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Establishment of Peritoneal and Hepatic Metastasis Mouse Xenograft Models Using Gastric Cancer Cell Lines

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Abstract. *Background/Aim: Establishment of mouse xenograft models is necessary for oncological research and depends on the characteristics of the cell lines and the immune system of the host. In this study, we describe the development of mouse xenograft models using human gastric cancer (GC) cell lines. Materials and Methods: MKN1 stably-expressing luciferase (MKN1-Luc), N87, KATO III, MKN45 stably-expressing luciferase (MKN45-Luc), NUGC4, and OCUM-1 human GC cell lines were injected intraperitoneally into mice to establish peritoneal metastasis models. MKN45-Luc were injected into subcutaneously implanted spleen, and MKN1-Luc and MKN45-Luc were injected directly into the portal veins of mice for the establishment of hepatic metastasis models. Results: Peritoneal metastasis was formed after implantation of MKN1-Luc, N87, KATO III, MKN45-Luc, and NUGC4 in nude mice, but not formed in OCUM-1 even in NOD/SCID mice. After intrasplenic injection of MKN45-Luc, we found no hepatic metastasis formation. We identified hepatic metastasis formation after direct injection of MKN45-Luc and MKN1-Luc into the portal veins of NOD/SCID mice. Conclusion: Peritoneal and hepatic metastasis mouse xenograft models were successfully established using several human GC cell lines.*

Gastric cancer (GC) is the fourth leading cause of cancer-related death worldwide (1). Perioperative adjuvant therapy has improved prognosis to a certain degree (2-4). However, it is still less than satisfactory in its therapeutic efficacy, because some patients with advanced GC often experience recurrence even after curative gastrectomy. To develop a therapeutic strategy, elucidation of the mechanisms of metastasis formation and identification of therapeutic targets are urgently required.

As previously reported, complex processes such as invasion into the circulation, survival in the circulation, adhesion, migration, invasion and proliferation in other organs are necessary for metastasis formation (5). Various molecules are involved in these processes (5-10). Analysis of certain molecules and development of therapeutic agents are undertaken using *in vitro* experiments. However, it is necessary to establish xenograft models for further molecular biological analysis and validation *via in vivo* experiments. Nonetheless, it is not always possible to develop xenograft models, because xenotransplantation results in the major histocompatibility complex (MHC) class II molecules of xenotransplanted cells and antigen-presenting cells phagocytosing the graft due to antigen presentation, thereby activating CD4-positive T cells. These activated CD4-positive cells also activate cell-mediated immunity and antibody-mediated immunity, which exclude implanted grafts (11, 12).

Herein we attempted to establish mouse xenograft models using several human GC cell lines. We hope that our experience will be useful for other researchers conducting *in vivo* studies.

Materials and Methods

Cell lines and cell culture. MKN1 cells stably expressing luciferase (MKN1-Luc), MKN45 cells stably expressing luciferase (MKN45-Luc), NUGC4, and OCUM-1 were obtained from the Japanese Collection of Research Bio Resources Cell Bank (JCRB) (Osaka, Japan). N87 and KATO III cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were incubated at 37˚C with 5% CO_2 in the recommended medium supplemented with 10% fetal bovine serum. The characteristics of the gastric cancer cell lines are listed in Table I.

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Key Words: Gastric cancer, peritoneal metastasis, hepatic metastasis, xenograft.

Table I. *Characteristics of gastric cancer cell lines.*

Cell line	Histological type	Collected tissue	Age	Gender	Race	Culture properties	Morphology	Recommended medium	Other
MKN1	Adenosquamous carcinoma	Liver	72	Male	Asian	Adherent	Epithelial	RPMI1640 + 10% FBS	ALP production +
N87	Well-differentiated adenocarcinoma	Liver	Unknown	Male	Caucasian	Adherent	Epithelial	RPMI1640 + 10% FBS	c-myc +, c-erb-B2 +, HER2 +, Expresses acetylcholine and muscarinic receptor
KATO III	Signet ring cell carcinoma	Pleural effusion	55	Male	Asian	Adherent and suspension	Spherical	RPMI1640 + 10% FBS	CEA production +
MKN45	Poorly differentiated adenocarcinoma	Liver	62	Female	Asian	Adherent	Spherical	RPMI1640 + 10% FBS	CEA production +
NUGC4	Signet ring cell carcinoma	Lymph node	35	Female	Asian	Adherent	Spherical	RPMI1640 + 10% FBS	HER2 +
OCUM-1	Poorly differentiated adenocarcinoma	Pleural effusion	38	Female	Asian	Adherent	Spherical	DMEM + 0.5 mM sodium pyruvate + 10% FBS	CEA, CA19-9, SPan-1 production + Expresses EGF receptor

RPMI: Roswell Park Memorial Institute medium; FBS: fetal bovine serum; DMEM: Dulbecco's Modified Eagle medium; ALP: alkaline phosphatase; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; EGF: epidermal growth factor.

Mice. All animal experiments conformed to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guideline and were approved by the Animal Research Committee of Nagoya University (IRB No. 29329) (13). Four-week-old male nude mice (BULB/cSlc-nu/nu) were obtained from Chubu Kagaku Shizai (Nagoya, Japan). Four-week-old male NOD/SCID mice (nod/shi-SCID) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Mice were housed and adapted to the breeding environment for two weeks before the experiment.

Mouse subcutaneous xenograft model. A total of 1×10^6 of MKN1, KATO III, and MKN45 cells were suspended in 100 μl of phosphate buffered saline (PBS) and subcutaneously injected into the bilateral flanks of nude mice.

Mouse peritoneal metastasis models. MKN1-Luc, N87, KATO III, MKN45-Luc, NUGC4, and OCUM-1 cells were injected into nude mice intraperitoneally with 1 ml PBS. A total of 1.0×10^6 OCUM-1 cells were injected into NOD/SCID mice intraperitoneally with 1 ml PBS. After each observation period, these mice were sacrificed and peritoneal metastasis formation was observed under direct viewing.

Mouse hepatic metastasis models. Under general anesthesia, we mobilized and excised the spleens of nude mouse, which were then subcutaneously implanted. A total of 0.5×10^6 MKN45-Luc cells were suspended in 100 μl of PBS and injected into the subcutaneously implanted spleens. For direct injection into the portal vein, nude mice and NOD/SCID mice were placed under general anesthesia and laparotomized. Then, 1.0×10^6 MKN45-Luc and 0.5×10^6 MKN1-Luc cells were suspended in 100 μl of PBS and directly injected into the portal veins of mice using a 35-gauge NanoNeedle (NIPPON Genetics, Tokyo, Japan). After injection of the cell suspensions, we oppressed the puncture site of the portal vein using SURGICEL (Johnson & Johnson, NJ, USA) for a few minutes. Twelve weeks after injection, these mice were killed and hepatic metastasis formation was observed under direct viewing.

In vivo imaging. The In Vivo Imaging System (IVIS) Lumina (Xenogen, Alameda, CA, USA) was employed every four weeks after injection to non-invasively measure the volumes of peritoneal and liver metastases. D-Luciferin (150 mg/kg) (Summit Pharmaceuticals International, Tokyo, Japan) was administered intraperitoneally and luciferase activity was measured using the IVIS at 15 min after injection of D-luciferin. We used Living Image Ver. 2.6 (Xenogen) software to acquire and analyze the data. Magnetic resonance imaging (MRS 3000, MR solutions, Guildford, UK) was employed twelve weeks after injection as an alternative approach to detecting the formation of hepatic metastasis.

Results

Subcutaneous tumor formation. Subcutaneous tumors were formed after subcutaneous injection of MKN1-Luc, KATO III, and MKN45-Luc cells. Previous reports have shown that N87, NUGC4 and OCUM-1 can form subcutaneous tumors in nude mice (14-18).

Peritoneal metastasis formation. Peritoneal metastasis formation was noted eight weeks after implantation of 0.5×10^6 and 1.0×10^6 MKN1-Luc cells in nude mice (Figure 1A and B). IVIS images of mice injected with 1.0×106

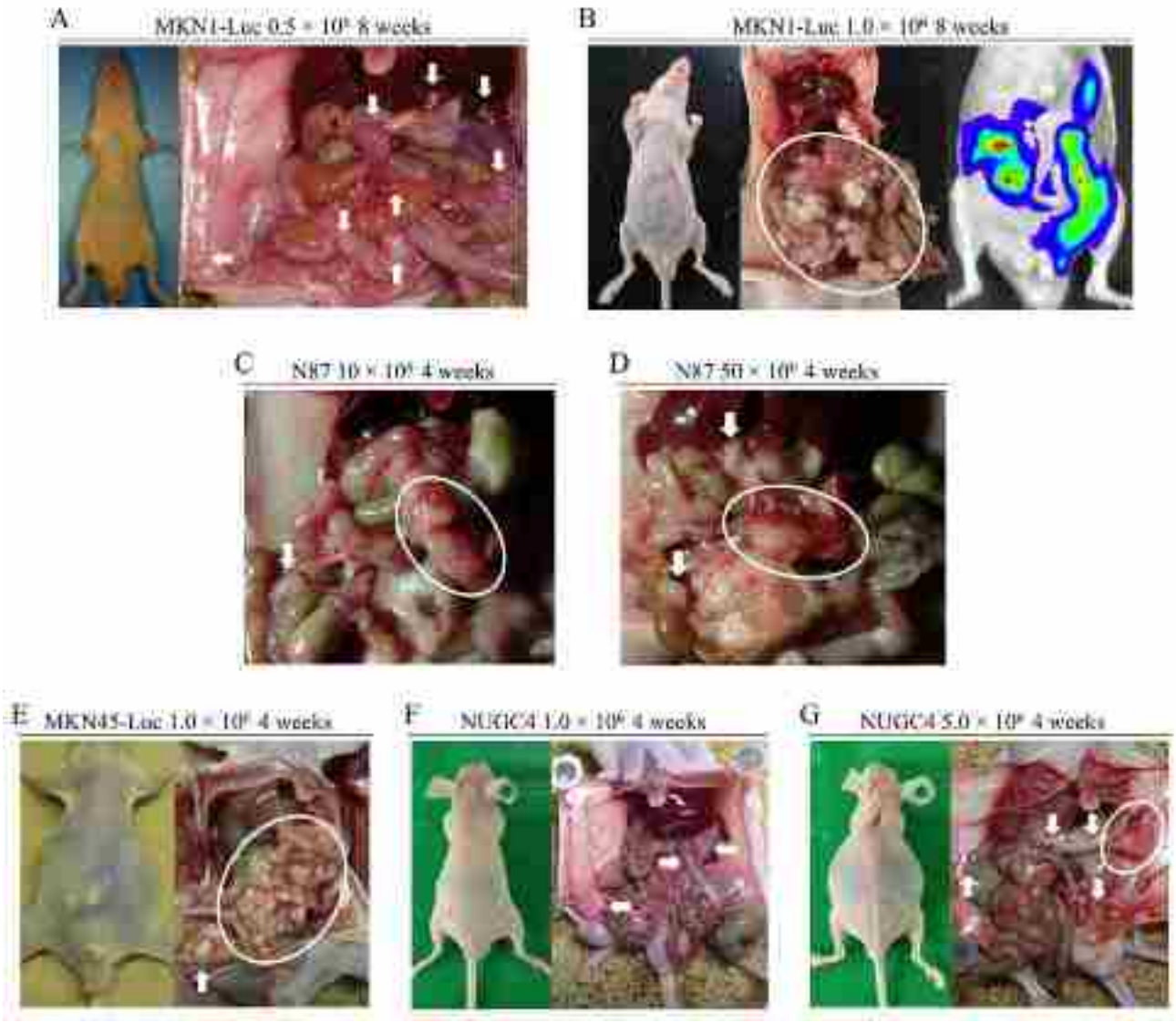

Figure 1. *Peritoneal metastasis formation in nude mice. (A) Eight weeks after injection with 0.5×10^6 MKN1-Luc cells. (B) Eight weeks after injection with 1.0×10^6 MKN1-Luc cells. (C) Four weeks after injection with 10×10^6 N87 cells. (D) Four weeks after injection with 50×10^6 N87 cells. (E) Four weeks after injection with 1.0×10^6 MKN45-Luc cells. (F) Four weeks after injection with 1.0×10^6 NUGC4 cells. (G) Four weeks after injection with 5.0×10^6 NUGC4 cells.*

MKN1-Luc cells are shown in Figure 1B. Four weeks after implantation of 10×10^6 and 50×10^6 N87 cells, peritoneal metastasis was observed in nude mice (Figure 1C and D). Four weeks after implantation of 1.0×10^6 MKN45-Luc and 1.0×10^6 and 5.0×10^6 NUGC4 cells, hemorrhagic ascites and peritoneal metastasis was also evident in nude mice (Figure 1E, F, G). Peritoneal metastasis formation was not detected eight weeks after implantation of 1.0×10^6 and 5.0×10^6 KATO III cells and four weeks after implantation of 10×10^6, 50×10^6, and 100×10^6 KATO III cells in nude mice (Figure 2A-E). However, nine weeks after implantation of 100×10^6 KATO III cells in nude mice, peritoneal metastasis was detected (data not shown). We could not identify peritoneal metastasis at four weeks after intraperitoneal injection of 1.0×10^6 or 5.0×10^6 OCUM-1 cells in nude mice, or 1.0×10^6 OCUM-1 cells in NOD/SCID mice(Figure 3A-C).

Hepatic metastasis formation. Subcutaneously implanted spleen data are shown in Figure 4A. Hepatic metastasis was not identified in nude mice, but splenic tumors were found twelve weeks after injection of 0.5×10^6 MKN45-Luc cells into subcutaneously implanted spleen (Figure 4B). The procedure

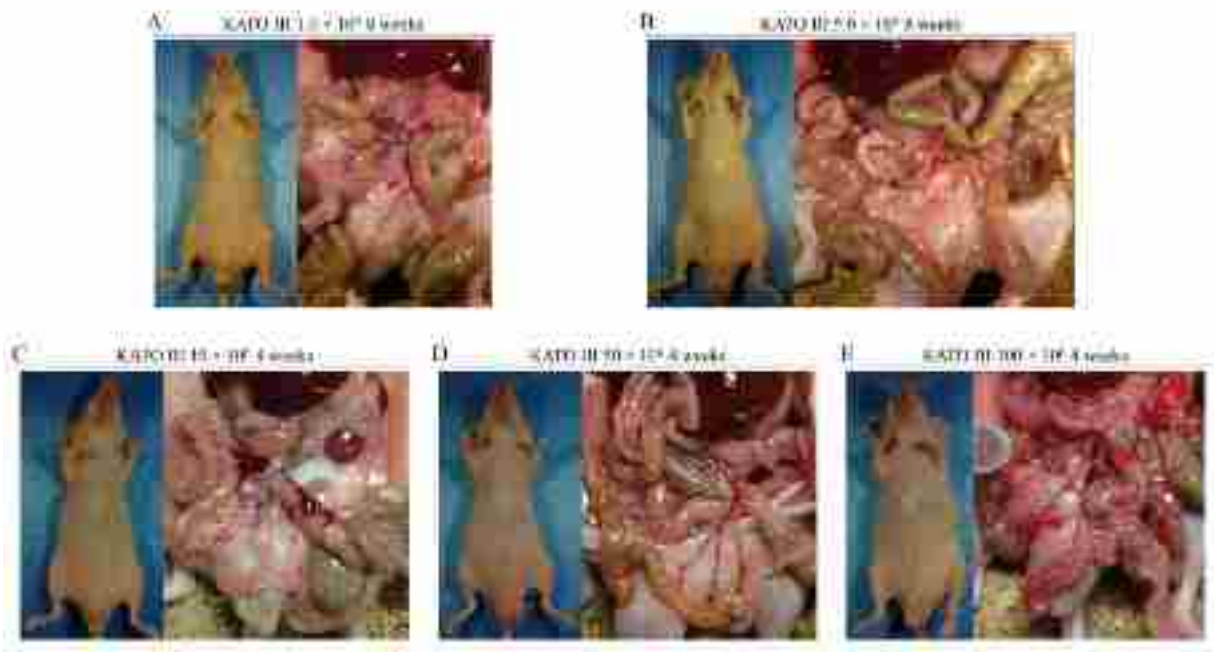

Figure 2. *Peritoneal metastasis formation in nude mice after intraperitoneal injection of KATO III cells. No peritoneal metastasis was found (A) eight weeks after injection with 1.0×10^6 cells, (B) eight weeks after injection with 5.0×10^6 cells, (C) four weeks after injection with 10×10^6 cells, (D) four weeks after injection with 50×10^6 cells, or (E) four weeks after injection with 100×10^6 cells.*

Figure 3. *Peritoneal metastasis formation after intraperitoneal injection of OCUM-1 cells. No peritoneal metastasis was found four weeks after injection of (A) 1.0×10^6 and (B) 5.0×10^6 OCUM-1 cells in nude mice. No peritoneal metastasis was found (C) four weeks after injection of 1.0×10^6 OCUM-1 cells in NOD/SCID mice.*

for direct injection into the portal veins of mice is shown in Figure 5A. In our early experiences, most of the mice died immediately after treatment if they underwent thoracotomy. Although another major cause of treatment-related death is hemorrhage from the portal vein after injection, it can be prevented by usage of a 35-gauge needle and astriction with an oxidized cellulose cotton. We did not find hepatic metastasis formation in nude mice at all. However, we identified hepatic metastasis formation twelve weeks after injection of 1.0×10^6 MKN45-Luc cells in the portal veins of NOD/SCID mice (Figure 5B). Hepatic metastasis was also detected twelve weeks after injection of 0.5×10^6 MKN1-Luc cells in the portal veins of NOD/SCID mice (Figure 5C). IVIS and MRI images of mice twelve weeks after injection are also shown in Figure 5C.

Discussion

Before conducting phase I studies, we need to validate the effects of novel pharmacological agents and treatment methods through the use of animal models (19). Experiments using mice are the first step in laboratory animal studies before the use of large-sized animals such as dogs and

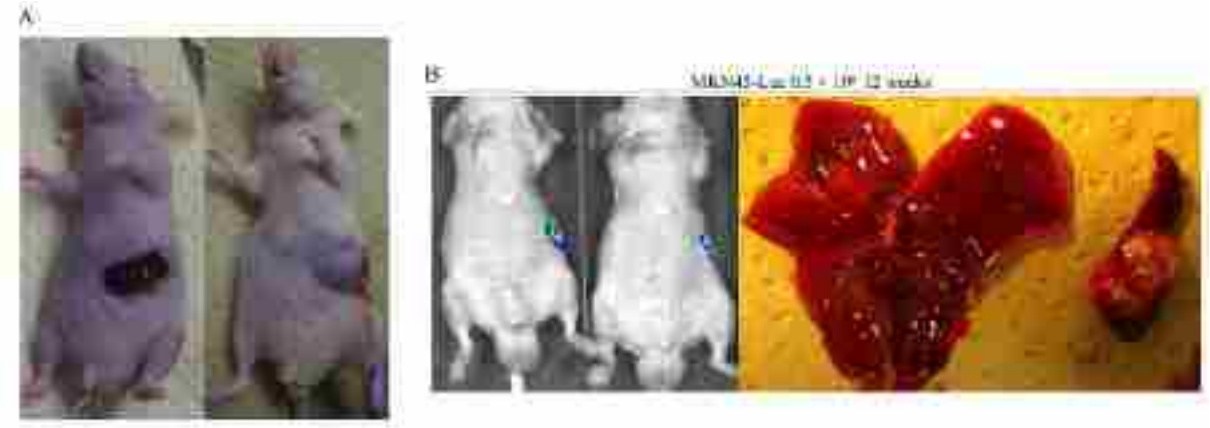

Figure 4. *Hepatic metastasis formation after intrasplenic injection in nude mice. (A) Subcutaneously implanted spleen. (B) Twelve weeks after injection with 0.5×10^6 MKN45-Luc cells.*

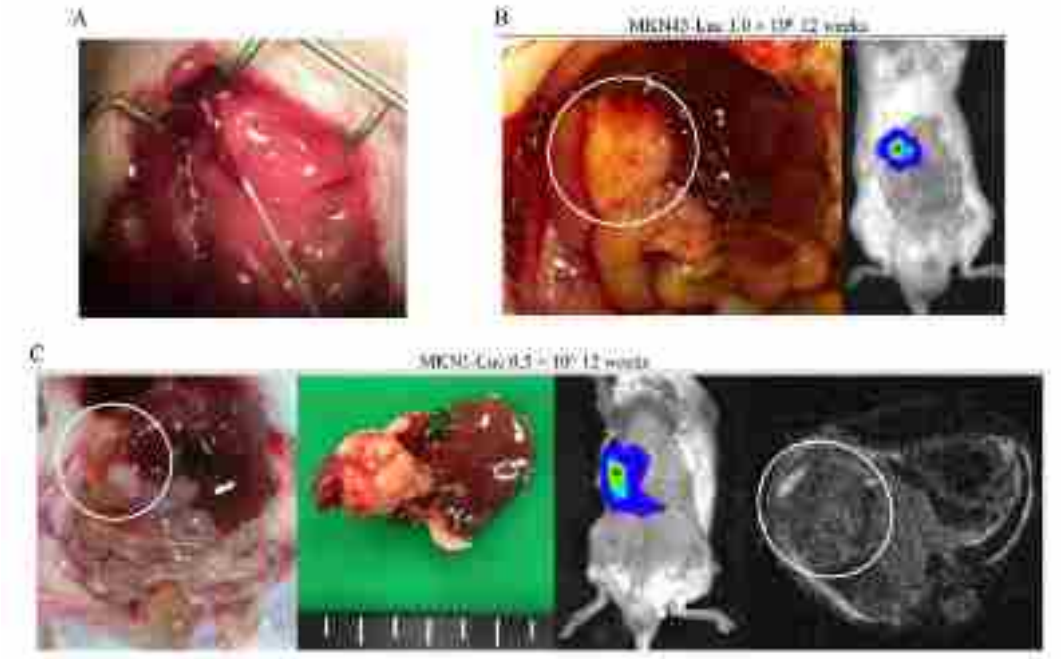

Figure 5. *Hepatic metastasis formation after direct injection of the portal vein in NOD/SCID mice. (A) Direct injection procedure into the portal veins of mice. (B) Twelve weeks after injection of 1.0×10^6 MKN45-Luc cells into the portal vein. (C) Twelve weeks after injection of 0.5×10^6 MKN1-Luc cells into portal vein.*

monkeys. Peritoneal metastasis and hepatic metastasis are the main reasons for gastric cancer-related death (20). In this study, we attempted to establish peritoneal and hepatic metastasis mouse xenograft models using several gastric cancer cell lines that can form subcutaneous tumors in nude mouse to generate tools, for examining the mechanisms of metastasis formation and for developing novel therapeutic strategies. A summary of this study is shown in Table II.

Generally, the host immune system rejects transplanted grafts in xenotransplantation (11, 12). Thus, immunocompromised animals are needed to establish xenograft models. In 1962, the nude mouse was detected as the first immunocompromised mouse. The nude mouse has no mature T cells due to the lack of a thymus gland, resulting in poor immunity (21). A number of human cancer cell lines were engrafted successfully in nude mice (22-24). These mice have subsequently been used in

Table II. *Summary of mouse xenograft models.*

Cell lines	Subcutaneous	Peritoneal			Liver		
		Implanted cells	Nude mouse	Nod-SCID mouse	Implanted cells	Nude mouse	Nod-SCID mouse
MKN1	Yes	$0.5\text{-}1.0\times10^6$	Yes	-	0.5×10^6	No	Yes
N87	Yes (ref 16, 17)	$10\text{-}50\times10^6$	Yes	-		Not performed	
KATO III	Yes	100×10^6	Yes	-		Not performed	
MKN45	Yes	1.0×10^6	Yes	-	1.0×10^6	No	Yes
NUGC4	Yes (ref 14, 18)	$1.0\text{-}5.0\times10^6$	Yes	-		Not performed	
OCUM-1	Yes (ref 15)	$1.0\text{-}5.0\times10^6$	No	No		Not performed	

cancer research and in the development of anticancer agents. However, the nude mouse has some immunocytes besides mature T cells, and many human cell lines cannot be engrafted successfully. Subsequently, the NOD/SCID mouse was developed as a immunocompromised mouse that lacks both T cells and B cells (25). The discovery of such mice has facilitated the establishment of xenograft models (26-28), although these animals do not absolutely lack immunity.

We observed peritoneal metastasis formation after injection of MKN1-Luc, N87, KATO III, MKN45-Luc, and NUGC4 cells. These cell lines can be disseminated in manageable nude mice and may be suitable for peritoneal metastasis xenograft models. However, OCUM-1 failed to form peritoneal metastases, even in the NOD/SCID mouse. This cell line has not been reported previously in peritoneal metastasis xenografts and is unsuitable for this practice.

We can generate hepatic metastasis mouse xenograft models by injecting cell suspensions directly into the liver. However, this procedure can only assess the survival and proliferation of cancer cells in hepatic tissue. Implantation by injection into the spleen is reportedly one of the ways to establish hepatic metastasis xenograft models (29-31). There have been several reports of successful establishment of hepatic metastasis models by subcapsular injection of murine cancer cell lines to the spleen (32-34). The procedure of intrasplenic injection is simple and convenient. However, this procedure does not reliably establish hepatic metastasis because cancer cells must migrate and invade into the splenic vein to reach the portal vein system. In our experience using human gastric cancer cell lines, we could not establish hepatic metastasis by intrasplenic injection of MKN45-Luc cells in nude mice. We conducted the procedure by directly injecting into the portal vein to ensure the cell suspension reached the portal vein system. However, it took some time to establish this procedure because of the difficulty of direct injection into the thin mouse portal vein, as well as the hemostasis after injection. In the early days of this study, hemostasis was not obtained after injection of the cell suspension into the portal vein and mice frequently died of blood loss. Finally, we were able to establish hepatic metastasis for a brief time in NOD/SCID mice after injection with 0.5×10^6 of MKN1-Luc and 1.0×10^6 of MKN45-Luc cells using our procedure.

Using immunosuppressed mice, human GC cell lines were successfully engrafted at different organs from the original organs from which the cell lines were derived. This enables us to use these cell lines properly according to the purpose of the animal experiment by applying several characteristics, such as cell differentiation and molecular expression. These findings contributed to our previous studies of the identification of molecules related to the formation of peritoneal and hepatic metastasis (35, 36).

In conclusion, we developed a procedure to establish peritoneal and hepatic mouse xenograft models with several human GC cell lines. This information will be useful for the elucidation of the mechanism of metastasis formation and the development of novel treatment approaches for GC.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

Mitsuro Kanda and Yasuhiro Kodera make substantial contributions to conception and design. Shinichi Umeda and Haruyoshi Tanaka and Dai Shimizu make substantial contributions to acquisition of data. Chie Tanaka and Daisuke Kobayashi and Masamichi Hayashi and Suguru Yamada and Goro Nakayama and Masahiko Koike make substantial contributions to interpretation of data.

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