZ* expression decreased further during *in vitro* cultivation, cell sorting could be performed once again.

It is important to note that introduction of foreign DNA into cells may change important biological characteristics of the cells. For example, in one study, the DNA transfection procedure alone altered the tumorigenicity and metastatic properties of the cells [22]. In another study, changes were observed in tumorigenicity and growth rate after transfections with either neo^R or growth factor genes into human cancer cells [23]. In our study, *lacZ*-transduced cells retained their tumorigenicity as well as their invasive phenotype *in vitro* and *in vivo*. This is in agreement with data presented by Lin *et al.* [12], who demonstrated that introduction of the *lacZ* gene by calcium phosphate precipitation into mouse 3T3 cells transformed by the Ha-*ras* oncogene did not alter the invasive and metastatic pattern of the cells.

The MDA-MB-231 and MDA-MB-435 human breast cancer cell lines are both known to be invasive in an artificial basement membrane [24] and to exhibit invasive properties when inoculated into nude mice [14]. These two cell lines thus seem appropriate models for experimental studies of the mechanisms involved in cancer cell invasion. When inoculated subcutaneously, BAG cell lines form tumours which grow invasively into the peritoneal wall. Secondary MDA-MB-231 BAG tumours are preferentially located in the pancreas, possibly reflecting sensitivity to insulin or insulin-like growth factors [25]. In contrast, secondary MDA-MB-435 BAG tumours were established in most of the intraperitoneal organs. We have previously observed that non-transduced MDA-MB-231 cells form secondary tumours by local extension through the peritoneal wall rather than by hematogenous dissemination [24]. This interpretation is based on a study in which non-transduced MDA-MB-231 cells inoculated subcutaneously into the neck region invaded locally into the underlying muscles, but produced no secondary tumours in the peritoneal or pleural cavities. However, the present study is in conflict with this interpretation, since secondary MDA-MB-231 BAG tumour formation as determined by X-gal staining was also found outside the peritoneal cavity, i.e. in the lungs, suggesting hematogenous spread of these cells. Histological examination of X-gal stained areas in lungs from mice carrying either of the tumours showed tumour tissue located in the blood vessels, which further indicates hematogenous dissemination.

Compared with the study published by Price *et al.* [14], our study revealed a higher incidence of lung metastases for both cell lines after subcutaneous tumour cell inoculation. Also, in our study of MDA-MB-231 BAG cells, lung metastases were detectable as early as 6 weeks after tumour cell inoculation, whereas Price *et al.* [14] first detected lung metastasis after 20 weeks. These differences in incidence and time probably reflect differences in detection methods: Price *et al.* [14] used the appearance of macroscopic metastases as end point, whereas our X-gal staining of BAG transduced cells was a highly sensitive method for an early detection of micrometastases.

Human malignant tumours are a mixture of epithelial tumour cells and non-malignant stromal cells. In xenotransplants of human tumours, the stromal elements are of mouse origin [26].

Different cell populations in a tumour can interact with each other [27, 28] in what seems to be a bidirectional interaction, but cell fusions between human and mouse cells in this model have never been observed [29]. After introduction of *lacZ* gene into human cancer cells, these cells can easily be discriminated from mouse host cells, thus allowing the study of tumour cell/stromal interaction *in vivo*.

The use of the *lacZ* gene to track tumour cells should have wide applicability in the study of invasion and metastasis. The high sensitivity of the X-gal staining enables the detection of microfoci of human tumour cells, thereby allowing a quantitative determination of the invasive and metastatic potential of different human cancer lines. A systematic transduction of different human cancer cell lines may reveal a higher proportion of metastatic cell lines than previously anticipated. As shown in this study, the model also allows for a discrimination between different types of metastatic behavior, e.g. direct extension, lymphatic spread vs. hematogenous dissemination. *lacZ*-Transduced cancer cells can also be injected intravenously or into specific organ sites and thereby provide a model for studying biological determinants for the distribution of metastases. With immunohistochemical staining and/or *in situ* hybridisation of X-gal stained areas, the complex interactions between tumour cells and non-malignant stromal components at a cell to cell level can be studied in detail. Transduction of mouse cells with the *lacZ* gene [12, 30], may be done to investigate the biology of invading host cells, e.g. tumour infiltrating lymphocytes, fibroblasts and macrophages. By subcutaneous injection of *lacZ*-transduced cancer cells with subsequent removal of the primary tumour, the response of micrometastases to adjuvant therapy can now be monitored more sensitively. Because of the high specificity and sensitivity of the X-gal staining and easy tumour-cell staining, the model may contribute significantly to future evaluation of new anti-metastatic drugs.

1. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEvan RN. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987, **47**, 3239–3245.
2. Mignatti P, Robbins E, Rifkin DB. Tumor invasion through the human amniotic membrane: requirement for a protease cascade. *Cell* 1986, **47**, 487–498.
3. Bassett P, Bellocq JP, Wolf C, *et al.* A new member of the metalloproteinase gene family is specifically expressed in stromal but not tumor cells of breast carcinomas. *Nature* 1990, **348**, 699–704.
4. Grøndahl-Hansen J, Ralfkiaer E, Kirkeby LT, Kristensen P, Lund LR, Danø K. Localization of urokinase-type plasminogen activator in stromal cells in adenocarcinomas of the colon in humans. *Am J Pathol* 1991, **138**, 111–117.
5. Dagg CP, Karnofsky DA, Roddy J. Growth of transplantable human tumors in the chick embryo. *Cancer Res* 1956, **16**, 589–594.
6. Kozlowski JM, Fidler IJ, Cambell DE, Xu ZL, Kaign ME, Hart IR. Metastatic behaviour of human tumor cell lines grown in the nude mouse. *Cancer Res* 1984, **44**, 3522–3529.
7. Fidler IJ. Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis. *Cancer Metastat Rev* 1986, 5, 29–49.
8. Neulat-Duga I,Sheppel A, Marty C, *et al.* Metastases of human tumor xenografts in nude mice. *Invasion Metast* 1984, **4**, 209–224.
9. Ossowski L, Reich E. Experimental model for quantitative metastasis. *Cancer Res* 1980, **40**, 2300–2309.
10. Ishikawa M, Fernandez B, Kerbel RS. Highly pigmented human melanoma variant which metastasizes widely in nude mice, including to skin and brain. *Cancer Res* 1988, **48**, 4897–4903.
11. Wilson EL, Gartner M, Champbell JAH, Dowdle EB. The metastasis of a human melanoma cell line in the nude mouse. *Int J Cancer* 1988, **41**, 83–86.
12. Lin W-C, Pretlow TP, Pretlow II TG, Culp LA. Bacterial *lacZ* gene as a highly sensitive marker to detect micrometastasis formation during tumor progression. *Cancer Res* 1990, **50**, 2808–2817.
13. Lin WC, Pretlow TP, Pretlow TG, Culp LA. Development of micrometastases earliest events detected with bacterial *lacZ* gene-tagged tumor cells. *J Natl Cancer Inst* 1990, **82**, 1497–1503.
14. Price JE, Polyzos A, Zhang RD, Daniels LM. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res* 1990, **50**, 717–721.
15. Rygaard K, Spang-Thomsen M. Growth—a computer program for determination of mean growth curves and calculation of therapy of solid tumor xenografts. In: Wu B-Q, Zheng J, eds. *Immune-deficient Animals in Experimental Medicine*. Basel, Karger, 1989, 301–306.
16. Price J, Turner D, Cepko C. Lineage analysis in the vertebrate system by retrovirus-mediated gene transfer. *Proc Natl Acad Sci USA* 1987, **84**, 156–160.
17. Miller AD, Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol Cell Biol* 1986, **6**, 2895–2902.
18. Nolan GP, Fiering S, Nicolas J-F, Herzenberg LA. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli lacZ*. *Proc Natl Acad Sci USA* 1988, **85**, 2603–2607.
19. Emerman M, Temin HM. High-frequency deletion in recovered retrovirus vectors containing exogenous DNA with promotors. *J Virol* 1984, **50**, 42–49.
20. Emerman M, Temin HM. Genes with promotors in retrovirus vectors can be independently suppressed by an epigenetic mechanism. *Cell* 1984, **39**, 449–467.
21. Palmer TD, Rosman GJ, Osborne WRA, Miller AD. Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc Natl Acad Sci USA* 1991, **88**, 1330–1334.
22. Kerbel RS, Waghorne C, Man MS, Elliott B, Breitman BL. Alteration of the tumorigenic and metastatic properties of neoplastic cells is associated with the process of calcium phosphate-mediated DNA transfection. *Proc Natl Acad Sci USA* 1987, **84**, 1263–1267.
23. Clarke R, Brünner N, Katz D, *et al.* The effects of a constitutive expression of transforming growth factor-α on the growth of MCF-7 human breast cancer cells *in vitro* and *in vivo*. *Mol Endocrinol* 1989, **3**, 372–380.
24. Thompson EW, Paik S, Brünner N, *et al.* Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human cell lines. *J Cell Physiol* 1992, **150**, 534–544.
25. Arteaga CL, Kitten LJ, Coronado EB, *et al.* Blockade of the type I somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. *J Clin Invest* 1989, **84**, 1418–1423.
26. Rygaard K. A rapid method for identification of murine cells in human malignant tumors grown in nude mice. In: Rygaard *et al.*, eds. *Immune-deficient Animals in Biomedical Research*. Basel, Karger, 1987, 268–272.
27. Hakim AA. Fibroblast function in paracrine interactions between breast cancer tissue and its stromal elements. *Naturwissenschaften* 1989, **76**, 329–331.
28. Champs JL, Chang S-M, Hsu TC, *et al.* Fibroblast-mediated acceleration of human epithelial growth *in vivo*. *Proc Natl Acad Sci USA* 1990, **87**, 75–79.
29. Povlsen CO, Visfeldt J, Rygaard J, Jensen G. Growth patterns and chromosome constitutions of human malignant tumors after long-term serial transplantation in nude mice. *Acta Path Microbiol Scand Sect A* 1975, **83**, 709–716.
30. Sanes JR, Rubenstein JLR, Nicolas J-F. Use of a recombinant retrovirus to study post-implantation cell lneage in mouse embryos. *EMBO J* 1986, 5, 3133–3142.

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