

Higher susceptibility of NOD/LtSz-*scid* *Il2rg*^{-/-} NSG mice to xenotransplanted lung cancer cell lines

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Purpose: No lung cancer xenograft model using non-obese diabetic (NOD)-*scid* *Il2rg*^{-/-} mice has been reported. The purpose of this study is to select a suitable mouse strain as a xenogenic host for testing tumorigenicity of lung cancer.

Materials and methods: We directly compared the susceptibility of four immunodeficient mouse strains, *c-nu*, C.B-17 *scid*, NOD-*scid*, and NOD/LtSz-*scid* *Il2rg*^{-/-} (NSG) mice, for tumor formation from xenotransplanted lung cancer cell lines. Various numbers (10¹–10⁵ cells/head) of two lung cancer cell lines, A549 and EBC1, were subcutaneously inoculated and tumor sizes were measured every week up to 12 weeks.

Results: When 10⁴ EBC1 cells were inoculated, no tumor formation was observed in BALB/*c-nu* or C.B-17 *scid* mice. Tumors developed in two of the five NOD-*scid* mice (40%) and in all the five NSG mice (100%). When 10³ EBC1 cells were injected, no tumors developed in any strain other than NSG mice, while tumorigenesis was achieved in all the five NSG mice (100%, *P*=0.0079) within 9 weeks. NSG mice similarly showed higher susceptibility to xenotransplantation of A549 cells. Tumor formation was observed only in NSG mice after inoculation of 10³ or fewer A549 cells (40% vs 0% in 15 NSG mice compared with others, respectively, *P*=0.0169). We confirmed that the engrafted tumors originated from inoculated human lung cancer cells by immunohistochemical staining with human cytokeratin and vimentin.

Conclusion: NSG mice may be the most suitable strain for testing tumorigenicity of lung cancer, especially if only a few cells are available.

Keywords: NOD-*scid* *Il2rg*(null), tumorigenicity, xenotransplantation, xenograft, mouse model

Introduction

The study of cancer in humans is impeded by several reasons, such as inaccessibility to the tumor sites, difficulty in the assessment of tumor biology, and ethical concerns. To overcome these restrictions, mouse models have been generated. Immunodeficient mice that cannot reject xenotransplanted cells have been shown to be the best living recipients for developing xenograft models of human cancer.¹ Immunodeficient mice have also been important for investigating carcinogenesis, cancer therapy, and imaging of tumor growth and metastasis.² To date, the most extensively used immunodeficient mouse strains for developing lung cancer xenograft models include nude,³ *scid*,⁴ and non-obese diabetic (NOD)-*scid*⁵⁻⁷ mice.

The interleukin-2 receptor (IL-2R) γ -chain is known as the common cytokine receptor γ -chain. The IL-2R γ -chain is a crucial component of the high-affinity receptors for IL-2, IL-4, IL-7, IL-9, and IL-15, and is required for signaling through

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these receptors.^{8,9} Absence of the IL-2R γ -chain in mice leads to severe impairments in T- and B-cell development and function, and completely prevents natural killer (NK) cell development.^{9–11} The immunodeficient strains of *Il2rg*^{-/-} mice include NOD·Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (abbreviated as NOD/LtSz-*scid* *Il2rg*^{-/-} and often referred to as NSG mice), NOD·Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug/Jic} mice (abbreviated as NOD/Shi-*scid* *Il2rg*^{-/-} and often referred to as NOG mice), C.Cg-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Sug} mice (abbreviated as BALB/c-*Rag2*^{-/-} *Il2rg*^{-/-} mice) and Stock (H2^d)-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Krf} mice (referred to as H2^d-*Rag2*^{-/-} *Il2rg*^{-/-} mice).²

NOG mice have shown superiority in cancer xenoplas-
tation systems compared with nude, *scid*, and NOD-*scid* mice, when human cervical cancer,¹² pancreatic cancer,¹³ and multiple myeloma¹⁴ cell lines were used. Similarly, higher tumorigenicity in NSG mice has been reported with neuroblastoma¹ and melanoma¹⁵ cells. Acute leukemia cells injected in NSG mice generated faster and more efficient leukemic characteristics compared with other NOD-*scid*-related strains.¹⁶ However, no lung cancer xenograft model using NOD-*scid* *Il2rg*^{-/-} mice (neither NSG nor NOG) has been reported. To select a suitable mouse strain as a xenogenic host for testing tumorigenicity of lung cancer, we directly compared the susceptibility of nude, *scid*, NOD-*scid*, and NSG mice for tumor formation from xenotransplanted lung cancer cell lines.

Materials and methods

Animals

NSG mice were originally generated at The Jackson Laboratory (Bar Harbor, ME, USA). BALB/c-*nu*, C.B-17 *scid*, NOD-*scid*, and NSG mouse strains (50 female mice in each strain) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and maintained in the Division of Animal Experiments, Life Science Research Center, Kagawa University (Kagawa, Japan), according to the Institutional Regulations for Animal Experiments. The regulations included the best considerations on animal welfare and good practice of animal handling contributing to the replacement, refinement, and reduction of animal testing (3Rs). All animals were housed in polycarbonate cages with white wood chips for bedding. Animals were given free access to drinking water, and a basal diet, Oriental MF (Oriental Yeast Co, Ltd, Tokyo, Japan), under controlled conditions of humidity (60%±10%), lighting (12-hour light/dark cycle), and temperature (24°C±2°C).

Cells

Two human lung cancer cell lines (EBC1, squamous cell carcinoma, and A549, adenocarcinoma) were obtained from

the Japan Cancer Research Bank (Tokyo, Japan). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and passaged on reaching 80% confluence as reported previously.¹⁷

Xenotransplantation in vivo

The protocols of the animal experiments were approved by the Animal Care and Use Committee for Kagawa University. For direct comparison of susceptibility to cancer cell engraftment, various numbers of EBC1 or A549 cells (10¹–10⁵ cells/head, suspended in 0.1 mL of serum-free medium) were subcutaneously inoculated into five mice of each condition in each strain when mice were aged 6 weeks. The mice were monitored daily and tumor sizes were measured every week with calipers. The tumor volume (TV) was calculated using the formula $TV = 1/2 \times A \times B^2$ (where A = length in millimeters and B = width in millimeters) according to the previous study.¹² The criteria for successive engraftment were as follows: progressive nodule growth at the site of injection and TV values exceeding 10 mm³.¹² Mice were monitored up to 12 weeks after inoculation, and mice that developed a successive engraftment were euthanized.

Histology and immunohistochemistry

The engrafted tumors were fixed with 10% phosphate-buffered formalin, and paraffin-embedded sections were stained using hematoxylin and eosin. Anti-human vimentin (Clone V9; #M0725; Dako, Glostrup, Denmark) and anti-human pancytokeratin (AE1/AE3; #M3515; Dako) antibodies were used for the confirmation of human cell-derived tumors. A staining without primary antibodies was also prepared as a control for nonspecific effects of primary antibodies. Secondary antibodies (I-VIEW DAB Universal Kit, #518100032) were purchased from Roche Diagnostics KK (Tokyo, Japan).

Statistical analyses

The differences in incidence of each engraftment were tested by Fisher's direct probability method with $P < 0.05$ as the cutoff for significance. All statistical analyses were performed using Excel Statistic 2012 (Social Survey Research Information Co, Ltd, Tokyo, Japan).

Results

Tumorigenicity of lung cancer cell lines

We directly compared the susceptibility of four immunodeficient mouse strains, such as nude (BALB/c-*nu*), *scid* (C.B-17 *scid*), NOD-*scid*, and NSG, to xenotransplanted human lung

cancer cell lines. When 10^5 EBC1 cells were inoculated subcutaneously, tumors formed in all mice tested within 9 weeks after inoculation (Figure 1A). In NSG mice, all tumor formations had been completed in 3 weeks. When 10^4 EBC1 cells were inoculated, no tumor formation was observed in nude or *scid* mice (Figure 1B). Tumors developed in two of the five NOD-*scid* mice (40%), while tumors formed in all the five NSG mice (100%). The superiority of NSG mice to xenotransplantation was clearer after 10^3 cells were inoculated (Figure 1C, $P=0.0079$). No tumors developed in any strain other than NSG mice, in which 100% tumorigenesis was still observed in 9 weeks. All tumors in NSG mice grew progressively (Figure 1D) and formed a large, hard, spheroid mass at 12 weeks (Figure 1E). Tumor formation developed in one of the five mice after inoculation of 10^2 EBC1 cells (Figure 1C).

Similar to EBC1 cells, a higher xenotransplantability of A549 cells was observed in NSG mice when compared with other strains. Although there was no statistical significance in final tumor number after inoculation of 10^5 and 10^4 A549 cells (Figure 2A and B, respectively), tumorigenesis

developed the fastest in NSG mice. After inoculation of 10^3 or fewer A549 cells, no tumor formation was observed in nude, *scid*, and NOD-*scid* mice. In NSG mice, however, tumors developed in 6 of 15 mice (40%) in conditions of less than 10^3 cells (Figure 2C), which is significantly higher when compared with other strains ($P=0.0169$). Unexpectedly, only ten A549 cells formed a tumor in one of the five NSG mice.

Pathology of the developed tumor

The pathologic findings of the developed tumor in the NSG mouse after inoculation of ten A549 cells showed, as expected, adenocarcinoma including glandular formation (Figure 3A). The tumor was positive for anti-human cytokeratin and anti-human vimentin (Figure 3B and C), suggesting that the developed tumor originated from a human source, that is, A549 cells. Tumors showed negative staining with an isotype control antibody, confirming accuracy of immunohistochemistry (Figure 3D). No metastatic tumor formation was observed in other organs including lungs and livers in all mice examined.

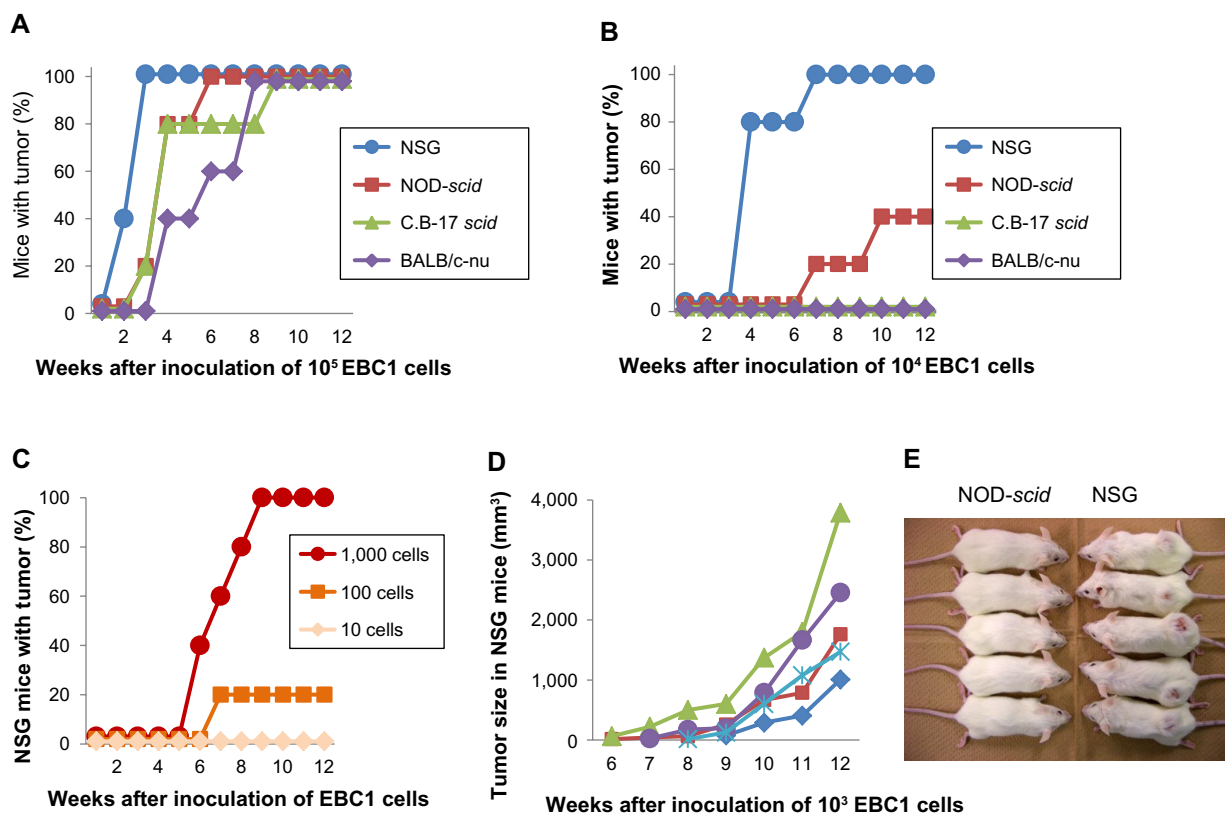


Figure 1 Tumorigenicity in mice after subcutaneous inoculation of EBC1 cells.

Notes: Tumorigenicity after inoculation of 10^5 (A) and 10^4 (B) EBC1 cells. (C) Tumorigenicity in NSG mice after inoculation of 10^3 EBC1 cells. X-axis shows period after inoculation of cells (weeks). Y-axis shows mice with tumor formation (%). (D) Tumor sizes in NSG mice after inoculation of 10^3 EBC1 cells. Each symbol represents an individual mouse. (E) At 12 weeks after inoculation of 10^3 EBC1 cells, mice were euthanized. NOD-*scid* and NSG mice are shown.

Abbreviations: NSG, NOD/LtSz-*scid* $Il2rg^{-/-}$; NOD, non-obese diabetic.

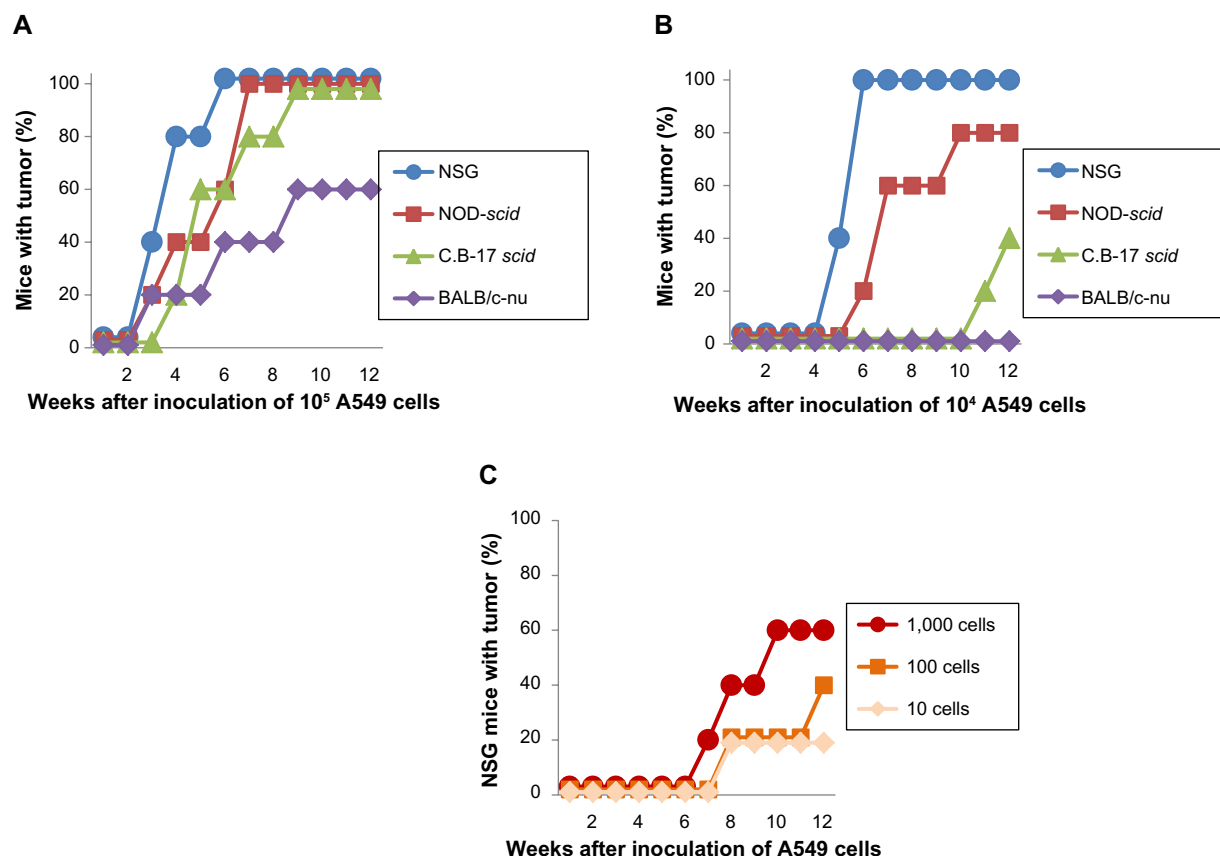


Figure 2 Tumorigenicity in mice after subcutaneous inoculation of A549 cells.

Notes: Tumorigenicity after inoculation of 10^5 (A) and 10^4 (B) A549 cells. (C) Tumorigenicity in NSG mice after inoculation of 10^3 A549 cells.

Abbreviation: NSG, NOD/LtSz-*scid* H2g^{-/-}.

Discussion

This study demonstrates that the highest susceptibility to xenotransplantation of two lung cancer cell lines was observed in NSG mice. In NSG mice, tumorigenesis appeared faster after inoculation of lung cancer cells, even with fewer cells.

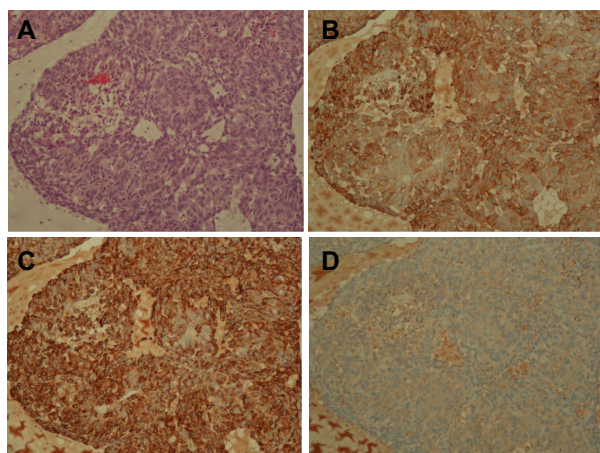


Figure 3 Pathology of developed tumor after inoculation of 10 A549 cells.

Notes: (A) Hematoxylin and eosin staining. (B) Immunostaining for anti-human pancytokeratin. (C) Immunostaining for anti-human vimentin. (D) Negative control without primary antibodies.

The first immunodeficient mouse model of cancer was developed in nude mice, which allowed the growth of human solid tumors, after inoculation of 1–20 million cells.¹⁸ Thereafter, several kinds of immunodeficient mice have been generated with more efficient xenotransplantability. Among them, NOD-*scid* mice showed a greater susceptibility to lung cancer cell lines and primary cells.^{5–7} The non-small cell lung carcinoma (NSCLC) xenografts from patients undergoing surgery were implanted into NOD-*scid* mice within 24 hours of surgery and they were successfully engrafted in 40% of cases.⁵ In this study, we demonstrated a dramatic improvement of xenotransplantation of fewer lung cancer cell lines in NSG compared with NOD-*scid* mice. When 10^3 or fewer cells were inoculated, tumors developed only in NSG mice and grew progressively, suggesting higher susceptibility of NSG than NOD-*scid* mice to xenotransplanted lung cancer cells. In addition, a subcutaneous tumor developed in one mouse that had been inoculated with only ten A549 cells.

The most commonly used procedure for diagnosing NSCLC is bronchoscopic examination,¹⁹ and only 20%–25% of patients have resectable disease because of the diagnosis in an advanced stage.²⁰ However, the number of cancer cells

obtained by a transbronchial biopsy (TBB) is usually quite few. Primary cell culture from patients with lung cancer has not fully been established, in particular, as the cell number is low, although many improvements in media used and culture technique have been reported for establishment of primary cell culture.²¹ If humanized mice can be available by successful xenotransplantation of lung cancer cells obtained by TBB, it might be useful for establishment of the best therapy for individual tumor. In this regard, NSG mice could be the best candidate. In fact, human breast cancers were propagated in SCID/Beige and NSG mice models.²² These models serve as a renewable, quality-controlled tissue resource for preclinical studies investigating metastasis and response to treatment.²² Development of xenografts platform from patients with NSCLC for drug testing in NSG mice models is also explored.²³ In this study, tumorigenesis appeared faster after inoculation of lung cancer cells in NSG mice, which might also be attractive for rapid assessment of individual tumor biology.

Compared with NOD-*scid* mice, NSG mice lack host NK cell activity and cytokines signaling are impaired. The contribution of IL-2 is well known to induce NK cell cytotoxicity against various types of malignancy.^{24–26} NK cell cytotoxicity for lung cancer cells was markedly augmented by stimulation with IL-2.^{27,28} Compared with normal subjects, patients with lung cancer consistently had higher levels of IL-2 in their bronchoalveolar lavage fluid and this titer correlated with an increase in NK cell activity.^{29,30} A differential composition of the immune cell infiltration was also assessed in malignant and non-malignant lung tissue areas associated with NSCLC.³¹ Interestingly, NK cells were almost absent in the malignant areas, while non-malignant counterparts were selectively populated by NK cells and those NK cells showed strong cytotoxic activity *ex vivo*.³¹ The higher tumorigenicity in NSG mice observed in this study might be due to, in part, lack of IL-2-dependent NK cell cytotoxicity, although cytokines other than IL-2 might also have a role.

The relevance of mouse models would further increase when assessed in an orthotopic organ site, ie, the lungs for lung cancer models, rather than assessment of subcutaneous growth. Orthotopic models would have some advantages compared to subcutaneous models. In the previous reports, some lung cancer cells inoculated intravenously into immunodeficient mice induced multiple lung nodules and malignant pleural effusion³² and murine lung cancer cells injected into the pleural space of mice developed malignant pleural effusion.³³ These models could make it possible to assess the therapeutic efficacy of cancer progression in a situation close to the actual target patients. On the other hand, in many

previous studies, subcutaneous models have been used for assessment of lung cancer, because of some advantages in subcutaneous models. First, tumor formation is recognized clearly at a glance. Second, it is easier to confirm the course of tumor progression, without use of radiological techniques or euthanasia. Third, subcutaneous inoculation of cells is an easier experimental procedure than intravenous inoculation or injection into the pleural space. Based on these advantages, we selected subcutaneous models in this study.

Conclusion

In summary, we directly compared the ability of four immunodeficient mice to successfully engraft lung cancer cells. NSG mice were more susceptible to tumor formation than other strains including NOD-*scid* mice. NSG mice could be a good choice in cases of limited cell numbers such as samples obtained by TBB or identification of a weakly tumorigenic phenotype.

Acknowledgment

There was no funding source for this study. We thank Ms Takimi Tamaki for long-term assistance with the animal experiments.

Disclosure

The authors report no conflicts of interest in this work.

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