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TECHNOLOGY REPORT

A Novel Reporter Rat Strain That Expresses LacZ Upon Cre-Mediated Recombination

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Summary: The recent widespread application of Cre/*loxP* technology has resulted in a new generation of conditional animal models that can better recapitulate many salient features of human disease. These models benefit from the ability to monitor the expression and functionality of Cre protein. We have generated a conditional (Cre/*loxP* dependent) *LacZ* reporter rat (termed the LacZ541 rat) to monitor Cre in transgenic rats. When LacZ541 rats were bred with another transgenic rat line expressing Cre recombinase under the control of the CAG promoter, LacZ/Cre double transgenic embryos displayed ubiquitous expression of LacZ, and when LacZ541 rats were bred with transgenic rats expressing Cre/*loxP*-dependent oncogenic H- or K-*ras*, LacZ was expressed in the lesions resulting from the activation of the oncogene. The LacZ541 rat enables evaluation of the performance of Cre-expressing systems which are based upon transgenic rats or somatic gene transfer vectors and provides efficient and simple lineage marking. genesis 00:00–00. © 2013 Wiley Periodicals, Inc.

Key words: rat; transgenic; reporter; β-galactosidase; Cre; loxP

The rat is an important murine model for studies in oncology, physiology, pathobiology, toxicology, neurobiology, and a variety of other disciplines (Jacob and Kwitek, 2002). The rat is of value in these fields because it is larger than the mouse and because a plethora of organ-specific physiologic and disease models have been developed for it over the last century. Surgical procedures can be performed more easily than in mice and disease models sometimes more closely reflect the situation encountered in humans. The importance of the rat as a biological model has led to an intense effort to also establish it as a strong genetic model.

Genetically engineered animals are invaluable in assessing the role of genes in complex processes such as tumorigenesis and embryonic development. The recent widespread application of Cre/*loxP* technology (Rajewsky *et al.*, 1996) has resulted in a new generation of conditional animal preclinical models that can better recapitulate many salient features of human disease. Cre expression achieved by classic transgenesis or targeting to an appropriate locus can be tissue specific, temporally restricted or inducible (Feil *et al.*, 1996). For example, we have established a transgenic rat carrying a human $Hras^{G12V}$ or $Kras^{G12V}$ oncogene regulated by the Cre/*loxP* system (Hras250 and Kras301 rats) (Tanaka *et al.*, 2010; Ueda *et al.*, 2006) in which

Additional Supporting Information may be found in the online version of this article.

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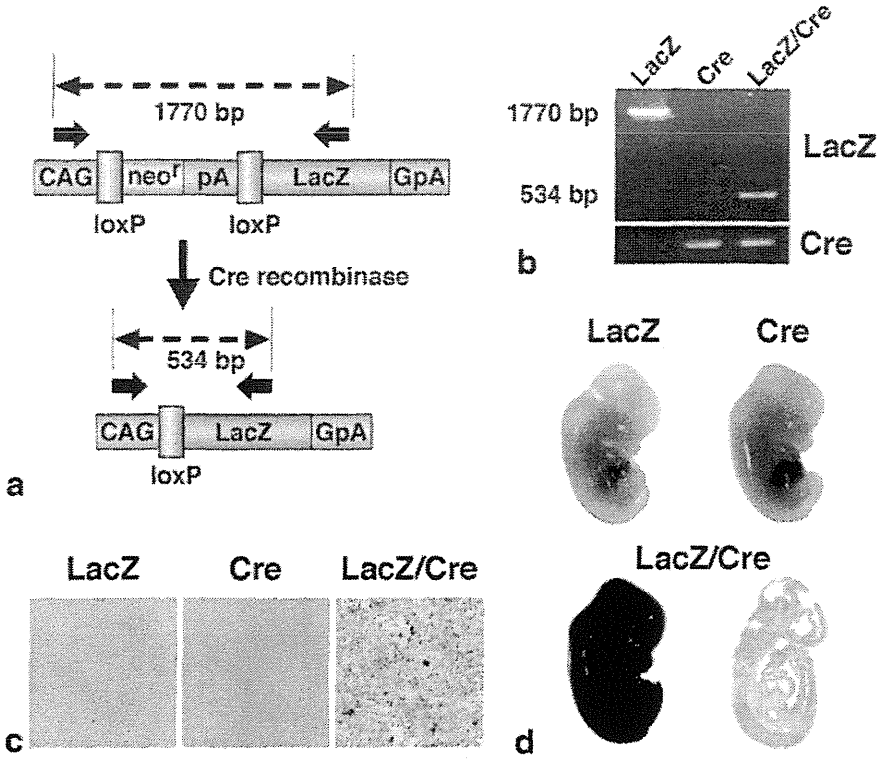

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FIG. 1. Cre-mediated activation of the lacZ gene in rats. (**a**) The transgene is comprised of a CAG promoter, a cassette for the neomycin resistance gene flanked by loxP sites, and a sequence containing the LacZ open reading frame. Cre recombinase activity results in Cre-mediated recombination of the transgene and removal of the neo-coding region and its associated mRNA polyadenylation signal, generating a functional LacZ expression unit. pA, SV40 early poly(A) site; GpA, rabbit-β-globin poly(A) site. Arrows indicate primers for the detection of recombination of the transgene. (**b**) Mating a heterozygous male LacZ541 transgenic rat with a homozygous female Cre-expressing transgenic rat resulted in progeny in which recombination of the LacZ transgene had occurred. The LacZ541 transgenic embryo was used as the negative control. PCR analysis showed that a 534-bp band is present in the LacZ/Cre double transgenic embryo, but not in the LacZ or Cre transgenic embryos. The neomycin cassette of the LacZ transgene in the LacZ/Cre embryo was removed by Cre recombinase. (**c**) X-Gal staining of fibroblast cells derived from LacZ (left), Cre (middle) and LacZ/Cre (right) heterozygous embryos. (**d**) Whole mount X-Gal staining of E14 LacZ (left), Cre (right) and LacZ/Cre (bottom) heterozygous littermate embryos. A sagittal section of the LacZ/Cre embryo is also shown.

pancreatic carcinogenesis is initiated by targeted activation of the transgene by injecting Cre-carrying adenovirus into the pancreatic ducts and acini through the common bile duct. This rat model provides a powerful research tool for examining the cytogenesis of pancreatic ductal adenocarcinoma.

In Cre/*loxP*-based experimental systems, it is important to monitor Cre activity at the desired time points and to verify the presence or absence of Cre activity during development. Such systems have been developed for the mouse: investigators have generated transgenic mouse lines in which β-galactosidase (lacZ) expression is conditional on Cre-dependent removal of an intervening segment (Akagi *et al.*, 1997; Araki *et al.*, 1995; Soriano, 1999; Tsien *et al.*, 1996), allowing Cre activity to be linked to lacZ activity. In the rat, a reporter line based on a DsRed/GFP double-reporter transgene under the control of the Cre/*loxP* system has also been established (Sato *et al.*, 2004). In this report we describe another reporter line, the LacZ541 rat, which carries a lacZ gene regulated by the Cre/*loxP* system. The advantage of LacZ is ease of visualization *in situ* and in section and whole mount preparations. The LacZ541 rat enables evaluation of the performance of Cre-expressing systems and provides efficient and simple lineage marking.

Reporter rats were generated by incorporating a transgene in which the CAG promoter is separated from a *lacZ* open reading frame by a stuffer sequence (neomycin resistant gene) flanked by *loxP* sites (Fig. 1A) which stops transcription of *lacZ*. A line was established (SD-Tg(CAG-lacZ)541Htsu, LacZ541) in which the transgene was transmittable to descendant generations. In these rats, *Cre*-mediated recombination removes the stop sequence, generating a functional LacZ expression unit and allowing expression of F1

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β-galactosidase in all cell types in which the CAG promoter is active. Heterozygous male LacZ541 transgenic rats were bred with homozygous female NCre rats (Sato *et al.*, 2004): The NCre rat was made by incorporating a transgene in which the CAG promoter directly controls expression of Cre, and consequently, NCre rats express Cre ubiquitously. When the NCre rat was bred to the DsRed/GFP reporter rat, Cre deleted the DsRed sequence in the progeny resulting in ubiquitous expression of GFP (Sato *et al.*, 2004). Results of crossing the deleter NCre rat line with heterozygous LacZ541 rats are shown in Figure 1. Genomic DNA was isolated from the embryonic yolk sac and subjected to PCR. In LacZ541 embryos, a 1770-bp band corresponding to the unmodified transgene was detected, while in LacZ/Cre compound embryos, PCR generated a 534-bp band corresponding to the recombinant transgene (Fig. 1b); Cre embryos do not have the LacZ transgene. Embryos were also collected at embryonic day 14 and stained with X-Gal for LacZ activity. Rat embryonic fibroblast cells and embryos heterozygous for both LacZ and Cre alleles displayed ubiquitous staining, whereas wild-type (data not shown), heterozygous LacZ and Cre embryos did not show any staining (Fig. 1c,d). These results demonstrate that in LacZ541 transgenic rats, Cre induces recombination of the transgene resulting in CAG promoter driven expression of the *lacZ* gene. The LacZ541 rats did not display an overt phenotype and were bred to obtain viable and fertile homozygous transgenic progeny. The LacZ541 transgenic rat is available from the National BioResource Project for the Rat in Japan (NBRP Rat No: 0569) (http://www.anim.med.kyoto-u.ac.jp/NBR/).

To examine the expression of LacZ in adult organs, LacZ/Cre double transgenic (LacZ/Cre) rats were generated by breeding heterozygous LacZ541 rats with homozygous female NCre rats. Major organs were removed from LacZ/Cre, LacZ, and Cre rats and the
F2 LacZ expression pattern and intensity was determined
F3 by X-Gal staining (Figs. 2 and 3, and Supporting Information Fig. S1). Skeletal muscle and myocardium exhibited strong LacZ expression in LacZ/Cre rats. The expression pattern and intensity of LacZ is summarized
T1 in Table 1. The expression pattern of LacZ in LacZ/Cre rats was almost the same as the expression pattern of LacZ in CAG/LacZ-DA rats: in CAG/LacZ-DA rats, the expression of LacZ is directly driven by the CAG promoter (Inoue *et al.*, 2005). The LacZ/Cre rat embryo clearly showed widespread lacZ expression. However, some adult organs, including the liver, were negative. Because CAG promoter can be activated in stem cells including fertilized eggs, Cre is expected to remove the stop sequence in the CAG-neo-LacZ transgene and allow *lacZ* transcription to occur ubiquitously in the early embryo, and PCR analysis confirmed that recombination had occurred in all of the organs listed in Table 1

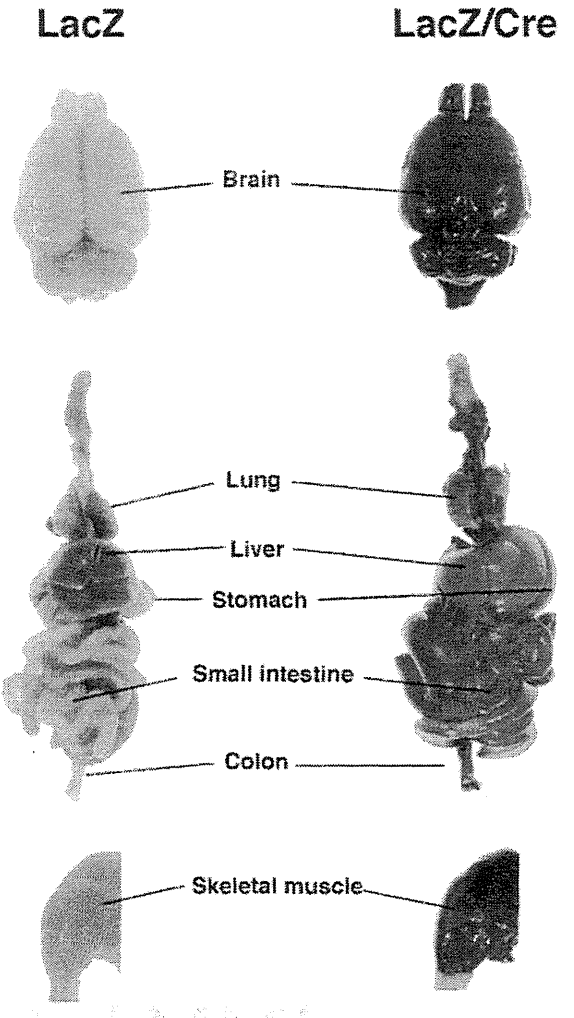

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FIG. 2. Cre-mediated recombination in the adult organs. Whole mount X-Gal staining of the adult organs of LacZ and LacZ/Cre double transgenic rats. Tissues were stained overnight (brain) or for 3 h (other organs). Note that the olfactory bulb and cerebellum of the LacZ rat brain is light green because of endogenous β-galactosidase activity; the olfactory bulb and cerebellum of the LacZ rat brain did not stain blue/green when staining was limited to 3 h (not shown).

(Supporting Information Fig. S2). This indicates that the recombination frequency in these tissues was not related with LacZ expression and activity. Lack of LacZ expression, for example in the liver, could be because the site of transgene integration is not permissive for expression in the adult liver, or CAG promoter activity might be low in the adult liver of LacZ541 rats.

To examine whether the LacZ541 rat is useful for carcinogenesis studies, we used the LacZ541 rat to investigate the expression of the oncogenic $Hras^{G12V}$ transgene in a rat model of pancreatic cancer. Our rat models of pancreatic cancer use three different Cre

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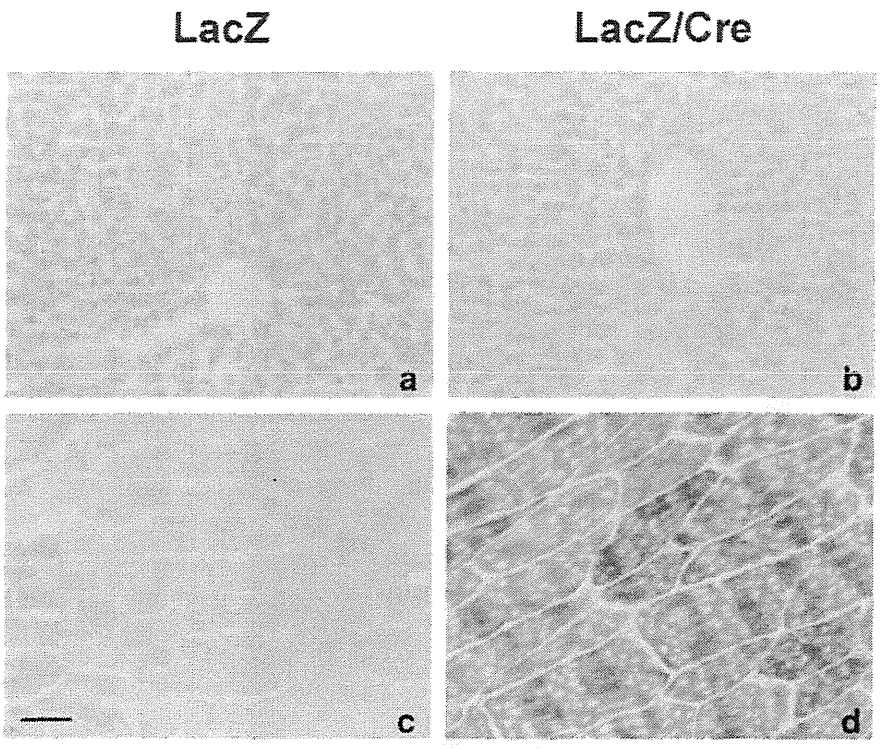

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FIG. 3. LacZ expression in the adult tissues of LacZ and LacZ/Cre rats. Frozen sections were stained with X-gal. (**a**) and (**b**), Liver; (**c**) and (**d**), Skeletal muscle. Bar = 50 μm.

regulated human ras^{G12V} transgenes to induce cancer, $Hras^{G12V}$, $Kras^{G12V}$, and HA-tagged $Kras^{G12V}$ (Fukamachi *et al.*, 2009; Tanaka *et al.*, 2010; Ueda *et al.*, 2006), with specific targeting of pancreatic cancer being achieved by injecting a recombinant adenovirus vector carrying Cre recombinase (AxCANCre) into the pancreatic duct via the common bile. Cells infected with AxCANCre express the oncogenic ras^{G12V} transgene when Cre removes the stop sequence which lies between the CAG promoter and the ras^{G12V} open reading frame. In the experiment described below, we used the $Hras^{G12V}$ (Hras250) and HA-$Kras^{G12V}$ (Kras301) rats.

In the HA-$Kras^{G12V}$ rat, expression of the oncogenic transgene can be investigated by techniques, such as immunohistochemistry, which target the HA tag (Fukamachi *et al.*, 2009; Tanaka *et al.*, 2010). In the $Hras^{G12V}$ rat, on the other hand, there are only two amino acid differences between the sequences of endogenous *ras* and the transgene; therefore, immunohistochemistry cannot be used to investigate the expression of the oncogenic transgene in this rat. We generated HA-$Kras^{G12V}$/LacZ and $Hras^{G12V}$/LacZ double transgenic rats (Kras/LacZ and Hras/LacZ rats) by breeding LacZ541 rats with Kras301 or Hras250 rats. Kras/LacZ and Hras/LacZ rats were injected with AxCANCre, and 3 weeks after injection, the animals were killed. Multiple grossly visible whitish nodules were observed throughout the pancreas in all of the Kras/LacZ and Hras/LacZ rats. Histological examination showed that these nodules were adenocarcinomas, as in our previous reports (Tanaka *et al.*, 2010; Ueda *et al.*, 2006). In Kras/LacZ rats, double immunofluorescence staining

Table 1
Pattern of LacZ Expression in Organs From LacZ/Cre-Tg Rats

Organ	LacZ expression
Brain	+
Myocardium	+++
Skeletal muscle	+++
Blood vessels	−
Lung	+
Liver	−
Spleen	−
Pancreas	±
Kidney	++
Adrenal grand	+
Stomach	+
Small intestine	+
Colon	+++
Ovary	+
Uterus	+

−, negative; ±, weakly positive; + mildly positive; ++, moderately positive; +++, strongly positive.

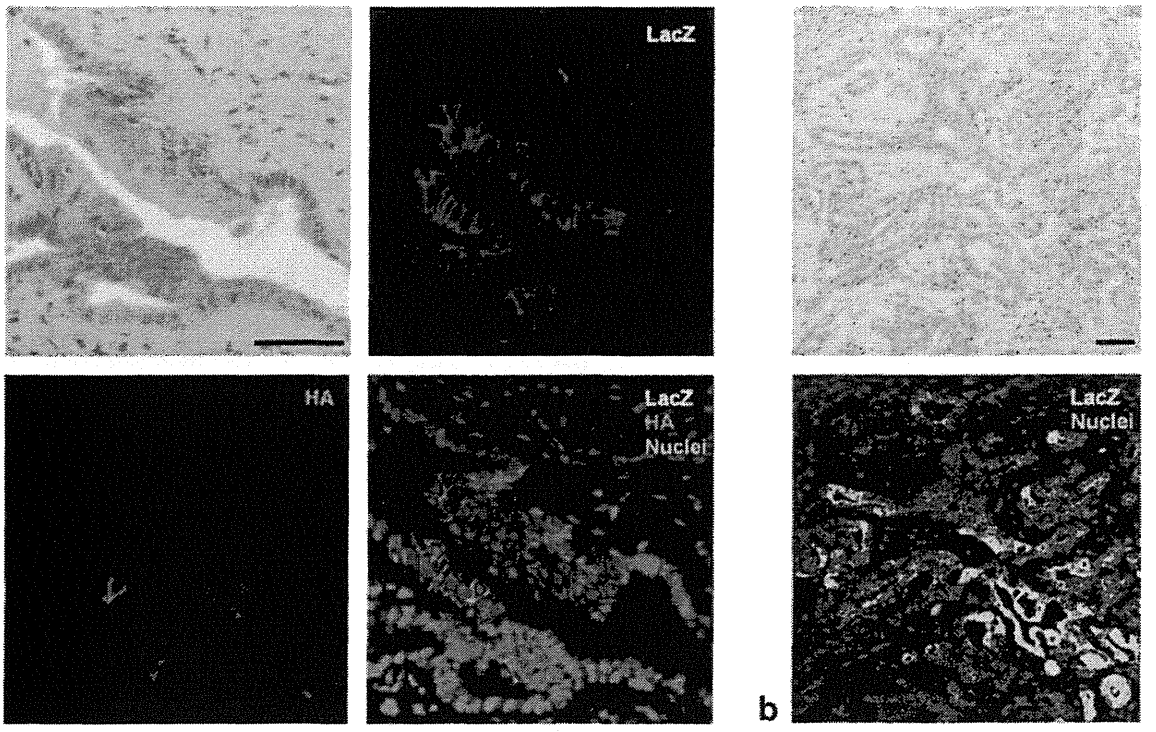

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FIG. 4. Analysis of LacZ expression in pancreas ductal adenocarcinoma of ras/LacZ double transgenic rats. (**a**) Colocalization of LacZ (green) and HA-*Kras*^{G12V} (red) in pancreatic lesions of the Kras/LacZ rat. (**b**) Immunofluorescence staining of β-galactosidase in the pancreas shows LacZ positive cells (green) clustered in ductal lesions of the Hras/LacZ rat. Bar = 50 μm.

for LacZ and HA showed that the expression pattern of LacZ closely resembled that of HA-*Kras*^{G12V} (Fig. 4a), demonstrating that in HA-*Kras*^{G12V}/LacZ double transgenic rats, the expression of the LacZ and HA-*Kras*^{G12V} transgenes is linked; in cells which are infected by AxCANCre and express Cre, Cre generally activates both the HA-*Kras*^{G12V} transgene and the LacZ transgene. Thus, LacZ can be used to monitor the expression of Cre/*loxP*-dependent transgenes. Therefore, the expression of the oncogenic transgene in H*ras*^{G12V} rats can be investigated using the LacZ541 reporter rat. In Hras/LacZ rats, immunostaining for LacZ revealed LacZ positive cells in the pancreas and these cells were clustered in ductal lesions (Fig. 4b). Because the cells expressing LacZ also express H*ras*^{G12V}, these results indicate that expression of oncogenic H*ras*^{G12V} gives rise to these lesions.

Until recently, a key genetic technology available for the mouse but not for the rat was the production of animals in which specified genes were disrupted (knockout animals) (Jacob and Kwitek, 2002). Pluripotent rat embryonic stem cell lines which are capable of producing genetically engineered rats have now been established (Buehr *et al.*, 2008; Kawamata and Ochiya, 2010; Li *et al.*, 2008). This crucial development, together with other recent advances in genetic engineering, such as the cloned rat (Zhou *et al.*, 2003), induced pluripotent stem (iPS) cells (Li *et al.*, 2009; Liao *et al.*, 2009), nucleases (ZFN/TALEN) (Geurts *et al.*, 2009; Tesson *et al.*, 2011; Tong *et al.*, 2012), and knockown/conditional knockdown by siRNA (Dann *et al.*, 2006), make genetically engineered rats powerful, innovative tools to advance biomedical research.

In conclusion, we have constructed a reporter line of rats that express LacZ only in cells expressing Cre recombinase and their daughter cells. The advantage of LacZ is the ease of visualization *in situ* and in section and whole mount preparations. The LacZ541 rat is an efficient and simple system for monitoring Cre expression and evaluating the performance of Cre-expressing systems which are based upon transgenic rats or somatic gene transfer vectors.

MATERIALS AND METHODS

Animals

For the generation of transgenic rats conditionally expressing LacZ, the CALNLZ switching unit was obtained from Riken Bioresources Center DNA Bank

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(RDB1680) (Kanegae *et al.*, 1995), and the purified cassette was injected into the pronuclei of Sprague-Dawley rat zygotes (CLEA Japan, Tokyo, Japan). Techniques used for the generation of transgenic rats were the same as those reported previously (Asamoto *et al.*, 2000). A total of 313 injected eggs were transplanted into pseudo-pregnant Sprague-Dawley rats. Of 53 potential transgenic rats screened, 8 female rats were shown by PCR to carry the transgene. Transgenic founder rats were mated with Sprague-Dawley rats, and offspring were screened for the presence of the transgene by PCR analysis of genomic DNA isolated from tail biopsies at the age of 3 weeks. Homozygous transgenic rats were identified by semiquantitave PCR, and then confirmed by genetic testing.

Transgenic rats expressing Cre recombinase regulated by the CAG promoter (W-Tg(CAG-cre)81Jmsk) (NCre rat) were supplied by the National BioResource Project for the Rat in Japan (Kyoto, Japan, http://www.anim.med.kyoto-u.ac.jp/NBR/). Because the transgene is located on the X chromosome, homozygous female NCre rats were used in this study.

Male HA-K*ras*^{G12V} or H*ras*^{G12V} transgenic (Kras301 or Hras250) rats were established in our laboratory previously (Fukamachi *et al.*, 2009; Tanaka *et al.*, 2010; Ueda *et al.*, 2006).

All animal experiments were conducted according to the "Guidelines for Animal Experiments of the Nagoya City University Graduate School of Medical Sciences."

Detection of Recombination of the Transgene

Genomic DNA was extracted using standard methods (Laird *et al.*, 1991). Genomic DNA was used as the template for PCR reactions for detecting transgene recombination. The primers (Fig. 1, arrows) used were: 5′-CGTGCTGGTTGTTGTGCTGTCT-3′ (in the CAG promoter region), 5′-TCCTGTAGCCAGCTTTCATC-3′ (in the LacZ coding region).

X-Gal Staining

Transgene expression in NCre x LacZ541 progeny was determined by X-gal staining. Embryos or dissected tissues were fixed in 4% paraformaldehyde for 1 hr at 4°C, and then washed three times in rinse solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 in PBS). Specimens were treated with staining solution (1 mg ml^{-1} X-Gal, 5 mM $K_3[FeCN_6]$, 5 mM $K_4[FeCN_6]3H_2O$ in rinse solution) overnight (brain) or for 3 h (other organs) at 37°C.

Frozen sections were fixed in fixative solution (0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA in PBS) for 5 min at 4°C, and then washed three times in rinse solution. Then they were treated with staining solution. The slides were counterstained with Kernechtrot solution (Nuclear fast red). After staining, samples were rinsed in distilled water three times, dehydrated with ethanol, cleared in xylene and mounted.

Cell Culture

Rat embryonic fibroblast cells (rEFs) were isolated from 14.5-day-postcoitum T_g rat embryos. Embryos were separated from maternal tissues and yolk sac and the internal organs were removed. The remaining tissues were finely minced and incubated with gentle agitation at 37°C for 10 min in 0.25% trypsin-EDTA. The cell suspension was then passed through an 18G needle and further incubated at 37°C for 15 min. The supernatant containing rEFs was plated in DMEM supplemented with 10% fetal bovine serum. The rEFs were fixed in formalin containing glutaraldehyde (2% formalin, 0.2% glutaraldehyde in PBS) for 5 min at 4°C. The fixed cells were treated with X-Gal staining solution at 37°C.

Tumor Induction, Immunohistochemistry and Immunofluorescence

Pancreas tumors were induced as described previously (Fukamachi *et al.*, 2009; Tanaka *et al.*, 2010; Ueda *et al.*, 2006). Briefly, purified adenovirus vector carrying Cre recombinase (AxCANCre) was injected into the pancreatic duct through the common bile duct. Animals were killed 3 weeks after injection of recombinant AxCANCre. Pathological examination was performed as described previously (Tanaka *et al.*, 2010). Paraffin section slides were treated with 0.1% trypsin for 20 min at 37°C and boiled for 10 min in citrate buffer before incubation with primary antibody: β-galactosidase (LacZ) antibody (AB9361, Abcam, Temecula, CA) diluted 1:100; HA-Tag antibody (6E2; Cell Signaling, Danvers, MA) diluted 1:100. Slides were incubated with secondary antibodies conjugated with Alexa Fluor488 (LacZ) and 546 (HA-Tag) (Molecular Probes, Eugene, OR). Nuclei were counterstained with TO-PRO-3 (Molecular Probes). Images were obtained with a FLUOVIEW FV300 confocal microscope (Olympus, Tokyo, Japan).

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Scientific Research

Animal Model of Lung Metastasis of Hepatocellular Carcinoma: A Tool for the Development of Anti-Metastatic Therapeutics*

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ABSTRACT

We observed that N-nitrosomorpholine (NMOR) given after a multi-carcinogenic treatment induces liver carcinomas with 56% lung metastasis. An additional treatment with diethylnitrosamine (DEN) with NMOR further enhanced the incidence of hepatocellular carcinoma (HCC) with lung metastasis. We have further revised the duration of NMOR treatment to establish an animal model with a simple experimental protocol and an appropriate experimental duration to facilitate investigation exploring the mechanisms of HCC metastasis and development of anti-metastatic therapeutics. We observed that DEN exposure followed by a 16-week treatment with NMOR to be a most efficient protocol for the induction of HCC metastasizing to the lung. In this review, we will discuss about the usefulness of animal models for induction of highly metastatic HCC and the assessment of the efficacy of anti-metastatic therapeutics. Additionally, we will also discuss use of these models in analysis of individual steps in the metastatic process by using non-steroidal anti-inflammatory drugs, aspirin and indomethacin, two nuclear factor kappa B (NF-κB) inhibitors, pentoxifylline and N-acetyl-L-cysteine.

Keywords: Lung Metastasis; Hepatocellular Carcinoma; NF-κB Inhibitor

1. Introduction

Despite the continuous improvements in early diagnosis and therapy for early stage cancer, most deaths from cancer occur due to metastases [1]. Once metastatic disease has developed, aggressive treatment such as systemic chemotherapy is required since surgical removal of all metastatic foci is not feasible [2]. Therefore, it is necessary to identify and develop novel treatment strategies for preventing cancer metastasis.

Tumor metastasis is a multistage process during which malignant cells spread from the primary tumor to discontiguous organs [3]. It involves invasion, transport, arrest, adherence, extravasation, growth in different microenvironments, which are treated clinically with different strategies depending on the tumor histiotype and metastatic location [4].

To study the mechanisms underlying metastasis, many tools and models have been developed. Most of them use cancer cell lines or transplantable tumors, injected into blood vessels or intraperitoneal cavity, or transplanted into the cecum, spleen or subcutis [5-7]. These models have provided very useful tools for analysis of individual steps in the metastatic process. However, in order to assess the efficacy of therapeutic treatments for advanced cancers with metastasis, it is necessary to develop animal cancer models for natural course of metastasis, which feature frequent metastasis of primary tumors to distant organs. Thus, comprehensive analysis is required to develop anti-metastasis agents.

2. Establishment of an *in Vivo* Highly Metastatic Rat HCC Model

We have previously shown by chance that N-nitrosomorpholine (NMOR) given after a multi-carcinogenic treatment with N-diethylnitrosamine (DEN), N-methylnitrosourea (MNU), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), 1, 2-dimethyl-hydrazine (DMH), and 2, 2'-dihydroxy-di-N-propylnitrosaminee (DHPN) induces liver carcinomas with frequent lung metastasis [8]. We attempted to establish an animal model with a simple experimental protocol and an appropriate experimental duration which would facilitate further study of the mechanisms of metastasis and antimetastatic agents (**Figure 1**) [9].

NMOR and DEN have been widely used as hepatocarcinogens in animal models, and the induced malignant

*The authors have declared that no conflict of interest exists.

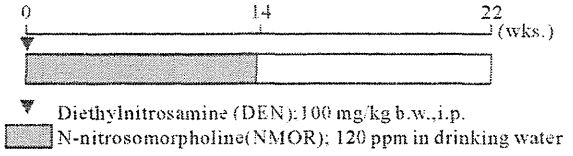

Figure 1. Protocol for an *in vivo* highly metastatic rat HCC model We had established an *in vivo* lung metastasis model of HCC induced by two hepato-carcinogens, DEN and 120 ppm NMOR. This model allows us to apply chemical substance in the intervening period to investigate modifying factors, particularly those leading to inhibition of lung metastasis formation. We attempted to establish an animal model with a simple experimental protocol and an appropriate experimental duration which would facilitate further study of the mechanisms of metastasis and antimetastatic agents.

tumors have been well characterized [10-12]. Lung metastasis by induced HCC in rats given either DEN or NMOR has been reported by Lijinsky *et al.* [13,14]. In our previous study, treatment with NMOR alone or with DEN followed by 8-weeks NMOR resulted HCC induction (**Figure 2(a)**) with only few lung metastases (**Figure 2(b)**) [9]. In contrast, DEN followed by 16 or 22-weeks NMOR treatment was associated HCC (**Figure 2(c)**) with higher frequencies of lung metastases (**Figure 2(d)**), with a duration dependence of NMOR treatment [9]. Histologically, we observed not only large metastatic nodules, but also extravasation in the lung at week 22. These findings suggest that a multi step process of metastasis (including invasion, transport, arrest, adherence, extravasation, and tumor cell proliferation) proceeded between weeks 16 and 22. Therefore, using this model, chemical substances could be applied in the intervening period to investigate modifying factors, particularly those leading to inhibition of lung metastasis formation.

Change in the expression of cadherin, a major adhesion molecule of epithelia [15-17], has been implicated in carcinogenesis because loss is frequent in human and murine high grade epithelial cancers [18-20]. In the previous study, we found that pan-cadherin expression to be decreased in the order of adenoma, HCC and advanced HCC. The quantitative difference of cadherin expression was observed between the HCC with metastasis and that without metastasis. These results suggest that down-regulation of cadherin expression may occur as an early event of carcinogenesis with decrease with in line with hyperplasia, adenoma and HCC.

Detection of circulating tumor cells in the blood may give us the evidence that tumor cells had already entered in the circulation before microscopic metastasis lesions were detected, and circulating tumor cells were also assessed in relation to HCC development and lung metastasis formation [21]. For detection of circulating

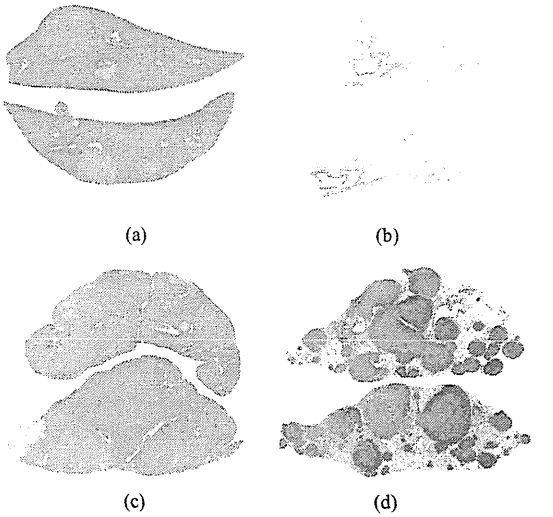

Figure 2. HCC and lung metastasis formation (a) Treatment with NMOR alone or with DEN followed by 8-weeks NMOR resulted HCC induction in week14; (b) In week 14, we observed only few lung metastases; (c) DEN followed by 14-weeks NMOR treatment induced multiple HCC; (d) We observed not only large metastatic nodules, but also extravasation in the lung at week 22.

tumor cells, RT-PCR has been utilized [22-24], and we found CK-8 expression have been demonstrated to be positive in blood. Through the travel in the circulation, only a small percentage of tumor cells (<0.01%) released from a primary tumor survive and arrest in the capillary beds of distant organs producing a successful metastasis [25]. Survival in the circulation appears to be responsible for this inefficiency due to immune factors in the blood, and this response may be the reason why tumor cells are circulating in the blood while no microscopic metastasis was found.

3. Suppression of Lung Metastasis by Aspirin but Not Indomethacin in an *in Vivo* Model of Chemically-Induced HCC

Because the metastatic cascade is a continuous process which begins with proliferation of the primary tumor and ends with proliferation of the metastatic foci [26], we hypothesized interference with cell proliferation might prevent metastasis formation. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin (ASP) and indomethacin (IM) are well known as potential chemopreventive agents through their modulation of levels of prostaglandins, PGE2, and cyclooxygenase (COX) in the colon and also other organs [27,28].

We have demonstrated that ASP but not IM significantly reduced the severity of lung metastasis, but not the average number. This indicates that the effect of ASP

was marginal [29]. We also demonstrated that only ASP suppressed lung metastasis formation although ASP and IM exerted inhibitory effects on cell proliferation of HCCs [29]. Thus, it is suggested that inhibition of cell proliferation per se may not be involved in the mechanism of inhibition of lung metastasis by ASP.

Epidemiological studies revealed that NSAIDs, such as ASP and IM, which suppress COX activity, possess considerable potential as chemopreventive agents for colorectal cancer [30,31]. Constitutive expression of COX-2 has been demonstrated to lead to phenotypic changes that alter the metastatic potential of colorectal cancer cells [32], and COX-2 inhibitor was found to exert inhibitory effects on metastasis formation of various cancer [33,34]. However, our data demonstrated that IM did not suppress lung metastasis formation in spite of down-regulation of COX-2 [29], indicating no direct involvement of this enzyme in the inhibitory effect on HCC metastasis. In addition, neither ASP nor IM exerted any apparent influence on cadherin expression within HCC [29]. Therefore, the mechanism of inhibition by ASP might be mainly in a stage of the metastatic cascade after the primary site, such as attachment to the vascular endothelium or re-invasion or re-proliferation in the lung.

The attachment of a cancer cell to the vascular endothelium is a complex phenomenon involving a number of cell adhesion molecules (CAMs). Among these latter, E-selectin, ICAM-1 and VCAM-1 are considered to play primary roles in hematogenous metastasis [35,36]. Induction of Eselectin, ICAM-1 and VCAM-1 is mediated by the transcription factor nuclear factor-kappa B (NF-κB) [37,38]. ASP has been shown to inhibit NF-κB dependent transcription [39], and these transcriptions appear not to be related to the inhibition of COX activity, since IM was ineffective [40]. In the previous study, ASP significantly suppressed the expressions of ICAM-1 and VCAM-1 [29], indicating a probable role of inhibition of attachment of tumor cells to the vascular endothelium. Therefore, a stronger inhibitor of NF-κB might be expected to have a stronger inhibitory effect on lung metastasis formation.

4. Suppression of Metastasis by Nuclear Factor KappaB Inhibitors in an *in Vivo* Lung Metastasis Model of HCC

In order to evaluate the suppressive effects of NF-κB inhibitors, we examined three examples, pentoxifylline (PTX) [41], Nacetyl-L-cysteine (NAC) [42], and ASP [39], in our *in vivo* lung metastasis model. PTX, widely used as a hemorheological agent in the treatment of peripheral vascular disease, was earlier shown to suppress lung metastasis formation by B16F10 melanoma [43] and NAC inhibits VEGF production in human melanoma cell lines [44], invasion of endothelial cells [45], and invasion of human bladder cancer cells through the suppression of MMP-9 [46]. ASP has been demonstrated to inhibit angiogenesis [47] and HGF- induced invasiveness of HepG2 human hepatoma cells [48].

Among the NF-κB inhibitors, PTX exerted the strongest effects on lung metastasis formation and NAC had rather less influence, while ASP did not significantly reduce lung metastasis [49]. Although PTX and NAC suppressed lung metastasis, they did not improve the survival rates. This was mainly because the increase in the mortality rates owing to bleeding from primary HCC diminished the decrease that resulted from suppression of lung metastasis. Thus, the increase and decrease were not significant, and treatment with NF-κB inhibitors did not affect the incidences and multiplicities of HCCs in liver. Therefore, further studies are necessary to elucidate the reasons why PTX and NAC did not affect the survival rates.

To evaluate the degree of inhibition of NF-κB transcription, inhibitor of κB (IκB) protein levels in HCCs were evaluated by western blotting. The IκB family has been shown to control the function of NF-κB complexes [50,51], and IkB protein has been shown to activate NF-κB when it is phosphorylated or cleaved by proteasomes through a ubiquitine- dependent pathway [52,53]. We demonstrated that IκB protein expression was suppressed by test compounds in the order of PTX, NAC and ASP. Therefore, these results suggest that the mechanism of reduction of lung metastasis formation observed in this study may involve inhibition of NF-κB transcription.

The contribution of NF-κB to the process of metastasis has been explored in relation to CAMs and VEGF expression was found to be significantly suppressed by NF-κB signaling blockade [54], and promoted by coactivation of NF-κB [55]. PTX significantly suppressed expression of VEGF-A splicing variants with heparin-, heparin-sulfate-, and extracellular matrix-binding domains. These results suggest that the mechanism of the suppression of lung metastasis by PTX involves suppression of VEGF-A with heparin-binding domains. On the other hand, NAC, which had less influence on lung metastasis formation than PTX, suppressed VEGF-A variants with and without the heparin-binding domain. Therefore, whether NF-κB controls only VEGF-A with heparin-binding domains remains to be elucidated.

5. Conclusions

Our rat model presented here provides an excellent tool for rapid induction of metastatic HCC. To our knowledge, this is the first model to reflect the natural course

