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Mature acinar cells are refractory to carcinoma development by targeted activation of Ras oncogene in adult rats

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Pancreatic ductal adenocarcinoma (PDA) is one of the most debilitating malignancies in humans. A thorough understanding of the cytogenesis of this disease will aid in establishing successful treatments. We have developed an animal model which uses adult Hras^{G12V} and Kras^{G12V} transgenic rats in which oncogene expression is regulated by the Cre/loxP system and neoplastic lesions are induced by injection of adenovirus-expressing Cre recombinase. When adenovirus with Cre recombinase under the control of the CMV enhancer/chicken β-actin (CAG) promoter (Ad-CAG-Cre) is injected into the pancreatic duct of these animals, pancreatic neoplasias develop. Pathologically, the origin of these lesions is duct, intercalated duct, and centroacinar cells, but not acinar cells. The present study was undertaken to test the effect of acinar cell-specific oncogenic *ras* expression. Adult transgenic rats were injected with adenovirus with Cre recombinase under the control of the acinar cell-specific promoters amylase (Ad-Amy-Cre) and elastase-1 (Ad-Ela-Cre) or under the control of the non-specific CAG promoter. Injection of either Ad-Amy-Cre or Ad-Ela-Cre into the pancreatic ducts of transgenic animals in which oncogenic *Kras* is tagged with hemagglutinin (HA), HA-Kras^{G12V} rats resulted in expression of oncogenic *ras* in acinar cells but not in duct, intercalated duct, or centroacinar cells. Notably, injected animals did not develop any observable proliferative or neoplastic lesions. In marked contrast, injection of Ad-CAG-Cre resulted in pancreatic cancer development within 4 weeks. These results indicate that adult acinar cells are refractory to Ras oncogene activation and do not develop neoplasia in this model. (*Cancer Sci* 2010; 101: 341–346)

Pancreatic ductal adenocarcinoma (PDA) is a highly lethal disease, which is usually diagnosed in an advanced state. Most patients die within 1 year of diagnosis, [1] and the 5-year survival rate is <5%.[2] Understanding of the cytogenesis of PDA offers new directions for targeted therapeutic approaches to combat this disease.

Previously, we reported on an animal model in which pancreatic neoplasia was induced in adult Hras^{G12V} transgenic rats by injection of adenovirus with Cre recombinase under the control of the CMV enhancer/chicken β-actin (CAG) promoter into the pancreatic duct.[3] In these animals, it was shown that duct, intercalated duct, centroacinar, and acinar cells were all infected with the adenovirus, but induced pre-neoplastic and neoplastic lesions were shown to express only duct cell-specific characteristics and not acinar cell-specific characteristics. Moreover, proliferative lesions were not observed in acinar cells. Therefore, we hypothesized that PDA does not develop from adult pancreatic acinar cells in this model.

The present study was undertaken to directly test the capability of mature acinar cells to develop into a neoplastic lesion. Transgenic rats with an Hras or hemagglutinin (HA)-tagged Kras oncogene were injected with Cre recombinase expressing adenoviruses in which Cre expression was under the control of promoters specifically active in acinar cells. Mature acinar cells in injected rats did express active Ras proteins, but did not develop any proliferative or neoplastic lesions.

Materials and Methods

Generation of transgenic rats. For the generation of transgenic rats conditionally expressing human Kras^{G12V}, we first made a cDNA fragment encoding the human Kras4B^{G12V} with a 3× HA tag sequence at its 5′ end (HA-Kras^{G12V}). The HA-Kras^{G12V} cDNA was subcloned into the SacI/KpnI site of pCALNL5 (DNA Bank, RIKEN Bio Resource Center, Ibaraki, Japan)[4,5] to produce pCALNLHAKras. pCALNLHAKras was digested with SalI/HindIII. The purified cassette (Fig. 1A) was injected into the pronuclei of Sprague–Dawley rats (CLEA Japan, Tokyo, Japan). Techniques used for the generation of transgenic rats were the same as those reported previously.[3,6] A total of 265 injected eggs were transplanted into pseudo-pregnant Sprague–Dawley rats. Of 37 potential transgenic rats screened, four male and one female rat 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. The following primers were used: 5′-TCTGGATCAAATCCGAACGC-3′, 5′-TGACCTGCTGTGTC-GAGAAT-3′. Two founder rats carrying a CALNLHAKras^{G12V} transgene transmittable to descendent generations (Kras301and Kras327) and two founder rats (Kras409 and Kras417) carrying a non-tagged Kras^{G12V} transgene were established using the same cassette (data not shown). In this study, we used Kras301 and Kras327. Hras250 rats conditionally expressing human Hras^{G12V} were generated as previously described. [3] They were maintained in plastic cages in an air-conditioned room with a 12-h light/12-h dark cycle. All experiments were conducted according to the Guidelines for Animal Experiments of the Nagoya City University Graduate School of Medical Sciences.

Preparation of adenovirus vectors. Adenoviruses in which either the mouse amylase-2 or the rat elastase-1 promoter drove the expression of Cre recombinase (Ad-Amy-Cre or Ad-Ela-Cre) (Fig. 1B) were prepared as described previously.[7] Recombinant adenovirus vectors carrying the *Cre* gene (Ad-CAG-Cre) (Fig. 1B) and empty adenovirus vector were prepared as described previously.[3] Recombinant adenovirus vectors were amplified in HEK-293 cells and then purified using Vivapure

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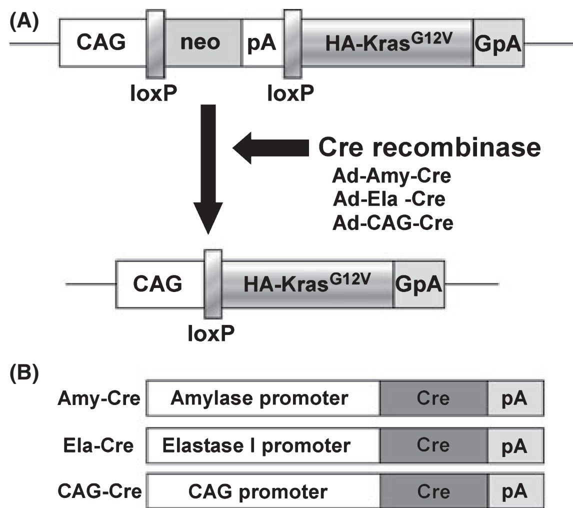

Fig. 1. Conditional expression of $Kras^{G12V}$ transgene. (A) The CALNL-$HAKras^{G12V}$ transgene is comprised of a hybrid CMV enhancer/chicken β-actin (CAG) promoter, a cassette for the neomycin resistance gene flanked by loxP sites, and a sequence containing a human $Kras^{G12V}$ with a hemagglutinin (HA)-tag. Infection with the Cre recombinase-expressing adenovirus results in Cre-mediated recombination of the transgene and removal of the neo-coding region and its associated mRNA polyadenylation signal, generating a functional HA-$Kras^{G12V}$ gene expression unit. GpA, rabbit- β-globin poly(A) site; pA, SV40 early poly(A) site. (B) Cre recombinase with nuclear localization signal expressing adenovirus in which Cre expression is under the control of three different promoters: the amylase promoter and the elastase-1 promoter which are active in acinar cells, and the CAG promoter which is a nonspecific promoter.

Adenopack (Vivascience, Hannover, Germany). The titer of the adenovirus was determined by using the Rapid titer kit (Clontech, Mountain View, CA, USA). The virus stock was concentrated to 1.0×10^{10} pfu/mL.

Induction of active Ras in the pancreas. Adenovirus vectors were injected into the pancreatic ducts of 12-week-old adult male rats through the common duct as previously reported. To induce active Ras specifically in acinar cells, adenoviruses (6×10^8 pfu/rat) in which the expression of Cre recombinase was under the control of acinar cell specific promoters, either the amylase-2 (Ad-Amy-Cre) or elastase-1 (Ad-Ela-Cre) promoter, were used. To induce active Ras non-specifically, adenoviruses (6×10^8 pfu/rat) in which the expression of Cre recombinase was under the control of the non-specific CAG promoter were used.

Western blotting. Western blot analysis and detection of activated Ras protein was performed using a Ras Activation Assay kit (Upstate, Lake Placid, NY, USA) as described previously.[3,8] Concentrations of the proteins were determined by Bio-Rad Protein assay. Proteins were separated by SDS-PAGE. After transfer to a polyviniliden defluoride membrane, the membrane was blocked with 5% nonfat milk and then incubated for 1 h at room temperature with primary antibodies. The following antibodies were used: anti-Ras, clone Ras10 (Upstate) diluted 1:4000; HA-probe (Y-11; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1,000; and monoclonal anti-β-actin (A5441; Sigma, St Louis, MO) diluted 1:10 000. The primary antibodies were detected using HRP-conjugated secondary antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) and ECL plus (GE Heallthcare UK, Buckinghamshire, UK).

Immunostaining. Tissues were fixed in 10% formalin or 4% paraformaldehyde fixative and embedded in paraffin. For Ki67, proliferating cell nuclear antigen (PCNA), and HA-tag staining, sections were boiled for 10 min in a 10-mM citrate buffer (pH 6.0) and then allowed to cool in PBS for 30 min before incubation with antibodies. For anti-α-amylase staining, section slides were incubated for 10 min in a 0.1% trypsin solution at 37°C and then washed in PBS for 5 min before incubation with antibodies.

Before staining, each section was blocked with 10% goat serum (Nichirei Bio Science, Tokyo, Japan) for 5 min at room temperature. The slides were incubated overnight at 4°C with primary antibodies against Ki67antigen (NCL-Ki67-p; Novocastra Laboratories, Newcastle, UK), diluted 1:3000; PCNA (clone PC10; DakoCytomation, Glostrup, Denmark), diluted 1:50; HA-Tag (6E2; Cell Signaling, Danvers, MA, USA), diluted 1:100; or anti-α-amylase (A8273; Sigma, St Louis, MO, USA), diluted 1:200. Slides were incubated with secondary antibodies conjugated with Alexa Fluor488, 546, and 647 (Invitrogen, Carlsbad, CA, USA), and images were obtained with a FLUOVIEW FV300 confocal microscope (Olympus, Tokyo, Japan) or a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Results

Targeted activation of HA-$Kras^{G12V}$ transgenes in mature acinar cells. Injection of transgenic rats with Cre recombinase expressing adenovirus resulted in excision of the stuffer DNA between the CAG promoter and the transgene and consequent expression of the transgene in infected cells (Fig. 1A). Kras301/ 327 rats were injected with Ad-Amy-Cre or Ad-Ela-Cre. Expression of HA-$Kras^{G12V}$ was observed only in amylase-positive acinar cells and not in duct, centroacinar, intercalated duct, or islet cells (Table 2) (Fig. 2A; data for Ad-Ela-Cre is identical to that of Ad-Amy-Cre). Some acinar cells with nuclei with an ''owl-eye'' or ''ground glass'' appearance, which are generally used for identification of virus-infected cells,[9] in rats treated with Ad-Amy-Cre or Ela-Cre were also positive for both amylase and HA (Fig. 2A-d,e). All acinar cells positive for HA were entirely negative for Ki67 (Fig. 2A).

Lack of PDA development by targeted activation of Ras^{G12V} in mature acinar cells. None of the Kras301/327 rats injected with Ad-Amy-Cre or Ela-Cre developed pancreatic lesions (Ad-Amy-Cre, 0 out of 5; Ad-Ela-Cre, 0 out of 7) after 8 weeks (Fig. 2B, Table 1). Similarly, none of the Hras250 rats injected with Ad-Amy-Cre or Ad-Ela-Cre (6×10^8 pfu/rat) developed pancreatic lesions (Ad-Amy-Cre, 0 out of 7; Ad-Ela-Cre, 0 out of 8) after 8 weeks (Table 1). In addition, Kras301/327 rats injected with higher titers of Ad-Amy-Cre (6×10^9 pfu/rat) did not develop pancreatic lesions (data not shown). Finally, tumor induction was not observed in injected Kras301/327 rats even after 6 months (data not shown).

Neoplasia development by activation of Ras^{G12V} transgenes in ductular cells. Both Kras301/327 and Hras250 rats injected with Ad-CAG-Cre (6×10^8 pfu/rat) developed pancreatic neoplasias: 22 of 22 Kras301/327 rats and 30 of 35 and Hras250 rats after 2 to 4 weeks (Table 1), as observed in our previous report.[3] Pancreatic neoplasias were also observed in Kras301/327 rats injected with lower titers of Ad-CAG-Cre (6×10^7 pfu/rat) (data not shown). Activation of the transgene in the pancreatic ductal lesions of Kras301/327 rats was shown by Western blotting using anti-HA antibody (Fig. 3). The expression of HA-$Kras^{G12V}$ was detected in pancreatic intraepithelial neoplasia (PanIN) and neoplastic lesions, but not in normal-looking pancreatic duct cells or stromal cells (Fig. 4A). Ki67 or PCNA and HA were positive in PanIN lesions (Fig. 4B) and in many neoplastic cells (Fig. 4C).

Discussion

The morphological and molecular signatures associated with human pancreas tumors suggests that duct epithelium is

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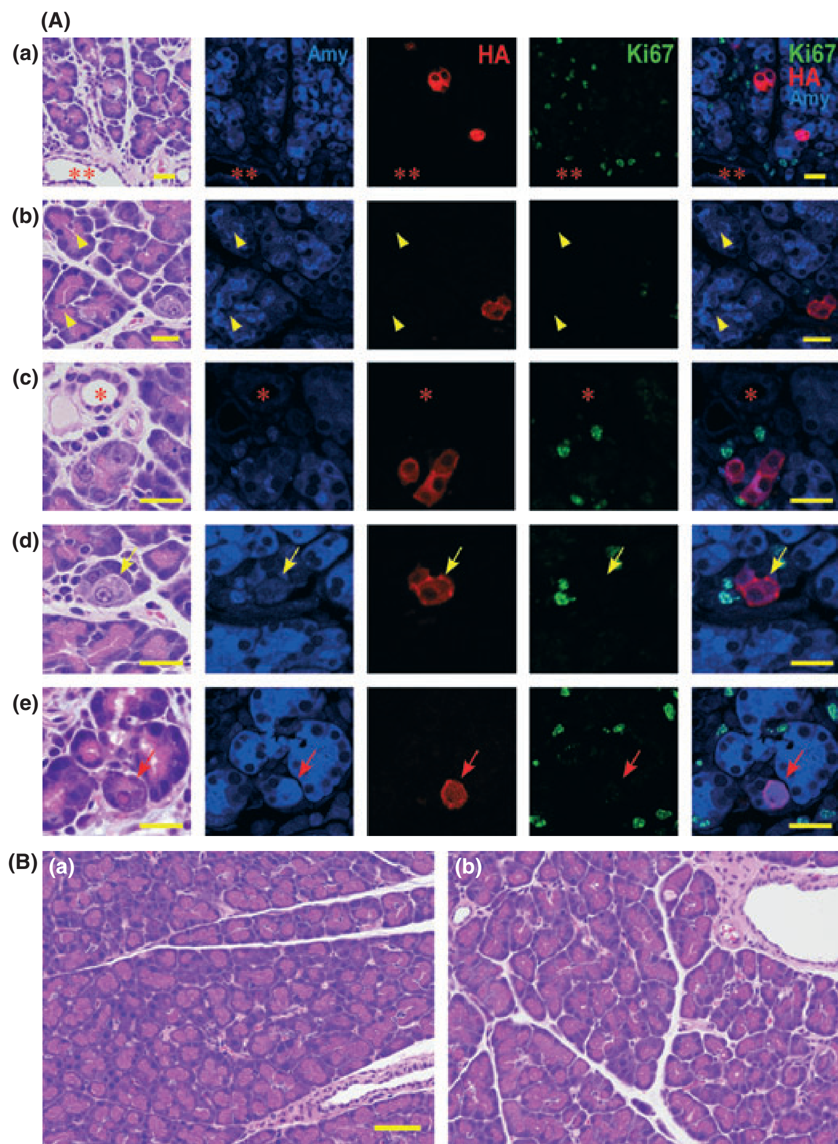

Fig. 2. Acinar cell-specific expression of hemagglutinin (HA)-Kras^{G12V}. (A) Localization of amylase (blue) protein, HA-Kras^{G12V} (red) and Ki67 (green) at 2 days after injection of virus with Ad-Amy-Cre (a, b, c, d) and Ela-Cre (e). All the HA-Kras^{G12V} positive cells (red) were acinar cells; expression was not observed in duct cells (**), centroacinar cells (yellow arrowhead), or small duct cells (*). Most virally infected acinar cells positive for HA-Kras^{G12V} were indistinguishable from non-infected acinar cells by hematoxylin–eosin staining. Some infected acinar cells have nuclei with a so-called ''owl-eye'' (yellow arrows) or ''ground glass'' (red arrows) appearance. Ki67 (green) is not present in the nuclei of the cells expressing HA-Kras^{G12V} (red). Bar, 20 μm. (B) None of the Ad-Amy-Cre (a) or the Ad-Ela-Cre (b) groups displayed any pancreatic lesions, even after 8 weeks. Bar, 50 μm.

responsible for the development of PDA, but it remains unclear whether other pancreatic cells might also contribute to the cytogenesis of these lesions. In our previous study using the Hras250 rat, 4 weeks after injection of adenovirus with Cre recombinase under the control of the constitutive CAG promoter, proliferative lesions in the duct epithelium, intercalated ducts, and centroacinar cells were widespread, but we could not detect any proliferative lesions in acinar cells; moreover, subsequent neoplastic lesions expressed only duct cell-specific characteristics and not acinar cell-specific ones.[3] We have obtained essentially identical results with Kras transgenic rats as we did with Hras250 rats (data not shown). These results suggest that PDAs may arise from centroacinar cells, intercalated duct, or pancreatic duct epithelium, but not from acinar cells.

The current study was undertaken to clarify whether mature acinar cells in adult rats could be induced to develop to PDA by targeted activation of oncogenic *ras*. Activation of oncogenic *ras* in acinar cells did not lead to the development of any observable pancreatic lesions, while nonspecific activation of oncogenic *ras* in the pancreas resulted in rapid development of

Table 1. Pancreas tumor induction by activation of Hras^{G12V} or hemagglutinin (HA)-Kras^{G12V} oncogene after Cre-adenovirus injection

Oncogene	Virus vector	Number of rats with tumor (%)
Hras^{G12V}	Amylase-Cre	0/7 (0)
	Elastase-Cre	0/8 (0)
	CAG-Cre	30/35 (87.5)
HA-Kras^{G12V}	Amylase-Cre	0/5 (0)
	Elastase-Cre	0/7 (0)
	CAG-Cre	22/22 (100)

Table 2. Target cell and tumor type in Hras^{G12V} and hemagglutinin (HA)-Kras^{G12V} transgenic rats

Virus vector	Target cells			Tumor yield	
	Acinar cells	Centroacinar cells	Duct cells	Acinar cells	Duct cells
Amy-Cre	+	–	–	–	–
Ela-Cre	+	–	–	–	–
CAG-Cre[3]	+	+	+	–	+

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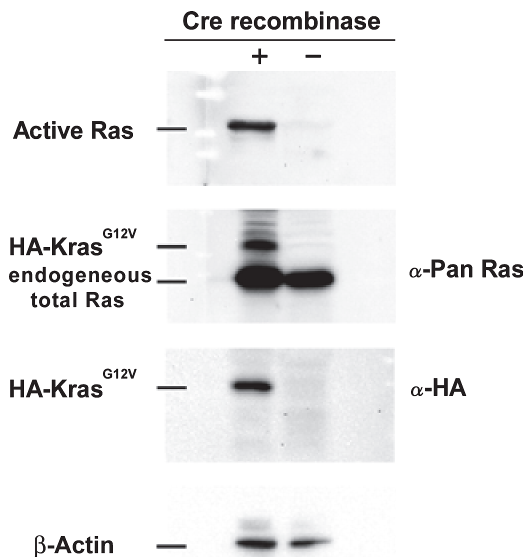

Fig. 3. Transgene activation in Kras301 and 327 by Western blotting. A high level of active Ras and hemagglutinin (HA)-Kras^{G12V} were detected in the pancreas of the Ad-CAG-Cre-treated rats. The amount of active Ras was analyzed by RBD (Ras-binding domain of Raf-1) pull-down assay followed by Western blotting with anti-pan Ras antibody. HA-Kras^{G12V} and endogeneous total Ras was detected using anti-Pan Ras antibody. HA-Kras^{G12V} was detected using anti-HA antibody. β-Actin was used as a loading control.

pancreatic neoplasias. Our results clearly show that conditional expression of oncogenic *ras* in acinar cells in fully developed pancreas tissue does not result in induction of neoplasia in this model (Table 1).

Previous reports in which Kras was activated in immature acinar cells during embryonic development[10,11] suggested that acinar-ductal metaplasia played a role in the development of PDA. In these models, premalignant acinar-ductal metaplasia and acinar tumor mixed with duct-like lesions developed in transgenic mice. This acinar-ductal metaplasia, however, may have occurred before the pancreas fully developed. Our model, on the other hand, targets mature acinar cells which express digestive enzymes, amylase and/or elastase, and these cells do not undergo acinar-ductal metaplasia in response to ras activation.

Our results are in agreement with a recent study in which the distribution of *K-RAS2* gene mutations was extensively examined in surgically resected pancreata from human patients and which concluded that ductal neoplasms of the human pancreas did not appear to arise from acinar cells.[12]

Kras mutations were not observed in pancreatic acinar cell carcinoma (ACC) induced in mature rats by administration of azaserine.[13] Furthermore, alterations in the APC/β-catenin pathway were detected in 23.5% of human ACC,[14] but mutation of Kras was not observed.[15,16] Thus, it is possible that APC/β-catenin or another pathway, but not necessarily Kras activation, is involved in ACC development.

While pancreas cancer in the hamster model is also believed to arise from ductal epithelial hyperplasia,[17,18] several studies using transgenic mice[11,19–24] suggest that PDA may develop from acinar cells. In most of these studies, however, oncogenic stimuli are present during embryonic development, prior to the development of a mature pancreas. Therefore, the acinar cells which were activated and developed into neoplasias in these models could very well be at a different developmental stage to the acinar cells which are present in a mature pancreas. This is important because the majority of PDA patients are 60 years of age or older. It is highly unlikely that an oncogenic insult occurring in the uterus is the root cause of most of these PDAs. Moreover, epidemiological studies indicate the incidence of PDA is closely related to lifestyle.[25] Therefore, PDA most likely develops from cells in the mature pancreas. Consequently, pancreas tumor models in which the oncogenic insult occurs during embryonic development are unlikely to be appropriate for determining the cytogenesis of human PDA.

Two models[22–24] use conditional activation of Cre recombinase to active oncogenic *ras* in adult animals: one model[22] uses the tet-off system to control expression of Cre recombinase and the other model[23,24] uses the tamoxifen-estrogen receptor system to control nuclear localization of Cre recombinase. These studies had slightly conflicting results. In one study, expression of oncogenic *ras* in adult acinar cells did not by itself induce pancreatic lesions; additional treatment causing chronic pancreatitis was also needed.[22] In the other study, expression of oncogenic *ras* was sufficient to induce PanIN-like lesions.[23,24] In our model, we clearly showed that while expression of oncogenic *ras* is sufficient to induce duct, intercalated duct, and/or centroacinar cells to develop into pancreatic cancers, it is not sufficient to induce acinar cells to develop into pancreatic cancers. Whether these discrepancies are due to experimental procedures, the nature of the Cre recombinase constructs used, or differences between mice and rats remains to be resolved. There are however, a few readily apparent differences. In our rat system, there is no expression of Cre recombinase in the animal until injection of adenovirus-expressing Cre recombinase, and the expression of Cre recombinase is transient. In the model which uses tamoxifen, on the other hand, Cre recombinase is expressed during embryonic development, but nuclear localization is regulated by tamoxifen.[23,24] In this model, however, there was a low level of tamoxifen-independent recombination events resulting in expression of oncogenic *ras* in embryonic acinar cells.[24] It is possible that embryonic acinar cells expressing oncogenic *ras* did not fully differentiate in the adult pancreas; for example, in the mouse colon expression of Kras^{G12V} inhibits differentiation.[26] Therefore, it is possible that in the tamoxifen-estrogen regulated model,[23,24] the acinar cells which were activated to undergo metaplasia to duct-like cells in the adult were not actually mature acinar cells. The other obvious difference is that in the model regulated by the tet-off system, two events were required to induce pancreas cancer: activation of oncogenic *Kras* and chronic pancreatitis.[22] Chronic pancreatitis would very likely result in the death of mature acinar cells and their replacement from a proliferative compartment. It is possible that these replacement cells are not fully mature acinar cells, again suggesting the possibility that the acinar cells which underwent metaplasia to duct-like cells were not actually mature acinar cells.

The primary aim of this study was to determine whether activation of oncogenic Kras in mature, digestive enzyme-secreting acinar cells would lead to pancreatic lesions. Our findings support our earlier hypothesis that PDA does not develop from Kras activation in mature acinar cells. It is possible, however, that PDA could develop from Kras activation in immature acinar cells, and in this regard we would like to emphasize the results of Guerra *et al.*[22] in which activation of Kras in the mature pancreas accompanied by chronic pancreatitis resulted in induction of PDA in transgenic mice. Importantly, chronic pancreatitis has been shown to be one of the main risk factors for PDA development in humans.[27,28]

Other factors which may influence PDA development in our model are inflammation and fibrosis. Shortly after infection of pancreatic tissue with Cre recombinase carrying adenovirus to activate the Kras transgene, infiltration of macrophages and lymphocytes could be observed. This infiltration is presumably in response to viral infection. A moderate degree of inflammation, however, was still observed in the stromal tissue surrounding the tumors when PDA developed. These finding suggest that

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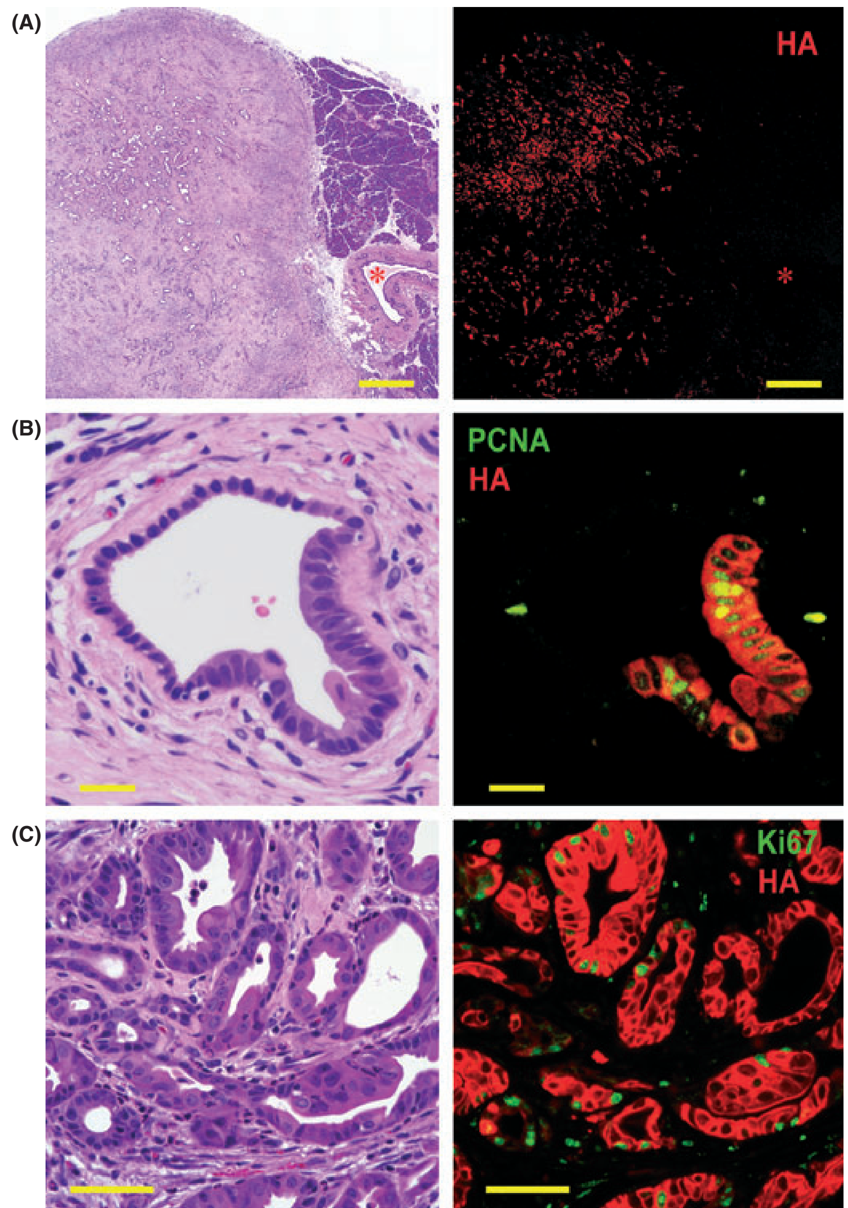

Fig. 4. Pancreatic ductal adenocarcinoma (PDA) induced by injection of Ad-CAG-Cre in Kras301/327 rats. (A) The expression of hemagglutinin (HA)-$Kras^{G12V}$ (red) was seen only in PDA lesions (on the left of photo), and not in stromal cells, acinar cells (on the right of photo), or normal pancreatic duct cells (*). Bar, 500 μm. (B) A pancreatic intraepithelial neoplasia (PanIN) lesion was surrounded by fibrous tissue with some infiltration of inflammatory cells. Expression of proliferating cell nuclear antigen (PCNA) (green) and HA protein (red) in a PanIN lesion in rats of the CAG-Cre group. PCNA is preferentially expressed in PanIN cells. Bar, 20 μm. (C) Expression of Ki67 (green) and HA protein (red) in PDA cells. Many PDA cells (red) are simultaneously positive for Ki67. Bar, 50 μm.

inflammation may play a role in PDA development in this model. However, interaction between the immune system and tumors is complex and whether inflammation actually promotes PDA development in this model remains to be examined.

A current study has demonstrated that the fibrous element accompanying inflammation can also play an important role in cancer development.[29] This aspect of PDA development in our model also remains to be examined.

In summary, while there are discrepancies between different animal models of pancreatic cancer, our results indicate that expression of oncogenic *ras* in fully mature acinar cells does not induce cell proliferation or result in development of any pancreatic lesions. Thus, we conclude that mature acinar cells are not the origin of PanIN or pancreatic neoplasia in this model.

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