function in amniotic fluid other than that associated with neutrophil activity.

In contrast to the nonpregnant female reproductive tract and amniotic fluid, antimicrobial levels of hLF are present in the cervical mucus plug of pregnant women. The origin of cervical mucus plug hLF (CMP-hLF) is unknown; however, it is likely to come from the neutrophils that are associated with the plug (Hein et al. 2005). Unlike tears and airway mucosal fluid, the cervical mucus plug is not continuously removed from the body; therefore, CMP-hLF does not function to suppress trapped microorganisms during their removal. Also, there is no evidence that hLF has an effect other than locally at the plug. In these conditions, it is plausible that the primary effect of hLF is to inhibit inflammatory responses as neutrophils kill microorganisms that penetrate into the plug. This assumption remains to be experimentally tested.

Summary and concluding remarks

The conserved, widespread expression of LF strongly suggests it has an important role in mammalian physiology, and the LF literature and the distribution profile of hLF suggest that it assists in the nonlethal removal of potentially pathogenic microorganisms from the body (or from spermatozoa). hLF sequesters iron away from target microorganisms and disrupts microbial factors that are involved in colonization and infection of target cells. Currently reported hLF targets are IgA1 protease, Hap adhesion, and type III secretion systems. hLF is also able to directly interfere with attachment of viruses to target surfaces. In addition, hLFcin may be microbicidal to specific target organisms, but this remains to be verified in vivo. Other antimicrobial activities of hLF, if they exist, remain to be identified.

For hLF to affect antimicrobial activity in mucosal fluids, it must be present at moderate to high levels. hLF is expressed at high levels in tear and airway mucosal fluids. Consequently, in these compartments, hLF levels are high enough to inhibit colonization and infection of the epithelial surface and inhibit the growth of microorganisms during the time required for their removal. hLF is also present at high levels in the intestine of the breast-fed infant. These high levels suggest that, similar to tear and airway fluids, hLF functions to prevent colonization and infection of the epithelium in the small intestine and to inhibit the growth of microorganisms as they are washed out of the upper small intestine with its exposed intestinal villi and deposited in the much better protected ileum and colon.

hLF is also present at relatively high levels in seminal plasma. Since spermatozoa are coated with hLF, one function of this hLF would appear to be protection of spermatozoa from infection by the vaginal microbiota. Seminal plasma hLF may also protect the vaginal epithelium from penis-introduced microorganisms; however, there are no reports of studies examining associations between vaginal infections and hLF levels in seminal plasma.

Finally, hLF is found at relatively high levels in the cervical mucus plug that develops after conception. Cervical plug hLF, unlike the hLF present in tears, airway fluids, breast milk, and seminal plasma, is likely to originate from neutrophils. Rather than functioning as an antimicrobial agent, a plausible supposition is that cervical plug hLF functions primarily to dampen inflammation as cervical mucus plug associated neutrophils protect the sterile uterus and the developing fetus by killing microorganisms that penetrate into the plug.

The role of hLF in saliva is problematical. On the one hand, it is present in saliva at concentrations much lower than in tears and airway fluids, suggesting that it may have little or no activity in the oral cavity. Since saliva is rapidly removed from the oral cavity, it is reasonable that the microbiostatic activity of saliva is of little importance compared with tears and airway fluids. On the other hand, it is secreted into saliva, suggesting that it does have a functional role in the oral cavity. One possibility is that its concentration in hLF-secreting glands may be much higher than in whole saliva, and its function may be to protect glandular acini and ducts rather than the oral epithelium from invading microorganisms. Another possibility is that while its levels in saliva are low, they may be sufficient to sequester iron away from invading microorganisms and inhibit their growth. Finally, if hLF is associated with the epithelium, it may be present at microbiostatic levels at the oral epithelial cell surface. The function of hLF in saliva requires further investigation.

hLF is found at low levels in the small intestine, the female reproductive tract (other than the cervical mucus plug), and in amniotic fluid. The function of hLF at these sites is unknown. The levels are far too low for hLF to have any antimicrobial effect in the overlying fluids. However, as suggested for the oral cavity, if hLF is associated with the epithelium, it may be present at microbiostatic levels at the epithelial cell surface.

hLF is the major LPS-binding protein in the mucosal fluids in which it is found and is consequently the major inhibitor of LPS-mediated inflammation. hLF also binds iron and inhibits generation of oxygen radicals and toxic metabolites. Finally, hLF binds to and activates LRP1 signaling. Therefore, at sites of tissue damage and high levels of neutrophil activity, hLF protects tissues by (*i*) dampening LPS-mediated inflammation, (*ii*) binding iron and inhibiting generation of oxygen radicals and toxic metabolites, and (*iii*) binding LRP1 and promoting tissue repair or slowing down tissue damage.

A very promising LF research tool is the LF-knockout mouse. For example, determining the effects of raising LF-knockout mice in barrier-free conditions would provide valuable information regarding the in vivo function of LF. Numerous other studies using these mice, for example, studies on infection of mucosal surfaces where LF is normally found at low levels in the overlying fluids and the effect LF-knockout has on wound healing, would also provide critical information regarding LF function. Other knockout mice can also be used to investigate the functions of LF. One example would be to determine whether neutrophils isolated from TLR-4 knockout mice and LF-knockout mice have the same defect in their oxidative burst response to PMA.

We would like to conclude this review with 3 points. (*i*) A striking fact about hLF is the paucity of direct experimental data about its in vivo functions and, consequently, definitive in vivo proof of hLF function is largely lacking. (*ii*) Statements of LF function in vivo based on in vitro evidence or based on experiments using exogenous LF should be made

with caution. (*iii*) hLF is a major component of biologically important mucosal fluids and of the specific granules of neutrophils, and delineating its biological function is essential for understanding neutrophil- and mucosal-mediated immunity.

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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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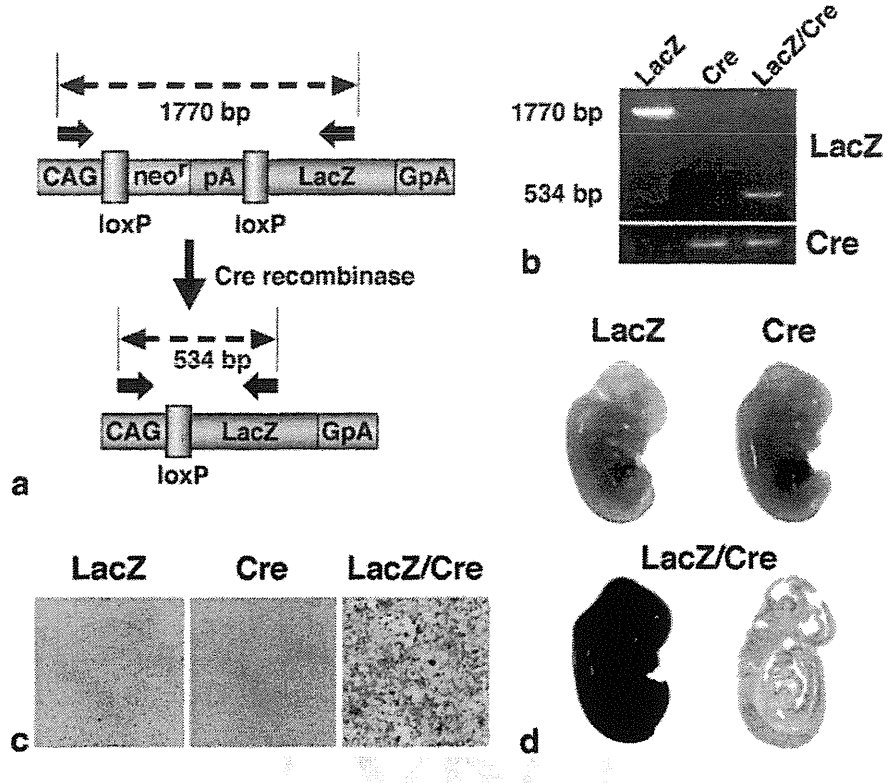

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FIG. 1. Cre-mediated activation of the lacZ gene in rats. **(a)** The transgene is comprised of a CAG promoter, a cassette for the neomycin resistance gene flanked by loxP sites, and a sequence containing the LacZ open reading frame. Cre recombinase 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 LacZ expression pattern and intensity was determined 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 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

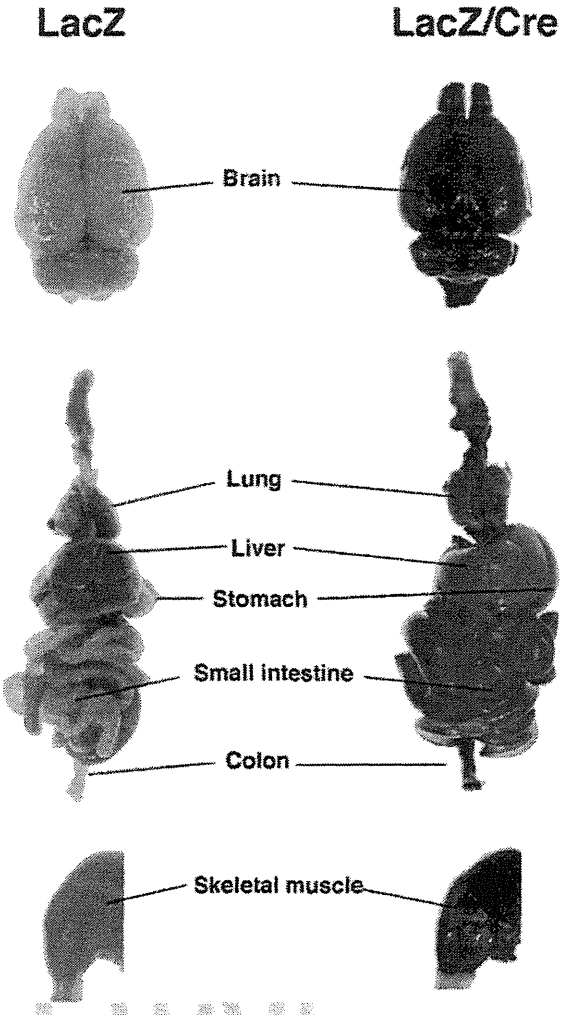

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FIG. 2. Cre-mediated recombination in the adult organs. Whole mount X-Gal staining of the adult organs of LacZ and LacZ/Cre double transgenic rats. Tissues were stained overnight (brain) or for 3 h (other organs). Note that the olfactory bulb and cerebellum 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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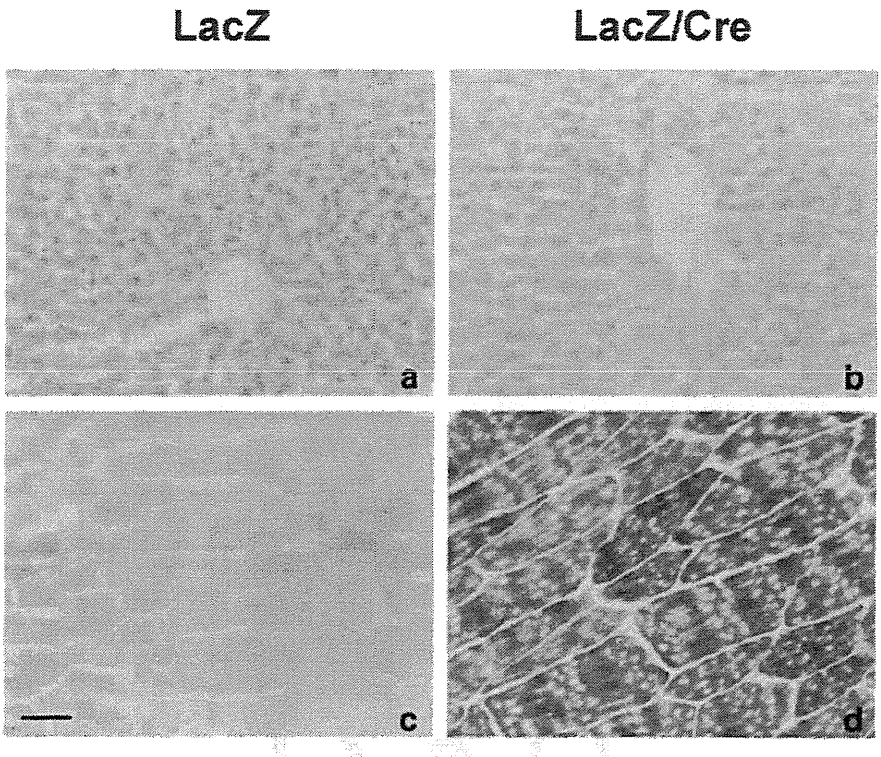

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FIG. 3. LacZ expression in the adult tissues of LacZ and LacZ/Cre rats. Frozen sections were stained with X-gal. (**a**) and (**b**), Liver; (**c**) and (**d**), Skeletal muscle. Bar = 50 μm.

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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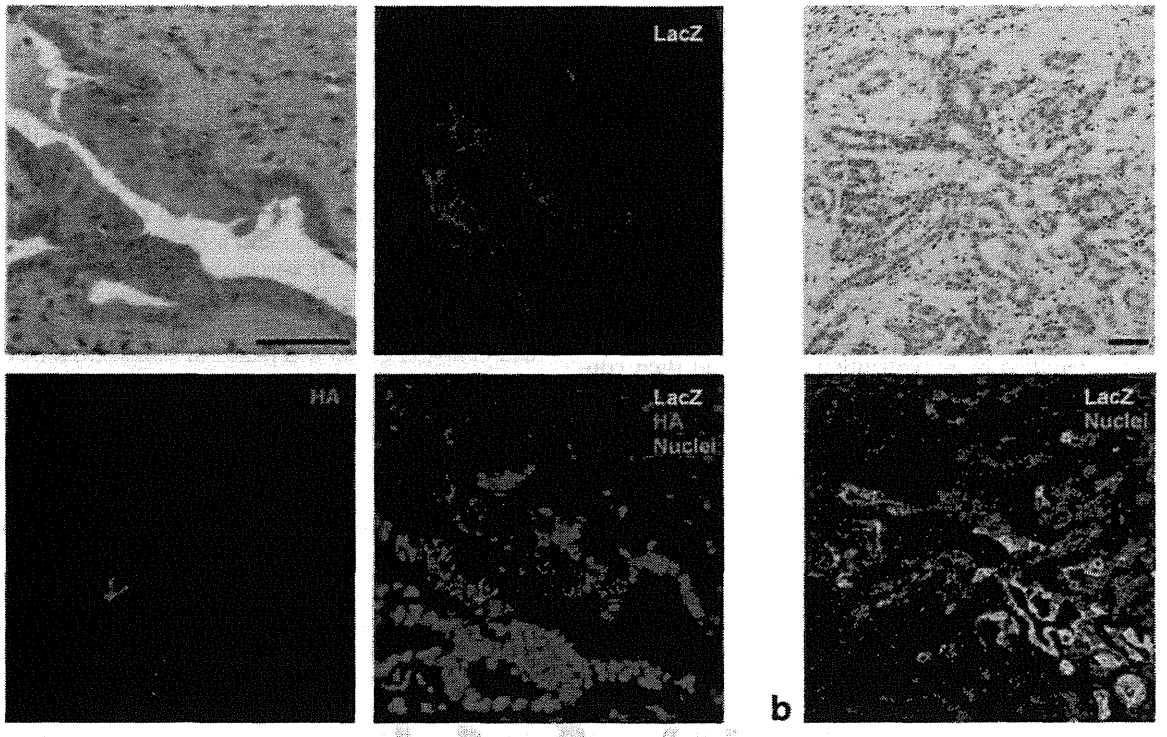

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FIG. 4. Analysis of LacZ expression in pancreas ductal adenocarcinoma of ras/LacZ double transgenic rats. **(a)** Colocalization of LacZ (green) and HA-K*ras*^{G12V} (red) in pancreatic lesions of the Kras/LacZ rat. **(b)** Immunofluorescence staining 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-K*ras*^{G12V} (Fig. 4a), demonstrating that in HA-K*ras*^{G12V}/LacZ double transgenic rats, the expression of the LacZ and HA-K*ras*^{G12V} transgenes is linked; in cells which are infected by AxCANCre and express Cre, Cre generally activates both the HA-K*ras*^{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.